RESEARCH ARTICLE

FOXO1 and *FYN* Expression Trends in Breast Cancer Stem Cells: An Integrative Study of Single Nucleotide Polymorphism (SNP) Array and Quantitative PCR (qPCR) Analysis

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Abstract

BACKGROUND: Currently, identification of breast cancer stem cells (BCSCs) commonly relies on $CD24^{-}/CD44^{+}$ expression profiles. However, few studies have integrated genomic mutation data with experimental gene expression validation in CSC and non-CSC populations. Genotyping results of $CD24^{-}/CD44^{+}$ MDAMB-231 cells revealed 36 mutations in BCSCs compared to non-BCSCs, with upregulated *FOXO1* and *FYN* that might represent promising candidate biomarkers for this subpopulation. Therefore, in this study, single nucleotide polymorphism (SNP) and quantitative polymerase chain reaction (qPCR) analysis were performed to assess the association between mutations and expression trends of *FOXO1* and *FYN* in MDAMB-231 cell, as breast cancer cell model with stem-like traits and well-characterized profile.
METHODS: Genomic DNA was isolated from BCSC and non-BCSC DNA from the MDAMB-231 cell line. Mutation analysis was conducted using PLINK, while gene expressions of *FOXO1* and *FYN* were quantified by one-step SYBR Green-based qPCR, using 18s rRNA as a reference. Data was then analyzed with the Livak ($2^{-\Delta\Delta Ct}$) method.
RESULTS: Among 36 mutations found in BCSCs of the MDAMB-231 cell line, *PTEN* (rs786204914) and *CHEK2* (rs587782401) were identified as pathogenic. While *FOXO1* (2.989±2.817 *vs.* 1.072±0.388) and *FYN* (1.405±0.072 *vs.* 0.855±0.140) mRNA levels were found to be higher in CSCs compared to non-CSCs, though these differences was not statistically significant.
CONCLUSION: Pathogenic mutations in *CHEK2* and *PTEN* were detected within BCSC population, implying a potential influence on the expression of *FOXO1* and *FYN*, though not statistically significant. These findings suggest a possible, but as yet unverified, association between gene mutations and expression patterns, emphasizing the importance of further functional studies to validate *FOXO1* and *FYN* as biomarkers for BCSCs.
KEYWORDS: breast cancer stem cells, *FOXO1*, *FYN*, *PTEN*, *CHEK2*, mutation, biomarker

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Introduction

Breast cancer is one of the most commonly diagnosed malignancies among women and remains the leading contributor to cancer-related deaths worldwide.(1,2) Emerging evidence highlights that breast cancer stem cells (BCSCs) are pivotal in reducing treatment efficacy, promoting disease relapse, and facilitating metastatic spread.(1) These BCSCs represent a small population of malignant cells that resemble normal stem cells in function but possess a markedly higher capacity to initiate tumors.(3)

The presence of BCSCs predicts a poor prognosis (3), with some underlying causes of BCSCs include DNA mutations. Currently, the identification of breast cancer stem cells commonly relies on cluster of differentiation $CD24^{-}/CD44^{+}$ expression profiles and aldehyde dehydrogenase (ALDH) activity. However, ongoing research is still assessing the precision and selectivity of these markers.(3) Therefore, our previous study attempted to search for BCSCs biomarker candidates from the secretome. Cells emit a mixture of molecules called the secretome to communicate and alter their environment, which may impact cancer development. (4-6) Understanding how cells communicate via signaling pathways may be critical to identifying distinct markers for BCSCs.(5)

High-throughput single nucleotide polymorphism (SNP) array technologies enable the detection of genetic alterations that may regulate the secretome. Specific mutations have been shown to correlate with secretome components in breast cancer stem cells.(7) Since only few studies have integrated genomic mutation data with experimental gene expression validation in CSC and non-CSC populations, the present study integrated genomic and proteomic approaches to explore the association between gene mutations and secretome-derived biomarkers as potential indicators of BCSCs.

The previous genotyping results of $CD24^{-}/CD44^{+}$ MDAMB-231 cells revealed 36 mutations in BCSCs compared to non-BCSCs, with upregulated Forkhead box protein O1 (*FOXO1*) and Fyn proto-oncogene Src family tyrosine kinase (*FYN*) that might represent promising candidate biomarkers for this subpopulation. *FOXO1* functions as a transcription factor involved in apoptosis, oxidative stress response, and metabolic control, while *FYN*, a non-receptor tyrosine kinase, modulates signaling pathways linked to cell adhesion and migration. Both are essential in maintaining CSC phenotypes, including quiescence, plasticity, and resistance to environmental stressors. Hence, this current study's framework focuses on *FOXO1* and *FYN*, two proteins with critical roles in cellular regulation.

Recent studies have highlighted the importance of characterizing expression patterns in CSCs, given their roles in tumor initiation, progression, and resistance to therapy. This study was conducted to enhance early detection strategies and inform the development of CSC-targeted therapies by examining how pathogenic mutations, particularly in Phosphatase and TENsin homolog (*PTEN*) and Checkpoint kinase 2 (*CHEK2*) that might influence the relative expression of *FOXO1* and *FYN* in CSC-enriched compared to the non-CSC subpopulations. This study combined SNP array and qualitative quantitative polymerase chain reaction (qPCR) analysis to explore the association between mutations and expression trends of *FOXO1* and *FYN* in the MDAMB-231 model, as a basis for identifying candidate biomarkers. MDAMB-231 cell line was utilized as it is known as breast cancer cell model with stem-like traits and well-characterized profile. This integrative analysis aimed to uncover novel mechanistic insights and potential targets in breast cancer stem cell biology.

Methods

Cell Culture and Isolation of BCSC and Non-BCSC

$CD24^{-}/CD44^{+}$ (BCSCs) and $CD24^{+}/CD44^{-}$ (non-BCSCs) were isolated from the MDAMB-231 cell line using flow cytometry with CD24 PE-A and CD44 FITC-A markers. MDAMB-231 cells were obtained from the Laboratory of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. Initially, BCSCs were cultured in serum-free Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium (Gibco, Waltham, MA, USA), while non-BCSCs were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco). Both were supplemented with 30 mM sodium bicarbonate ($NaHCO_3$), 1% Amphotericin B, and 1% Penicillin/Streptomycin. Once both cell populations reached 70–80% confluency, they were cultured in serum-free DMEM/F12 medium for 24 hours, followed by cell harvesting.

DNA Isolation

DNA was isolated from each cell culture using the Genomic DNA mini kit for blood/cultured cell (GB100/300; Gene Aid, New Taipei City, Taiwan). Then, the DNA isolates were quantified using a Nanodrop at the absorbance ratio of 260/280 for concentration accuracy. Double-stranded DNA (dsDNA) was quantified using the Qubit 3.0 fluorometer combined with the Broad Range (BR) assay kit (Thermo Fisher Scientific, Waltham, MA, USA).(8)

Preparation of a Microarray for DNA Mutation Analysis

The concentration of each isolate DNA in the SNP array study was adjusted to 50 ng/µL before processing with the Infinium Asian Screening Array (ASA)-24v1.0, a genotyping panel featuring 659,184 SNP markers tailored for Asian populations. Microarray steps include amplification, enzymatic fragmentation, alcohol precipitation, DNA resuspension, hybridization, incubation, followed by

enzymatic extension, fluorescence staining, and iScan Illumina reading of fluorescence intensity.

Bioinformatics Analysis of Microarray

The output data from the tool is in the form of .idat files for each sample, which are then analyzed using the gtc to vcf software to convert the .idat files into .vcf before performing control analysis using PLINK (v1.9) (https://github.com/freeseek/gtc2vcf) (https://zzz.bwh.harvard.edu/plink/). The desired quality control screening thresholds for genotyping studies are as follows: genotyping rate (>98%), SNP missingness (<0.02), individual missingness (<0.02), minor allele frequency (MAF; >0.01), Hardy-Weinberg Equilibrium (HWE; <0.001), heterozygosity rate deviation (<3).(9) Genetic mutations that passed quality control (QC) were then analyzed using single nucleotide variants (SNV) calling. Detailed SNP analysis for mutation detection was performed on post-QC data with a *p*-value<0.01 using PLINK (v1.9) to generate data .bim, .bed, .fam.(10) The final mutation list was cross-validated using the Catalogue of Somatic Mutations in Cancer (Cosmic) database to confirm clinical relevance and known oncogenic profiles (http://cancer.sanger.ac.uk/cosmic/).

***In silico* Analysis for Candidate Biomarkers Secretome of BCSCs**

GSE7513 and GSE7515 gene expression profiles were acquired from the gene expression omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/browse/), which provides access to high-throughput gene expression datasets along with relevant biological annotations.(11) GSE7513 comprises 14 $CD24^{-}/CD44^{+}$ and 15 non-$CD24^{-}/CD44^{+}$, while GSE7515 comprises 15 cancer mammospheres and 11 non-mammosphere primary breast cancer, respectively. Differentially expressed genes (DEGs) were selected based on a |Log2 fold change|>1.0 and a *p*-value<0.05. For the enrichment gene ontology study, Enrichr software was utilized (https://maayanlab.cloud/Enrichr/).(11,12) Protein-protein interactions were further analyzed using the String database (https://string-db.org/). The interaction significance was determined based on a combined score range of 0.95-0.99. Cytoscape software version 3.9.1 (https://cytoscape.org/) was used as a visualization and network analysis tool to interpret protein interaction networks. Subsequent analysis involved hub node filtering based on centrality assessment using five components: degree (D), betweenness centrality (BC), closeness centrality (CC), stress (S), and average shortest path length (ASPL). K-means clustering was applied during the in-silico validation process.(11,13,14)

RNA Isolation and RNA Concentration Measurement

Total RNA was extracted using TriPure isolation reagent (ATB2700-50), following the manufacturer's instructions, to ensure high-quality RNA suitable for downstream gene expression analysis. RNA purity and concentration were assessed by Nanodrop One Spectrophotometer (Thermo Scientific), ensuring RNA integrity for qPCR.

qPCR Analysis

qPCR was conducted using SYBR Green chemistry to quantify the *FOXO1*, *FYN*, and *18S* as an internal control for normalization in the comparison between CSC and non-CSC groups (Table 1). The reaction mixture consisted of 5 µL of 2× SensiFAST SYBR Master Mix (One step kit), 0.1 µL of reverse transcriptase enzyme, 0.2 µL of RNase inhibitor, 0.4 µL each of 10 µM forward and reverse primers, 1.9 µL of DEPC-treated RNase-free water (BIO-72005), and 2 µL of RNA (containing 100 ng of template RNA), making a final reaction volume of 10 µL. The amplification protocol was optimized as follows: incubation at 45°C for 10 min, an initial denaturation at 95°C for 2 min, followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 30 s, with a final extension at 60°C for 1 min. All samples were analyzed in triplicate. The expression profiles of *FOXO1* and *FYN* were analyzed using the Livak ($2^{-\Delta\Delta Ct}$) method with *18S* as an internal control.

Results

Genotyping Analysis Results

In this genotyping analysis with SNP arrat, a search was conducted for mutations in the BCSCs ($CD24^{-}/CD44^{+}$) from the MDAMB-231 cell line. Based on the parameters used, there were 36 different mutations in BCSC (*ACP3, USP17L15, PPID, LINC02899, TXLNB, STX5, LRRIQ1, DHX37, CDH2, SERPINB11, MRI1, ZNF687, FH, MSH6, SCN1A, TTN, CPS1, RAF1, CC2D2A, APC, SYNE1, BRCA, DNAH11, KCNH2, TG, CDH23, PTEN, CHEK2, MYBPC3, CDON, PKP2, EXOSC8, PSEN1, SNHG14, PAFAH1B1,* and *GAA*) compared to non-BSCS. Three of

Table 1. Primer sequences.

Primer	Forward (5'-3')	Reverse (5'-3')
18s	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
FYN	AGTTGCGCCATCTGTCAGGA	AACCTCGCCTCTACTCTCGC
FOXO1	AGACAACGACACATAGCTGG	AGGGAGTTGGTGAAAGACAT

Table 2. Pathogenic mutation in CSC of the cell line MDAMB231.

rsID	Mutation				
	Gene	Chromosome	Position	Alel Changes	Mutation Type
rs786204914	*PTEN*	10	87894024	G > C	Splice
rs587782401	*CHEK2*	22	28734401	A > T	Splice

the 36 mutations were pathogenic (*BRCA*, *CHEK2*, and *PTEN*). Of the three pathogenic mutations identified, this study focused on *CHEK2* (rs587782401) on NM_007194 and *PTEN* (rs786204914) on NM_000314 because both were involved in pathways that regulate *FOXO1* and *FYN*, such as Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT) and DNA damage response. Their roles in stemness, cell proliferation, and therapy resistance in BCSCs made them more relevant for expression analysis. *BRCA*, although pathogenic, was not prioritized due to its broader link to hereditary breast cancer and lack of direct association with the target genes. Verification through the Cosmic database indicated that *PTEN* was a recognized somatic mutation, while *CHEK2* was not listed as a documented somatic mutation in the database.

PTEN, a phosphatase gene on chromosome 10, commonly mutates, with X27_splice (rs786204914) being an oncogenic truncating mutation (Table 2 and Figure 1). *CHEK2*, a tumor suppressor and intracellular kinase, with X107_splice (rs587782401) was a truncating mutation in a tumor suppressor gene (Table 2 and Figure 2). This confirmed the pathogenic characteristics of these to genes that might be oncologic.

FOXO1 and *FYN* as Candidate Protein Biomarkers Secretome of BCSCs

We identified 681 and 1294 up-regulated genes in GSE7513 and GSE7515, respectively. A Venn diagram displayed 65 common DEGs (Figure 3A), with a network of 11 nodes and 11 edges (Figure 3B). The medium confidence cut-off was 0.40, identifying key nodes through interactions, with protein-protein interactions filtered by Stringdb score 0.95-0.99. Based on the centrality analysis results, two candidate upregulated protein biomarkers found in the secretome are FOXO1 and FYN (Table 3).

Upregulated Expression of *FOXO1* and *FYN*

In Figure 4, the findings regarding the differences in *FOXO1* and *FYN* expression levels between CSC and non-CSC groups were presented. *FOXO1* mRNA expression was higher in CSCs (2.989±2.817) compared to non-CSCs (1.072±0.388) (Figure 5). *FYN* also showed elevated mRNA expression in CSCs (1.405±0.072) compared to non-CSCs (0.855±0.140). The data distribution for *FOXO1* and *FYN* mRNA expression was normal (p>0.05), but the differences mentioned above were not statistically significant based on the independent t-test analysis (p>0.05).

Discussion

In this study, MDAMB 231 was selected as the sole *in vitro* model for triple-negative breast cancer (TNBC) due to its stem-like traits and well-characterized profile. This cell line is highly invasive and metastatic. It shows a mesenchymal phenotype with over 90% $CD24^{-}/CD44^{+}$ cells, traits associated with cancer stemness. Compared to other TNBC cell lines, MDAMB 231 is extensively studied

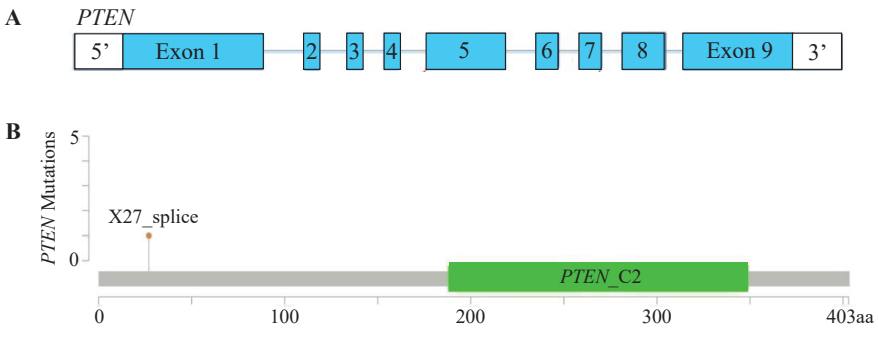

Figure 1. *PTEN* analysis results. A: Normal structure of the *PTEN* gene. B: *PTEN* gene mutations shown with Lollipop Plot.

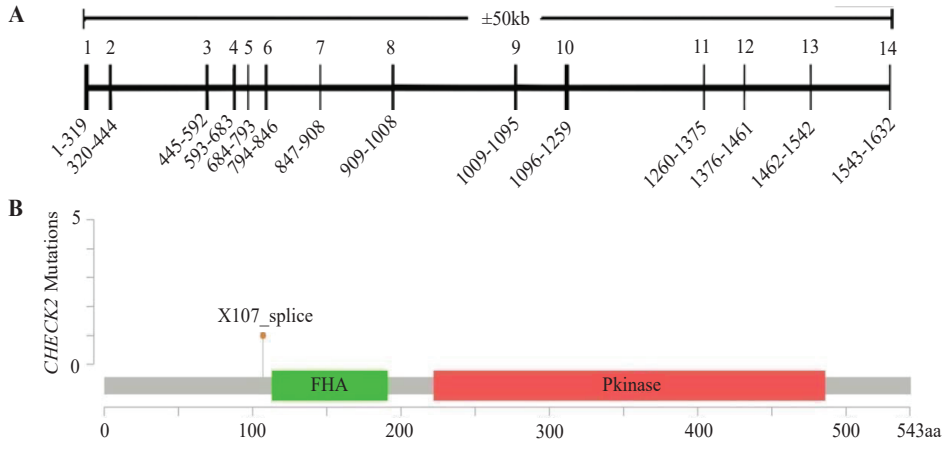

Figure 2. *CHEK2* analysis results. A: Normal structure of the *CHEK2* gene. B. *CHEK2* gene mutations shown with Lollipop Plot.

and well-characterized, offering consistent, reproducible data.

Both *CHEK2* and *PTEN* are well-established tumor suppressor genes known to regulate essential cellular processes such as DNA repair, cell cycle arrest, and apoptosis, mechanisms frequently disrupted in cancer. *CHEK2*, a tumor suppressor and intracellular kinase, has germline mutations on chromosome 22 linked to increased risks of breast, prostate, and colorectal cancers. The *CHEK2* X107_splice (rs587782401) is a truncating mutation in a tumor suppressor gene, and therefore is likely oncogenic. (15) *CHEK2*, increases breast cancer risk by affecting cell cycle arrest, DNA repair, and apoptosis.(16-17) *CHEK2* expression slows cell growth, promoting senescence and apoptosis, which reduces cancer cell survival.(18) *CHEK2* phosphorylates proteins like p53, which is upregulated in early cancer stages. *CHEK2* mutations link to breast cancer development, as its decreasing expression during tumor progression diminishes regulatory control over cancer stem cells.(19-20) *CHEK2* expression is crucial for regulating cellular responses to DNA damage, leading to cell cycle arrest, DNA repair, or apoptosis depending on damage severity.(21-23) *CHEK2* is upregulated in early cancer stages, regulating cell proliferation, but downregulation later diminishes its control over cancer stem cells.(19,22)

In parallel, *PTEN* inactivation is linked to tumor development in various cancers, including breast cancer. (24) *PTEN*, a phosphatase gene on chromosome 10, commonly mutates, with X27_splice (rs786204914) being an oncogenic truncating mutation. Truncating mutations in *PTEN* can lead to different forms of C-terminally truncated *PTEN* proteins, affecting its phosphatase function and regulation of the PI3K/AKT pathway.(25) *PTEN* regulates cell motility, growth, survival, and DNA repair by converting dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2), inhibiting the PI3K/AKT pathway. Its loss promotes uncontrolled growth, stem-like states, drug resistance, and cancer progression.(24) *PTEN* regulates cell movement through its lipid and protein phosphatase activity, involving Rac1, Src kinases (including FYN), and the PI3K/AKT/mTOR pathway.(26) c-Src participates in proliferation, differentiation, survival, and migration. *PTEN* diminishes Akt activity by PIP3 to PIP2, limiting the proliferation of

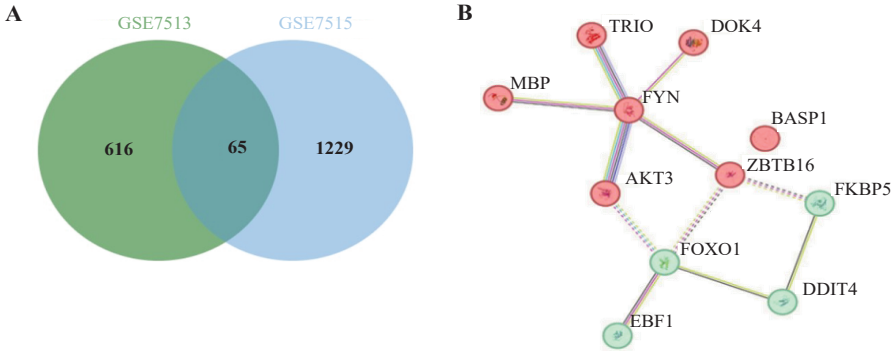

Figure 3. Analysis of candidate protein biomarkers secretome for BCSCs. A: Venn diagram depicted the common up-regulated genes |log2FC|>1.0; p<0.05. B: K-means clustering of 11 up-regulated proteins using the STRING online database.

Table 3. Centrality measurement of the two up-regulated.

	Protein	Description	D	BC	CC	S	ASPL
Up-regulated	FOXO1	Forkhead box O1	5	0.67	0.5	68	2
	FYN	Src family tyrosine kinases	4	0.67	0.5	70	2

BC: betweenness centrality; CC: closeness centrality; D: degree; S: stress; ASPL: average shortest path length.

glioblastoma stem cells while facilitating c-Src activation. (27) *PTEN* mutations decrease *PTEN* activity, activating the PI3K/AKT pathway and promoting cancer growth and survival.(28) Active AKT phosphorylates *FOXO1*, leading to its degradation and reduced tumor suppression function. (29) *FOXO1* activates sex determining region Y-box 2 (*SOX2*), triggering its transcription in feedback loop.(30) *FOXO1* regulates Octamer-binding transcription factor 4 (*OCT4*), likely enhancing cancer aggressiveness.(31)

In this study, we observed that the relative expression of *FOXO1* and *FYN* was higher in CSCs than in non-CSCs. This finding is consistent with our *in silico* predictions, which indicated that *FOXO1* and *FYN* are upregulated in CSCs. *FOXO1* mRNA expression in CSCs is higher than in non-CSCs (1.072), indicating significant transcriptional upregulation. This suggests that *FOXO1* is crucial in maintaining CSC stem-like characteristics, such as self-renewal, therapy resistance, and tumor recurrence. Consistent with previous findings, *FOXO1* has been identified as a key regulator of stem cell renewal, dormancy, and resistance to therapy. Its role in CSC biology is particularly important due to its influence on metabolic balance, apoptosis, and immune responses. *FOXO1*, a tumor suppressor in cancers like breast cancer, often shows abnormal regulation in these environments.(32) Expression of *FOXO1* is upregulated in metastatic TNBC.(33) *FOXO1* regulates differentiation, survival, metabolism, stress resistance, and tumor suppression. *FOXO1* interacts with $CD8^+$ T and natural killer (NK) cells, emphasizing its crucial role in immune responses.(34,35) *FOXO1* is enriched in specific tissues and acts as a tumor suppressor. High *FOXO1* expression is linked to cancer, contributing to epithelial-mesenchymal transition (EMT) under high glucose conditions.(21) Elevated *FOXOs* enhance tumor growth, reduce apoptosis through the insulin like growth factor 1 (IGF-1)/Akt pathway, activate antioxidant enzymes, and support cancer stem cells.

A similar trend was observed in the case of *FYN*, where the CSC group exhibited increased mRNA expression compared to the non-CSC group. Elevated *FYN* mRNA expression despite reduced protein levels in CSCs suggests complex post-transcriptional regulation. Elevated *FYN* mRNA may still influence stemness through non-coding, which regulates genes like *OCT4*, *SOX2*, and Nanog homeobox gene (*NANOG*). Targeting FYN's post-transcriptional regulators could be a promising therapy. Src kinase inhibitors show potential in reducing CSCs, but combined targeting of transcriptional and translational pathways may be needed for sustained efficacy.

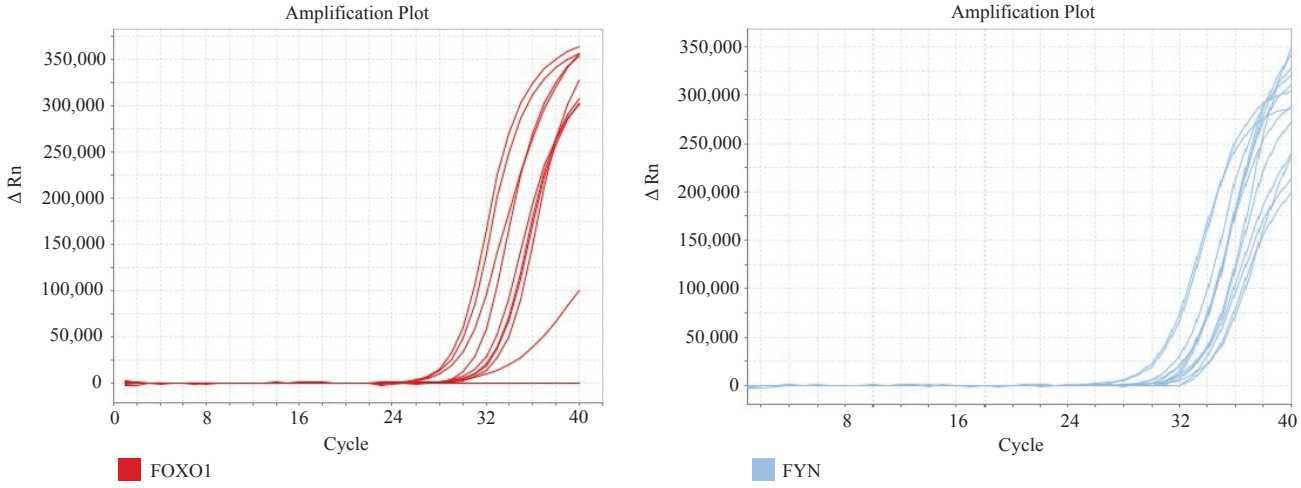

Figure 4. Representative amplification plots obtained from a 10-fold serial dilution. A: *FOXO1* gene; B: *FYN* gene.

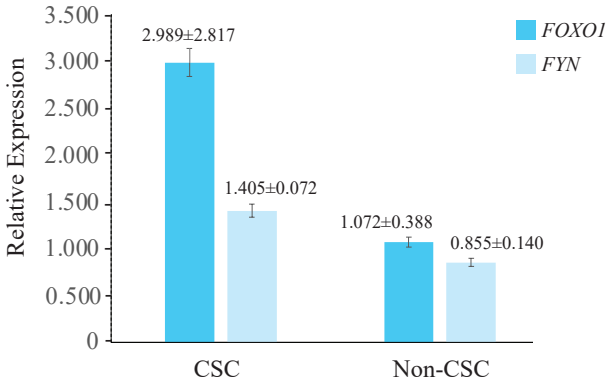

Figure 5. Comparison of *FOXO1* and *FYN* relative expression between CSC and non-CSC of MDAMB-231 cell. Analyzed with independent t-test.

FYN, a member of the Src family kinase, is known to enhance cancer cell proliferation, invasion, and drug resistance.(36) *FYN* is classified as a non-receptor tyrosine kinase within the oncogenic protein tyrosine kinase family and contributes to cancer progression through its pro-tumorigenic activity.(37) It is linked to cell motility and proliferation and is overexpressed in the MDAMB-231 cell line. Elevated *FYN* expression activates PI3K/AKT, mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT) pathways, promoting cell proliferation, migration, invasion, and EMT, while inhibiting apoptosis.(38) In breast cancer, elevated *FYN* expression activates PI3K/AKT and ERK/MAPK pathways, promotes EMT, and is more expressed in aggressive cell lines like MDAMB-231 than in MCF-7 cell (39), since MCF-7 is known to be more not as invasive (40). *FYN* is more abundant in invasive breast cancer cells like MDAMB-231 than in MCF-7 or MCF-10A.(21,37) *FYN* upregulation increases Snail family transcriptional repressor 1 (*SNA1*) expression, influencing EMT. Furthermore, *FYN* is expressed in drug-resistant cancer cells. *FOXO1* controls the transcriptional activity of *FYN* and facilitates fibroblast growth factor 2 (FGF2)-stimulated epithelial-mesenchymal transition through the activation of PI3K/AKT and ERK/MAPK signaling pathways.(16,37)

This study integrates SNP array and *in silico* analyses to identify mutations and predict stem cell-specific protein biomarkers (Figure 6). This approach improves understanding of CSC-related changes and supports translational potential. Future research should validate these findings in clinical samples to assess *FOXO1* and *FYN* as stemness biomarkers and therapeutic targets.

Conclusion

MDAMB-231 cell line, implying a potential influence on the expression of *FOXO1* and *FYN*. Although both genes exhibited elevated mRNA levels in CSCs compared to non-CSCs, the differences were not statistically significant. These findings suggest a possible, but as yet unverified, association between gene mutations and expression patterns, emphasizing the importance of further functional studies to validate *FOXO1* and *FYN* as candidate biomarkers for BCSCs.

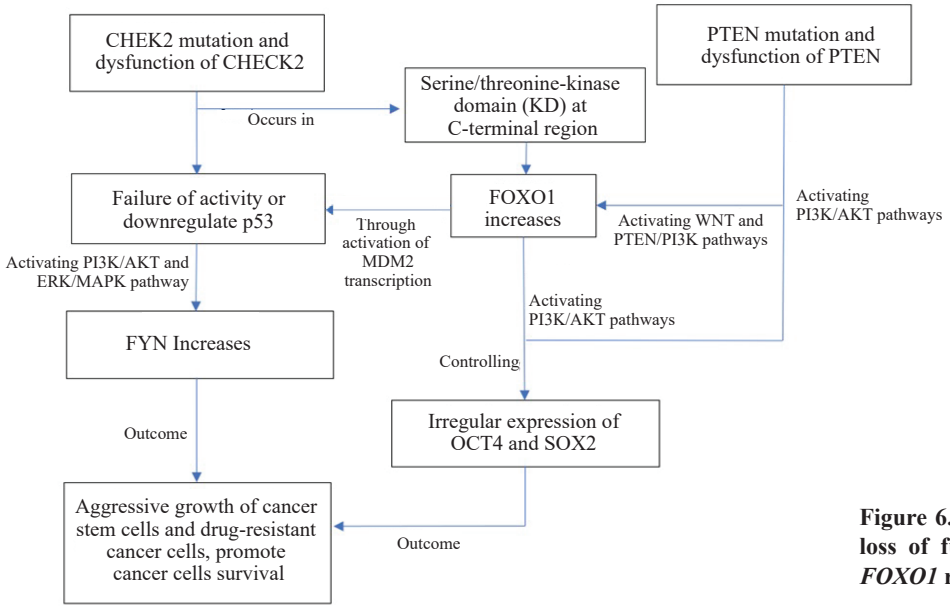

Figure 6. Summary of *CHEK2* and *PTEN* loss of function algorithms on *FYN* and *FOXO1* regulation.

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Authors Contribution

SIW contributed to study design, data collection, interpretation, manuscript preparation, supervision, and funding acquisition. FF participated in data collection, study design, statistical analysis, and supervision. RIP participated in data collection and data interpretation. ALM contributed to study design, data collection, statistical analysis, data interpretation, manuscript preparation, literature search, and funding acquisition.

Conflict of Interest

The authors declare no conflicts of interest or competing interests related to the content of this manuscript.

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