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Research Article

A Systems Biology Approach to Validate Potential Biomarkers for Early Detection and Personalized Therapy for BRCA1-Associated TNBC

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Abstract

Triple-negative breast cancer (TNBC), which accounts for 15-20% of all breast cancer cases, is a particularly aggressive form of breast cancer that disproportionately affects young African-American (AA) women. Our previous work has shown wildtype BRCA1/1a proteins interact with E2 SUMO-conjugating enzyme Ubc9 and function as a growth/tumor suppressor in BRCA1 mutant TNBC and ovarian cancer cells, unlike the disease-associated pathogenic variants. We have also found high levels of expression of Ubc9 in TNBC cells and breast tumor tissues. This study is based on the hypothesis that high Ubc9 protein expression observed in patient-derived BRCA1 mutant TNBC cell line HCC1937 should correlate with high UBE2I RNA levels. We tested this hypothesis by isolating total RNA from both vector and BRCA1a-transfected stable HCC1937 cells. The RNA was subsequently subjected to mRNA sequencing, and the most significant transcripts were identified via bioinformatics analysis using Ingenuity Pathway Analysis. Our mRNA sequencing analysis could not detect a significant fold change in UBE2I nor another BRCA1/1a downstream target SIRT1's RNA expression; however, we found variations in associated network maps active in both stable cell lines, leading to the identification of several potential downstream targets (GABRA3, Mitochondrial complex I, GnRH, TP63, ETV6, NGF, and TGFB2). This study will lead in the future to the development of potential molecular mechanism-based personalized biomarkers for early detection and targeted therapies for TNBC, thus reducing the high mortality rates associated with these aggressive cancers in AA women, leading to health equity.

Keywords: TNBC; BRCA1; BRCA1a; Ubc9; UBE2I; SIRT1; Biomarkers; Bioinformatics; Personalized medicine; Targeted therapy

Abbreviations: TNBC: triple-negative breast cancer; BRCA1: breast cancer susceptibility gene 1; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; Ubc9: SUMO-conjugating enzyme 9; SUMO: small ubiquitin-like modifier; IPA: Ingenuity Pathway Analysis

Introduction

Breast cancer is the most commonly diagnosed, as well as the leading cause of death from cancer in women worldwide and is the second most common cause of death from cancer in women in the United States [1]. According to the American Cancer Society, an estimated 43,250 women in the United States are expected to die from breast cancer in 2022 alone [2]. Most breast cancers in the United States are classified as invasive and can be divided into four molecular subtypes: luminal A, luminal B, HER2-positive, and basal-like [3]. Triple-negative breast cancer (TNBC), a type of basal-like breast cancer, is a heterogeneous disease based on gene

expression profiling and accounts for 15-20% of all breast cancers [4]. TNBCs are known for being aggressive cancers with higher mean tumor sizes, higher grade tumors, higher rates of node positivity, high likelihoods of recurrence, distinct metastasis patterns, and poorer survival compared to other breast cancers [5]. They have also been characterized by the absence of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) protein observed in other breast cancer subtypes, leaving them insensitive to endocrine therapy or HER2-targeted therapies [6]. This leaves affected patients relying on cytotoxic chemotherapy as the mainstay treatment for TNBC, as there currently remains a lack of effective targeted therapies available [7]. Lehmann et al. have classified TNBC into six molecular subtypes to better identify targeted therapies: basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) [8]. Basal-like TNBCs, the most aggressive subtype, tend to be associated with mutations in the BRCA1 gene. BRCA1, cloned by Miki et al. in 1994, is a tumor suppressor gene located on chromosome 17q21 [9]. In TNBC, BRCA1 is mutated, expressed at low levels, or abnormally localized [10]. We have identified and cloned two major isoforms of BRCA1, BRCA1a/p110 and BRCA1b/p100, which are expressed at reduced levels in breast tumors compared to normal mammary cells [11,12]. We have also found BRCA1a to possess anti-tumor activity in human ER-α positive breast cancers, hormone-independent ovarian and prostate cancer cells, and TNBC [13]. Our previous work suggested a molecular mechanism as to how BRCA1 mutations result in TNBC [14]. BRCA1/1a proteins interact with nuclear chaperone Ubc9, a SUMO E2-conjugating enzyme, thereby activating ER-α and functioning as a tumor suppressor, unlike the pathogenic mutants which are unable to bind Ubc9 and are stalled in the cytoplasm, resulting in TNBC [15]. We have shown deregulated levels of Ubc9 trigger proliferation, migration, and metastasis of TNBC cells, which have consequently been linked to poor response to chemotherapy and poor clinical outcomes [16,17]. We have also found Ubc9 to be expressed at elevated levels in tumor tissue obtained from an AA woman with BRCA1 mutant TNBC and high mammographic density compared to matched benign tissue, suggesting a potential role for Ubc9 in triggering BRCA1-associated TNBC development [18]. These levels have also been shown to correlate with poor clinical outcomes in Nigerian women with TNBC from a study by Agboola et al., which agrees with our findings [19]. Additionally, high levels of Ubc9 have also been observed in other cancers such as ovarian, lung, head and neck cancers, and advanced melanomas [17,20].

Recently, we have found SIRT1 to be a new downstream target of BRCA1 [5]. Sirtuin 1 (SIRT1) is an NAD+-dependent enzyme involved in several functions such as genome stability, apoptosis, proliferation, and tumorigenesis [21]. We have shown BRCA1 to bind Ubc9, resulting in increased SIRT1 expression and facilitated BRCA1 to translocate to the nucleus, activate ER-α, and function as a tumor suppressor [5,14,15]. Mutations in BRCA1 in TNBC were shown to impair tethering to Ubc9, resulting in cytoplasmic localization of BRCA1 proteins and inhibited SIRT1 expression, leading to TNBC and other diseases such as cardiac disease [14,22].

This study is based on the hypothesis that high levels of Ubc9 protein expression observed in patient-derived BRCA1 mutant HCC1937 TNBC cells should correlate with elevated levels of UBE2I RNA expression. As mentioned previously, we have already shown Ubc9 to be expressed at high levels in several TNBC cell lines and tumor tissues, suggesting that Ubc9 could be used as a potential mechanism-based protein biomarker for TNBC. Therefore, validating UBE2I as an RNA biomarker could provide additional diagnostic and prognostic value, as RNA biomarkers are more sensitive, specific, and cost-effective compared to protein biomarkers [23]. We plan to test this hypothesis by using HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable TNBC cell lines and investigating their transcriptome. This study, if successful, will allow us to study whether UBE2I, SIRT1, or other downstream targets can be used in the future as potential mechanism-based biomarkers for early detection or targeted therapies for TNBC, which will assist in the advancement of precision oncology and reduce cancer health disparities, saving countless lives worldwide.

Materials and Methods

Development of Stable Cell Lines

HCC1937 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Both HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable cell lines were established and cultured as described previously [5].

RNA Isolation

RNA was isolated from HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable cells using the RNeasy mini kit (Qiagen, Germantown, MD, USA). The cells were trypsinized and collected as a cell pellet via centrifugation for 5 minutes at 300 RPM. 600 µL of RLT buffer was added to disrupt the cells and then vortexed. The lysate was pipetted into a QIA shredder spin column and placed into a 2 mL collection tube. The lysate was then centrifuged for 2 minutes at full speed. 350 µL of 70% ethanol was added to the lysate and vortexed. 700 µL of the resulting sample was placed into an RNeasy spin column placed in a 2 mL collection tube. This mixture was centrifuged at ≥8000 x g for 15 seconds. After the resulting flow through was discarded, another 700 µL of buffer was added into the spin column and centrifuged at ≥8000 x g for 15 seconds. The flow through was again discarded, and then 500 µL of buffer was added to the spin column and centrifuged at ≥8000 x g for 15 seconds. Another 500 µL of buffer was added to the spin column after discarding the flow through and centrifuged for 2 minutes at ≥8000 x g. The RNeasy spin column was placed into a new 2 mL collection tube and centrifuged for 1 minute at full speed. The spin column was placed into a new 1.5 mL collection tube, and then 30-50 µL of RNase-free water was added directly to the spin column membrane. This mixture was then centrifuged at ≥8000 x g for 1 minute.

RNA Quality Testing

Total RNA concentration and purity were assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) at the University of Alabama at Birmingham's Genomics Core Facility. RNA from both HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable cells with an RNA integrity number of 7.0 or above was used for library preparation for sequencing.

mRNA Sequencing and Analysis

mRNA sequencing was performed at the University of Alabama at Birmingham's Genomics Core Facility using the Illumina NextSeq500 (Illumina, San Diego, CA, USA). The Agilent SureSelect Strand Specific mRNA library kit was used as per the manufacturer's instructions [24]. To construct the library, it began with two rounds of polyA selection using oligo dT-containing magnetic beads. The resulting mRNA, using cations and heat, was randomly fragmented. First-strand synthesis was completed using random primers with the addition of 2.4 ng/μL of Actinomycin D. Second-strand cDNA production was conducted using standard techniques, then the ends of the resulting cDNA were made blunt, A-tailed, and adapters were ligated for indexing to allow for multiplexing during sequencing. Using a Roche LightCycler 480, cDNA libraries were quantitated via qPCR using the Kapa Biosystems kit (Kapa Biosystems, Wilmington, MA, USA). Cluster generation was performed as per the manufacturer's instructions for onboard clustering [25]. Paired-end 75 base pair sequencing runs were completed to allow for better alignment of the sequences to the reference genome. STAR was used to align the raw RNA sequencing FASTQ reads to the reference genome from Gencode [26]. After the alignment, Cufflinks was used to assemble transcripts, estimate their abundances, and test for differential expression and regulation [27]. Normalization and differential expression were applied using Cufflinks' Cuffdiff package.

Ingenuity Pathway Analysis

The resulting Excel dataset, which contained several genes and their expression values from our stable cells, was uploaded into Ingenuity Pathway Analysis (IPA). A fold change cutoff of ≥±2 and a p-value of <0.05 was set to identify analysis-ready molecules that were significantly differentially expressed in our HCC1937 pcDNA3 and/or HCC1937 pcDNA3+BRCA1a cells. Molecules which met both specified cutoff requirements (fold change of ≥±2 and p-value of <0.05) and were associated with diseases and/or functions in IPA's Knowledge Base were considered for our analysis.

Results and Discussion

UBE2I RNA was Not Expressed at High Levels in HCC1937 Cells

As mentioned earlier, our previous work has shown Ubc9 to be expressed at elevated levels in BRCA1 mutant HCC1937 TNBC cells, suggesting that it could play a significant role in BRCA1-loss mediated TNBC [16]. To test whether UBE2I RNA was also expressed at high levels in HCC1937 cells, mRNA sequencing analysis was performed. Fold change between both stable cell lines was indicated to measure differences in the level of expression of each gene under wildtype and mutant conditions. The resulting analysis exhibited a fold change of -1.33 and a p-value of 0.48 for UBE2I RNA between HCC1937 pcDNA3 and HCC1937 pcDNA3 +BRCA1a cells, showing no significant fold change or p-value for UBE2I RNA expression in either cell line. Since we have found several other downstream targets of BRCA1/1a, such as SIRT1, we decided to examine SIRT1 RNA expression. Our analysis once again showed no significant fold change (1.18) or p-value (0.78) for SIRT1 RNA levels between the two cell lines.

Identification of Significantly Involved Diseases and Functions in HCC1937 and HCC1937+BRCA1a Cells

Our previous studies have elucidated the significance of downstream targets of BRCA1/1a such as Ubc9 and SIRT1. SIRT1 has been implicated in diseases such as obesity, heart disease, and cancer [28]. However, due to insignificant UBE2I and SIRT1 RNA expression in our results, we explored several other possible downstream pathways to identify potential targets. IPA characterized our data into "Top 5" categories that displayed the top biological diseases and functions associated with the genes in our stable cells (Tables 1 & 2). These results indicated that numerous molecules found in our stable cells were related to conditions or functions that fall into our Ubc9/SIRT1 pathway, such as cancer, reproductive system disease, and cardiovascular disease.

Table 1: Top 5 biological diseases associated with our HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable cells. The p-value represents statistical significance (<0.05) and the # of molecules represent the number of molecules found in our dataset associated with that disease.

Biological Diseases	p-value	# of Molecules
Cancer	1.59E-04 - 2.51E-28	752
Organismal Injury and Abnormalities	1.59E-04 - 2.51E-28	764
Gastrointestinal Disease	1.13E-04 - 8.04E-23	676
Reproductive System Disease	6.57E-05 - 2.62E-20	499
Dermatological Diseases and Conditions	1.39E-04 - 1.50E-17	501

Table 2: Top 5 network functions associated with our HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable cells. The higher the score, the more likely the genes in our dataset are associated with those functions.

Associated Network Functions	Score
Cardiovascular Disease, Congenital Heart Anomaly, Developmental Disorder	46
Dermatological Diseases and Conditions, Infectious Diseases, Inflammatory Disease	37
Cell Morphology, Cell-to-Cell Signaling and Interaction, Cellular Assembly and Organization	37
Cancer, Connective Tissue Disorders, Organismal Injury and Abnormalities	37
Cell Morphology, Cellular Development, Developmental Disorder	35

Identification of Significant Networks in HCC1937 and HCC1937+BRCA1a cells

To study which differentially expressed genes had the most significant association with our Ubc9/SIRT1 pathway, IPA was used to generate multiple network maps consisting of molecules found in our dataset which have a known relationship to each other based on literature findings from public databases found in IPA's Knowledge Base. The first network map of significance to us contained molecules involved in cell morphology, development, and developmental disorders (Figure 1). From this map, we chose GABRA3 and Mitochondrial complex I as potential targets. Gamma-aminobutyric acid type A receptor subunit alpha-3 (GABRA3) is a subunit of the $GABA_A$ receptor linked to GABA, the major inhibitory neurotransmitter found in vertebrate brains. This was of interest to us as a previous study by Li et al. showed GABRA3 to be overexpressed in TNBC tissues and cell lines [29]. Mitochondrial complex I (MCI), an enzyme complex involved in oxidative phosphorylation, was selected as SIRT1 enhances MCI activity [30].

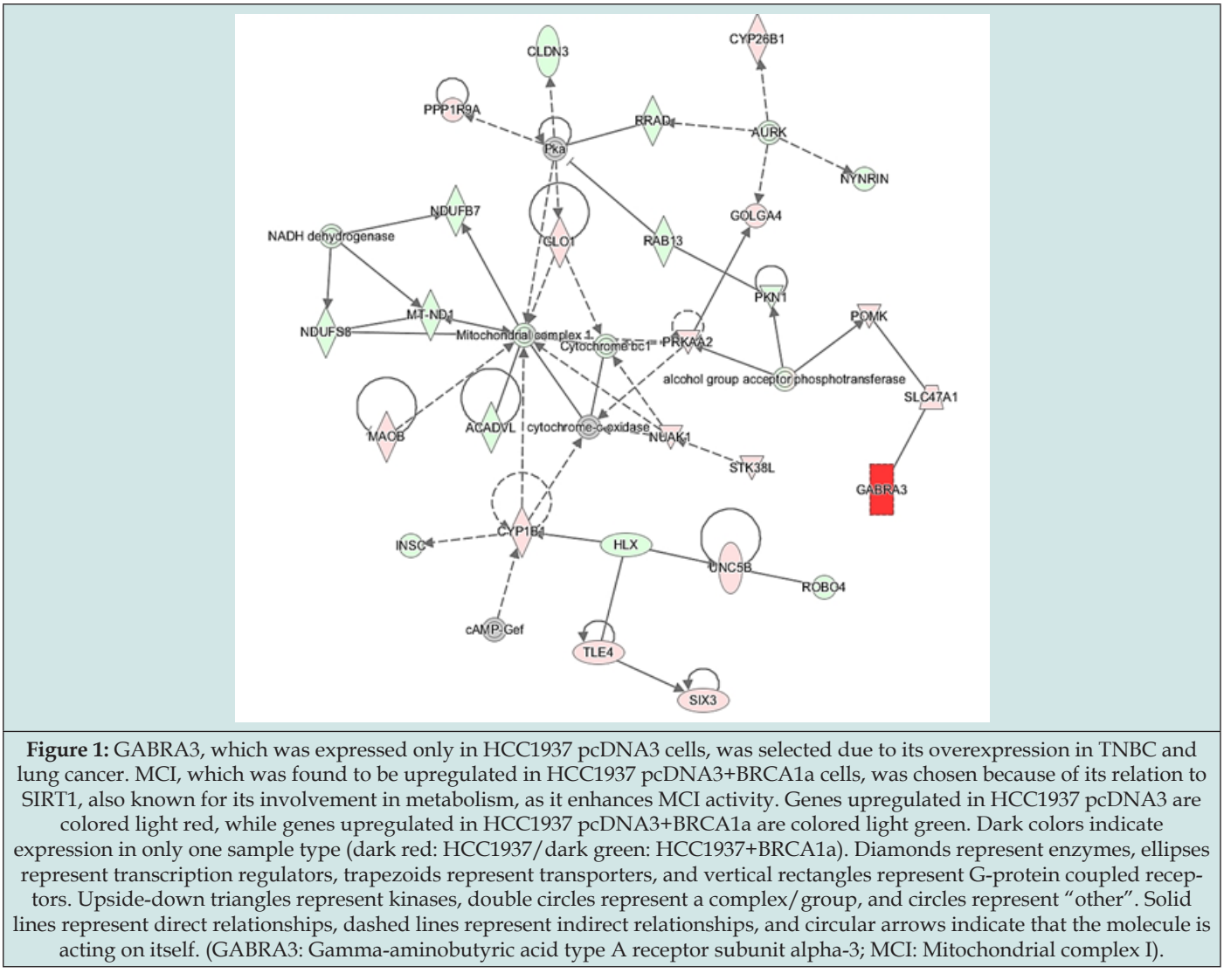

Figure 1: GABRA3, which was expressed only in HCC1937 pcDNA3 cells, was selected due to its overexpression in TNBC and lung cancer. MCI, which was found to be upregulated in HCC1937 pcDNA3+BRCA1a cells, was chosen because of its relation to SIRT1, also known for its involvement in metabolism, as it enhances MCI activity. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937/dark green: HCC1937+BRCA1a). Diamonds represent enzymes, ellipses represent transcription regulators, trapezoids represent transporters, and vertical rectangles represent G-protein coupled receptors. Upside-down triangles represent kinases, double circles represent a complex/group, and circles represent "other". Solid lines represent direct relationships, dashed lines represent indirect relationships, and circular arrows indicate that the molecule is acting on itself. (GABRA3: Gamma-aminobutyric acid type A receptor subunit alpha-3; MCI: Mitochondrial complex I).

The next network map encompassed molecules involved in cancer, organismal injury and abnormalities, and cellular growth and proliferation (Figure 2). Potential targets chosen from this map were GnRH and TP63. Gonadotropin-releasing hormone (GnRH) regulates the synthesis and secretion of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary, and was selected by us as Di Sante et al. showed that SIRT1 induces GnRH neuronal migration, which has been shown to exhibit significance in early development [31]. The next potential target, tumor protein 63 (TP63), known for its involvement in functions such as differentiation and stem cell self-renewal, was picked by us as previous studies have suggested that overexpression of an isoform of TP63, ΔNp63α, induces loss of cell adhesion in TNBC and downregulation of SIRT1 [32,33]. This is significant as SIRT1 expression is usually inhibited in TNBC.

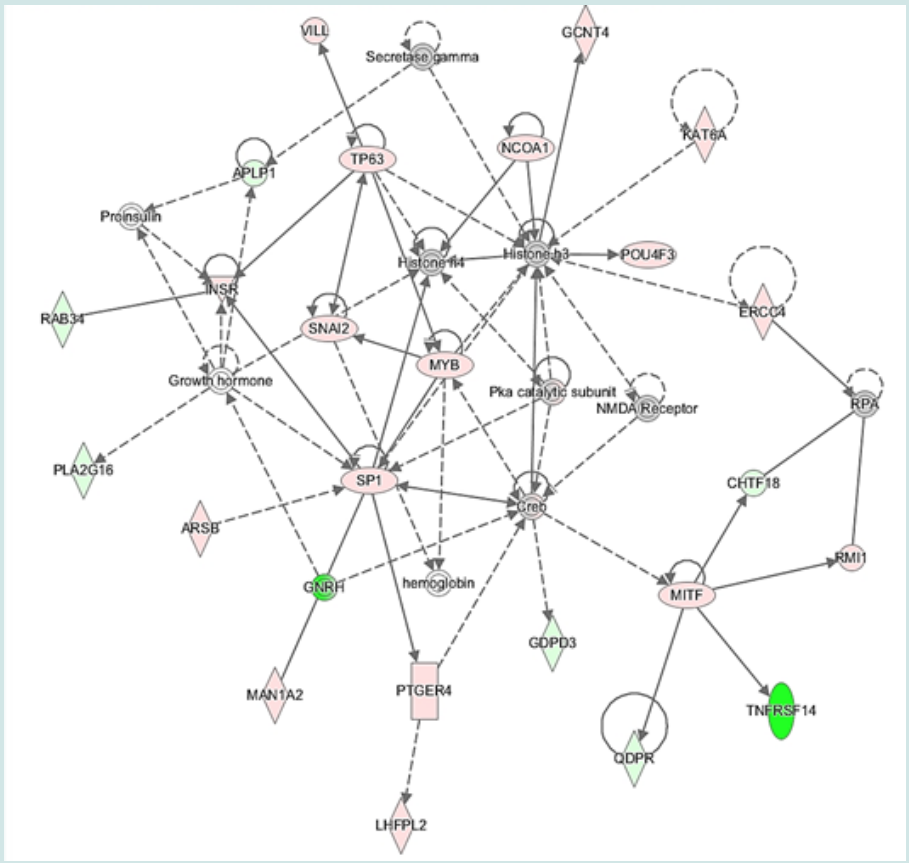

Figure 2: GnRH, which was expressed only in HCC1937 pcDNA3+BRCA1a cells, was selected due to its relation to SIRT1, as SIRT1 induces GnRH. TP63 was also chosen because of its relation to SIRT1, as overexpression of the TP63 isoform ΔNp63α down-regulates SIRT1, whose expression is usually inhibited in TNBC. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937/dark green: HCC1937+BRCA1a). Diamonds represent enzymes, ellipses represent transcription regulators, ovals represent transmembrane receptors, and vertical rectangles represent G-protein coupled receptors. Upside-down triangles represent kinases, double circles represent a complex/group, and circles represent "other". Solid lines represent direct relationships, dashed lines represent indirect relationships, and circular arrows indicate that the molecule is acting on itself. (GnRH: Gonadotropin-releasing hormone; TP63: Tumor protein 63).

ETV6 was chosen from the next map, which included molecules involved in skeletal and muscular system development and function, skeletal and muscular disorders, and cardiovascular system development and function (Figure 3). ETS variant transcription factor 6 (ETV6) is a transcriptional repressor that has demonstrated the ability to fuse with other genes, leading to potential tumorigenesis [34]. ETV6 was selected by us as Ubc9 and ETV6 have been shown to interact with each other, resulting in its relieved transcriptional repression [35]. The next network map consisted of molecules involved in developmental disorders, hereditary disorders, and metabolic diseases (Figure 4). From this map, we chose NGF as the potential target. Nerve growth factor (NGF), a neurotrophic factor that plays a role in the development and survival of several types of neurons, was of significance to us as Sugino et al. have shown SIRT1 influences NGF-induced neurite outgrowth, a process in which young neurons generate new axons or dendrites as they grow in response to their respective guidance cues, which is essential in nervous system development [36].

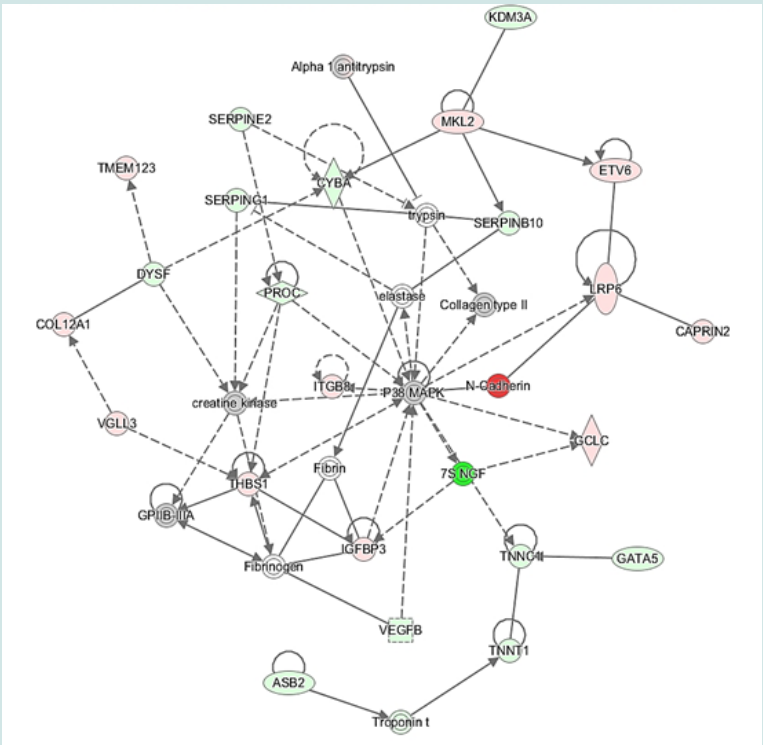

Figure 3: ETV6 was upregulated in HCC1937 pcDNA3 cells and was selected due to its relation to Ubc9, as their interaction relieves transcriptional repression of ETV6. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937/dark green: HCC1937+BRCA1a). Diamonds represent enzymes, ellipses represent transcription regulators, and ovals represent transmembrane receptors. Rhombuses represent peptidases, dashed squares represent growth factors, double circles represent a complex/group, and circles represent "other". Solid lines represent direct relationships, dashed lines represent indirect relationships, and circular arrows indicate that the molecule is acting on itself. (ETV6: ETS variant transcription factor 6).

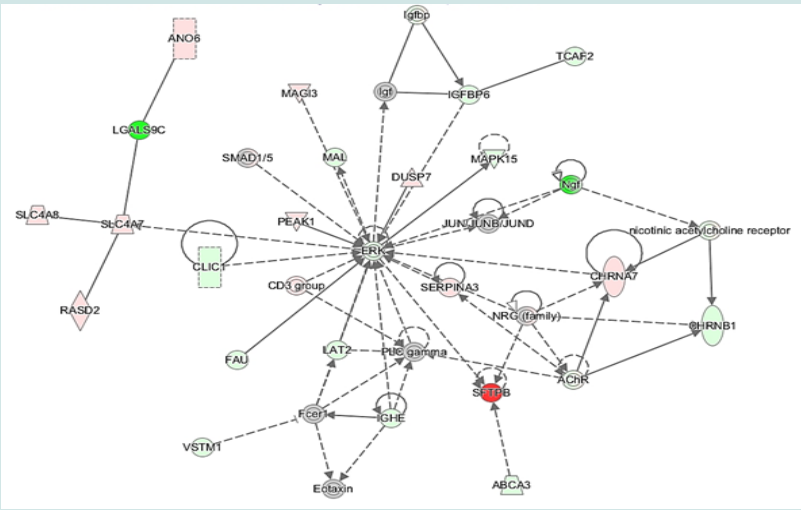

Figure 4: NGF, which was expressed only in HCC1937 pcDNA3+BRCA1a cells, was selected due to its relation to SIRT1, as SIRT1 is known to influence NGF-induced neurite outgrowth. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937/dark green: HCC1937+BRCA1a). Diamonds represent enzymes, ovals represent transmembrane receptors, and trapezoids represent transporters. Upside-down triangles represent kinases, triangles represent phosphatases, and dashed rectangles represent ion channels. Solid lines represent direct relationships, dashed lines represent indirect relationships, and circular arrows indicate that the molecule is acting on itself. (NGF: Nerve growth factor).

The last network map we appointed incorporated molecules involved in cell morphology, cell-to-cell signaling and interaction, and cellular assembly and organization (Figure 5). The potential target chosen from this map was TGFB2. Transforming growth factor beta 2 (TGFB2), involved in functions such as proliferation, differentiation, and apoptosis, was of significance to us as a previous study by Chanda et al. showed the SUMO system, which also includes Ubc9, and TGFB have been involved in the regulation of epithelial-to-mesenchymal transition, leading to potential implications for TGFB in cancer formation and progression [37].

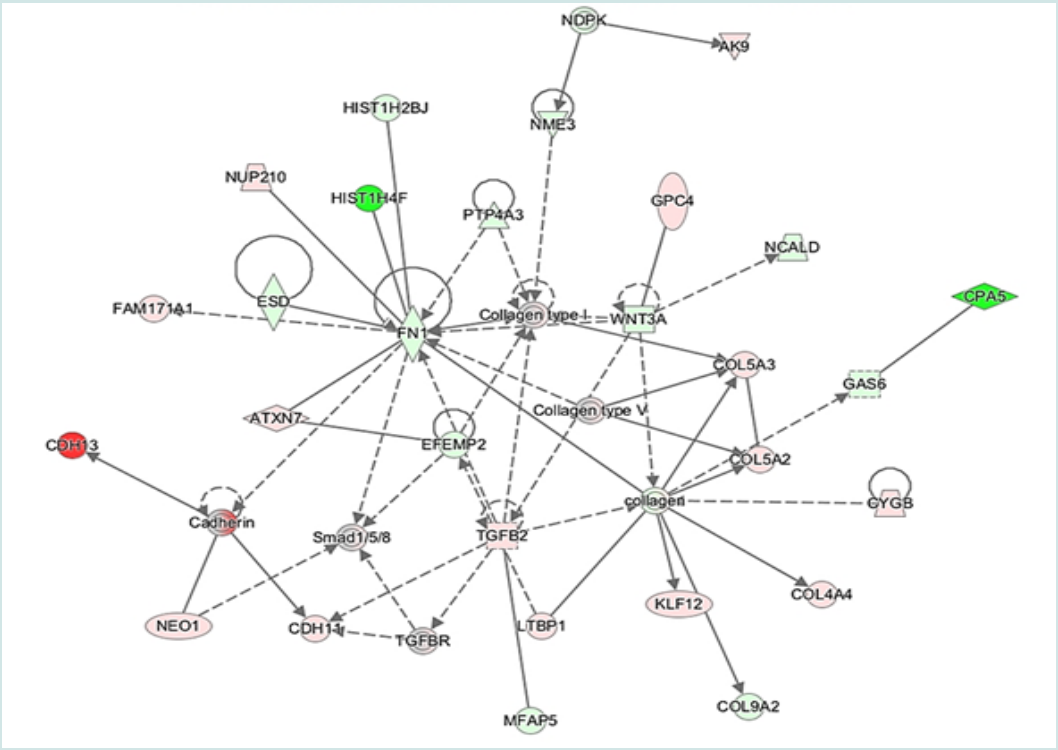

Figure 5: TGFB2 was upregulated in HCC1937 pcDNA3 cells and was selected due to its relation to the SUMO system, as both have been implicated in cancer formation and progression. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937/ dark green: HCC1937+BRCA1a). Diamonds represent enzymes, ellipses represent transcription regulators, and trapezoids represent transporters. Upside-down triangles represent kinases, rhombuses represent peptidases, double circles represent a complex/group, and circles represent "other". Solid lines represent direct relationships, dashed lines represent indirect relationships, and circular arrows indicate that the molecule is acting on itself. (TGFB2: Transforming growth factor beta 2).

Conclusions and Future Directions

It is widely accepted that TNBC is one of the hardest-to-treat aggressive subtypes, often associated with poor patient outcomes due to therapy resistance and metastases to distant organs such as the lungs and the brain [38]. Compared to other types of breast cancer, TNBC has limited treatment options, is prone to recurrence and metastasis, and has poor prognosis. The main reason for this is that they lack both hormone receptors (ER, PR) and HER2 overexpression, hence no effective targeted therapies being available for this disease. This lack of effective treatment options leads to a greater need for the development of molecular mechanism-based biomarkers and therapeutic targets, as cytotoxic chemotherapy remains the current systemic treatment method.

Our analysis did not show a significant fold change for UBE2I RNA expression in either HCC1937 pcDNA3 or HCC1937 pcDNA3 +BRCA1a stable cell line. This could be due to UBE2I RNA having a high turnover rate or a short half-life, so we decided to look at SIRT1, a known downstream target of BRCA1/1a that has extensively been studied by us. Our results showed no significant fold change for SIRT1 RNA expression in our stable cells. This led us to investigate possible downstream targets of Ubc9 and/or SIRT1. Our study identified several potential downstream targets for BRCA1-associated TNBC: GABRA3, Mitochondrial complex I, GnRH, TP63, ETV6, NGF, and TGFB2. The major limitation of our study was sample size as we were only able to utilize one cell line. We used HCC1937 cells as HCC1937 is currently the only available BRCA1 mutant TNBC cell line that has a basal-like phenotype and was shown by us to express high Ubc9 protein in several of our previous studies. Despite these limitations, our discoveries will lead in the future to the development of molecular mechanism-based personalized diagnostics and targeted therapies for TNBC, thus reducing the high mortality rates seen in young AA women and

leading to health equity. Future work will be geared towards utilizing patient-derived BRCA1 mutant TNBC tumor samples to validate our results.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

Acknowledgments

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