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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 invasive breast cancer cases, is a particularly aggressive form of breast cancer that disproportionately affects young African-American (AA) women. Approximately 75-80% of breast cancer patients who have BRCA1 mutations will develop TNBC in their lifetime. Our previous work has shown wildtype BRCA1/1a proteins to bind to 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 level 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 then 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 its downstream target SIRT1 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 mortality associated with these aggressive cancers in AA women, leading to health equity.

Keywords: TNBC; BRCA1; BRCA1a; Ubc9; UBE21; 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 leading cause of death from cancer in women worldwide and 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 [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-enriched, 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]. TNBC is notable for being an aggressive cancer with higher mean tumor size, higher grade tumors, higher rates of node positivity, high likelihood of recurrence, distinct metastasis patterns, and poorer survival compared to other breast cancers [5]. TNBC is also characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) commonly observed in other breast cancer subtypes; thus, they are not sensitive to endocrine therapy or HER2 treatment, leaving cytotoxic chemotherapy as the mainstay treatment for TNBC patients, as there are currently no targeted therapies available [6,7] 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]. TNBCs with BRCA1 mutations are normally associated with the BL1 and BL2 subtypes. 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 [10,11]. We have identified and cloned two major isoforms of BRCA1, namely BRCA1a/p110 and BRCA1b/p100, which are expressed at reduced levels in breast tumors compared to normal mammary cells [5,12]. We have also found BRCA1a to possess anti-tumor activity in human ER-a positive breast cancers, hormone-independent ovarian and prostate cancer cells, as well as TNBC [13]. Our previous work suggested a molecular mechanism as to how BRCA1 mutation results in TNBC [14]. BRCA1/1a proteins interact with nuclear chaperone Ubc9, a SUMO E2-conjugating enzyme, thereby activating ER-a 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 Ubc9 levels to trigger proliferation, migration, and metastasis of TNBC cells, and high Ubc9 levels have been linked to poor response to chemotherapy and poor clinical outcome [16,17]. We have also found Ubc9 to be expressed at elevated levels in tumor tissue obtained from an AA woman with BRCA1-associated TNBC with high mammographic density compared to matched benign tissue, suggesting a potential role for Ubc9 in triggering BRCA1-associated TNBC development [18]. In addition, high Ubc9 protein expression has also been shown to correlate with poor clinical outcomes in Nigerian Black women with TNBC from a study by Agboola et al., which agrees with our findings [19]. In addition to TNBC, high expression of Ubc9 has also been observed in other cancers such as ovarian, lung, head and neck cancers, and advanced melanomas [20,21].

Recently, we have found SIRT1 to be a new downstream target of BRCA1 [5]. SIRT1 is a class-III histone deacetylase (HDAC), an NAD+-dependent enzyme involved in gene regulation, genome stability, apoptosis, autophagy, senescence, proliferation, aging, and tumorigenesis [22]. We have shown BRCA1 to bind to Ubc9, resulting in increased SIRT1 expression and this facilitated BRCA1 to translocate to the nucleus, activate ER- a and function as a tumor suppressor [5,14,15]. Mutations in BRCA1 in TNBC was shown to impair tethering to Ubc9, resulting in cytoplasmic localization of BRCA1 proteins inhibiting SIRT1 expression leading to TNBC [14] and other diseases such as myocardial infarction, cardiovascular disease, and tumorigenesis [23].

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 earlier 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 more cost-effective compared to protein biomarkers [24,25]. We plan to test this hypothesis by using HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable TNBC cell lines and investigated their transcriptomes. This study, if successful, will allow us to study if UBE2I, SIRT1, or other downstream targets can be used in the future as potential mechanism-based biomarkers for early detection or targeted therapies for BRCA1-associated TNBC, which will assist in the advancement of precision oncology and reduce cancer health disparities saving countless lives.

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 [15].

RNA Isolation

RNA was isolated from HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a stable cells using the RNeasy mini kit (Qiagen, Germantown, MD). 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, then 50 µL of RNase-free water was directly added 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 was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the University of Alabama Birmingham's Genomics Core Facility (University of Alabama at Birmingham, Birmingham, AL). 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). The Agilent SureSelect Strand Specific mRNA library kit was used as per the manufacturer's instructions. 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 then randomly fragmented. First strand synthesis was then completed using random primers with the addition of 2.4 ng/μL of Actinomycin D. Second strand cDNA production was done using standard techniques, then the ends of the resulting cDNA were made blunt, A-tailed, and adaptors 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). Cluster generation was then performed as per the manufacturer's instructions for onboard clustering. 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 (GRCh38) from Gencode. After the alignment, Cufflinks was used to assemble transcripts, estimate their abundances, and test for differential expression and regulation. Lastly, normalization and differential expression was applied using Cufflinks' Cuffdiff package.

Ingenuity Pathway Analysis

A dataset containing 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 that met both cutoff requirements and were associated with biological 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

Ubc9 is the sole E2 SUMO conjugase essential for protein SUMOylation and is a multifunctional protein which is overexpressed in several cancers like colon, prostate, breast, lung, ovarian, melanomas, and head and neck cancers [26]. 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 critical 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 the gene under wildtype and mutant conditions. The resulting differential gene expression analysis exhibited a p-value of 0.4829 and a fold change of -1.3273 for UBE2I RNA between HCC1937 pcDNA3 and HCC1937 pcDNA3+BRCA1a cells, showing no significant fold change for UBE2I RNA expression in either cell line. Since we have found several other downstream targets like SIRT1, we decided to examine its RNA expression. Our analysis once again showed no significant fold change (1.1764) or p-value (0.7844) 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. As previously mentioned, SIRT1 has been implicated in numerous physiological and pathological functions such as life-span extension, neurodegeneration, age-related disorders, obesity, heart disease, inflammation, and cancer [27]; however, due to insignificant UBE2I and SIRT1 RNA expression in our results, we explored several other possible downstream pathways to identify potential druggable targets for TNBC. 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 cells are related to conditions or functions that fall into our Ubc9/SIRT1 pathway, such as cardiovascular diseases, dermatological diseases, and cancer

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 network 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 that 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, cellular development, and developmental disorders (Figure 1). From this map, we chose GABRA3 and Mitochondrial complex I (MCI) as potential targets for BRCA1-associated TNBC. GABRA3 is a subunit of GABA, the major inhibitory neurotransmitter in the vertebrate brain. This molecule was of interest to us as previous studies have shown GABRA3 to be overexpressed in TNBC and lung cancers [28,29]. MCI, a multimeric enzyme complex involved in oxidative phosphorylation, was selected as it has been shown to interact with SIRT1, as SIRT1 enhances MCI activity [30].

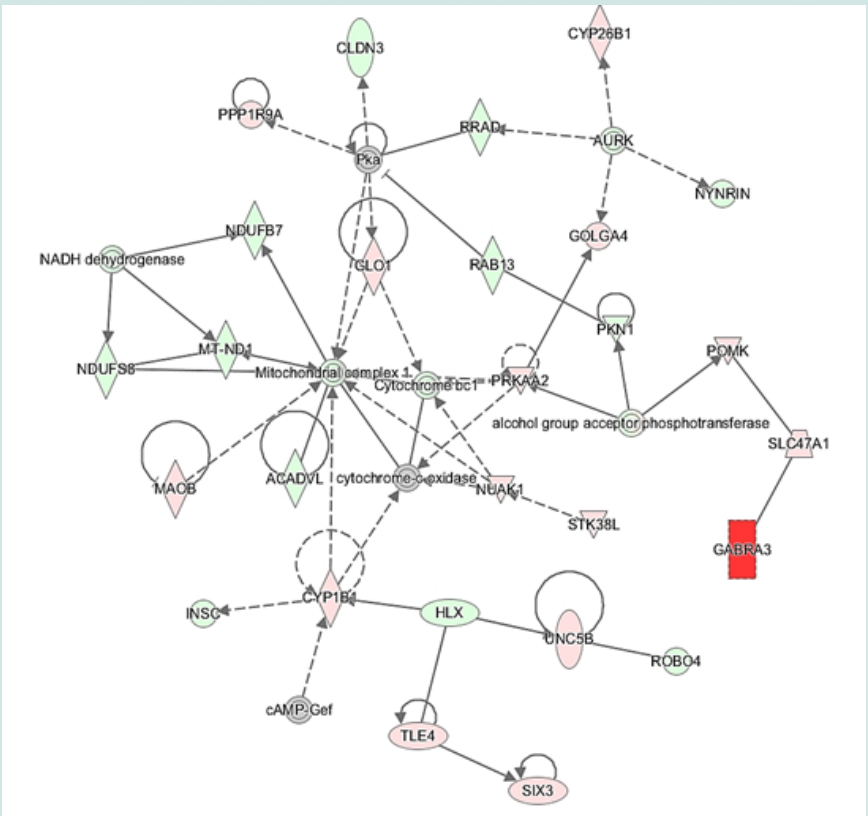

Figure 1: GABRA3, which was expressed only in HCC1937 pcDNA3 cells, was selected because of its high expression in TNBC and lung cancers. MCI was found to be upregulated in HCC1937 pcDNA3+BRCA1a cells and was chosen because of its relation to SIRT1, also known for its involvement in metabolism, as it 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 we chose encompassed molecules involved in cancer, organismal injury and abnormalities, as well as cellular growth and proliferation (Figure 2). Potential targets chosen from this map were GnRH and TP63. Gonadotropin-releasing hormone (GnRH), which is found in the hypothalamus, regulates synthesis and secretion of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary gonadotrophs, and was selected because Di Sante, et al. showed that SIRT1 catalytic function induces GnRH neuronal migration [31]. The next potential target, TP63, which has important functions in tumorigenesis, epidermal differentiation, and stem cell self-renewal, was picked due to its relationship with SIRT1. A study by Sommer et al. suggested that the overexpression of an isoform of TP63, ΔNp63a, induces loss of cell adhesion in TNBC and induces downregulation of SIRT1 in mice [32,33]. This is significant as SIRT1 expression is normally inhibited in TNBC.

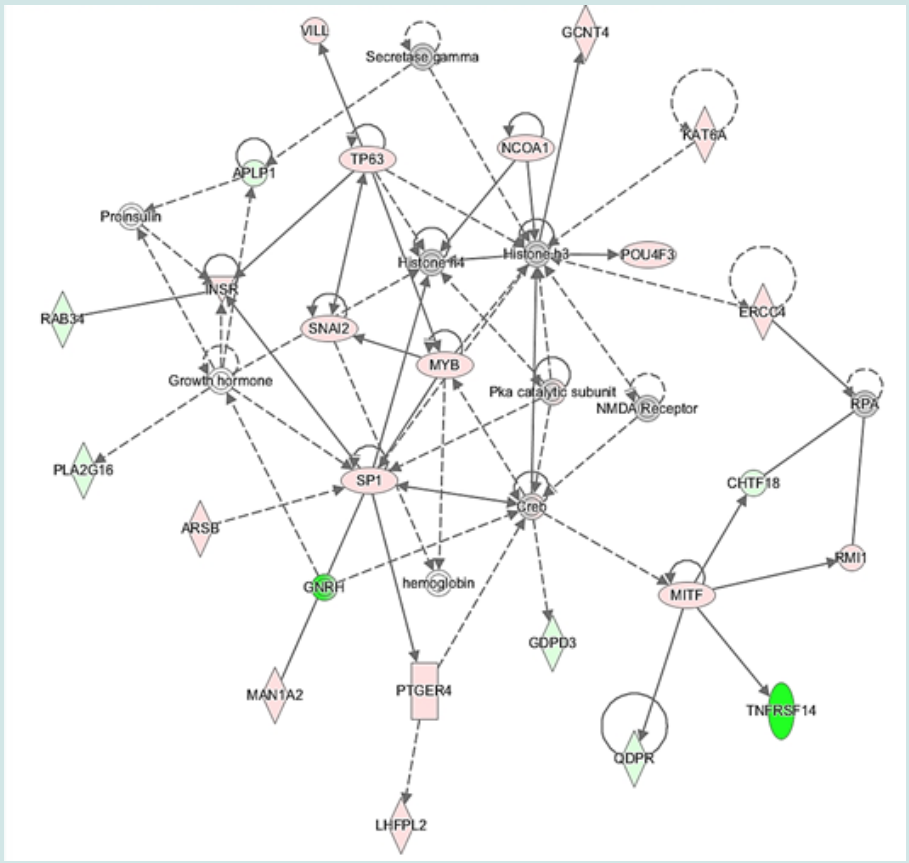

Figure 2: GnRH, which was expressed only in HCC1937 pcDNA3+BRCA1a cells, was chosen because of its relation to SIRT1, as SIRT1 induces GnRH. TP63 was also selected due to its relation to SIRT1, as overexpression of the TP63 isoform ΔNp63α down-regulates SIRT1, whose expression is typically inhibited 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). Circles represent "other", double circles represent a complex/group, diamonds represent enzymes, and ellipses represent transcription regulators. Upside-down triangles represent kinases, vertical rectangles represent G-protein coupled receptors, and ovals represent transmembrane receptors. 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 contained molecules involved in skeletal and muscular system development and function, skeletal and muscular disorders, and cardiovascular system development and function (Figure 3). ETV6 is a transcriptional repressor mainly involved in hematopoiesis and maintenance of vascular networks and has developed to be a major oncogene with the potential ability of forming fusion partners with many other genes with carcinogenic consequences [34]. This gene was selected as Ubc9 has been shown to physically interact with it by aiding in relieving transcriptional repression of ETV6, and their interaction also leads to the covalent modification of ETV6 by the small ubiquitin like modifier SUMO-1 [35]. The next network map consisted of molecules involved in developmental disorders, hereditary disorders, and metabolic diseases (Figure 4). NGF was chosen as the sole potential target from this map. Nerve growth factor (NGF), a neurotrophic factor that plays an essential role in the development and survival of various types of neurons, was of significance to us as Sugino et al. have shown cytoplasmic SIRT1 to promote NGF-induced neurite outgrowth, a process in which developing neurons produce new projections as they grow in response to guidance cues, in PC12 cells in mice [36].

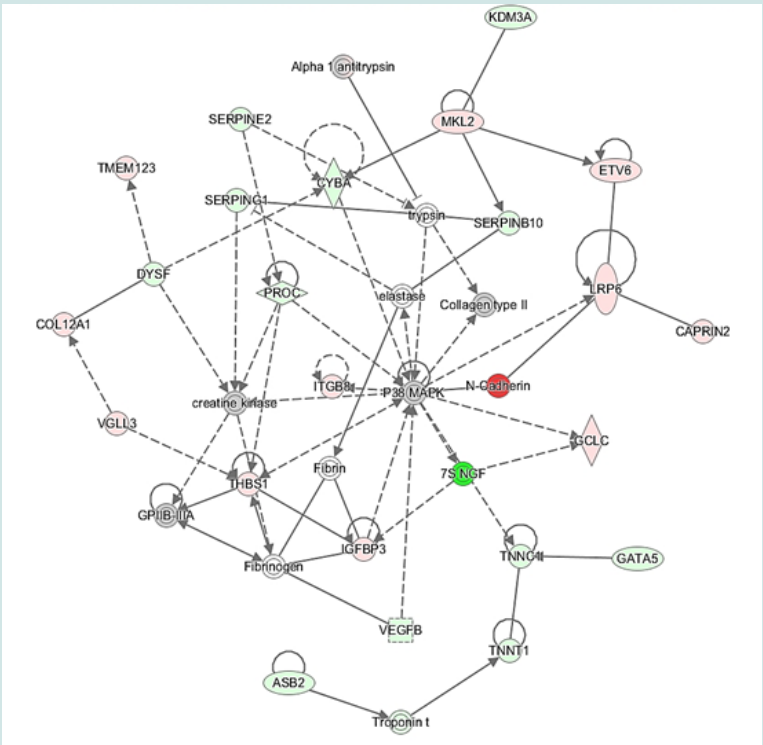

Figure 3: ETV6 was upregulated in HCC1937 pcDNA3 cells. ETV6 was selected because of its interaction with Ubc9, which relieves transcriptional repression of ETV6. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937; dark green: HCC1937+BRCA1a). Circles represent "other", double circles represent a complex/group, diamonds represent enzymes, and ovals represent transmembrane receptors. Ellipses represent transcription regulators, rhombuses represent peptidases, and dashed squares represent growth factors. 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).

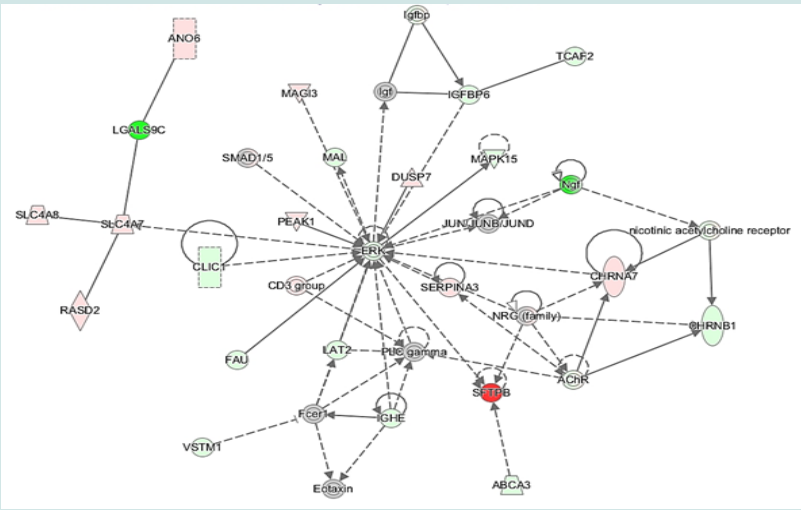

Figure 4: NGF was expressed only in HCC1937 pcDNA3+BRCA1a cells. NGF was chosen because SIRT1 is known to increase NGF-induced neuritogenesis. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937; dark green: HCC1937+BRCA1a). Diamonds represent enzymes, ovals represent transmembrane receptors, and trapezoids represent transporters. Triangles represent phosphatases, upside-down triangles represent kinases, 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 selected included 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. TGFB2, involved in several essential biological functions such as cell growth, proliferation, differentiation, and apoptosis, was of significance to us as it has been shown that the SUMO system, which includes Ubc9, and TGFB signaling interplay in the regulation of epithelial-mesenchymal transition, leading to implications for cancer formation and progression [37].

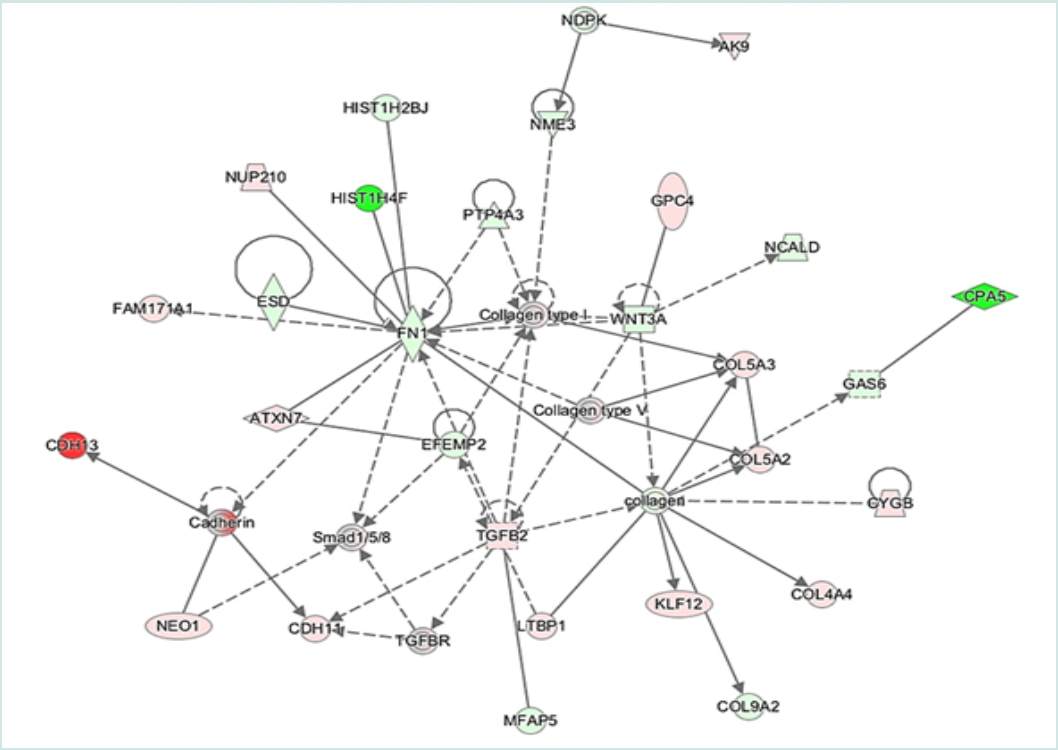

Figure 5: TGFB2 was upregulated in HCC1937 pcDNA3 cells. TGFB2 was selected because TGFB and the SUMO system have been implicated in cancer formation and progression. Genes upregulated in HCC1937 pcDNA3 are colored light red, while genes upregulated in HCC1937 pcDNA3+BRCA1a are colored light green. Dark colors indicate expression in only one sample type (dark red: HCC1937; dark green: HCC1937+BRCA1a). Circles represent "other", double circles represent a complex/group, trapezoids represent transporters, and upside-down triangles represent kinases. Diamonds represent enzymes, rhombuses represent peptidases, and ellipses represent transcription regulators. 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 outcome 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 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 method of treatment.

Our analysis did not show a significant fold change for UBE2I RNA expression in either HCC1937 or HCC1937+BRCA1a stable cell lines. 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 Ubc9 that has been extensively studied by us. Our results once again showed no significant fold change for SIRT1 RNA expression in our stable cells. This led us to investigate potential downstream targets of Ubc9 and/or SIRT1. Our study identified several potential downstream targets for BRCA1 mutant 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 (BL1) phenotype and 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 mortality rates in young African American women with BRCA1-

associated TNBC, 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.

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