T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MOLECULAR EFFECTS OF PLANT-BASED DRUGS ON BREAST CANCER BY MAPPING TRANSCRIPTOME DATA ON PROTEIN-PROTEIN INTERACTIONS

RONALD REGAN ODONGO
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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YÜKSEK LİSANS TEZİ

MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

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SUMMARY

Breast cancer is the second most commonly diagnosed cancer in females. Despite improvements in knowledge of molecular mechanisms of carcinogenesis and availability of treatment options, it's still the major contributor of fatalities globally. Reductionism is the dominant drug design and evaluation approach, however, the molecular complexity in cancer requires robust and holistic approaches to account for all the molecular players. Thus, systems pharmacology is increasingly being preferred. Carcinogenesis overrides normal cellular growth control mechanisms through genetic and epigenetic dysregulations. Cell signaling pathways is one of the main targets of such deregulations. Exquisitely targeting single proteins in signaling networks has largely failed to improve disease prognosis and compounds with multiple targets in perceived oncogenic signaling pathways are preferred. Fortunately, plant-based drugs, known to simultaneously target multiple ligands, are increasingly gaining attention as anti-cancer alternatives. Here, a systems pharmacology approach was applied to extract drug-specific subnetworks. KeyPathwayMiner was used to map transcriptome data on PPIN. Subsequently, a set of bioinformatics tools were used to infer perturbed signal transduction pathways. Three breast cancer subtypes under actein, indole-3-carbinol, compound kushen injection and Withaferin A plant-based drugs were studied. In line with multi-target approach, this work shows that, with the exception of MDA-MB-157 cell line, these compounds/formulations target multiple oncogenic signal transduction networks regulating various carcinogenesis processes. The novelty in subnetwork approach is the reliance on interconnected entities over differentially expressed genes to infer biological context. This holistic approach could inform smarter experimental designs and drug mechanism of action from enriched signaling pathways.

Keywords: Breast cancer, bioinformatics, systems biology, plant-based drugs, signal transduction networks, network pharmacology, protein interactions, transcriptome.

ÖZET

Meme kanseri kadınlarda en sık rastlanan kanserdir. Moleküler karsinogenez bilgisindeki gelişmeler ve çeşitli tedavi seçenekleri bulunmasına rağmen hala dünya çapında ölümlerin önemli nedenleri arasında yer almaktadır. Redüksiyonizm, baskın ilaç tasarımı ve değerlendirme yaklaşımıdır, ancak bu tip kanserlerdeki moleküler karmaşıklık sistemdeki tüm moleküler bileşenler için sağlam ve bütünsel bir yaklaşımı gerektirmektedir. Bu kapsamda, sistem farmakolojisi global moleküler resmin belirlenmesinde tercih edilen bir yöntem haline gelmektedir. Karsinogenez, normal hücre büyüme kontrol mekanizmalarının genetik mutasyonlar ve epigenetik düzensizlikler aracılığıyla geçersiz sayılmasıyla ilişkilidir. Hücresel sinyal yolakları bu tür deregülasyonların temel hedeflerinden biridir. Sinyal ağındaki tek bir proteini hedeflemek hastalığın ilerleyişini iyileştirmede önemli ölçüde başarısız olmuştur ve onkogenik sinyal yollarındaki çoklu hedefleri olan ürünler tercih edilmektedir. Öte yandan, çoklu bileşenleri hedef aldığı bilinen bitkisel kaynaklı ilaçlar anti-kanser alternatifler olarak önem kazanmaktadır. Bu nedenle bu tezde, transkriptom ifade verilerinden ilaca spesifik protein-protein etkileşim modüllerini alt ağları oluşturmak için bir ağ farmakoloji yaklaşımı uygulanmış ve bunlar bozulan sinyal ileti yollarını açığa çıkarmak amacıyla kullanılmıştır. Bitkisel kaynaklı ilaçlar olan actein, indole-3-carbinol, Kushen enjeksiyonu bileşiği ve Withaferin A ile muamele edilen üç tip meme kanserini incelemek amacıyla KeyPathwayMiner ve bir dizi biyoinformatik araç kullanılarak etkileşim modülleri elde edilmiştir. Çok hedefli yaklaşıma uygun olarak bu çalışma, MDA-MB-157 hücre hattı hariç, bu ilaçların çeşitli karsinogenez işlemlerini düzenleyen çoklu onkogenik sinyal ileti ağlarını hedeflediğini göstermektedir. Alt ağ analizi yaklaşımındaki yenilik, biyolojik önemi ortaya çıkarmak için alanlı değişen genlerin tespit edilmesi yerine birbiriyle etkileşen ve alanlı değişen proteinler grubunun dikkate alınmasıdır. Bu kapsamlı analiz, daha akıllı deney dizaynları için öncü olabilir ve sinyal ileti yolaklarında ilaçların etki mekanizmasını detaylandırabilir.

Anahtar kelimeler: meme kanser, biyoinformatik, system biyolojisi, bitkisel kaynaklı ilaçlar, sinyal transdüksiyon ağları, ağ farmakolojisi, protein etkileşimi, transkriptom.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		Explanation
Akt	:	Protein kinase B
AMPK	:	AMP- activated protein kinase
ATM	:	Ataxia-telangiectasia mutated
BCF	:	B-cell receptor
CDK	:	Cyclin dependent kinase
CKI	:	Compound kushen injection
ER	:	Oestrogen receptor
ERK	:	Extracellular regulated kinase
GPCR	:	G-protein coupled receptor
HER	:	Human epidermal receptor
I3C	:	Indole 3 Carbinol
IkB	:	Inhibitor of kappa B
IL	:	Interleukin
IL	:	interleukin
INF	:	Interferon
JNK	:	c-Jun N-terminal kinase
KEAP	:	Kelch-like ECH associated protein
KPM	:	KeyPathwayMiner
LABC	:	Luminal A breast cancer
MAPK	:	Mitogen activated protein kinase
MKK, MKKK	:	MAPK kinase, MAPK kinase kinase
mTOR	:	Mammalian target of rapamycin
NF-kB	:	Nuclear factor kappa B
NRF2	:	Nuclear factor erythroid 2 - related factor 2
PDGF(R)	:	Platelet derived growth factor (receptor)
PI3K	:	Phosphoinositide 3-kinase
PPIN	:	Protein-protein interaction network
PR	:	Progesterone receptor
PTEN	:	Phosphatase and tensin homolog

RNA	:	Ribonucleic acid
STAT3	:	Signal transducer and activator of transcription 3
TCR	:	T-cell receptor
TGF-beta	:	Transforming growth factor beta
TLR	:	Toll like receptor
TNBC	:	Triple negative breast cancer
TNF	:	Tumor necrosis factor
VEGF(R)	:	Vascular endothelial growth factor (receptor)
WA	:	Withaferin A
Wnt	:	Wingless integrated

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1. INTRODUCTION

1.1. Breast Cancer

Of all cancers, breast cancer is the most debilitating cancers in women. It is the second most prevalent cancer in females and globally contributed over 2 million new cases and 600,000 fatalities in 2017 alone [1]. Many factors contribute to transition from normal to malignant cells including radiation, mutations, viruses, and epigenetics. Hence, cancers of this type are highly heterogenous complicating treatment approaches. Breast cancers are molecularly classified into three groups based on progesterone receptor (PR), human epidermal growth factor receptor (HER) and oestrogen receptor (ER) expression: luminal (ER+, PR+, HER-), HER2 positive (+) and triple negative (TNBC) (ER-, PR- and HER2-) [2]. Overall, the underlying pathogenesis in the different breast cancer (BC) subtypes involves the hijacking of the normal cellular processes and uncontrolled progression to malignancy.

1.2. Breast Cancer Treatment Approaches

Standard treatment approaches for breast cancer patients typically involve surgery to remove the tumor, radiation therapy, hormone therapy, chemotherapy and targeted therapy [3]. Thus, to date, a number of drugs have been introduced in the clinical management of BC which are broadly categorized into cytotoxic, hormonal and single molecule targeted therapies (mainly monoclonal antibodies). TNBC exhibit more aggressive clinical phenotype and is associated with metastasis to distant organs, resistance to chemotherapy and overall low 5-year survival [4]. On the other hand, the luminal and HER2+ harbouring patients largely respond to hormonal treatment and have better prognosis [3].

Signal transduction is the mechanistic transmission of extracellular or intracellular generated signals through a cell using a cascade of protein post-translational modifications, with the net result being expression of targeted genes. Malignant cells seize these cascades through activating/deleterious mutations and

epigenetic control of the rate limiting proteins. In effect, this limits the ligand binding response regulation. These dysregulations have been used as drug targets for decades [5]. The activity and utility of any particular cascade is BC subtype specific occasioned by the underlying genetic phenotype. Hence, a new and effective drug should be able to target disease specific networks across multiple subtypes or within the various subtypes. Current approaches are increasingly being developed to target multiple targets on a disease specific signaling network [5], [6].

2. LITERATURE REVIEW

2.1. Signal Transduction Pathways in Breast Cancer Carcinogenesis for Targeted-Therapy

Concerted efforts by different groups have greatly increased our understanding of signal transduction networks in breast cancer. The association of these networks to carcinogenesis processes has been useful in prioritization of drug targets in onco-therapy. The main biological processes hijacked by malignant cancer cells to advance carcinogenesis are cell cycle (proliferation) and death, inflammation, metastasis and angiogenesis. Each of these processes are further driven by a network of signal transduction networks and promiscuity of these networks is common.

Recently, a consortium of oncology researchers from the TCGA (The Cancer Genome Atlas) PanCancer Atlas Initiative classified oncogenic signaling pathways into 10 frequently altered canonical pathways based on a profiling analysis of over 9000 samples from 33 tumors [6]. This novel template is useful while working with signaling pathways in cancer cells for precision medicine and oncology research. However, not factored in this classification are the primordial roles of tumor microenvironment and mechanisms regulating development of new blood vessels (angiogenesis). Below, we summarise current knowledge on dysregulated canonical signaling in breast cancer, including the two additional signaling pathways regulating the missing processes.

2.1.1. Receptor Tyrosine Kinase/Ras/Mitogen Activated Protein Kinase (MAPK)

The receptor tyrosine kinase (RTK) is an important pathway in breast cancer therapy and comprises a family of closely related receptors with an intracellular tyrosine kinase regulator domain. It plays very important roles in breast cancer carcinogenesis through regulation of cell proliferation, differentiation, death and survival processes. Over the years, a number of sub-family members have been discovered due to their importance in various cancers including HER-1, HER-2, HER-3 and HER-4. These receptors are stimulated by ligand binding to the extracellular domain, resulting in receptor dimerization. The type of dimers formed determine the

type of downstream effectors induced. Dimerized receptors induce phosphorylation of the intracellular tyrosine kinases and consequently downstream kinases resulting in dysregulation of several cascades including MAPK and PI3K (Figure1.1). RTK has also been shown to crosstalk with TGF-ß [7], Notch [8], ER and insulin like growth factor signaling [9].

HER-2 (ErbB-2 or HER-2/neo) is the most dysregulated receptor in breast cancer and is associated with tumorigenesis progression. Furthermore, most of the HER receptors are understood to dimerize with HER-2 receptor to affect biological functions pointing to its importance in cell function. In this respect, a number of targeted therapeutic antibodies against pathway proteins have been successfully developed. For instance, Herceptin (trastuzumab) and Perjeta (pertuzumab) binds to the receptor preventing dimerization and canertinib, erlotinib and Iressa (gefitinib) are small molecule inhibitors that act as tyrosine kinase inhibitors [5], [7], [10].

The mitogen activated protein kinase network is composed mainly of three members: c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK) and the p38 MAPK which mediate cell growth or cell death depending on the signal. While ERK is modulated by growth factors, JNK and p38 MAPK are activated by cellular stress, growth factor and cytokines. A signalling molecular binding to a receptor at the cell surface triggers a phosphorylation cascade through MAP kinase kinase kinase (MKKK), which in turn phosphorylates and activates MAP kinase kinase (MKK) (Figure 1.1) [11]. Depending on the activating signal, MKK activates either ERK, JNK or p38. Activated ERK specifically drives cell growth while JNK and p38 can either mediate cell growth or death by targeting genes involved in the respective networks for activation or destruction. In addition, JNK can target c-Jun and IkB-α and ERK can also activate the NF-kB thereby affecting cellular processes.

The role of this network in breast cancer carcinogenesis has been studied by different groups and targeted therapies have since been developed. The most well understood mechanism is the regulation of the MAP kinase phosphatases (MKP) intrinsic regulator. MKP-1 is the most studied MKP subtype and has been reported to be controlled by p53. Hence, induction by p53 can directly regulate the G1 phase of the cell cycle. It is believed that developing drugs that can augment this inhibitor can override the pathological phenotype associated with this pathway [12].

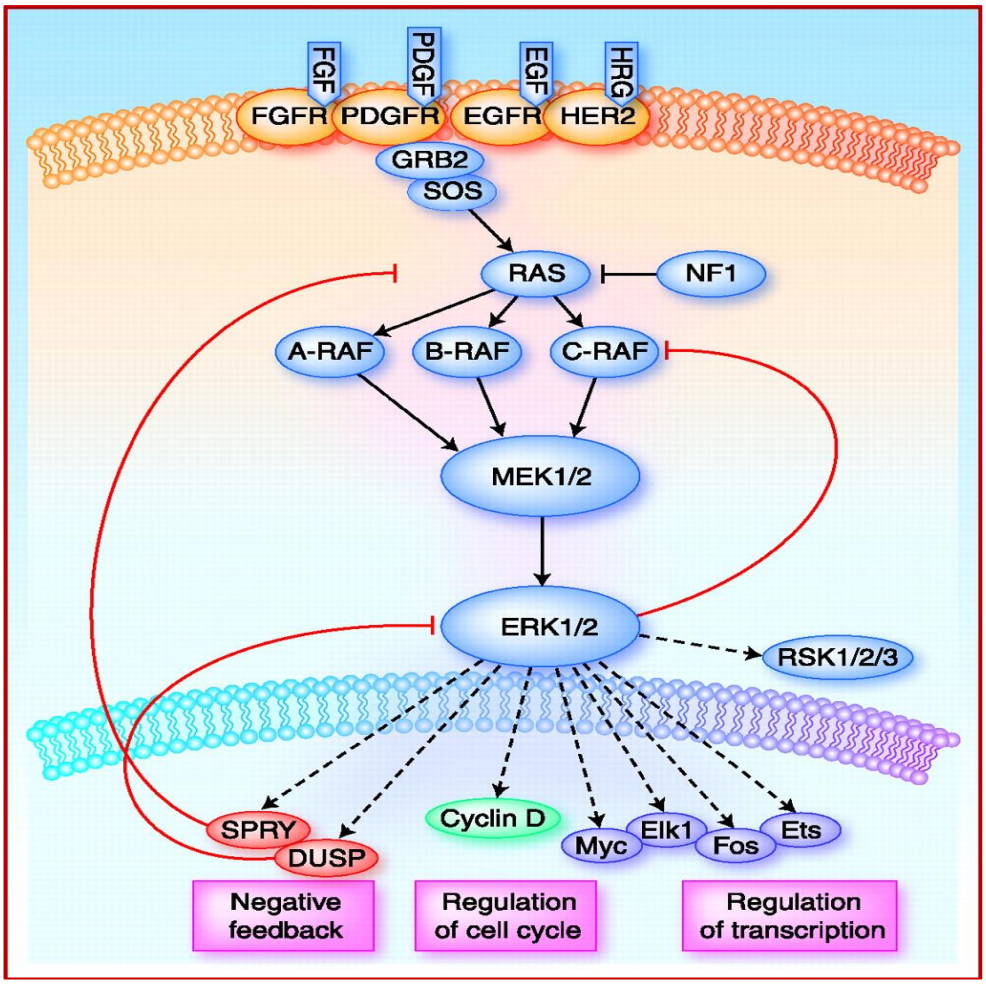


Figure 2.1: The RTK-Ras-MAPK signaling pathway.

2.1.2. The PI3K-Akt-mTOR Pathway

Response to growth inducers, hormones and nutrients to regulate tumor cell proliferation and growth in breast cancer is chiefly mediated by signal transduction through phosphoinositide 3-kinase (PI3K)-Akt (PKB, protein kinase B)-mammalian target of rapamycin (mTOR). Upstream activation by receptor tyrosine kinase (RTK) of PI3K leads to homodimer formation and concomitant activation. PI3K phosphorylates and activates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,4-triphosphate (PIP3), which in turn activates Akt through phosphorylation (Figure 1.2) [13]. The tumor suppressor, phosphatase and tensin homolog (PTEN) controls the PIP2-to-PIP3 activation and is thus mutated or deleted in most proliferating breast tumors. Akt performs important biological functions through regulation of cell cycle, survival and growth. Its overexpression is linked to poor disease outcomes in breast cancer. Finally, activated Akt activates mTOR by

phosphorylating and inhibiting tuberous sclerosis 1/2(TSC1/2); a tumor suppressor. An active mTOR mediates increased cell growth and metabolism, thus contributing to increased carcinogenesis (Figure 1.2). It is also known that Akt can directly activate NF-kB in tumors.

Clinically, unregulated signaling through PI3K-Akt-mTOR has been linked to trastuzumab resistance in HER-2 over-expressing breast cancers and endocrine resistance in HR-positive breast cancers as it can directly activate ER, independent of oestrogen. Thus, gaining control of this network is an area under active research with different drug candidates targeting one or multiple signaling molecules [14].

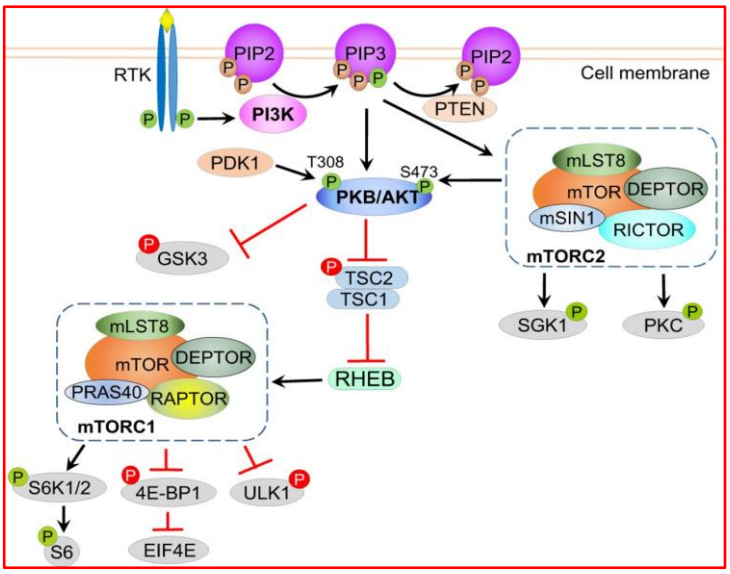


Figure 2.2: The PI3K-Akt-mTOR signaling pathway

2.1.3. The Transforming Growth Factor -beta (TGF-ß) Pathway

TGF-ß is a cytokine with promiscuous cellular roles in breast cancer. It regulates cell cycle and induces apoptosis during early stages of tumorigenesis by targeting cyclin dependent kinases and checkpoint regulators while accelerating cell cycle progression, motility, invasion and metastasis in late tumors. TGF-ß has five members, and signaling through this circuit is orchestrated by ligand binding to the receptor (TßR-I, II and III). TßR-I and TßR-II are transmembrane receptors with serine-threonine kinase intracellular domains and are auto-phosphorylated and activated by ligand binding. The activated TßR-II then recruits TßR-I to the complex. The activation signal is relayed via the Smad family cascade. Phosphorylation and

activation of the R-Smads (Smad 2 and 3) is the first step followed by heterodimer formation through binding of Smad4 (co-Smad) to form a functional transcription factor which migrates to the nucleus to influence the expression of the responsive genes (Figure 1.3) [15]. Negative regulation of this cascade is achieved through I-Smads (Smad 6 and 7), which form stable inhibitory complexes with TßRI. Additionally, the active complex has been shown to directly interact with MAPK and NF-kB. This forms the canonical signaling pathway. In non-canonical signaling, activated receptor complex can directly interact with ERK 1/2 and p38 MAPK, thus controlling cell migration and invasion [7], [16].

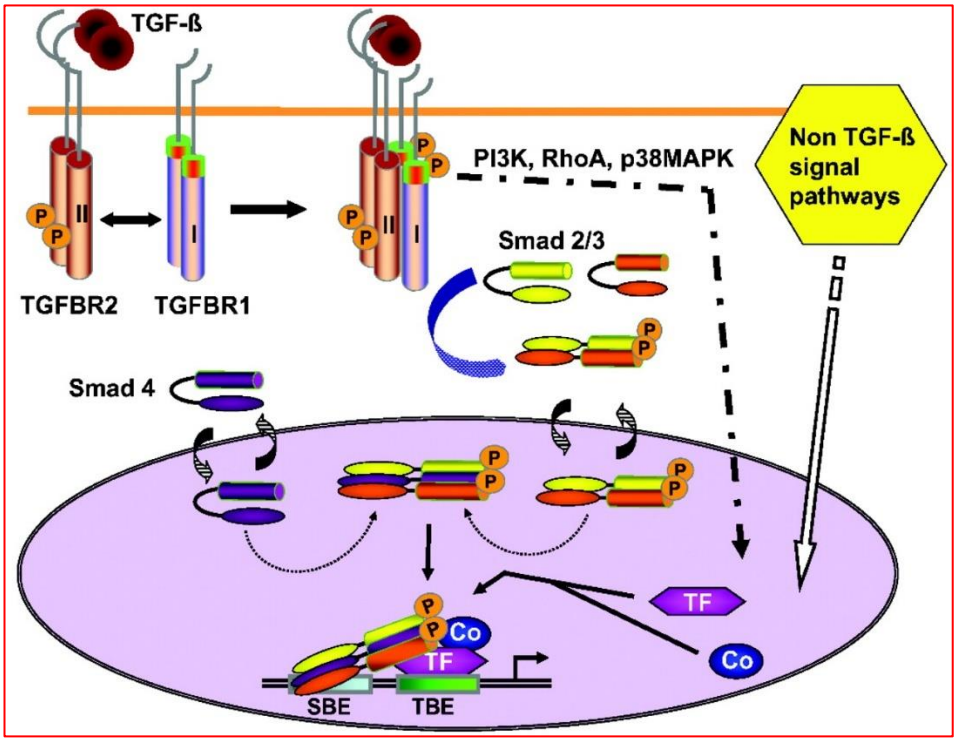


Figure 2.3: The TGF-beta pathway

2.1.4. The Cell Cycle and P53 Pathway

The cell cycle pathway controls cell proliferation through sequential regulation of the key events in the production of daughter cells. Through epigenetic and mutational dysregulation of the control points, normal cells can be transformed into malignant highly proliferative cells with reduced/no response to regulatory factors. The molecular control of the cycle through G1, S, G2, M and G0 has been well established (Figure 1.4)[17]. The dysregulations contributing to cancer involve overriding the checkpoint regulators that inhibit cyclin dependent kinases (p15, p16,

p18, p21, p27 and p57) and/or chromosomal deletion or functional mutation of the tumor suppressor genes: retinoblastoma (Rb) and p53 pathway (Figure 1.5) [18]. Hence, regaining control of the cell cycle through up-regulating the checkpoint inhibitors and the tumor suppressor genes has been a highly lucrative approach in anticancer drug development [19], [20]. It is worth noting that the cell cycle regulatory genes are directly/indirectly controlled by PI3K/Akt/mTOR, TGF-ß and MAPK signaling pathways in the context of cancer.

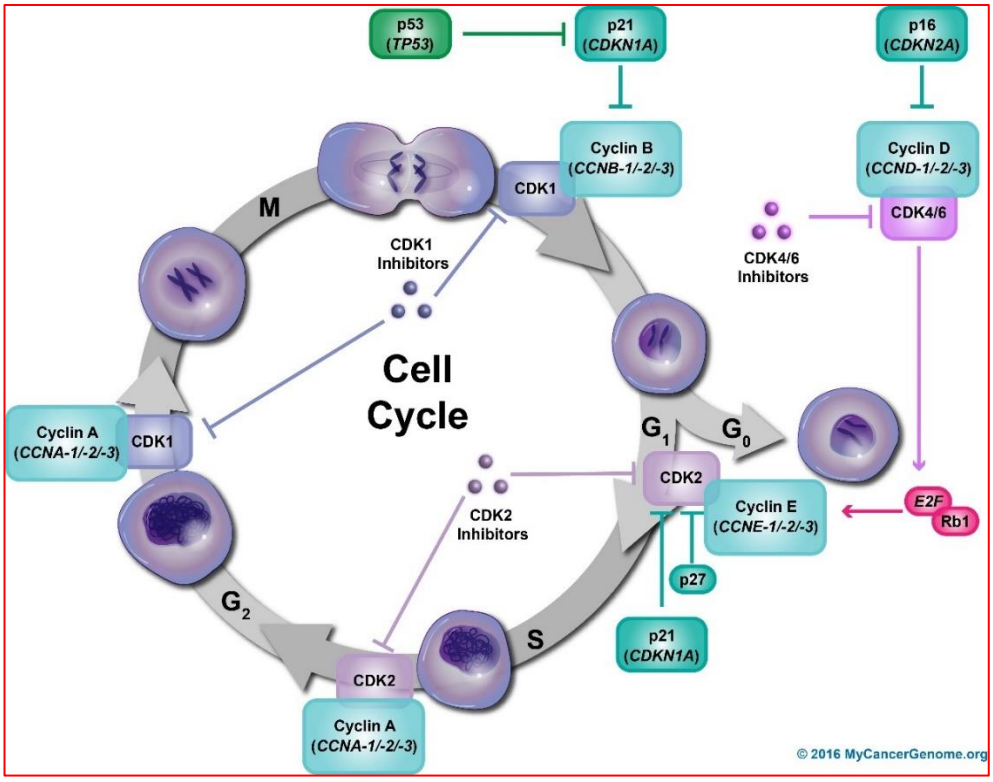


Figure 2.4: The cell cycle pathway

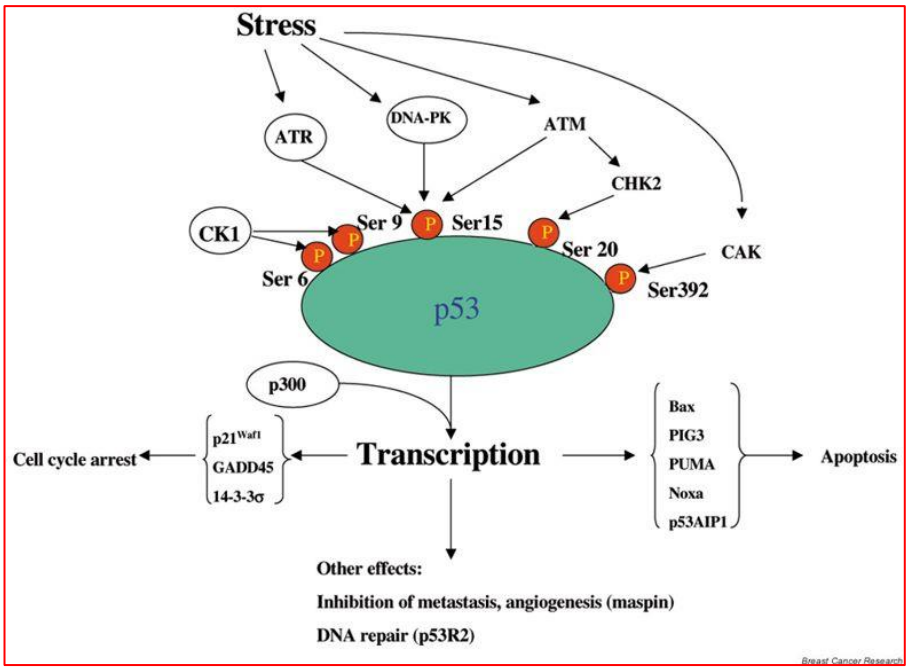


Figure 2.5: The P53 signaling pathway

2.1.5. Wingless Integrated (Wnt)/ß-Catenin

Owing to its pivotal roles in regulating cell proliferation, apoptotic cell death, migration and cell fate decisions, aberrant expression of Wnt associated genes is linked to several cancers including that of the breast [21]. Signaling through this pathway entails ligand binding of the cell surface receptors at the cysteine rich domain, inducing a structural change in the cytoplasmic domain. This activates the receptor, leading to the stabilization of the intracellular ß-catenin protein. Accumulation of cytosolic ß-catenin translocates to the nucleus, forming complexes with TCF/LEF1 transcription factor families to induce the transcription of responsive genes. Intracellular levels of ß-catenin are controlled through ubiquitination by GSK3ß, Axin and APC (Figure 1.6) [22]. Dysregulation of the Wnt receptor, Wnt and the negative regulators of the ß-catenin thus are attractive targets in breast cancer [21], [23].

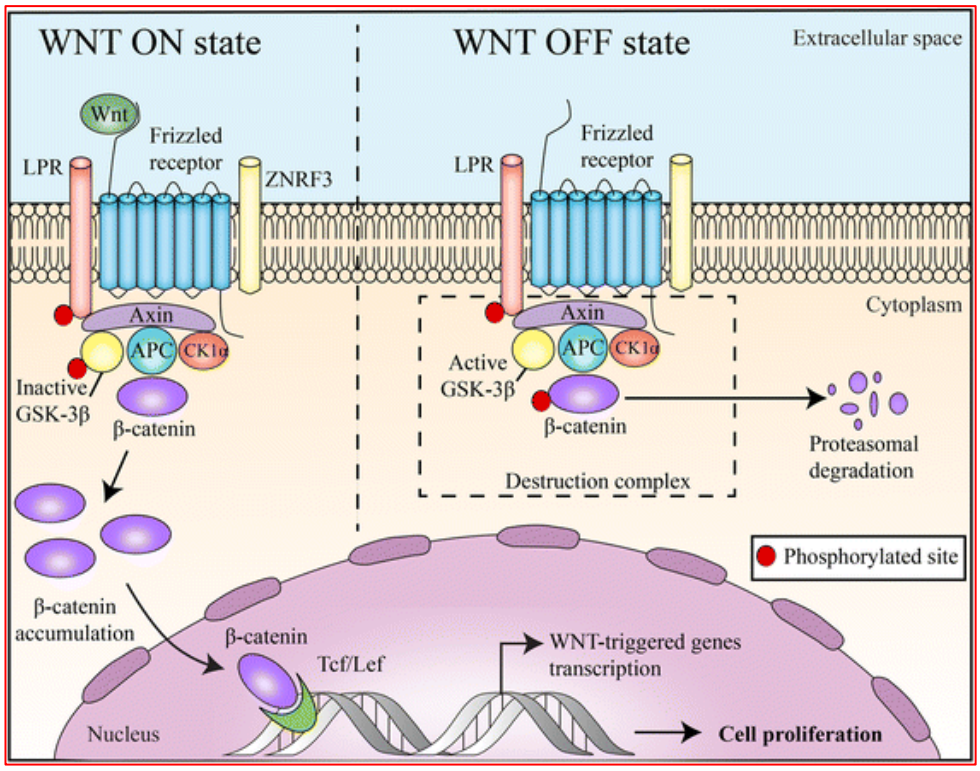


Figure 2.6: The Wnt/Beta Catenin signaling pathway showing the active and inactive states.

2.1.5. Notch Pathway

Notch signaling is one of the most conserved pathways. Its role in regulating cell survival, angiogenesis, stemness and migration has made it an important target in anticancer drug research in breast cancer [24]. Signaling is initiated by receptor (four exists: Notch 1-4) interaction with the transmembrane ligands (five DSL ligands: Jagged 1,2 and Delta-like 1,3 and 4) from an adjacent cell. Proteolytic cleavage by ADAM 10 and 17 of the S2 site and subsequently by gamma-secretase releases the Notch intracellular domain (NICD) which translocates to the nucleus. NICD associates with DNA binding protein RBPj and further Mastermind-like (MAML) transcription coactivator to initiate the transcription of the Hes, Hey, Cyclin D1 and Slug target genes (Figure 1.7) [25]. In breast cancer, aberrant expression of the Notch receptors, NICD, target genes and the negative regulator, NUMB, have been reported and correlates with poor prognosis [26]. Specifically, overexpression of Notch1 is implicated in HER+ and TNBC while elevated Notch is observed following ER-α treatment. Activating mutations in the PEST domain of Notch 1, 2 and 3 have also been found in TNBC [24], [26].

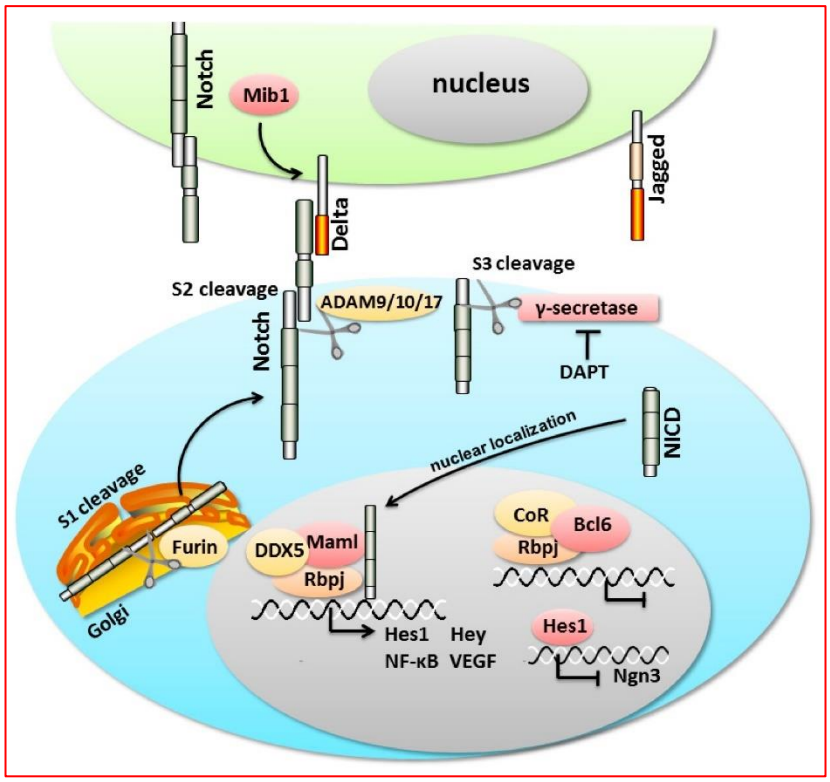


Figure 2.7: Notch signaling pathway

2.1.6. Hippo Pathway

The Hippo signaling pathway regulates mammary gland development and has been associated with carcinogenesis events in breast cancer. Specifically, it has been found to modulate cell proliferation, metastasis, stemness and drug resistance in established tumors. Several signals, including cell-cell contact, extracellular matrix, stress, the GPCR and PI3K can activate the pathway. Following activation, a cascade involving an activation through interaction and complex formation between the phosphorylated mammalian sterile 20-like kinase 1/2 (Mst 1/2) and Sav1 leads to the phosphorylation of the large tumor suppressor 1 and 2 (LATS 1/2) and MOBKL1 A/B. Subsequently, the activated proteins form another kinase complex which phosphorylates and inhibits the transcription coactivators: YAP and TAZ, preventing their translocation to the nucleus. The Hippo pathway, thus, controls cellular features through the interaction of the activated YAP and TAZ with TEAD1-4, p73 and Smads (Figure 1.8) [27]. An activated YAP/TAZ inhibits apoptosis and drives stemness and cell proliferation [28], [29].

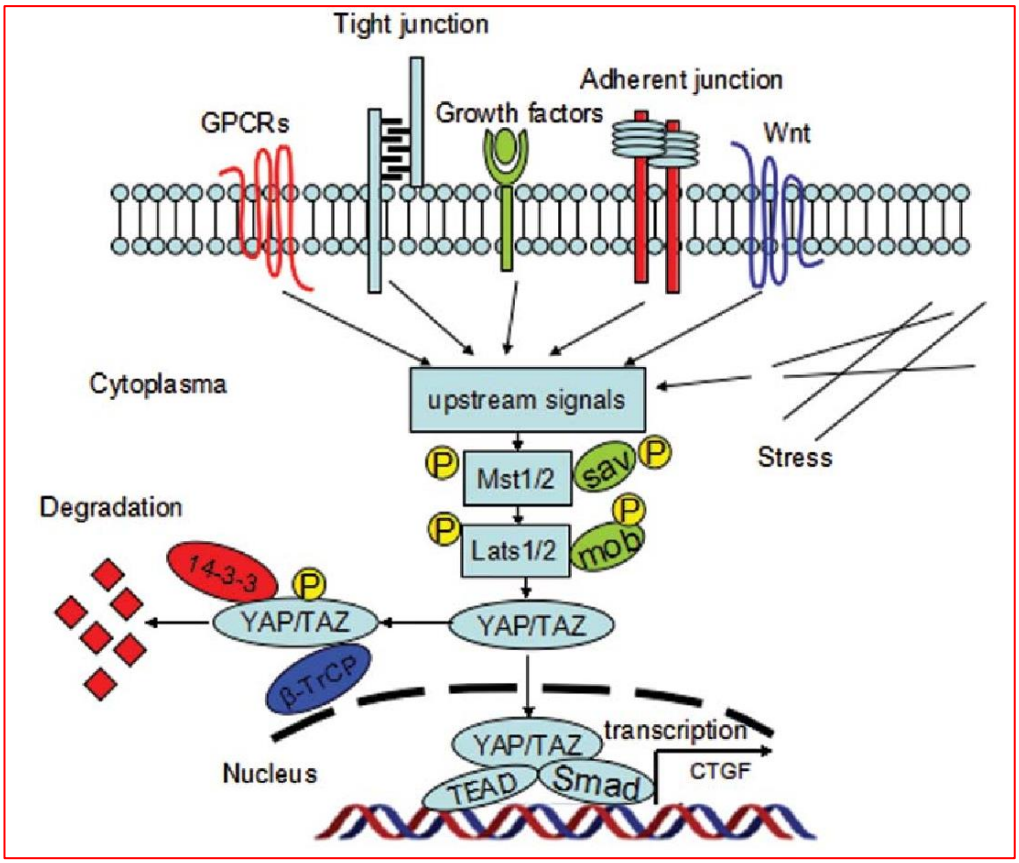


Figure 2.8: Hippo signaling pathway

2.1.7. The Myc Pathway

Aberrant expression of the Myc proto-oncogene is a normal phenomenon in many types of cancers. By acting as a transcription factor as dimer with another protein, MAX, the Myc proteins regulate transcription of cell fate decisions, including growth, proliferation and apoptosis. The positional role as a transcription factor imbues it as a target of many signaling pathways, which deregulate its cellular level to regulate pivotal cellular processes (Figure 1.9) [30]. Indeed, aggressive breast cancer subtypes (TNBC) overexpress Myc and are associated with a poor disease outcome. However, to date, finding a plausible drug target has remained a challenge due to its effector overlapping role in regulating normal cell functions [6], [31]. Since this pathway was not significantly affected by the compounds studied in this work, the interplay of the various molecular players involved have been intentionally not included.

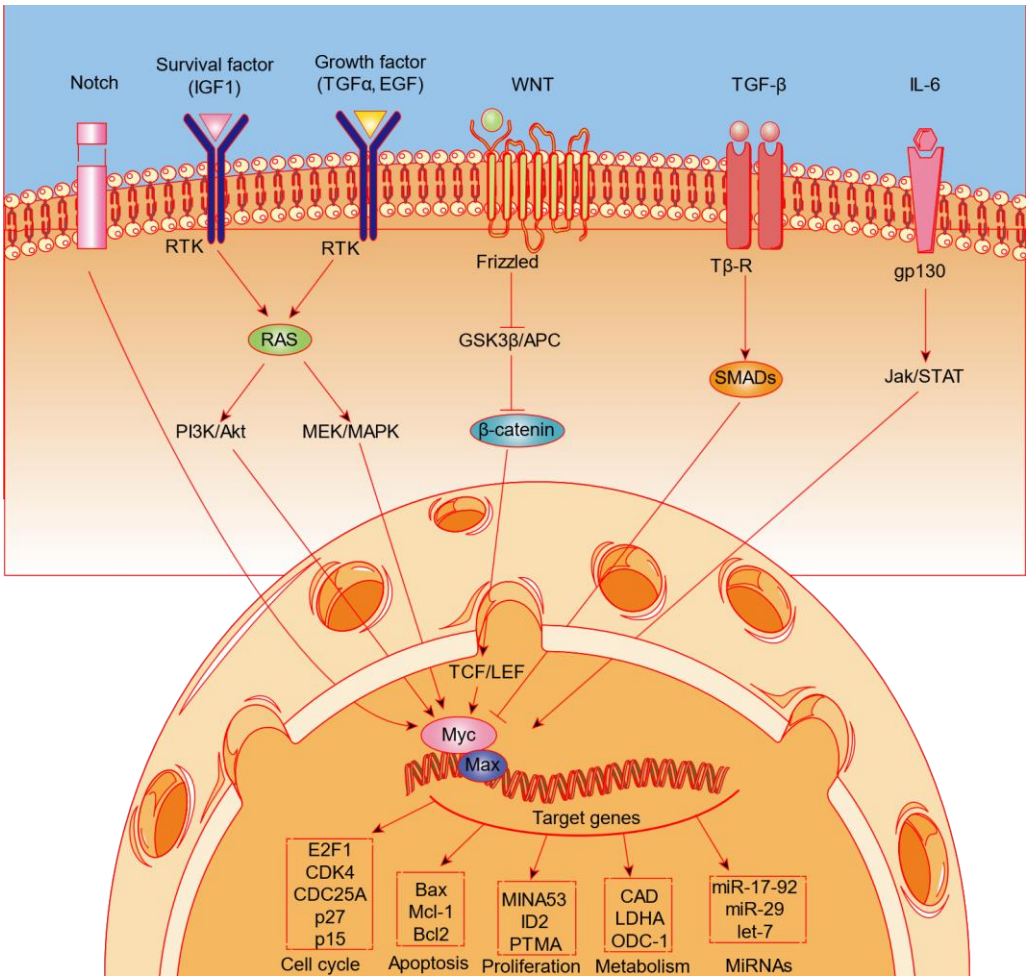


Figure 2.9: The convergence of multiple signaling pathways on Myc signaling pathway

2.1.8. The NRF2 Pathway

Dynamic response to cellular oxidative stress is mainly regulated by signaling through nuclear factor erythroid 2-related factor (NRF2). Following activation, NRF2 dissociates from its negative regulator, KEAP, and acts as a transcription factor by binding to the antioxidant response elements at the promoter region leading to the expression of the respective genes (Figure 1.10) [32]. Further, the biological effects of this pathway have been demonstrated to be enhanced through pathway crosstalk with the AMPK, MAPK, PI3K/Akt and mTOR pathways. It has been observed that NRF2 possesses promiscuous functions by both aiding progression to carcinogenesis as well as acting as tumor suppressor. Hence, developing drugs targeting this network has largely been unsuccessful [6], [33].

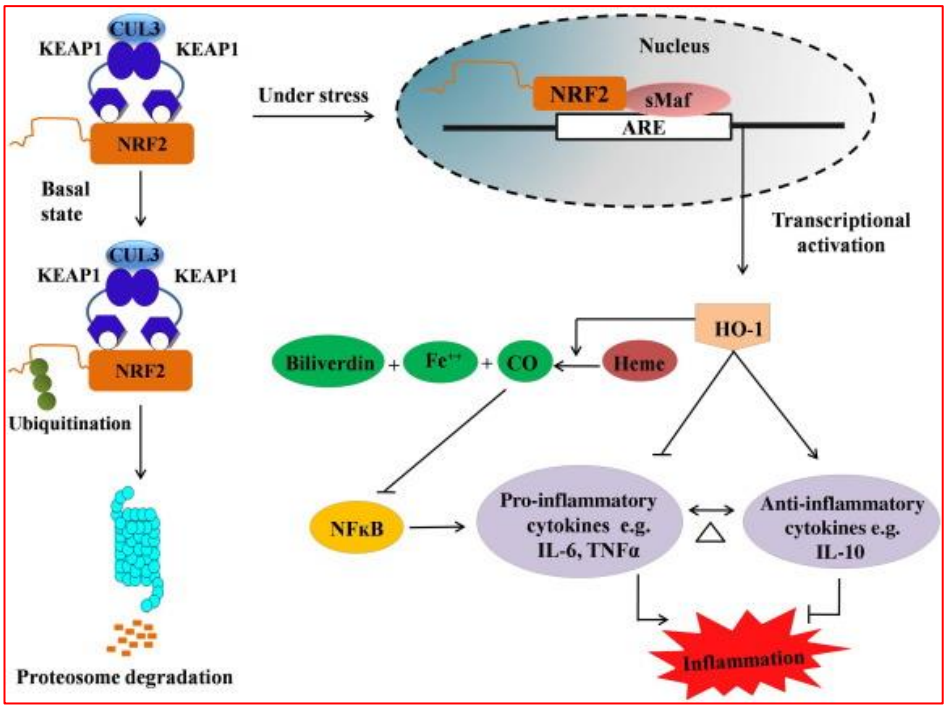


Figure 2.10: The NRF2-KEAP1 pathway

2.1.9. Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF) Pathway

Advanced primary tumors are characterized by increased demand for nutrients required for growth. Thus, angiogenesis (development of new blood vessels) plays a pivotal role in this response by regulating signaling through VEGFR and PDGF receptors. These receptors belong to the receptor tyrosine kinase group, and various family members (VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α and PDGFR-ß) have been reported to date. Upon activation by extracellular ligands (VEGF, PDGF-A, B, C, D, angiopoietin and ephrins), these receptors regulate important processes including migration, proliferation, survival and vascular permeability (Figure 1.11). It has been further determined that these processes are achieved through the regulation of PI3K-Akt and p38 MAPK networks. Angiogenesis in breast cancer has been directly linked to over-expression of VEGFR-2, which mediates growth and permeability activities of VEGFR. PDGFR activation equally contributes to angiogenesis as well as regulating cell proliferation, migration, invasion and metastasis. Thus, anti-cancer drugs targeting the angiogenesis process have largely been targeted at these receptors and it has been appreciated that bifunctional drugs are more effective in targeting cancers [34], [35].

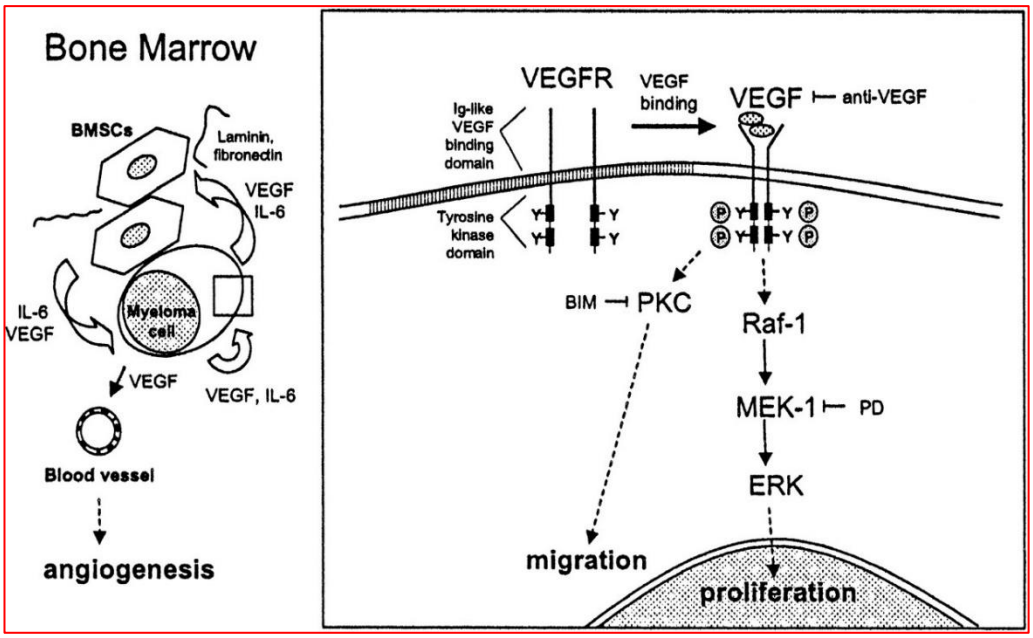


Figure 2.11: The VEGFR signaling pathway

2.1.10. Immune Response Signaling

The tumor micro-environment plays an important role in regulating carcinogenesis. Immune cells, largely regulating the inflammatory processes, play paradoxical roles in cancer, wherein they can contribute to the eradication of early neoplastic cells as well as to the proliferation and development of epithelial cancers. In this context, molecular crosstalk between innate and adaptive immunity at the network level has been associated with the regulation of neoplastic transformation and tumor progression. The intricate interplay of the various immune components is very important in cancer and the balance of which determines cancer prognosis. Pro-inflammatory pathways lead to cancer progression while the anti-inflammatory pathways plays inhibitory roles. Thus, it has been suggested that immunity modulating drugs can synergise anti-tumor response and help in prognosis prediction. Central to the inflammatory response are NF-kB and STAT3 transcription factors and the inflammatory cytokines (IL-1ß, IL-6, IL-23 and TNF-α). Hence, in breast cancer, oncogenic signaling pathways converge at these components to promote tumorigenesis and targeting them has been pursued for treatment purposes [36].

2.2. Plant-Derived Drugs in Breast Cancer Therapy

The study and use of plant derived drugs, pharmacognosy, has been reported in a number of cancers [37], [38]. It is currently understood that the anti-cancer effectiveness of some drugs can match or even surpass conventional drugs. In fact, some of the current medications are formulations derived from plant sources. The use of these drugs in breast cancer has been a subject of intense investigation; subsequently leading to the clinical inclusion of synthetic chemical analogues[37]. The unique advantage of plant derived drugs/formulations is their pleiotropic nature in molecular targeting which is in line with the concept of poly-pharmacology as advanced in complex diseases like cancer. Perhaps the most widely studied tubulin inhibitor, taxol, from the yew tree discovered in the 1960s acted as an eye-opener to the immense potentials of plant derived drugs. Following this, vincristine from *Vinca rosea* and its isoforms were successfully adopted in the clinical management of breast cancer.

Compounded by the large number of currently available plant-derived pharmacologic compounds, a new school of thought of drug-repurposing has been proposed. In this approach, effective dosages of such compounds are tested on cancer cell lines through high throughput screening. This approach has further accelerated the adoption of plant derived drugs as alternative anticancer treatments. However, still, since this approach is reductive it is one-sided and fails to report important pharmacodynamic properties of the drugs. In the context of this thesis, focus has been placed on four compounds based on the availability of corresponding high-quality transcriptomic data: indole 3-carbinol, actein, compound kushen injection and withaferin A.

2.2.1. Indole 3-Carbinol

Indole 3-carbinal (I3C) (Figure 1.12) [39] is a phytohormone derived from cruciferous vegetables and is a breakdown product of glucosinate 3-ylmethylglucosinate compound. Previous studies have documented I3C's good pharmacological profile in humans upon oral administration. It has also been reviewed for the treatment of many human cancers [40].

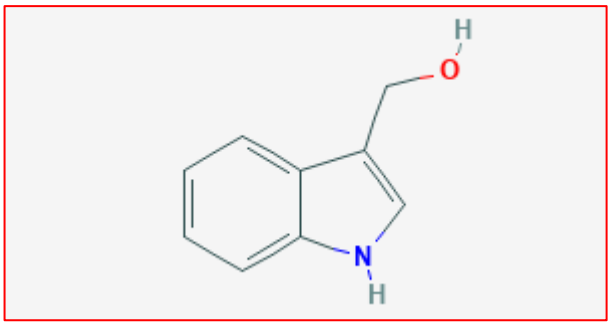


Figure 2.12: 2D structure of I3C.

2.2.2. Actein

Actein (Figure 1.13) [41], a derivative of the *Cimicifuga* species roots *(also known as black cohosh)*, is a natural triterpene glycoside that has been used by different civilizations in North America, Asia and Europe for centuries to treat different ailments [42]. Since its initial evaluation in breast cancer, separate studies have tried to decipher its mechanism of action using in vivo approaches [42]–[44].

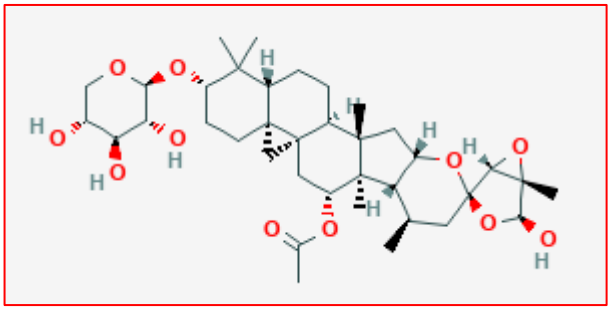


Figure 2.13: 2D structure of actein.

2.2.3. Compound Kushen Injection

Compound kushen injection (CKI) is an ancient formulation in the Chinese pharmacopoeia and is derived from a mixture of *Radix sophorae flavescentis* and *Rhizoma smilactis glabrae* herbs. It has been extensively studied and applied in the treatment of several tumors and tumor associated pains. Molecular and genomic studies have established its roles in targeting cell cycle, apoptosis, energy metabolism, DNA repair and cytokine signaling [45]. A recent study revealed that the mixture consists of 22 distinct chemical constituents that mediate its biological roles [45].

2.2.4. Withaferin A

Withaferin A (Figure 1.14) [46] is a steroidal lactone belonging to the withanolide group of compounds; a plant-derived natural compound from *Withania somnifera*. It is a vital component of the Indian Ayurvedic medicine. It has been investigated in a large number of inflammatory diseases, and in vivo studies in cancer have established some of its target molecular networks [47]–[51].

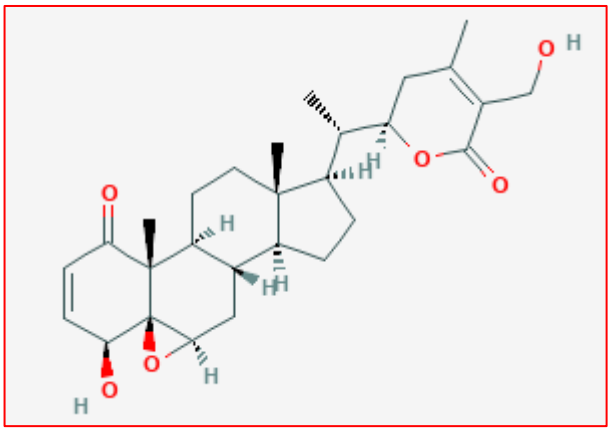


Figure 2.14: 2D Chemical structure of Withaferin A.

2.3. Transcriptomics in Systems Biology

Systems biology is a research approach focusing on the study of complex biological systems and processes as a whole under different states at the molecular level. The appreciation of the genetic complexity of different cancers, including breast cancer, means reductive molecular biology techniques are insufficient, as they only provide a partial explanation of the underlying biological processes. The introduction and increasing application of high-throughput techniques such as microarrays and next-generation sequencing through RNA-sequencing to generate transcriptomic profiles has led to the identification of novel drug targets [52]–[55]. Moreover, a number of biological mysteries has been uncovered. In this context, transcriptomics provides a picture of all the genes expressed at a given time. Over the past decade, there has been a number of studies that examined the global effects of candidate drugs on the gene expression pattern in cancer and other diseases to discover new therapeutic and diagnostic biomarkers. Transcriptomics provides the systemic view of RNA

transcripts expressed under a set of conditions at an instance, contributing to a holistic picture of the biological system at transcriptional level [56].

2.4. Systems Pharmacology/Network Pharmacology

Network biology is a concept that originated from other scientific disciplines upon the appreciation of the fact that a pathological phenotype is not the result of an individual gene but rather a complex interaction of biological processes forming a network [52]. Hence, human diseases can today be studied using metabolic, protein-protein interaction, genetic interaction and gene regulatory networks. This perspective in pathology has found its way into drug discovery research for complex diseases challenging the traditional highly selective approach to drug development and evaluation. The concept of network/system pharmacology is increasingly being accepted. Poly-pharmacologic drugs possess superior therapeutic index [57], [58]. Through network pharmacology, synthetic lethality of multiple gene targets has demonstrated positive results in a number of studies with various cancers [59]. Coincidentally, plant-derived drugs and concoctions have shown multi-targeting in various diseases and cancer [38]. In fact, a number of groups have evaluated genome-wide effects of these drugs using various omics strategies. Surprisingly, despite their appreciation of the demonstrated broad spectrum activity, no attempt has been made to apply the currently available robust computational systems biology methods to decipher their molecular effects. Therefore, network pharmacology hopes to identify drug targets, predict toxic effects and resistant subtypes, enabling decisive experimental plans and personalization of treatments. We can, thus, increase drug efficacy and clinical trial success rates, and reduce drug-associated side effects in patients.

While predicting biological systems requires the integration of multiple biological data associated with a disease phenomenon, current methods are still incomplete at addressing such systems, and there is insufficient high-throughput data publicly available to date. In the context of this thesis, therefore, we focus on one level of omics to draw our biological conclusions on the systemic effects of the drugs under consideration.

2.5. Protein-Protein Interaction Networks (PPIN)

Owing to their central roles in mediating all cellular functions and biological processes, the study of protein-protein interaction networks (PPIN) has been an important subject in systems biology, where proteins and their interactions are depicted as a network. PPINs enable visualization, depiction and quantification of the functioning and importance of individual proteins in a cell. Importantly, by taking systems view of the PPIN of a cell under a defined condition, it is possible to infer novel functions, quantify positional importance of proteins and enable prognostic evaluation of a cell perturbation [60]. By taking this approach, the pleiotropy of proteins in complex diseases can be addressed at the network level [61]–[63].

To date, a number of online curated organism-specific PPIN databases have been established. While they all hold important protein network information, they differ in terms of size, method used for network construction and organism coverage. The main methods for network construction are experimental (high-throughput and traditional methods) and computational techniques. For instance, the current release of BioGRID [64], HPRD [65], and STRING [66] contain differing number of proteins and interactions. Thus, the decision on which database to select is largely based on the user's preference, the quality of the PPIN based on the bioinformatic method to be applied and the biological question. However, despite improvements in the knowledge of the human interactome, available networks still suffer from sparsity and biases introduced during curation. Still, emphasis should be placed on selecting high-confidence PPIN, which are largely manually curated from experimental evidence.

2.6. Protein-Protein Interaction Network Integration Methods

A major hinderance to the complete adoption of network pharmacology has been the stochasticity of most network enrichment methods. The dynamic nature of biological systems, however, means that an individual node can interact with one or multiple other proteins creating an interconnected condition-specific network. To address this, concerted efforts in developing deterministic network integration methods based on node scoring algorithms have contributed to a number of

bioinformatic tools. The highly significant proteins based on the scores is then used to identify a condition specific active subnetwork within the web of all PPIN, by integrating curated PPIN with scored proteins from high-throughput data, mostly from transcriptome experiments.

Network mapping techniques for condition-specific transcriptome datasets have been applied in different complex diseases like cancer and Parkinson's disease with novel discoveries on mechanistic drug action, synergistic drug combinations and druggable targets [67]–[69]. This systems-level approach is superior over other gene set enrichment analyses as it does not only provide in-depth knowledge of the perturbed networks but also relies on efficient un-supervised and empirical inference-directed algorithms to model perturbation networks from gene expression data [70]. Furthermore, pathway-pathway crosstalk and unearthing of previously un-characterised pathways and sub-pathways is possible from protein-protein interaction networks [71].

To date, a substantial number of tools are available. A recent benchmarking report by Batra et al. [71] evaluated the performance of various network integration methods currently available, including BioNet, COSINE, GiGA, KeyPathwayMiner, DEGAS and PinnacleZ. The advantage of these tools is the reliance on experimental data to derive condition specific subnetworks. Of the highly reliable and less stochastic tools, BioNet and KeyPathwayMiner (KPM) exhibited higher performance. However, despite being heuristic methods, they follow different approaches in subnetwork extraction. BioNet [72] models subnetwork identification as a prize collecting Steiner Tree problem and solves it using integer linear programming from a predefined false discovery rate cut-off and extracting the maximal scoring subgraph (Heinz algorithm) [72]. KPM [73], on the other hand, finds the subnetwork where all proteins but K exceptions are significantly changed using Greedy, exact branch and bound and Ant Colony Optimization (ACO) algorithms from a binarized expression dataset [73]. Considering these two approaches, the optimal graph solutions are intuitive and optimizations must be performed to comprehensively extract the systemic biological phenomenon under study [71]. Thus, to penalize and consider only highly significantly perturbed proteins, KPM is preferable even though it may suffer from node prioritization problem due to the binarized input experimental data. Furthermore, in our optimization studies, we observed that while repeatability of BioNet's graph solution is low for small problems, KPM is largely consistent over all graph problems.

KPM has been successfully applied in a number of studies with important findings on underlying subnetworks. Recently, Frisch et al [74] integrated human PPIN with RNA-Seq data to delineate potential druggable targets in multiple sclerosis using KPM. The role of immune-modulation networks during bacterial and fungal infection of human monocytes treated with vitamins A and D was discovered in another study. To identify the relevant genes targeted during oligodendrocyte differentiation, Cantone et al [75] integrated PPIN with RNA-Seq transcriptomics data using KPM to identify SOX10, OLIG2 and TCF7I2 as the main transcription factors in the extracted subnetwork. Another study to elucidate the molecular mechanisms of liver fibrosis applied KPM to identify the pathways and networks associated with chemically induced liver fibrosis by integrating PPIN with rat transcriptome data [76]. These studies demonstrate the reliability and robustness of KPM in integrating experimental omic transcriptome data with priori molecular interaction networks to identify conditionally perturbed subnetworks.

2.7. Aim of the Thesis

It is well appreciated that epigenetic and mutational modifications drive tumor progression in breast cancer by hijacking normal cell signal transduction pathways [5], [77]. The use of single drug targets has largely failed due to target mutations, target by-pass and utilisation of alternative signaling mechanisms [62], [78], [79]. It is understood that within a single tumor numerous genetic modifications are present at the same time. This diseasome network further complicates the exquisite ligand-targeted single drug design paradigm [59]. However, by employing a multi-target strategy, several studies have registered better treatment outcomes and thus, drug combinations is a prevalent strategy today [3], [77]. Coincidentally, plant-derived drugs have been investigated in a number of anti-cancer drug research studies, and their pleiotropic effects were demonstrated [38].

Despite technological advancements in drug research, reductionist molecular biology studies are still the preferred method of choice in prioritising new drug candidates. This has not only slowed drug research but also led to high attrition rates of drug candidates at the clinical trials stage [58], [59], [61], [68]. In the recent past, network pharmacology has emerged as a holistic approach to address this limitation

by considering all the molecular players following drug treatment. To identify perturbed networks, *de novo* network enrichment methods that integrate *priori* interaction networks with experimental data to extract condition-specific subnetworks have been used [71]. Interestingly, though, there is no standard method widely accepted by the scientific community on this subject, with different groups applying different methods.

It is currently understood that an effective anti-cancer drug candidate should possess multiple targets and able to perturb multiple carcinogenesis signaling networks [69], [55]. Hence, in this thesis, a holistic method of subnetwork discovery is implemented by mapping breast cancer transcriptome treated with plant-derived drugs onto high-confidence human protein-protein interaction network and infer signal transduction pathway networks. This approach unravels novel perturbed signaling pathways unique to the drug and the treated cancer cell response. Through this approach, the pleiotropic effects of plant-derived drugs/compounds are demonstrated; a resistant cancer subtype protein network is analysed and the associated mechanisms and drug targets identified. Additionally, commonly targeted pathways by plant-derived drug/compounds are determined.

3. METHODOLOGY

A graphical summary of the computational analysis pipeline applied in this study is depicted in Figure 3.1. We provide detailed description of these steps in the subsequent sections below.

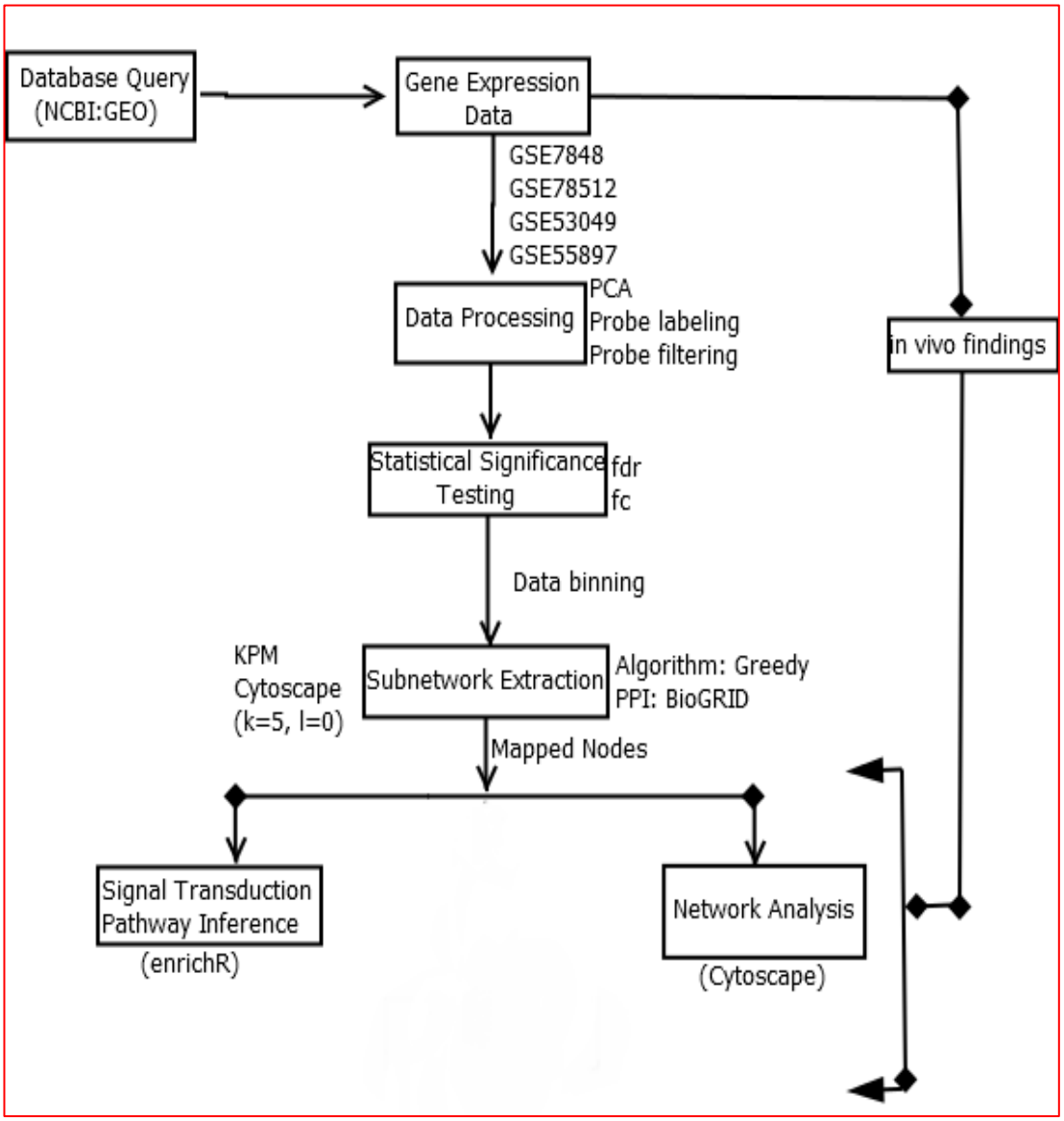


Figure 3.1: Analysis pipeline summarising the steps followed in the data analysis.

3.1. Dataset Extraction and Outlier Analysis using Principal Component Analysis

The Gene Expression Omnibus (GEO) database maintained by the NCBI is a public repository for high-throughput gene expression and genomics datasets, with advanced data search application interface [80] For this study, a structured query, below, was developed to filter the available datasets in the database:

(breast cancer) AND (human[organism]) AND (plant*)

Pre-processed transcriptome datasets (series matrix) meeting the search criteria were downloaded. Minimum sample size of 3 for both control and treatment were considered for subsequent analysis. Principal component analysis (PCA) is a widely used unsupervised high dimension data reduction method in statistical analysis [81]. Given the large number of data points in genomic analysis, PCA provides a global picture of the variations contributed by the samples in the dataset. To determine the grouping pattern of the control and treatment samples PCA was performed using princomp R base function and ggfortify package in RStudio [82]. The former calculates the eigenvalue and eigenvectors while the later makes a loadings plot, allowing for the identification of similarly behaving samples before subsequent analysis can proceed.

3.2. Identification of Drug-Specific Differentially Expressed Genes

Microarray derived gene expression data are coded by unique platform-specific identifiers called probes. Thus, for these datasets, the corresponding platform annotation files were downloaded, probes matched and the respective official gene names used in subsequent analyses. The multiple probe identifiers problem for some genes, common in array designs, was resolved by taking the maximally expressed ones. Next, for the RNA-Seq datasets, genes with zero expression values in at least 2 samples were removed. For all the datasets, log2 transformation was used to make the expression values closer to normal and allow for cross-platform statistical analysis.

Linear models for microarray (limma) [83] data analysis method has been extensively applied in microarray [84] and RNA-Seq [84], [85] data for differential gene expression analysis. Limma is available as an R package. Using this tool, the

annotated data was fitted to a linear model in limma, contrasts for the two conditions (control versus drug-treated) performed, Bayesian-based t-test significance testing carried out, and to reduce the number of false positives common in multiple testing, the p-values were adjusted using Benjamini-Hochberg method [85]. It is imperative to note that gene fold change was used in all decision tests.

3.3. Protein-Protein Interaction Network (PPIN)

The current release of undirected human PPIN from the Biological General Repository for Interaction Datasets (BioGRID, v3.5.173) [64], [86] was downloaded and used. The human PPIN in this release (*March 2019*) consists of 22435 proteins with 478529 interactions. The tab delimited file was pre-processed by removing unnecessary header lines that are not readable by the Cytoscape import function.

3.4. Drug-perturbed subnetwork extraction from priori PPIN

KeyPathwayMiner [73] is a heuristic enrichment tool that integrates PPIN with binarized input gene expression data and finds the maximal connected subnetworks using Greedy, Ant Colony Optimization and Exact optimization algorithms. Greedy algorithm treats the subnetwork extraction as an optimization problem, finding local best solutions with the hope of making the best global solution. To this end, it is worth noting that the choice of KPM in this study stems from a prior analysis on the reproducibility of graph-solutions in both KPM and BioNet using one of the datasets, where reproducibility was observed in the former. In this work, KPM, as a Cytoscape (v3.7.1) app, was used [87].

The problem and solution to the subnetwork extraction in this work is described: with a labelled *priori* undirected PPIN (BioGRID) graph, $G = (V, E, d)$, with V vertices (proteins), E edges (interactions) and a mapping function, $d: V \rightarrow \{1, 0\}^q$, and a gene expression matrix (adjacency matrix) defined by:

$$C_{p \times q} = \begin{cases} 1: differentially\ expressed \\ 0: non - differentially\ expressed \end{cases} \tag{3.1}$$

Here, the objective is to extract a maximally connected clique, $S(U)$, from an exception vertex set $C(G, l)$ derived from G, with k vertices to get a (K, l) component. Any vertex $v \in V$ with $d(v) \leq l$ is an exception matrix.

This presents an optimisation problem and amongst the previously proposed algorithms, here it is solved using Greedy algorithm. In this approach, a set W_u is iteratively constructed from $W_u = \{u\}$ for each vertex, u. A vertex v from $C(G, l)$ which is adjacent (in $C(G, l)$) to W_u and maximizes $|S(W_u \cup \{v\})|$ is added to the set Wu. At $|W_u| = K$, the maximal solution for u, $S(W_u)$ found is returned as the optimal sub-graph [88].

After simulations with different noise levels ($K = 1, 2, 4, 5, 8, 10$), $K = 5$ was used as the optimal allowable insignificantly/non-differentially expressed, but highly connected, genes (noise) in the network and INES (Individual Node Exceptions) as the search strategy. Since only one case was considered, the default ($l = 0$) was used. Thus, $K\ and\ l$ are the defined constraints applied in this work. In addition to mapping all the significantly expressed genes (both up and down), the up- and down-regulated genes were also mapped separately. To include only highly significant genes in the subnetwork solutions, both gene fold change and FDR adjusted p-value were used to select condition-activated genes. With this approach, noise in the sub-graph solution is believed to be significantly curtailed. The unique advantage of this method in pathway analysis is that the resulting network consists of only the directly interacting biomolecules [73].

3.5. Centrality analysis of subnetworks and survival analysis

To quantitatively identify the biologically essential genes enriched in the derived subnetworks, two main centrality measures were applied: betweenness and degree centralities. Betweenness centrality measures the number of short paths through a given node (gene) in a network and reflects the load on the gene. Degree centrality determines the number of nodes (genes) connected to a given node (gene) in a network [89]. For this, CytoNCA [90], a widely used network topology analysis tool available as an app in Cytoscape was used. Subsequently, to determine the biological significance of the identified genes in breast cancer carcinogenesis, the web-based

KM-Express bioinformatic tool was used. In this approach, the biological roles of the top 5 significant genes, based on centrality measures, on overall survival and phenotype association in breast cancer was analysed. The breast cancer specific patient RNA-Seq gene expression data from the TCGA database, that is categorized into high and low expression groups. In this work, classification into the two groups was based on the median gene expression cut-off [6], [91]. Kaplan-Meier and disease-phenotype association in box-plots were used to interpret the results. Statistical significance was evaluated based on hazard-ratio, which indicates the propensity of death between the high and low gene expression disease groups, and log-rank p-value.

3.6. Signal transduction network inference

Pathway enrichment is the statistical method to determine the existence of biological pathways in a gene list than would occur by chance [92]. To date, a number of tools have been developed, including g:Profiler, GSEA, WebGestalt [92], Enrichr and PANTHER for this type of analysis. However, the comprehensives, ease of use as an R package and great interactive visualization of EnrichR results sets it apart over other tools [93]. To infer perturbed signaling pathways from the subnetwork, the latest releases of KEGG (2019), Reactome (2016), WikiPathways (2019) and GO Biological processes (2018) databases were used. Only terms with “signal” or “pathway” or “apoptosis” or “cell cycle” were selected in this work. Further, the identified terms were considered to be significantly enriched if the FDR<0.05 and with at least 3 overlapping genes.

4. RESULTS

4.1. Datasets used in this study

From the database search strategy described previously, six transcriptome datasets were identified from GEO database, all specific for breast cancer lines treated with plant-derived compounds. Subsequent analysis by PCA resulted in the elimination of 2 datasets and 3 groups in actein dataset, in which no clear separation of controls and treated groups could be observed. The PCA analysis results are electronically available as supplementary. As a result, 4 high quality datasets: GSE7848, GSE78512, GSE53049, and GSE55897 belonging to actein [94], compound kushen injection (CKI) [95], Withaferin A (WA) [96] and indole 3-carbinol (I3C) [97] plant-derived compounds and formulations respectively were used in subsequent analysis. These datasets represent 7 breast cancer cell lines (triple negative, human epidermal receptor-2-positive and luminal A), each harbouring a specific mutation. The triple negative subtype lacks the oestrogen, progesterone and human epidermal receptors; luminal A expresses only oestrogen and progesterone receptors; and HER2-positive expresses only the HER2 receptor [2]. These characteristics are detailed in Table 4.1 below. Henceforth, LA, TN and HER2+ will be used to refer to luminal A, triple negative and human epidermal receptor-2-positive breast cancer subtypes.

Table 4.1: Summary of datasets used and the molecular profile of the cell lines included. The columns Controls and Treatments list the number of samples. (HER2+: human epidermal receptor 2 positive, LA: luminal A, and TN: triple negative, AC: adenocarcinoma, IDC: invasive ductal carcinoma, MC: medullary carcinoma, Wt: wild type, Mut: Mutant, Del: deleted).

Drug	Platform	Cell line	Subtype	Pathology	BRCA1	P53	Controls	Treatments
Actein	Affymetrix Human Array	MDA-MB-453	HER2+	AC	Wt	Del	4	3
CKI	Illumina HiSeq 2500	MCF-7	LA	IDC	Wt	Wt	3	3
I3C	Illumina beadchip	MCF-7	LA	IDC	Wt	Wt	3	3
		T47D	LA	IDC	Wt	Mut	3	3
		ZR751	LA	IDC	Wt	Wt	3	3
		MDA-MB-231	TN	MC	Wt	Mut	3	3
		MDA-MB-157	TN	AC	Wt	Mut	3	3
		MDA-MB-436	TN	AC	Mut	Mut	3	3
WA	Illumina beadchip	MDA-MB-231	TN	MC	Wt	Mut	3	3
		MCF-7	LA	IDC	Wt	Wt	3	3

4.2. Plant-derived drugs affect differing number of genes from differential gene expression analysis

Determining treatment responsive genetic features depends on the choice of, with high confidence, threshold in differential comparison of the transcriptomic datasets. In this study, limma package was used to both adjust the Bayesian t-test p-values and determine the gene fold change. Different FDR cut-offs and a standard fold change cut-off of 2 was applied across all the datasets. However, in order to make the effect of a drug on different cell lines comparable, the same FDR was used for the datasets of the same drug Table 4.2, below, summarises the number of differentially expressed genes for the different drugs. The FDR cut-offs were chosen to represent at most 10% of all covered genes in the data. Actein and CKI induced differential expression of HER2+ (MDA-MB-453) and LA (MCF-7) genes respectively. Comparatively, under I3C treatment, more genes are regulated in luminal A cells (T47D, MCF-7 and ZR751) than triple negative cells (MDA-MB-231, MDA-MB-157

and MDA-MB-436) (Table 4.2). Conversely, triple negative (MDA-MB-231) genes under WA are more responsive that luminal A (MCF-7) genes (Table 4.2). CKI dataset includes treatment data for four different doses of the drug (1mg/24hr, 2mg/24hr, 1mg/48hr and 2mg/48hr). Since the others led to extremely low numbers of differentially expressed genes, subsequent analyses focused on the 2mg/24hr dosage data.

Table 4.2: Summary of differential expression analysis results. The number of differentially expressed genes under different dosages of the respective plant-derived drugs/compounds are given in the table. DEG: differentially expressed genes, FDR: false discovery rate, FC: fold change

Drug/Compound	**Dosage**	**Cell Line**	**DEGs**	**FDR cut-off**	**FC**
Actein	40µg/ml	MDA-MB-453	1008	0.05	2
CKI	2mg	MCF-7	1661	0.01	2
I3C	200µM	MCF-7	3115	0.005	2
		T47D	2462	0.005	2
		ZR751	2125	0.005	2
		MDA-MB-231	202	0.005	2
		MDA-MB-157	430	0.005	2
		MDA-MB-436	869	0.005	2
WA	700nM	MDA-MB-231	1247	0.001	2
		MCF-7	452	0.001	2

4.3. PPIN mapping reveals drug-specific subnetwork enrichment for the different breast cancer subtypes

The main aim of this study was to map transcriptome data from breast cancer cell lines treated with plant-derived drugs on PPIN to extract highly affected subnetworks. Among a number of de novo network enrichment methods available, KPM [73], [88] was used in this study. Thus, the FDR and FC were binarized, based on the set cut-offs (as illustrated in differential gene expression analysis Table 4.2 above). In the resulting expression matrix, ‘1’ represents active gene while ‘0’ represents an inactive gene as described [88]. Next, the condition (drug)-specific subnetworks were extracted by solving the Greedy optimisation problem and

integrating the high-confidence BioGRID [64] PPIN to extract the drug-perturbed subnetwork for the different cell lines.

The Table 4.3 illustrates the number of genes and their interactions in the extracted subnetworks. To aid in the elucidation of the networks whose activity increased/decreased, the down/up-regulated genes were separately mapped to the PPIN using KPM, again using similar constraints and the results reported in Table 4.4. The subnetworks presented here represent the systemically targeted PPIN in the respective cells. It can be seen that same drug/compound (CKI and WA) affected subnetworks with different number of proteins and interactions in LA and TN subtypes and within LA subtypes for CKI (T47D, ZR751 and MCF-7) (Table 4.3). This demonstrates differential targeting of protein-protein interaction networks by the drug/compound on different breast cancer subtypes harbouring different genetic mutations.

Table 4.3: The extracted drug-specific protein-protein interaction subnetworks. The proteins represent the network nodes while the interactions represent the network edges.

Drugs	**Cell Lines**	**Genes**	**Interactions**
Actein	MDA-MB-453	829	3858
CKI	MCF-7	1332	9331
I3C	MCF-7	1974	10684
	T47D	1681	7050
	ZR751	1403	5457
	MDA-MB-231	93	126
	MDA-MB-157	86	110
	MDA-MB-436	541	1275
WA	MCF-7	333	941
	MDA-MB-231	998	3277

Table 4.4: The extracted drug-specific protein-protein interaction subnetworks for up-/down-regulated proteins. The proteins represent the network nodes while the interactions represent the network edges.

Drugs	**Cell Lines**		**Genes**	**Interactions**
Actein	MDA-MB-453	Up	327	687
		Down	455	2166
CKI	MCF-7	Up	933	2838
		Down	304	1676
I3C	MCF-7	Up	453	1162
		Down	1399	6816
	T47D	Up	620	1324
		Down	959	3254
	ZR751	Up	545	1105
		Down	961	6323
	MDA-MB-231	Up	17	17
		Down	86	111
	MDA-MB-157	Up	18	19
		Down	75	106
	MDA-MB-436	Up	98	120
		Down	402	932
WA	MCF-7	Up	117	353
		Down	202	564
	MDA-MB-231	Up	456	1011
		Down	480	1208

4.4. Subnetwork centrality analysis and *in silico* survival and disease phenotype association predictions

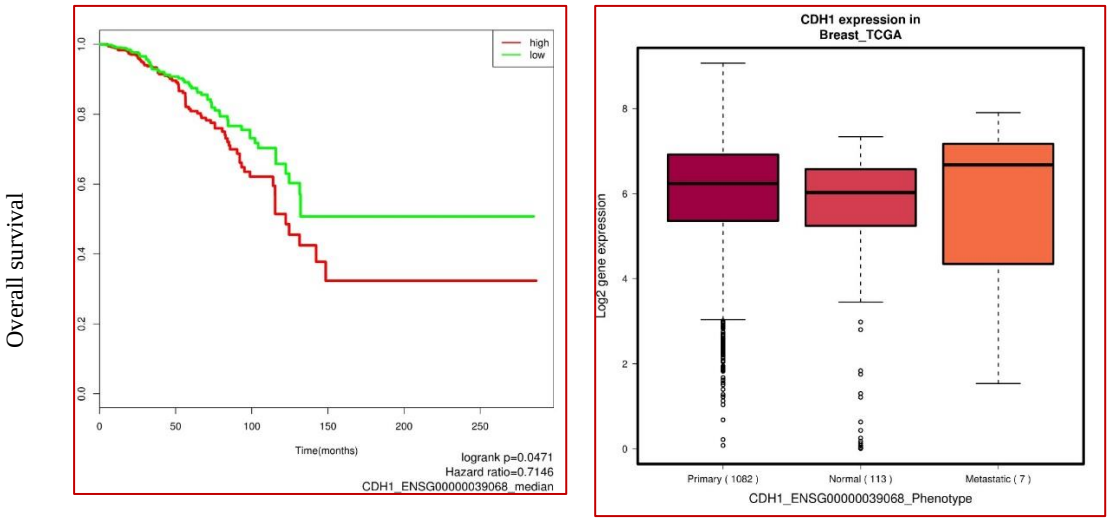
Apart from predicting the mechanism of action of a drug, network pharmacology can be used to identify drug targets [58], [74], [98]. To achieve this, network centrality measures are frequently used to determine genes in a network whose elimination would destabilize the network topology. A number of network topology analysis tools are available as Cytoscape apps for this purpose and CytoNCA [90] offers both network centrality measures and a user-friendly visualization interface. In this approach, betweenness centrality measures the influence a gene has on the whole subnetwork while degree centrality measures the number of genes connected to a given gene. This app was used to determine degree and betweenness centrality measures of all the subnetworks. Table 4.5 provides the top 5 highly significant genes from this analysis based on degree and betweenness scores.

One of the main sources of predictive data is scientific literature reports and expert-curated biological databases. To determine the biological role of the identified genes in relation to overall cancer prognosis, an *in-silico* overall survival and phenotype association analysis was performed. Kaplan-Meier plot illustrates the fraction of surviving cases over a follow-up period. In this analysis, publicly available curated breast cancer gene expression data from TCGA (943 breast cancer samples) accessible through KM-Express tool was used. The tool generates several figures for each gene; two of them gives the Kaplan-Meier plot for overall survival together with the corresponding Cox hazard ratio and log-rank; and the gene-pathology association as boxplots derived from analyses of normal, metastatic and primary cases. The results from this analysis are as shown in Figure 4.3 A-D (p-value<0.05 for significance), presenting the two figures for each gene. Of all the identified 11 unique (HNRNPL, TRIM25, ESR2, CUL3, BAG3, ELAVL1, CDH1, APP, RNF4, EGLN3 and MCM2) genes based on centrality measures (Table 4.5), four genes were found to be significantly related to overall survival (CDH1, TRIM25, APP and ELAVL1) and a further two were associated with metastatic tumor phenotype (CDH1 and TRIM25). High expression of CDH1, APP and TRIM25 genes is associated with reduced overall survival (hazard-ratio: 0.7146, 0.6346 and 0.6054 respectively) and the reverse is true for ELAVL1 (hazard-ratio: 1.4731) (Figure 4.3 A-D). The enrichment of these genes in the various subnetworks is illustrated in Table 4.5 below. The plant-derived drugs/compounds display a differential targeting of these genes in the different breast cancer subtypes. For instance, CDH1 is only targeted by I3C in MDA-231 cell line and I3C has insignificant effect on TRIM25 in the same cell line. When the p-values and fold changes of these genes are checked, most are not differentially expressed and are rather introduced to the subnetworks as a result of the K parameter.

Table 4.5: Degree and betweenness centrality scores of subnetworks and the GO annotation of the top 5 high scoring genes. The table has been truncated to show only the top 10 genes from the network centrality analysis.

Cell line/Drug	Gene	Degree	Betweenness	Gene Annotation
MDA-157/I3C	HNRNPL	37	4424.167	heterogeneous nuclear ribonucleoprotein L
	TRIM25	21	2166.897	tripartite motif containing 25
	ESR2	18	2260.333	estrogen receptor 2
	CUL3	13	1284.508	cullin 3
	BAG3	6	827.1667	BCL2 associated athanogene 3
MDA-231/I3C	HNRNPL	47	5145.078	heterogeneous nuclear ribonucleoprotein L
	ELAVL1	24	2545.711	ELAV like RNA binding protein 1
	ESR2	22	2154.689	estrogen receptor 2
	CUL3	14	1563.87	cullin 3
	CDH1	10	1066.333	cadherin 1
MDA-231/WA	ELAVL1	213	204362.3	ELAV like RNA binding protein 1
	TRIM25	199	212504.2	tripartite motif containing 25
	ESR2	182	159988.9	estrogen receptor 2
	APP	156	149501.2	amyloid beta precursor protein
	RNF4	147	114686	ring finger protein 4
MDA-453/Actein	TRIM25	181	111875.336	tripartite motif containing 25
	ESR2	171	94044.89	estrogen receptor 2
	APP	168	114215.73	amyloid beta precursor protein
	EGLN3	152	66460.44	egl-9 family hypoxia inducible factor 3
	ELAVL1	148	98134.93	ELAV like RNA binding protein 1
MCF-7/I3C	ESR2	419	549778.8	estrogen receptor 2
	TRIM25	416	666503.6	tripartite motif containing 25
	ELAVL1	383	574226	ELAV like RNA binding protein 1
	HNRNPL	319	476118.7	heterogeneous nuclear ribonucleoprotein L
	APP	300	458041.3	amyloid beta precursor protein
MCF-7/WA	TRIM25	79	25750.4	tripartite motif containing 25
	ESR2	74	20150.8	estrogen receptor 2
	APP	69	23955.31	amyloid beta precursor protein
	ELAVL1	67	19761.62	ELAV like RNA binding protein 1
	MCM2	47	8673.256	minichromosome maintenance complex component 2
MCF-7/CKI	ELAVL1	235	450995	ELAV like RNA binding protein 1
	APP	192	331986.8	amyloid beta precursor protein
	HNRNPL	184	335120.6	heterogeneous nuclear ribonucleoprotein L
	TRIM25	180	301207.5	tripartite motif containing 25
	RNF4	127	189331.5	ring finger protein 4
ZR751/I3C	HNRNPL	268	385008.5	heterogeneous nuclear ribonucleoprotein L
	TRIM25	267	369696.4	tripartite motif containing 25
	ELAVL1	242	300361.7	ELAV like RNA binding protein 1
	APP	198	268438.6	amyloid beta precursor protein
	RNF4	157	160883.6	ring finger protein 4
T47D/I3C	TRIM25	338	481338.8	tripartite motif containing 25
	HNRNPL	330	518969.9	heterogeneous nuclear ribonucleoprotein L
	ELAVL1	311	449179.8	ELAV like RNA binding protein 1
	ESR2	292	347761.2	estrogen receptor 2
	APP	230	330949.3	amyloid beta precursor protein

A. CDH1



B. ELAVL1

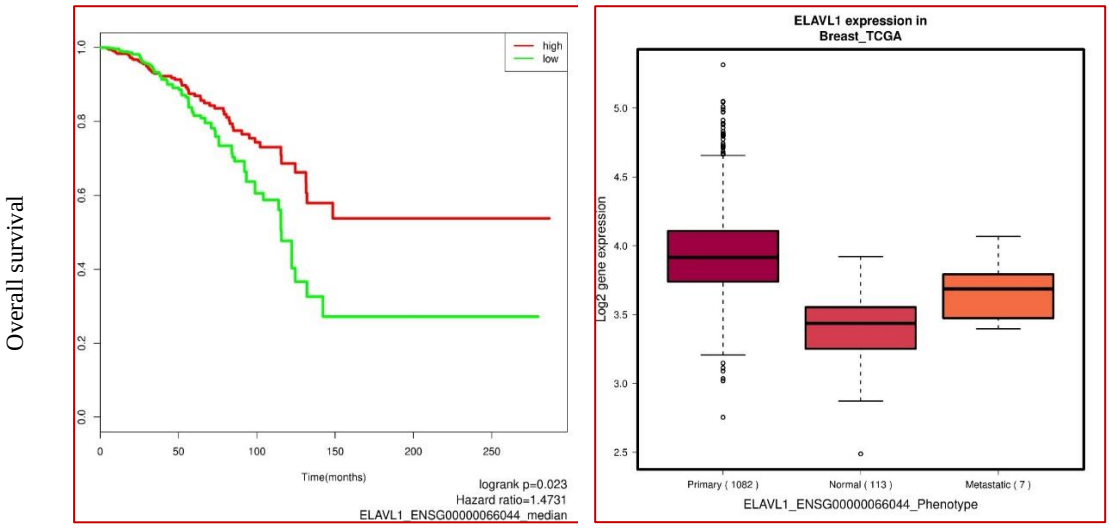
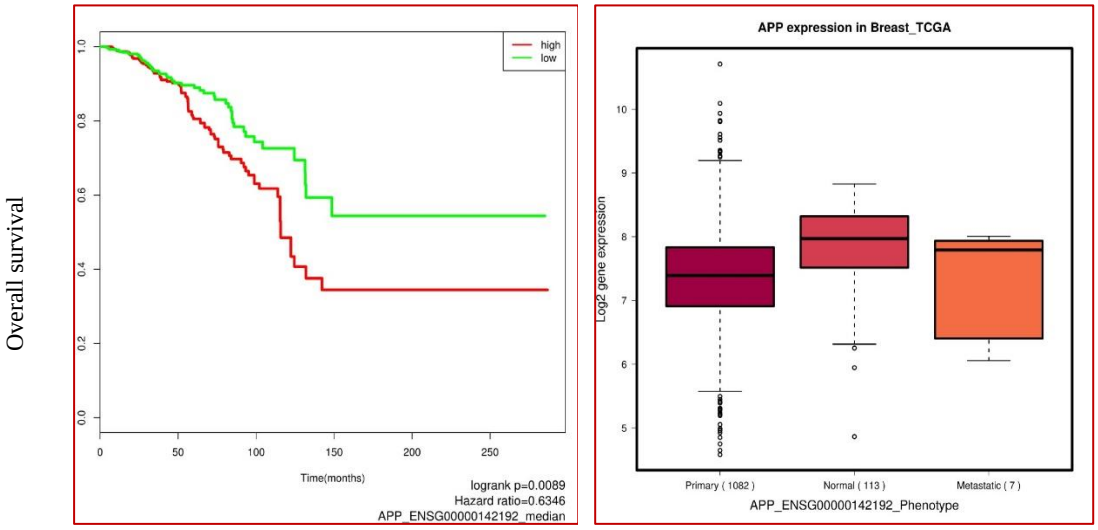


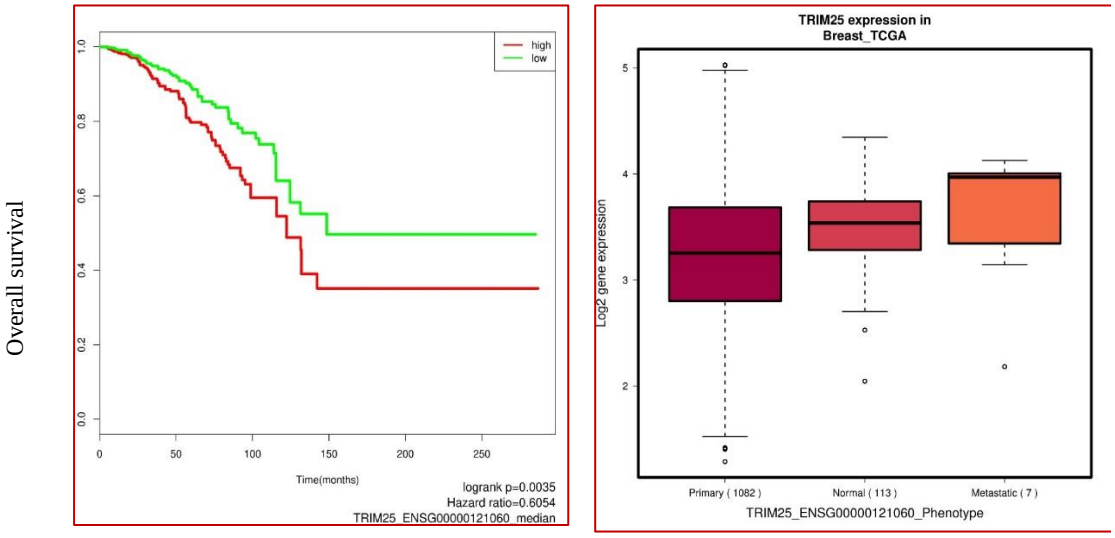
Figure 4.1: Survival and phenotype association analysis of A) CDH1, B) ELAVL1, C) APP and D) TRIM25 genes: Kaplan Meier plot and the corresponding hazard ratio for the probability of fatality and log rank p value illustrating the significance of the difference in overall survival between the high and low gene expressors; and boxplot of gene-phenotype association for primary, normal and metastatic tumors ($P < 0.05$).

Continuation of Figure 4.1

C. APP



D. TRIM25

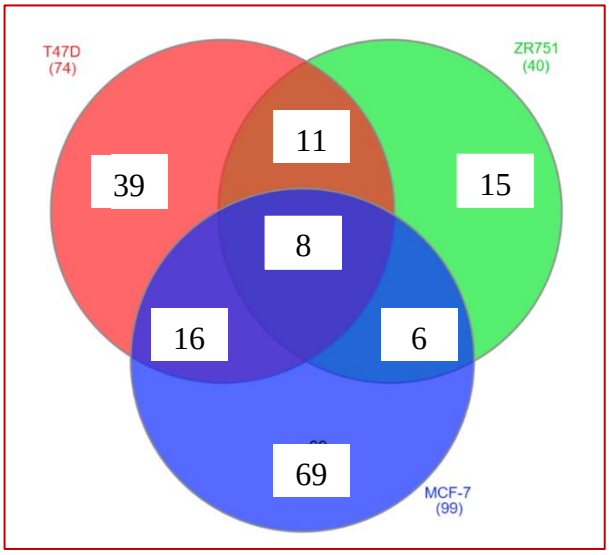


4.5. Plant-derived drugs/compounds display pleiotropic effects by targeting multiple carcinogenesis related oncogenic signaling pathways in TNBC, LABC and HER2+ breast cancer

To determine the enriched signal transduction pathways in order to provide a biological inference, the respective subnetwork genes were used to perform pathway enrichment analysis using EnrichR package [93] in RStudio.

Overall, significant enrichment (FDR<0.05) of signal transduction pathways perturbed by actein, CKI, I3C and WA in the different subnetworks were found. Supplementary Tables 1.1-1.27 details the pathway enrichment results from KEGG, Reactome, WikiPathways and GO Biological process [93]. The presented pathways belong to the main oncogenic signaling pathways [6] and both downstream effector and perceived cross-talk pathways [26], [99]–[101]. The pathways are arranged based on the FDR values.

A. LA under I3C



B. TN under I3C

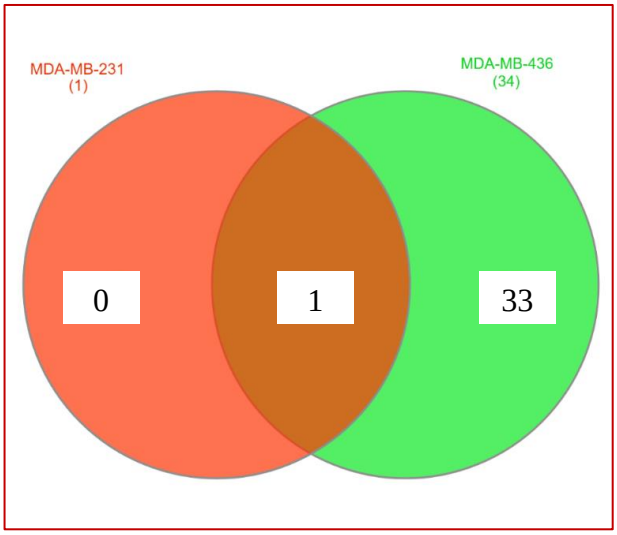
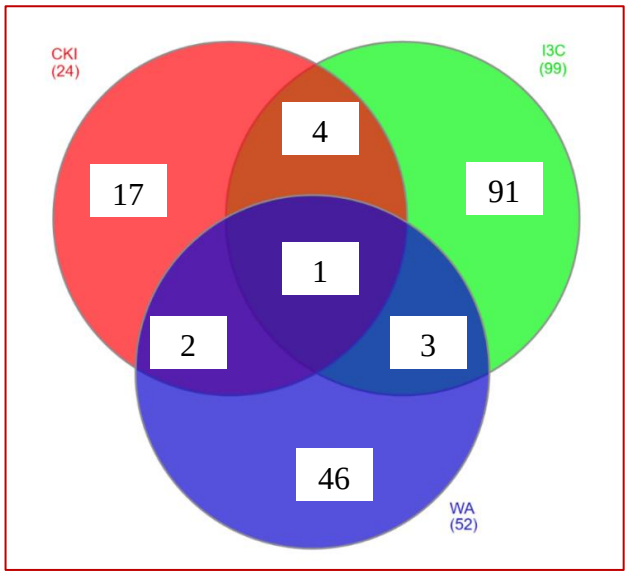


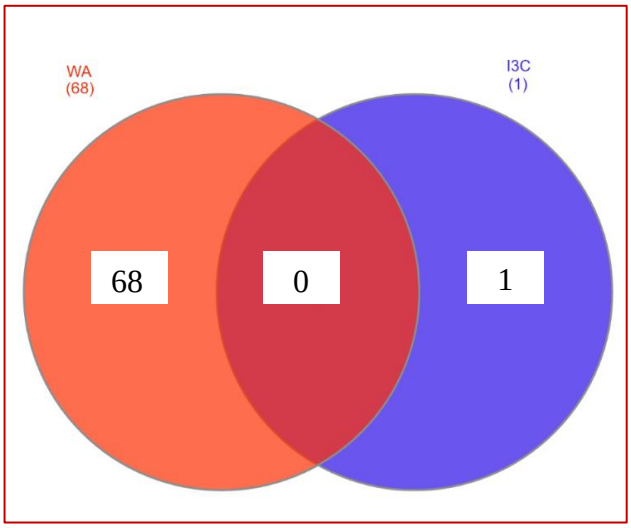
Figure 4.2: Venn diagrams showing the number of signal transduction terms enriched in the related subnetworks. A) LA under I3C, B) TN under I3C C) MCF-7 targeted pathways by WA, I3C and CKI, D) MDA-MB-231 under I3C and WA.

Continuation of Figure 4.1

C. MCF-7 under CKI, I3C and WA



D. MDA-MB-231 under WA and I3C



More oncogenic signaling pathways are targeted in LA (Figure 4.4A) than in TN (Figure 4.4 B) under I3C. Comparatively, the greatest number of targeted unique pathways in LA are in MCF-7 (69) then T47D (39) and ZR751 (15) respectively. Additionally, more common targeted pathways are between MCF-7 and T47D (16), followed by T47D and ZR751 (11) and ZR751 and MCF-7 (6) (Figure 4.4A). MCF-7 under I3C, CKI and WA shows that 1 pathway is targeted by such a combination and I3C targets the greatest number of unique pathways (91), followed by WA (46) and CKI (17). CKI and I3C targets the highest number of common pathways (4), followed

by I3C and WA (3) and WA and CKI (2) (Figure 4.4C). On the other hand, no common signaling pathway is targeted by I3C and WA in MDA-MB-231, with each drug targeting unique pathways and WA targets the highest number (68 versus 1) (Figure 4.4D).

Intriguingly, no significant signal transduction term in MDA-MB-157 subnetwork under I3C was found. TN subtype is one of the most clinically aggressive breast cancers and the observed phenomenon is acceptable [77]. Clearly, therefore, I3C does not target genes coding for oncogenic signaling pathway related proteins in this cell line.

To simplify the enrichment analysis results, the pathways were grouped based on the biological roles of the enriched genes in regulating a carcinogenesis process. Carcinogenesis is known to go through three main phases: initiation, promotion and finally progression [102]. These phases are occasioned by genetic and epigenetic dysregulations closely related to cell cycle (proliferation) and death, inflammation, metastasis and angiogenesis. These four groups represent the main biological processes associated with carcinogenesis and are directly/indirectly regulated by the main known oncogenic signaling related pathways. Furthermore, these are the main indicators of effectiveness in ascertaining the biological effect of a drug candidate. To determine whether a pathway is activated or inactivated, the pathway enrichment results from the up-/down-regulated subnetwork genes was used. The Table 4.6 below details the oncogenic signaling pathways under the different carcinogenesis processes. It can be seen that cell proliferation and death has the highest number of perturbed pathways across all the drugs/compounds studied. Detailed information used to generate this table is available externally as supplementary Table 1.10-1.27. The most targeted carcinogenesis process across all the datasets is cell cycle/proliferation and apoptosis through regulation of related oncogenic signaling pathways (Figure 4.5).

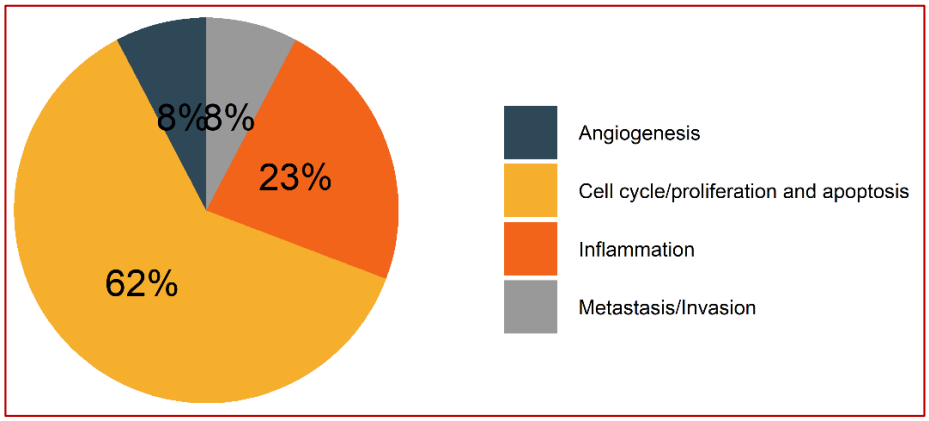
Table 4.6: Oncogenic signaling pathway-carcinogenesis process grouping. This table illustrates the different carcinogenesis processes they are involved in based on the enriched genes as well as whether the genes are up-/down-regulated. U: up, D: down.

Drug/ Compound	**Cell Line**	**Carcinogenesis Process**			
Actein		**Cell cycle/Proliferation and Apoptosis**	**Metastasis and invasion**	**Inflammation**	**Angiogenesis**
	MDA-MB-453	TGF-(D) Apoptosis(D) Cell cycle(D) INF(D) FasL(D)	-	-	-
		PI3K-Akt-mTOR(U) NRF2(U)	-	-	-
CKI	MCF-7	ATM(D) P53(D) Intrinsic apoptosis(D)	-	-	-
		FoxO(U) PI3K-Akt-mTOR(U) ERBB/EGFR(U) NRF2(U) ATM(U)	TGF-beta(U)	BCR(U) TCR(U) FCR(U)	VEGFA-VEGFR2(U)
WA	MCF-7	ATM (D) P53 (D) AMPK (D)	Rho GTPase (D)	Cytokine (D)	TGF-beta (D)
		NRF2 (U) MAPK (U) P53 (U) Intrinsic apoptosis (U)	-	-	-
	MDA-MB-231	NRF2(D) FoxO (D) ATM (D) MAPK (D) AMPK (D) ErbB (D) P53 (D) TGF-beta (D) Notch (D)	Wnt (D)	IL-4, IL-17 (D)	-
		PI3K-Akt-mTOR (U)	-	INF gamma (U) TNF (U)	VEGF (U) Notch (U) TGF-beta (U) HIF-1 (U)

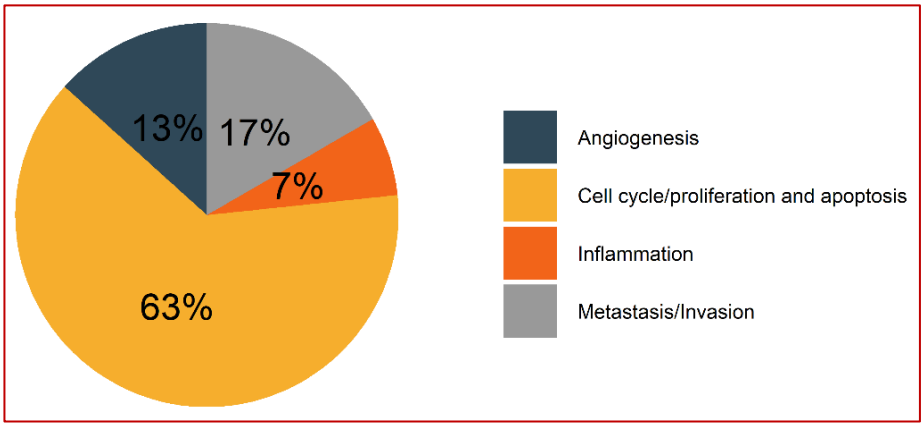
Continuation of Table 4.6

I3C	MCF-7	P53 (D) EGFR/ErbB4(D) Apoptosis(D) PI3K-Akt-mTOR(D) MAPK(D) Wnt(D)	TGF-beta(D)	TCR(D) IFN(D) TNF-alpha(D) BCR(D)	-
		Apoptosis(U) Cell cycle(U)	-	-	-
	T47D	Cell cycle, G2/M (D) ErbB4(D) PI3K-Akt-mTOR(D)	Notch1(D) Wnt(D) TGF-beta(D)	Chemokine IFN-1, IL-7(D)	Rho GTPase(D) VEGFA-VEGFR2(D) PDGF(D)
		RIG-I like receptor(U) Genotoxicity(U) Apoptosis (U) ATM(U) MAPK(U) IFN(U) TGF-beta(U)	-	-	-
	ZR751	ATM(D) EGF(D) FasL(D) Notch(D) TGF-beta(D) Apoptosis(D) RIG-I(D)	Wnt(D)	-	VEGF(D)
		IFN(U) NRF2 Apoptosis(U) MAPK(U)	-	-	-
	MDA-MB-231	-	Hippo (D)	-	VEGFA-VEGFR2(D)
		NRF2 (U)	-	-	-
	MDA-MB-157	-	-	-	-
		-	-	-	-
	MDA-MB-436	EGFR/ErbB (D) PI3K-Akt(D) MAPK(D)	Rho GTPase(D) Wnt(D) Hippo(D)	TCR(D)	PDGF(D) TGF-beta(D)
		Apoptosis (U) TGF-beta(U)	-	TLR4(U)	-

A. CKI on MCF-7



B. I3C on LA



C. I3C on TN

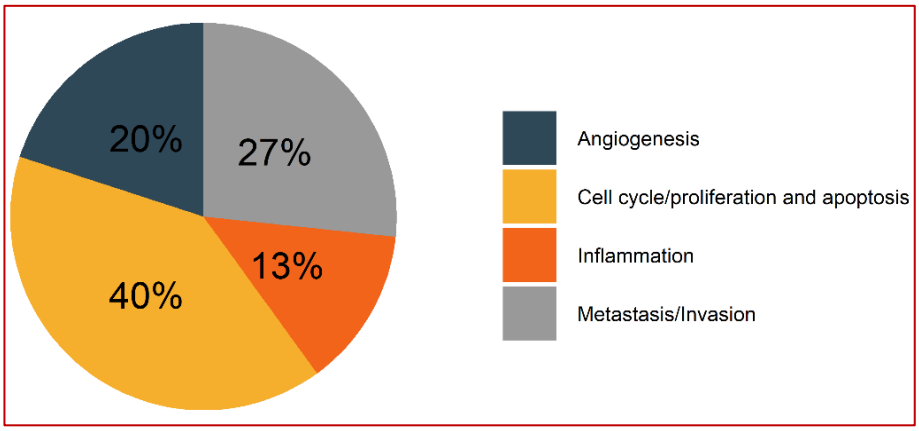
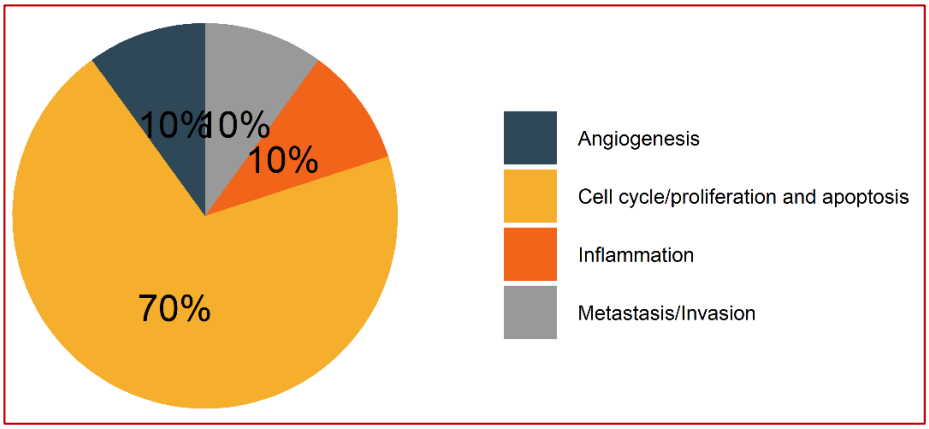


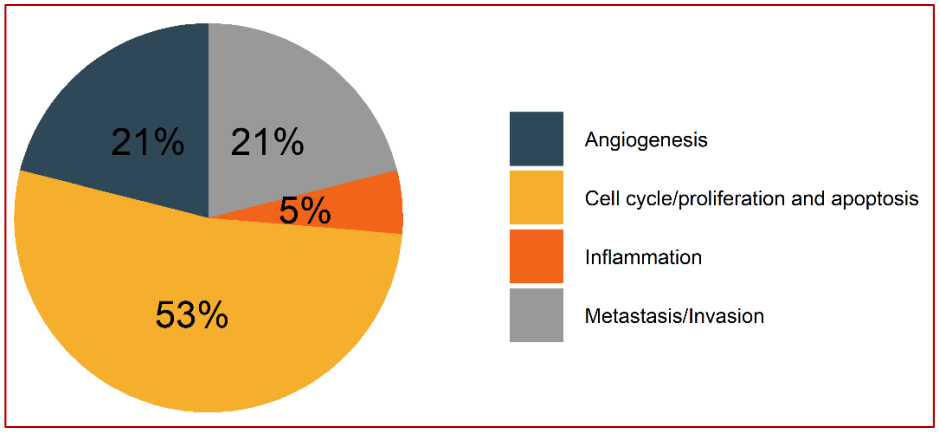
Figure 4.3: Pie charts illustrating the proportion of oncogenic signaling pathways involved in the regulation of angiogenesis, cell cycle/proliferation and apoptosis, inflammation and metastasis/invasion as determined from enrichment analysis of A) MCF-7 under CKI, B) LA under I3C, C) TN under I3C, D) MCF-7 under WA and E) MDA-MB-231 under WA.

Continuation of Figure 4.3

D. WA on MCF-7



E. WA on MDA-MB-231



4.6. I3C targets metastasis/invasion regulating hippo signaling network in TN

From the pathway enrichment, it was apparent that TN cell lines were comparatively less affected by I3C treatment at the subnetwork level (Table 4.3 and Supplementary Tables 1.6 and 1.7). The common targeted oncogenic signaling pathway between MDA-MB-231 and MDA-MB-436 was identified to be 'hippo signaling pathway' (Figure 4.2B). To understand the regulatory mechanism of this pathway in the two cell lines further, the gene expression fold changes of the enriched genes in the pathway were plotted (Figure 4.4). GNAQ, PRKCA and PRKCE genes

are commonly deregulated in the two cell lines. This observation illustrates a distinct cancer subtype-specific targeting of the metastasis/invasion regulating pathway by I3C.

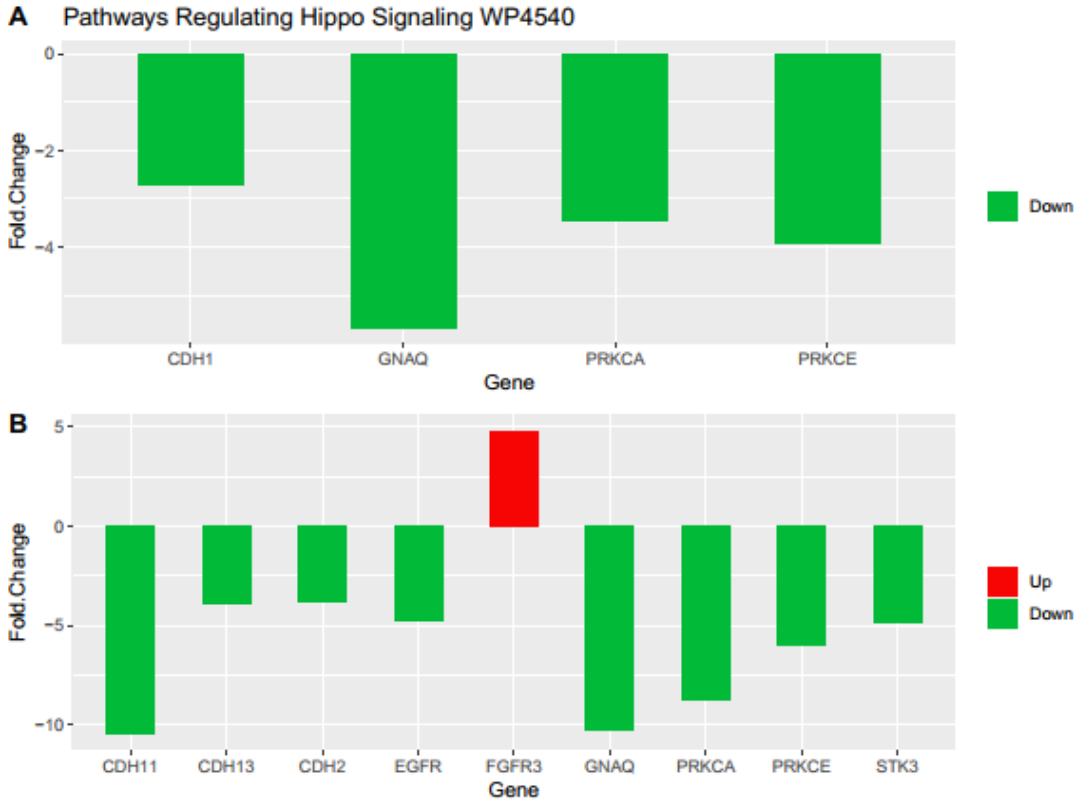


Figure 4.4: Bar chart plots illustrating differential gene regulation of Hippo signaling in MDA-MB-231 (A) and MDA-MB-236 (B) under I3C.

4.7. Analysis of common pathways in MCF-7 under I3C, WA and CKI identifies TGF-beta pathway as the common oncogenic signaling pathway.

Anti-cancer drug combinations to enhance therapeutic efficacy in drug resistant cancer types is a common clinical application. In this approach, drugs targeting different carcinogenesis pathways are used together to prevent tumor cell survival from alternate pathways [52], [59], [103]. Thus, to investigate a potential drug/compound combination that could synergistically/antagonistically target oncogenic signaling pathways in LA MCF-7 cell line, common pathways were identified. The Venn diagram (Figure 4.2C) indicates that combined, the three drugs/compounds target TGF-beta signaling pathway. Paradoxically, the enriched genes in this pathway under different drugs show differential deregulation of

metastasis and invasion (CKI and I3C) and angiogenesis (WA) carcinogenesis processes (Table 4.6). Conspicuously, there are unique pathways targeted by the different drugs/compounds in the same cancer subtypes (MCF-7) (Figure 4.4C).

5. DISCUSSION

In the past decade, the gradual paradigm shift from reductionism to system pharmacology approaches in drug discovery, repurposing and evaluation has accelerated the elucidation of drug mechanism of action. This shift has been supported by the appreciation of the need to treat a disease pathology as a system rather than focusing on single genetic targets driving disease-phenotype in multi-factorial/complex diseases such as cancer [52], [53], [55]. Therefore, multi-targeting of oncogenic signaling pathways is a common strategy. Fortunately, the discovery of poly-pharmacologic effects of plant-derived drugs/compounds from separate reductionist experiments begs the question as to their systemic effects on oncogenic pathways in multifactorial diseases. To date, with the need to account for all the molecular players under a drug treatment, several data integration methods have been proposed. Thus, the reconstruction of drug perturbed subnetworks is possible and has been previously explored [55], [58], [104]. While separate studies have reported targeting of several genes in different signal transduction networks by plant derived drugs [59], [104], leveraging the current technological advances in protein-protein interaction network extraction methods from omics data is still less well explored. In light of this, a *de novo* network enrichment method and gene-pathway enrichment methods have been used to illuminate the pleiotropic effects of plant-derived drugs on signal transduction pathways in LA, TN and HER2+ breast cancers. The comprehensive analysis of the most frequently altered oncogenic signaling pathways as reported in TCGA have been used to guide this discussion [6].

Drug/compound-specific perturbed subnetworks for LA, TN and HER2+ breast cancer subtypes were reconstructed by integrating the related transcriptome data with BioGRID [64] protein-protein interaction network. These subnetworks possess different topological features that are drug/compound and cell-type specific. This difference in the number of genes and interactions in the networks indicates differential targeting of these cells based on the underlying genetic and pathologic differences (Table 3.1).

Network topology-based centrality analysis identified the most connected genes in the subnetworks for all the cell lines studied. These genes also represent the information transit nodes in the respective subnetworks (Table 3.5). Subsequent

survival analysis using Kaplan-Meier plot and phenotype association prediction from box-plots revealed biologically important genes: TRIM25 [105], CDH1 [106] and APP [107]. ELAVL1 gene is responsible for the expression of the RNA-binding HuR protein and has been shown to be involved in multiple processes associated with carcinogenesis [108]. Significant high expression of CDH1, APP and TRIM25 genes is associated with reduced overall survival. Conversely, significant low expression of ELAVL1 is associated with low overall survival (Figure 3.3 A-D). These findings are in agreement with previous observations on the biological role of these genes in cancer [111-114] and indicate that the studied drugs perturb the underlying diseasome network by targeting known oncogenesis specific genes and their interacting genes.

Actein is one of the least comprehensively studied plant derived compounds, but has recently attracted attention in breast cancer due its effects on various biological processes in cancer [42]–[44], [94], [109]. In this work, actein is shown to target 48 oncogenic signaling pathways in HER2+ (MDA-MB-453) subtype, of which 5 (Cell cycle, PI3K-Akt-mTOR, EGFR and TGF-beta) are canonical oncogenic pathways [6]. The enriched genes in these pathways mainly regulate cell proliferation and death (Table 3.6). Network analysis reveals that cell death and cell cycle arrest-related genes in TGF-beta, PI3K-Akt-mTOR and NRF2 pathways are up-regulated while cell cycle and proliferative genes in TGF-beta are down-regulated (Table 3.6). Additionally, the tumor microenvironment regulation through cytokine signaling represented by interferon signaling pathway is down-regulated (Table 3.6). In agreement with this explanation, in reports on breast and other cancers, actein has been shown to target apoptosis [43], [44], cell adhesion [109] and migration [43], [109]. Thus, from these findings, actein mainly targets cell proliferation and apoptosis, regulating signal transduction pathways in HER2+ breast cancer.

CKI is a formulation with 22 chemical constituents [45]. The molecular effects of compound kushen injection on breast cancer has largely provided mixed results [110]. At the network level, CKI perturbs 24 pathways. P53 (down-regulated), RTK/RAS (EGFR, p38 and ErbB), PI3K-Akt-mTOR, NRF2 and TGF-beta (up-regulated) (Table 3.6) are the defined canonical oncogenic signaling pathways [6] in MCF-7. Based on enriched genes, these pathways regulate cell proliferation and apoptosis (P53, RTK/RAS, PI3K-Akt-mTOR and NRF2) and metastasis/invasion (TGF-beta) (Table 3.6). Moreover, CKI also targets angiogenesis and tumor microenvironment regulating pathways through VEGFA/VEGFR2 and cytokine

signaling (B cell receptor, T cell receptor and FC-epsilon signaling) respectively (Table 3.6), which is consistent with a previous report [111]. The down-regulation of P53 pathway is in line with a previous observation of P53 independent apoptotic cell death [95]. Reports from other groups have shown that CKI directly regulates hepatocellular carcinoma (HCC) cell proliferation [45], cell migration in HCC, colon and breast cancer [45], [111]; and apoptosis in breast cancer [111]. The results in this work, therefore, extends the global effects of CKI on signal transduction pathways associated with MCF 7 cells, and defines cell cycle/proliferation and apoptosis, metastasis/invasion, inflammation and angiogenesis as targeted carcinogenesis processes (Table 3.6).

Indole-3-carbinol is a widely studied plant phytohormone and its effectiveness is well defined in ER driven cancers [40], [97], [112], [113]. Thus, it is not surprising to see higher number of signal transduction terms in LA than TN subtypes. Importantly, in LA, TGF-beta, Notch, cell cycle and Wnt were identified as the commonly targeted oncogenic signaling pathways (Figure 3.4A). These pathways regulate cell proliferation and apoptosis (Wnt, cell cycle, Notch and TGF-beta) and invasion/metastasis (TGF-beta, Wnt and Notch) (Table 3.6). Taking into account the enriched genes in the different pathways, dramatic observations can be made on TGF-beta, whose metastasis/invasion promoting genes are down-regulated in T47D and MCF-7 while cell death related genes are up-regulated in T47D and down-regulated in ZR751 (Table 3.6). Thus, I3C targets 8 signaling pathways to exert its anti-cancer activity across LA subtype. These pathways are spread across cell cycle/proliferation and apoptosis, metastasis/invasion, inflammation and angiogenesis as targeted carcinogenesis processes (Table 3.6).

The role of I3C on TN is less well elaborated and was noted to be less effective [97]. Indeed, in this study no oncogenic signaling pathway was enriched in MDA-MB-157 subnetwork; illustrating an I3C non-responsive subtype at the protein-protein interaction network level.

An important finding in the responsive TN cells is the common targeting of invasion/metastasis processes through down-regulation of the hippo signaling pathway in MDA-MB-231 and MDA-MB-436 (Figure 3.4). Further, I3C perturbs this pathway through CDH1, GNAQ in MDA-MB-231, CDH11, CDH13, CDH2, EGFR, FGFR3 and STK3 in MDA-MB-436 and commonly through GNAQ, PRKCA and PRKCE. The effect of I3C on cancer cell invasion has been reported for LA elsewhere [114].

Overall, more MDA-MB-436 signaling pathways are targeted by I3C (Supplementary Table 3.7), and these pathways control carcinogenesis by regulating cell cycle/proliferation and apoptosis, metastasis/invasion, inflammation and angiogenesis processes (Table 3.6).

The characteristic anti-cancer effects of Withaferin A are well anchored in scientific reports [48], [49], [79], [96], [115]–[122]. Multiple carcinogenesis processes have been proposed to be affected in breast cancer by WA [49], [51], [96], [115], [116]. Here, MAPK, TGF-beta, NRF2 and P53 oncogenic signaling pathways are targeted in both TN and LA (Table 3.6). Differential targeting of Wnt, Notch, VEGFA-VEGFR2 and PI3K-Akt-mTOR in TN and cytokines in LA was identified (Table 3.6). Moreover, WA also targets cytokine mediated signaling in both cells (Table 3.6). The up-regulation of NRF2 pathway genes (Table 3.6) is consistent with in vivo findings of induction of oxidative stress in the two cell lines [49], [123]. These results illustrate multi-targeting of several carcinogenesis processes including cell proliferation and death, inflammation, metastasis/invasion and angiogenesis (Table 3.6) in both TNBC and LABC to produce the phenotypes anchored in *in vitro* studies.

Drug combination in cancer is a novel and common approach in targeting multiple oncogenesis processes, especially in resistant cancer subtypes. This is currently highly pursued approach in network medicine [52], [69], [124], [125]. In this work, the common oncogenic signaling pathways targeted by CKI, I3C and WA in MCF-7 were determined to be TGF-beta (Figure 3.4C); while no common pathway was found for I3C and WA in MDA-MB-231 cell line (Figure 3.4D). Given the independent targeting of oncogenic pathways in MDA-MB-231, the two drugs could complement each other to regain control of multiple signaling pathways that are independently targeted. However, given the complex nature in pharmacodynamics and pharmacokinetics under multiple drug conditions, it can be hypothesized that different targeted pathways could be obtained from combination studies.

Even though the current approach applied involves using one omic level data (gene expression) to make systemic inference, this study has laid the foundation for future studies in biological data integration and perturbed oncogenic signal transduction pathway inference from protein-protein interaction network reconstruction. The differential effects observed across the different tumor subtypes under different drugs and literature concurrence illustrate the robustness of the implemented method. Whether the demonstrated perturbations are as a result of direct

drug-protein(s) interaction or drug-rate-limiting protein (network hub protein) interaction cannot be clearly deduced from this work and demands future attention.

6. CONCLUSION

In this thesis work, the network/systemic molecular effects of actein, indole-3-carbinol, compound kushen injection and withaferin A, as plant derived drugs, on oncogenic signaling pathways in triple negative, luminal A and HER2-positive breast cancer have been reconstructed from transcriptome data and protein-protein interaction network. It has been established that these drugs/compounds have multiple oncogenic-related signaling pathway targets, and the enriched proteins in the pathways interact in a network. Also, the targeted signaling pathways are tumor subtype specific. Therefore, this method of network mapping could facilitate prioritisation of anti-cancer drug/compounds based on the underlying perturbed oncogenic signaling pathway. Furthermore, the mechanism of action and molecular targets of a drug/compound can be elucidated using this approach. Across the datasets analysed, differential cell response was observed. This means that for every treatment/experimental approach, it is important to treat each sample as unique and avoid generalization bias.

7. RECOMMENDATIONS

In this study PPIN has been used to infer perturbed signaling networks. However, signaling pathways do not only affect protein expression. Thus, given the complexity and interrelatedness of the different OMICs data, further integrative work is required to definitively ascertain the different observations on the role of the drugs studied in this work. Also, given the tissue specific differential gene expression patterns, a more accurate predictive study would employ a breast specific protein-protein interaction network as well as considering their phosphorylation status since active/active signaling proteins are post-translationally modified. Such PPINs can be derived through text mining coupled with manual curation from experimental observations. Equally important is the delineation of a pathway activity under drug treatment, which was painstakingly manually done in this study. Towards this, the application of machine learning algorithms, as has been applied in other biological studies [70], [126], can be utilized to scientifically predict the pathway status by taking experimental evidence from perturbation experiments.

Greedy algorithm is believed to be efficient in subnetwork extraction. Much more accurate algorithm in KPM is ACO, though it is computationally intensive and hence not preferable [71], [88]. Again, one limitation of the KPM approach is the fact that network scoring is based on binary matrix for gene expression. An alternative to this approach is the Heinz algorithm in BioNet which scores a network based on the false discovery of gene expression, effectively ignoring the direction of the gene expression. Therefore, a solution to this would be implementing a scoring algorithm which both factors in the magnitude of the false discovery rate and direction and magnitude of gene fold change. This would take into account the fact that some proteins contribute more to a drug-effect network phenotype than the rest.

Given that this is the first attempt to systemically evaluate the role of plant-derived drugs on oncogenic signaling pathways, future studies should strive to integrate drug-ligand binding and cell fate observations to authoritatively determine the mechanism of action of a drug.

One of the main resources used in this work is the curated signaling pathway from KEGG, WikiPathways, Reactome and GO Biological Processes. While they are instrumental in pathway enrichment analysis in the determination of underlying

molecular process, their main problem is redundancy. Each of these databases classify proteins under pathway names which might be similar, different or subsets of major known biological pathways. It is thus imperative that future studies should seek to resolve cross-database pathway enrichment analysis.

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BIOGRAPHY

Regan Odongo was born in 1989 in Kenya. He graduated with a BSc in Biochemistry and thereafter worked for two years in the pharmaceuticals sector. Regan would like to study biological networks and their applications in systems pharmacology for targeted therapeutics for oncogenic signal transduction pathways in cancer and cancer stem cells, computational prediction of pathways' role in cell fate through biomedical data integration methods.

APPENDICES

Appendix A: Related conference attendance

Odongo, R., Çakır, T., Demiroğlu-Zergeroğlu, A., (2019), "Mapping Breast Cancer Transcriptome Data onto PPI Network Reveals Multi-targeting of Carcinogenesis Related Signal Transduction Pathways by I3C", Lisansüstü Araştırmalar Sempozyumu ve Tanıtım Günleri, 150, Gebze Technical University, Gebze, 17-18 June.

Appendix B: Supplementary tables

Tables of pathway enrichment analysis for the different extracted subnetwork genes

Table B3.1: Actein targeted signal transduction pathways in the subnetwork for HER2+ MDA-MB-453 cell line.

Signaling Pathways	**FDR**
regulation of G2/M transition of mitotic cell cycle (GO:0010389)	0.000164
regulation of mitotic cell cycle (GO:0007346)	0.000214
ATR Signaling WP3875	0.000249
regulation of cell cycle (GO:0051726)	0.000404
positive regulation of cell cycle arrest (GO:0071158)	0.001027
ATM Signaling Network in Development and Disease WP3878	0.001057
apoptotic process (GO:0006915)	0.001057
regulation of apoptotic process (GO:0042981)	0.002147
miRNA regulation of p53 pathway in prostate cancer WP3982	0.003818
APC/C-mediated degradation of cell cycle proteins_Homo sapiens_ R-HSA-174143	0.004199
Regulation of mitotic cell cycle_Homo sapiens_R-HSA-453276	0.004199
positive regulation of apoptotic process (GO:0043065)	0.004567
positive regulation of mitotic cell cycle phase transition (GO:1901992)	0.004940
regulation of mitotic cell cycle spindle assembly checkpoint (GO:0090266)	0.005245
positive regulation of mitotic cell cycle (GO:0045931)	0.006040
regulation of metaphase/anaphase transition of cell cycle (GO:1902099)	0.006306
Interferon Signaling_Homo sapiens_R-HSA-913531	0.007398
TGF-beta Signaling Pathway WP366	0.010990
EGF/EGFR Signaling Pathway WP437	0.010990
PI3K-AKT-mTOR signaling pathway and therapeutic opportunities WP3844	0.011528
mir-124 predicted interactions with cell cycle and differentiation WP3595	0.011529
negative regulation of pathway-restricted SMAD protein phosphorylation (GO:0060394)	0.012793
intrinsic apoptotic signaling pathway in response to DNA damage (GO:0008630)	0.013892
Apoptosis WP254	0.018195
Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling WP3612	0.018682
Downregulation of TGF-beta receptor signaling_Homo sapiens_ R-HSA-2173788	0.018719
Target Of Rapamycin (TOR) Signaling WP1471	0.021716
Notch Signaling Pathway WP61	0.022409
extrinsic apoptotic signaling pathway (GO:0097191)	0.025832
Genotoxicity pathway WP4286	0.025839
regulation of cell cycle checkpoint (GO:1901976)	0.027681
positive regulation of extrinsic apoptotic signaling pathway via death domain receptors (GO:1902043)	0.027681
regulation of G1/S transition of mitotic cell cycle (GO:2000045)	0.035702
negative regulation of intracellular signal transduction (GO:1902532)	0.036064
negative regulation of G1/S transition of mitotic cell cycle (GO:2000134)	0.037442

Continuation of Table B3.1

negative regulation of B cell apoptotic process (GO:0002903)	0.038684
regulation of intrinsic apoptotic signaling pathway (GO:2001242)	0.038727
Fas Ligand (FasL) pathway and Stress induction of Heat Shock Proteins (HSP) regulation WP314	0.041397
TGF-beta receptor signaling activates SMADs_Homo sapiens_R-HSA-2173789	0.041659
NRF2 pathway WP2884	0.042693
Signaling by TGF-beta Receptor Complex_Homo sapiens_R-HSA-170834	0.045418
positive regulation of G2/M transition of mitotic cell cycle (GO:0010971)	0.048516

Table B3.2: CKI targeted signaling pathways in the subnetwork for LA MCF-7 cell line

Signaling Pathways	**FDR**
ATM Signaling Pathway WP2516	0.000226
EGF/EGFR Signaling Pathway WP437	0.0017
EPHB-mediated forward signaling_Homo sapiens_R-HSA-3928662	0.00364
PI3K-AKT-mTOR signaling pathway and therapeutic opportunities WP3844	0.009488
Aryl Hydrocarbon Receptor Pathway WP2873	0.010227
Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling WP3612	0.010227
NRF2 pathway WP2884	0.01023
ErbB Signaling Pathway WP673	0.011746
VEGFA-VEGFR2 Signaling Pathway WP3888	0.012339
TGF-beta Signaling Pathway WP366	0.012727
EPH-Ephrin signaling_Homo sapiens_R-HSA-2682334	0.013565
RAC1/PAK1/p38/MMP2 Pathway WP3303	0.016496
Tie2 Signaling_Homo sapiens_R-HSA-210993	0.018594
ERBB signaling pathway (GO:0038127)	0.02541
transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)	0.028166
ephrin receptor signaling pathway (GO:0048013)	0.028166
G13 Signaling Pathway WP524	0.031902
Canonical and Non-Canonical TGF-B signaling WP3874	0.03503
Constitutive Signaling by EGFRvIII_Homo sapiens_R-HSA-5637810	0.03531
Signaling by EGFRvIII in Cancer_Homo sapiens_R-HSA-5637812	0.03531
Signaling by ERBB2_Homo sapiens_R-HSA-1227986	0.03788
Signaling by FGFR3 fusions in cancer_Homo sapiens_R-HSA-8853334	0.041502
B cell receptor signaling pathway	0.044712
p53 signaling pathway	0.048133

Table B3.3: I3C targeted signaling pathways in the subnetwork for LA MCF-7 cell line

Signaling Pathways	**FDR**
TGF-beta Signaling Pathway WP366	0.000228
Signaling by Rho GTPases_Homo sapiens_R-HSA-194315	0.000235
regulation of mitotic cell cycle (GO:0007346)	0.000258
Apoptosis_Homo sapiens_R-HSA-109581	0.000367
positive regulation of apoptotic signaling pathway (GO:2001235)	0.000386
regulation of mitotic cell cycle phase transition (GO:1901990)	0.000422
Signaling by the B Cell Receptor (BCR)_Homo sapiens_R-HSA-983705	0.000663
Fas Ligand (FasL) pathway and Stress induction of Heat Shock Proteins (HSP) regulation WP314	0.000904
Apoptosis WP254	0.001253
G2/M transition of mitotic cell cycle (GO:0000086)	0.001308
intrinsic apoptotic signaling pathway (GO:0097193)	0.001334
cell cycle G2/M phase transition (GO:0044839)	0.001349
Signaling by EGFR_Homo sapiens_R-HSA-177929	0.001441
Signaling by NOTCH_Homo sapiens_R-HSA-157118	0.001441
Signaling by TGF-beta Receptor Complex_Homo sapiens_R-HSA-170834	0.001622
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest (GO:0006977)	0.001823
Signaling by ERBB4_Homo sapiens_R-HSA-1236394	0.001895
cell cycle G1/S phase transition (GO:0044843)	0.001904
signal transduction involved in mitotic G1 DNA damage checkpoint (GO:0072431)	0.002119
negative regulation of apoptotic process (GO:0043066)	0.002356
G1 to S cell cycle control WP45	0.002431
DNA damage response, signal transduction by p53 class mediator (GO:0030330)	0.002559
Downstream signaling events of B Cell Receptor (BCR)_Homo sapiens_R-HSA-1168372	0.002593
Signaling by NOTCH1_Homo sapiens_R-HSA-1980143	0.003091
Insulin Signaling WP481	0.003170
Signaling by SCF-KIT_Homo sapiens_R-HSA-1433557	0.003289
negative regulation of cell cycle (GO:0045786)	0.003462
Cell Cycle Checkpoints_Homo sapiens_R-HSA-69620	0.003554
Apoptosis Modulation by HSP70 WP384	0.003824
T-Cell Receptor and Co-stimulatory Signaling WP2583	0.004260
Apoptosis-related network due to altered Notch3 in ovarian cancer WP2864	0.005823
Fc epsilon receptor (FCERI) signaling_Homo sapiens_R-HSA-2454202	0.006553
positive regulation of cell cycle arrest (GO:0071158)	0.006592
regulation of G2/M transition of mitotic cell cycle (GO:0010389)	0.006793
IL-4 Signaling Pathway WP395	0.006958
Signaling by FGFR3_Homo sapiens_R-HSA-5654741	0.007524
Signaling by FGFR_Homo sapiens_R-HSA-190236	0.007820

Continuation of Table B3.3

Notch Signaling Pathway WP61	0.0080173
negative regulation of mitotic cell cycle (GO:0045930)	0.0091501
Signaling by FGFR2_Homo sapiens_R-HSA-5654738	0.0092866
G1/S transition of mitotic cell cycle (GO:0000082)	0.0105152
Signaling by PDGF_Homo sapiens_R-HSA-186797	0.0110098
G13 Signaling Pathway WP524	0.0112213
Signaling by FGFR4_Homo sapiens_R-HSA-5654743	0.0113228
regulation of canonical Wnt signaling pathway (GO:0060828)	0.0116853
Apoptosis Modulation and Signaling WP1772	0.0132289
Signaling by Wnt_Homo sapiens_R-HSA-195721	0.0134704
Signaling by FGFR1_Homo sapiens_R-HSA-5654736	0.0137164
H19 action Rb-E2F1 signaling and CDK-Beta-catenin activity WP3969	0.0137862
Downstream signaling of activated FGFR2_Homo sapiens_R-HSA-5654696	0.013795
epidermal growth factor receptor signaling pathway (GO:0007173)	0.0157379
Downstream signaling of activated FGFR1_Homo sapiens_R-HSA-5654687	0.0159297
PI3K-AKT-mTOR signaling pathway and therapeutic opportunities WP3844	0.0170538
negative regulation of Wnt signaling pathway (GO:0030178)	0.0175333
TCF dependent signaling in response to WNT_Homo sapiens_R-HSA-201681	0.0185266
TCR signaling_Homo sapiens_R-HSA-202403	0.0216495
TRIF-mediated TLR3/TLR4 signaling_Homo sapiens_R-HSA-937061	0.0223544
Constitutive Signaling by NOTCH1 HD+PEST Domain Mutants_Homo sapiens_R-HSA-2894862	0.023179
Signaling by Robo receptor_Homo sapiens_R-HSA-376176	0.0231794
CD209 (DC-SIGN) signaling_Homo sapiens_R-HSA-5621575	0.0231794
TP53 regulates transcription of additional cell cycle genes whose exact role in the p53 pathway remain uncertain_Homo sapiens_R-HSA-6804115	0.0231794
positive regulation of canonical Wnt signaling pathway (GO:0090263)	0.0238908
Genotoxicity pathway WP4286	0.0238946
APC/C-mediated degradation of cell cycle proteins_Homo sapiens_R-HSA-174143	0.0254569
Regulation of mitotic cell cycle_Homo sapiens_R-HSA-453276	0.0254569
Caspase activation via extrinsic apoptotic signalig pathway_Homo sapiens_R-HSA-5357769	0.0254569
NIK/NF-kappaB signaling (GO:0038061)	0.0256562
PI3K events in ERBB4 signaling_Homo sapiens_R-HSA-1250342	0.026443
PIP3 activates AKT signaling_Homo sapiens_R-HSA-1257604	0.026443
RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways_Homo sapiens_R-HSA-168928	0.0269128
TNF alpha Signaling Pathway WP231	0.0277115
Bone Morphogenic Protein (BMP) Signalling and Regulation WP1425	0.0304220
Canonical and Non-Canonical TGF-B signaling WP3874	0.0314138
Kit receptor signaling pathway WP304	0.0314138
Cytokine Signaling in Immune system_Homo sapiens_R-HSA-1280215	0.0332534
regulation of cell cycle process (GO:0010564)	0.0346069
positive regulation of Wnt signaling pathway (GO:0030177)	0.0354920

Continuation of Table B3.3

Signaling by Insulin receptor_Homo sapiens_R-HSA-74752	0.0355391
TP53 Regulates Transcription of Genes Involved in G2 Cell Cycle Arrest_Homo sapiens_R-HSA-6804114	0.0361099
TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest_Homo sapiens_R-HSA-6804116	0.0362023
Myometrial Relaxation and Contraction Pathways WP289	0.0367856
Activated TLR4 signalling_Homo sapiens_R-HSA-166054	0.0375829
regulation of epidermal growth factor receptor signaling pathway (GO:0042058)	0.0396817
Cell-type Dependent Selectivity of CCK2R Signaling WP3679	0.0401413
positive regulation of mitotic cell cycle phase transition (GO:1901992)	0.0408936
negative regulation of epidermal growth factor receptor signaling pathway (GO:0042059)	0.0408936
Beta-catenin independent WNT signaling_Homo sapiens_R-HSA-3858494	0.0415134
TNF related weak inducer of apoptosis (TWEAK) Signaling Pathway WP2036	0.0421357
AMP-activated Protein Kinase (AMPK) Signaling WP1403	0.0426044
Structural Pathway of Interleukin 1 (IL-1) WP2637	0.0445468
MAPK6/MAPK4 signaling_Homo sapiens_R-HSA-5687128	0.0448451
MAPK family signaling cascades_Homo sapiens_R-HSA-5683057	0.0463484
TP53 regulates transcription of several additional cell death genes whose specific roles in p53-dependent apoptosis remain uncertain_Homo sapiens_R-HSA-6803205	0.0465501
Constitutive Signaling by AKT1 E17K in Cancer_Homo sapiens_R-HSA-5674400	0.0466047
regulation of intrinsic apoptotic signaling pathway (GO:2001242)	0.0471004
regulation of mitotic cell cycle spindle assembly checkpoint (GO:0090266)	0.0483295
positive regulation of DNA damage response, signal transduction by p53 class mediator (GO:0043517)	0.0483295
ERBB signaling pathway (GO:0038127)	0.0483295
PI3K/AKT Signaling in Cancer_Homo sapiens_R-HSA-2219528	0.0492115

Table B3.4: I3C targeted signaling pathways in the subnetwork for LA T47D cell line

Signaling Pathways	**FDR**
positive regulation of apoptotic process (GO:0043065)	0.00017
Notch Signaling Pathway WP61	0.00020
Signaling by Rho GTPases_Homo sapiens_R-HSA-194315	0.00031
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway WP3932	0.00071
MAPK Signaling Pathway WP382	0.00118
Cell Cycle, Mitotic_Homo sapiens_R-HSA-69278	0.00130
TGF-beta Signaling Pathway WP366	0.00229
Genotoxicity pathway WP4286	0.00351
Apoptosis WP254	0.00351
VEGFA-VEGFR2 Signaling Pathway WP3888	0.00351
NRF2 pathway WP2884	0.00358

Continuation of Table B3.4

Cytokine Signaling in Immune system_Homo sapiens_R-HSA-1280215	0.00464
regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043281)	0.00497
Signaling by TGF-beta Receptor Complex_Homo sapiens_R-HSA-170834	0.00521
ATM Signaling Pathway WP2516	0.00563
Interferon type I signaling pathways WP585	0.00593
Signaling by ERBB4_Homo sapiens_R-HSA-1236394	0.00613
Interferon alpha/beta signaling_Homo sapiens_R-HSA-909733	0.00613
ErbB Signaling Pathway WP673	0.00634
AMP-activated Protein Kinase (AMPK) Signaling WP1403	0.00634
Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling WP3612	0.00641
epidermal growth factor receptor signaling pathway (GO:0007173)	0.00851
negative regulation of apoptotic process (GO:0043066)	0.00857
type I interferon signaling pathway (GO:0060337)	0.00970
PI3K-Akt Signaling Pathway WP4172	0.00975
ATM Signaling Network in Development and Disease WP3878	0.01036
cytokine-mediated signaling pathway (GO:0019221)	0.01044
regulation of small GTPase mediated signal transduction (GO:0051056)	0.01103
G2/M transition of mitotic cell cycle (GO:0000086)	0.0118
cell cycle G2/M phase transition (GO:0044839)	0.0124
Fc epsilon receptor (FCERI) signaling_Homo sapiens_R-HSA-2454202	0.01442
Interferon Signaling_Homo sapiens_R-HSA-913531	0.01442
AXIN mutants destabilize the destruction complex, activating WNT signaling_ Homo sapiens_R-HSA-4839735	0.01569
TCF dependent signaling in response to WNT_Homo sapiens_R-HSA-201681	0.01589
RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways_ Homo sapiens_R-HSA-168928	0.01878
Wnt Signaling Pathway and Pluripotency WP399	0.01885
Signaling by Wnt_Homo sapiens_R-HSA-195721	0.01998
Activated TLR4 signalling_Homo sapiens_R-HSA-166054	0.01998
Signaling by Robo receptor_Homo sapiens_R-HSA-376176	0.01998
miRNA regulation of p53 pathway in prostate cancer WP3982	0.02236
B Cell Receptor Signaling Pathway WP23	0.02236
Rho GTPase cycle_Homo sapiens_R-HSA-194840	0.02310
Cell Cycle Checkpoints_Homo sapiens_R-HSA-69620	0.02406
DNA damage response, signal transduction by p53 class mediator (GO:0030330)	0.02494
Signaling by WNT in cancer_Homo sapiens_R-HSA-4791275	0.02653
small GTPase mediated signal transduction (GO:0007264)	0.02772
intrinsic apoptotic signaling pathway in response to DNA damage (GO:0008630)	0.02772
regulation of cell cycle (GO:0051726)	0.02772
regulation of protein kinase B signaling (GO:0051896)	0.02772
regulation of cell cycle G2/M phase transition (GO:1902749)	0.02839
Signaling by SCF-KIT_Homo sapiens_R-HSA-1433557	0.03003
IGF1R signaling cascade_Homo sapiens_R-HSA-2428924	0.03003

Continuation of Table B3.4

Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)_ Homo sapiens_R-HSA-2404192	0.03003
Signaling by the B Cell Receptor (BCR)_Homo sapiens_R-HSA-983705	0.03003
PDGF Pathway WP2526	0.03051
IL-4 Signaling Pathway WP395	0.03141
Signaling by VEGF_Homo sapiens_R-HSA-194138	0.03246
Signaling by NOTCH1_Homo sapiens_R-HSA-1980143	0.03246
Cell Cycle WP179	0.03369
Chemokine signaling pathway WP3929	0.03369
VEGFA-VEGFR2 Pathway_Homo sapiens_R-HSA-4420097	0.03438
TP53 Regulates Transcription of Genes Involved in G2 Cell Cycle Arrest_Homo sapiens_R-HSA-6804114	0.03505
signal transduction in response to DNA damage (GO:0042770)	0.03896
G alpha (12/13) signalling events_Homo sapiens_R-HSA-416482	0.04114
IL-6 signaling pathway WP364	0.04772
Nuclear signaling by ERBB4_Homo sapiens_R-HSA-1251985	0.04805
Interferon gamma signaling_Homo sapiens_R-HSA-877300	0.04842
positive regulation of transmembrane receptor protein serine/threonine kinase signaling pathway (GO:0090100)	0.04931

Table B3.5: I3C targeted signaling pathways in the subnetwork for LA ZR751 cell line

Signaling Pathways	**FDR**
Cell Cycle WP179	0.000280
Cell Cycle Checkpoints_Homo sapiens_R-HSA-69620	0.000587
Signaling by Rho GTPases_Homo sapiens_R-HSA-194315	0.000587
regulation of cell cycle (GO:0051726)	0.000588
Interferon alpha/beta signaling_Homo sapiens_R-HSA-909733	0.000709
Interferon Signaling_Homo sapiens_R-HSA-913531	0.000780
Integrated Cancer Pathway WP1971	0.000783
cell cycle G1/S phase transition (GO:0044843)	0.000937
ATM Signaling Network in Development and Disease WP3878	0.000948
G1/S transition of mitotic cell cycle (GO:0000082)	0.001691
apoptotic process (GO:0006915)	0.004276
Interferon gamma signaling_Homo sapiens_R-HSA-877300	0.005031
ATM Signaling Pathway WP2516	0.005071
EGF/EGFR Signaling Pathway WP437	0.005410
NRF2 pathway WP2884	0.006950
VEGFA-VEGFR2 Signaling Pathway WP3888	0.006950
Apoptosis WP254	0.007677
extrinsic apoptotic signaling pathway via death domain receptors (GO:0008625)	0.007962
Notch Signaling Pathway WP61	0.009858
intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress (GO:0070059)	0.015662

Continuation of Table B3.5

regulation of mitotic cell cycle (GO:0007346)	0.016476
TGF-beta Signaling Pathway WP366	0.017552
positive regulation of apoptotic signaling pathway (GO:2001235)	0.017614
negative regulation of apoptotic process (GO:0043066)	0.020292
Apoptosis-related network due to altered Notch3 in ovarian cancer WP2864	0.025074
Notch signaling pathway (GO:0007219)	0.025219
MAPK Signaling Pathway WP382	0.028800
DNA damage response, signal transduction by p53 class mediator (GO:0030330)	0.031929
Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling WP3612	0.034676
negative regulation of protein kinase B signaling (GO:0051898)	0.034757
Wnt Signaling WP428	0.037622
Leptin signaling pathway WP2034	0.037622
extrinsic apoptotic signaling pathway (GO:0097191)	0.038232
TCF dependent signaling in response to WNT_Homo sapiens_R-HSA-201681	0.039778
miRNA regulation of prostate cancer signaling pathways WP3981	0.040011
Bone Morphogenic Protein (BMP) Signalling and Regulation WP1425	0.042644
Wnt/beta-catenin Signaling Pathway in Leukemia WP3658	0.043482
Fas Ligand (FasL) pathway and Stress induction of Heat Shock Proteins (HSP) regulation WP314	0.043482

Table B3.6: I3C targeted signaling pathways in the subnetwork for TN MDA-MB-231 cell line

Signaling Pathways	**FDR**
Pathways Regulating Hippo Signaling WP4540	0.03004

Table B3.7: I3C targeted signaling pathway in the subnetwork for TN MDA-MB-436 cell line

Signaling Pathways	**FDR**
MAPK Signaling Pathway WP382	0.00112
Signaling by EGFR_Homo sapiens_R-HSA-177929	0.00212
ErbB Signaling Pathway WP673	0.00234
TGF-beta Signaling Pathway WP366	0.00267
Rho GTPase cycle_Homo sapiens_R-HSA-194840	0.00299
regulation of apoptotic process (GO:0042981)	0.00353
G Protein Signaling Pathways WP35	0.00451
Signaling by Rho GTPases_Homo sapiens_R-HSA-194315	0.00608
negative regulation of Ras protein signal transduction (GO:0046580)	0.00840
regulation of cell cycle (GO:0051726)	0.00996
Signaling by Robo receptor_Homo sapiens_R-HSA-376176	0.01208
regulation of small GTPase mediated signal transduction (GO:0051056)	0.01235
T-Cell Receptor and Co-stimulatory Signaling WP2583	0.01345

Continuation of Table B3.7

Pathways Regulating Hippo Signaling WP4540	0.01707
regulation of ERBB signaling pathway (GO:1901184)	0.02067
DAP12 signaling_Homo sapiens_R-HSA-2424491	0.0214
PI3K/AKT Signaling in Cancer_Homo sapiens_R-HSA-2219528	0.02148
Signaling by PDGF_Homo sapiens_R-HSA-186797	0.0227
PI3K events in ERBB4 signaling_Homo sapiens_R-HSA-1250342	0.0288
PIP3 activates AKT signaling_Homo sapiens_R-HSA-1257604	0.0288
DAG and IP3 signaling_Homo sapiens_R-HSA-1489509	0.02880
Apoptosis-related network due to altered Notch3 in ovarian cancer WP2864	0.02986
regulation of mitotic cell cycle (GO:0007346)	0.03004
negative regulation of cell cycle (GO:0045786)	0.03241
positive regulation of apoptotic process (GO:0043065)	0.03241
PLC-gamma1 signalling_Homo sapiens_R-HSA-167021	0.03272
G13 Signaling Pathway WP524	0.03363
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway WP3932	0.03363
regulation of epidermal growth factor receptor signaling pathway (GO:0042058)	0.03691
negative regulation of ERBB signaling pathway (GO:1901185)	0.03923
PI3K-Akt Signaling Pathway WP4172	0.04219
PDGFR-beta pathway WP3972	0.04822
Genotoxicity pathway WP4286	0.04822
Signaling by SCF-KIT_Homo sapiens_R-HSA-1433557	0.04921

Table B3.8: WA targeted pathways in the subnetwork for TN MDA-MB-231 cell line

Signaling Pathways	**FDR**
TCF dependent signaling in response to WNT_Homo sapiens_ R-HSA-201681	2.36E-05
Genotoxicity pathway WP4286	2.80E-05
Signaling by Wnt_Homo sapiens_R-HSA-195721	0.000275
Notch Signaling Pathway WP61	0.000339
FoxO signaling pathway_Homo sapiens_hsa04068	0.000709
TGF-beta Signaling Pathway WP366	0.000947
NRF2 pathway WP2884	0.001031
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway WP3932	0.001262
Estrogen signaling pathway_Homo sapiens_hsa04915	0.001921
Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling WP3612	0.002317
Signaling by NOTCH1_Homo sapiens_R-HSA-1980143	0.003185
p75NTR recruits signalling complexes_Homo sapiens_R-HSA-209543	0.003971
VEGFA-VEGFR2 Signaling Pathway WP3888	0.004773
Notch signaling pathway (GO:0007219)	0.005049
Constitutive Signaling by NOTCH1 HD+PEST Domain Mutants_ Homo sapiens_R-HSA-2894862	0.005778

Continuation of Table B3.8

Constitutive Signaling by NOTCH1 PEST Domain Mutants_ Homo sapiens_R-HSA-2644606	0.005778
Signaling by NOTCH1 HD+PEST Domain Mutants in Cancer_ Homo sapiens_R-HSA-2894858	0.005778
Signaling by NOTCH1 in Cancer_Homo sapiens_R-HSA-2644603	0.005778
Signaling by NOTCH1 PEST Domain Mutants in Cancer_Homo sapiens_R-HSA-2644602	0.005778
AMPK signaling pathway_Homo sapiens_hsa04152	0.005847
Recruitment and ATM-mediated phosphorylation of repair and signaling proteins at DNA double strand breaks_Homo sapiens_R-HSA-5693565	0.006396
p53 signaling pathway_Homo sapiens_hsa04115	0.006858
intrinsic apoptotic signaling pathway (GO:0097193)	0.007681
Photodynamic therapy-induced AP-1 survival signaling. WP3611	0.008723
p75NTR signals via NF-kB_Homo sapiens_R-HSA-193639	0.009535
PI3K-Akt signaling pathway_Homo sapiens_hsa04151	0.010249
regulation of I-kappaB kinase/NF-kappaB signaling (GO:0043122)	0.010298
Alpha 6 Beta 4 signaling pathway WP244	0.011266
TNF signaling pathway_Homo sapiens_hsa04668	0.011575
HIF-1 signaling pathway_Homo sapiens_hsa04066	0.015133
regulation of tumor necrosis factor-mediated signaling pathway (GO:0010803)	0.016324
insulin receptor signaling pathway (GO:0008286)	0.017294
regulation of TOR signaling (GO:0032006)	0.017294
Target Of Rapamycin (TOR) Signaling WP1471	0.017413
Apoptosis Modulation and Signaling WP1772	0.017413
ErbB Signaling Pathway WP673	0.017413
PI3K-Akt Signaling Pathway WP4172	0.017413
MAPK signaling pathway_Homo sapiens_hsa04010	0.017726
positive regulation of I-kappaB kinase/NF-kappaB signaling (GO:0043123)	0.017871
cytokine-mediated signaling pathway (GO:0019221)	0.017871
Notch signaling pathway_Homo sapiens_hsa04330	0.018072
AMP-activated Protein Kinase (AMPK) Signaling WP1403	0.018717
mTOR signalling_Homo sapiens_R-HSA-165159	0.0251
ATM Signaling Pathway WP2516	0.025625
NF-kB is activated and signals survival_Homo sapiens_R-HSA-209560	0.026535
Notch signaling involved in heart development (GO:0061314)	0.027859
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest (GO:0006977)	0.028238
positive regulation of cytokine-mediated signaling pathway (GO:0001961)	0.030255
negative regulation of intracellular signal transduction (GO:1902532)	0.030255
signal transduction involved in mitotic G1 DNA damage checkpoint (GO:0072431)	0.030255
mTORC1-mediated signalling_Homo sapiens_R-HSA-166208	0.033523
Notch Signaling WP268	0.040067
extrinsic apoptotic signaling pathway (GO:0097191)	0.041324
ID signaling pathway WP53	0.04199
Signaling by Rho GTPases_Homo sapiens_R-HSA-194315	0.042583

Continuation of Table B3.8

intrinsic apoptotic signaling pathway in response to oxidative stress (GO:0008631)	0.043923
extrinsic apoptotic signaling pathway via death domain receptors (GO:0008625)	0.043923

Table B3.9: WA targeted signaling pathways in the subnetwork for LA MCF-7 cell lines

Signaling Pathways	**FDR**
DNA damage response, signal transduction by p53 class mediator (GO:0030330)	1.18E-05
p53 signaling pathway_Homo sapiens_hsa04115	1.19E-05
Interferon Signaling_Homo sapiens_R-HSA-913531	7.39E-05
ATM Signaling Pathway WP2516	9.68E-05
Signaling by Rho GTPases_Homo sapiens_R-HSA-194315	0.000174
Resolution of AP sites via the multiple-nucleotide patch replacement pathway_Homo sapiens_R-HSA-110373	0.000306
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest (GO:0006977)	0.0005
signal transduction involved in mitotic G1 DNA damage checkpoint (GO:0072431)	0.000518
NRF2 pathway WP2884	0.000572
ATM Signaling Network in Development and Disease WP3878	0.001423
Negative regulators of RIG-I/MDA5 signaling_Homo sapiens_R-HSA-936440	0.001848
Cytokine Signaling in Immune system_Homo sapiens_R-HSA-1280215	0.002372
RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways_Homo sapiens_R-HSA-168928	0.002604
regulation of signal transduction by p53 class mediator (GO:1901796)	0.00335
NF-kB activation through FADD/RIP-1 pathway mediated by caspase-8 and -10_Homo sapiens_R-HSA-933543	0.005956
Interferon alpha/beta signaling_Homo sapiens_R-HSA-909733	0.006138
cytokine-mediated signaling pathway (GO:0019221)	0.00908
AMP-activated Protein Kinase (AMPK) Signaling WP1403	0.010534
negative regulation of extrinsic apoptotic signaling pathway in absence of ligand (GO:2001240)	0.01196
negative regulation of signal transduction in absence of ligand (GO:1901099)	0.011956
intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress (GO:0070059)	0.013129
type I interferon signaling pathway (GO:0060337)	0.013966
regulation of extrinsic apoptotic signaling pathway in absence of ligand (GO:2001239)	0.01708
interferon-gamma-mediated signaling pathway (GO:0060333)	0.01751
MAPK signaling pathway_Homo sapiens_hsa04010	0.018406
FoxO signaling pathway_Homo sapiens_hsa04068	0.021969
Interferon gamma signaling_Homo sapiens_R-HSA-877300	0.023853
negative regulation of extrinsic apoptotic signaling pathway (GO:2001237)	0.024507

Continuation of Table B3.9

MAPK Signaling Pathway WP382	0.02491
Type II interferon signaling (IFNG) WP619	0.026935
positive regulation of cytokine-mediated signaling pathway (GO:0001961)	0.02935
regulation of epidermal growth factor receptor signaling pathway (GO:0042058)	0.035461
Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways_Homo sapiens_R-HSA-168643	0.036926
Longevity regulating pathway - multiple species_Homo sapiens_hsa04213	0.039072
TGF-beta Signaling Pathway WP366	0.046347
Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling WP3612	0.04827