Computational Approach to Identify Regulatory Biomarkers in the Pathogenesis of Breast Carcinoma

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***Abstract*—Breast Cancer is reckoned amongst the most common cause of morbidity and mortality among women, adversely affecting female population irrespective of age. The poor survival rate reported in invasive carcinoma cases demands the identification of early developmental stage key markers. MicroRNAs are contributing a critical role in gene regulation potential markers. Over 2000 miRNAs have been identified and considered to offer a unique opportunity for early detection of diseases. In this study, a gene-miRNA-TF interaction network was constructed from the differentially expressed genes obtained from the invasive lobular and invasive ductal carcinoma samples. The network consists of experimentally validated miRNAs and transcription factors were identified for the target genes, followed by thermodynamics studies to identify the binding free energy between mRNA-miRNA. Our analysis identified miRNA; hsa-miR-28-5p binds with MAD2L1 with unexpectedly high binding free energy equivalent to -92.54kcal/mol and also makes canonical triplex with hsa-miR-203a, which acts as a catalyst to initialize the MAD2L1 regulation. For the identified regulatory elements, we proposed a mathematical model and feed-forward loops that may serve in understanding the regulatory mechanisms in breast cancer pathogenesis and progression.**

Keywords*—Breast cancer; invasive lobular carcinoma; invasive ductal carcinoma; biomarkers; MicroRNA; transcription factors; feed forward loops

I. Introduction

Breast cancer is the major type of cancer affecting female population in both developed and developing countries. Breast carcinoma can be classified into ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). In the initial stages of breast cancer (i.e., DCIS), the abnormal cells are restricted to the lining of breast ducts, which may lead to invasive cancer if not treated, “In situ” type tumor is confined to the epithelial layer, where it leads to DCIS and LCIS [1]. When the tumor cells burst out from the tissues, these can be Invasive Ductal Carcinoma (IDC) and Invasive Lobular Carcinoma (ILC). IDC accounts for almost 80% of the diagnoses of breast cancer cases, while ILC is composed of another 5%-15% [2].

Most breast cancers begin as asymptomatic lump or tumor that originates from either glandular tissue of breast i.e. lobules or in the ducts that connect these lobules. Molecular subtypes of cancer have been identified by biomarkers including hormones (Estrogen (E), progesterone (P)), and receptors (ER, PR, HER1 and HER2). In 2019, almost 62,930 and 268,600 new cases of DCIS and IDC were reported respectively in United States. Breast cancer is associated with mutations in genes like BRCA1/2, p53, ATM, and CHD1. Nearly 5-10% of breast cancers are linked to the inheritance of gene mutations, mostly BRCA1/2 mutations. Women with BRCA1/2 mutations have a 45-65% higher chance of developing breast cancer [3]. Non-genetic risk factors include first-degree family history, race and ethnicity, lifestyle, exposure to radiation, alcoholism, obesity, and hormone replacement therapies. Few reports suggest that metabolism, particularly fatty acid oxidative metabolism plays a significant role in cancer progression by reprogramming their signaling pathways [4,5].

MADL21 gene is known as mitotic arrest deficient 2 like 1 or mitotic spindle assembly checkpoint protein MAD2. Aliases of MAD2L1 include or HSMAD2 and MAD2. MADL21 plays an essential role in supervising cell cycle regulation, G1/S checkpoint, cell growth and death, Oocyte meiosis and progesterone-mediated oocyte maturation [6]. Studies have reported that chromosomal instability due to aberrant expression of MAD2L1 may promote tumorigenesis, where upregulation of MAD2L1 has been reported in breast, lung, liver and stomach cancer [7,8,9].

MicroRNAs (miRNAs) are small, 18-22 nucleotide long, non-coding RNA molecules. They are the key players in RNA interference pathways causing silencing of their associated target genes. miRNAs regulate the expression of 30-50% of genes and target various elements of signaling pathways and cellular networks. Recent studies have established their role as oncogenes or anti-oncogenes in regulating multiple cellular pathways involved in breast cancer pathogenesis. Therefore, miRNA may aid in developing new methods for cancer diagnostics and therapy.

II. Related Work

The expression profile of miRNAs differs in normal and tumor tissues. miRNA profiling has identified that miR-99a was dysregulated in breast cancer tissues as compared to normal tissue [10]. Furthermore, numerous studies have reported that miRNAs are dysregulated at various stages of breast cancer [11]. Therefore, they have been used to identify characteristic miRNA signatures in human breast cancers. To decipher and analyze the differential changes of oncogenes

and tumor suppressor genes, transcriptome analysis with the help of microarray expression-based studies would help in the identification of novel markers associated with the cancer progression [12].

The role of miR-28-3p is well established in different cancer types including breast cancer, colorectal cancer, B-cell lymphoma, glioma and renal cell carcinoma. Liang Ma et al. identified the inhibition property of miR-28-3p by regulating WSB2 in breast cancer [13], while in other cancer it has been found to target TRPM7, Rap1b and cyclin D1 [14, 15]. However, the potential role of miR-28-3p targeting MAD2L1 is not yet reported in breast cancer.

III. Materials and Method

A. Mining of Differentially Expressed Genes

In the present study, breast cancer dataset GSE36295 was retrieved from Gene Expression Omnibus database of NCBI and analyzed to identify DEGs. The dataset encompasses 50 Saudi Arabian subjects with 5 control samples, 5 ILC (Invasive Lobular Carcinoma samples), 34 IDC (Invasive Ductal Carcinoma) and the remaining 6 samples were poorly differentiated (not included in the study). Expression profiling was conducted on Human Gene 1.0 ST GeneChip arrays (Affymetrix) testing platform. In order to calculate and analyze the DEGs between Control vs. ILC and Control vs. IDC samples, we have used Bioconductor packages in R namely affy, limma and other packages included in our customized pipeline. To calculate FDR and p-values, Benjamin and Hochberg (FDR) and t-test methods were utilized respectively [16]. A volcano plot and heatmap were constructed, which represent the distribution of p-value and fold change of DEGs using R.

B. Gene-Gene Interaction Network (GGIN) Analysis for Hub Nodes and Target Genes Identification

GeneMANIA [17] and Search Tool for Retrieval of Interacting Genes/Proteins (STRING) [18] were utilized to develop GGIN. The parameters used for network generation were; all prediction sources enabled medium confidence score≥0.40 and no interactors in the first and second shell. The final GGIN was downloaded in .txt format for visualization and analysis in Cytoscape 3.7.1 using CytoHubba [19]. In this study, identified hub genes were further subjected to the Oncoprint module in cBioPortal and the hub genes with the relative frequency of mutation and copy number variants < 1 were selected as target genes.

C. Enrichment Analysis of Target Genes and Functional Annotation

Erichr [20] identified the enriched terms in a given gene list and web-based gene set analysis toolkit (WebGestalt) [21] is a data mining system. It has four modules that aid in examining gene sets for Gene Ontology terms, metabolic and signaling pathways, tissue expression and chromosome distribution. The target genes were subjected to enriched pathways and biological process enrichment analysis through Enrichr and WebGestalt, respectively.

D. Identification of Validated miRNA for Target Genes

miRTarBase provides comprehensive data on experimentally validated miRNA and target interactions [22]. It consists of more than 13,404 validated miRNA and target interactions. In our study, the genes with a lesser frequency of mutations were subjected to regulatory analysis to identify the novel targets for validated miRNA. Furthermore, Regulatory Network Repository (RegNetwork) consisting of the integration of five-types of transcriptional and post-transcriptional regulatory relationships for mice and humans, was used to identify the experimentally validated Transcription Factors.

E. Thermodynamics Estimation of Gene-miRNA Interactions and miRNA-mRNA Duplex Binding Site Prediction

miRmap is an open-source software library that ranks potential targets on the basis of repression strength. It combines evolutionary, probabilistic, thermodynamics and sequence-based features [23]. It uses four approaches to examine feature correlations using experimental data from transcriptomics, proteomics and immunopurification studies. For binding site and thermodynamics estimation, the identified target gene-miRNA duplex was subjected to miRmap web.

F. miRNA Triplex, ODE and mRNA Regulation using Feed Forward Loop

TriplexRNA is a web resource that incorporates methods for triplex structure analysis, miRNA target prediction, simulation and c. The triplex formation of target genes obtained miRNA was examined through TriplexRNA [24]. An Ordinary Differential Equation (ODE) was proposed to explain the alteration of miRNA and Transcriptional Factors (rate of change) concerning the target gene in initiating the regulatory pathogenesis of the disease. The MAD2L1 regulatory mechanism was further explained with Feed Forward Loop.

IV. Results and Discussion

A. Identification of differentially Expressed Genes

The microarray expression dataset GSE36295 was obtained from GEO and was analyzed in R using bioconductor packages. The dataset comprised of 5 control, 5 ILC, and 34 IDC tissue samples of female breast cancer patients. The principal components analysis revealed the relatedness between samples of each category (Fig. 1A). The dataset was normalized using RMA normalization approach and DEGs were identified using the threshold of |logFC|≥1 and a Benjamini & Hochberg adjusted p-value cut-off of 0.05 (Fig. 1B). The principal components analysis plots clearly classify the distinct expression of genes in Control and Cancerous samples (IDC and ILC). Each cluster represents the sample with same feature within it and differentiates from the samples in other clusters. Thus, differentiating the gene expression in normal breast to the gene expression in breast cancer samples. 1509 DEGs were identified that were further used for network construction.

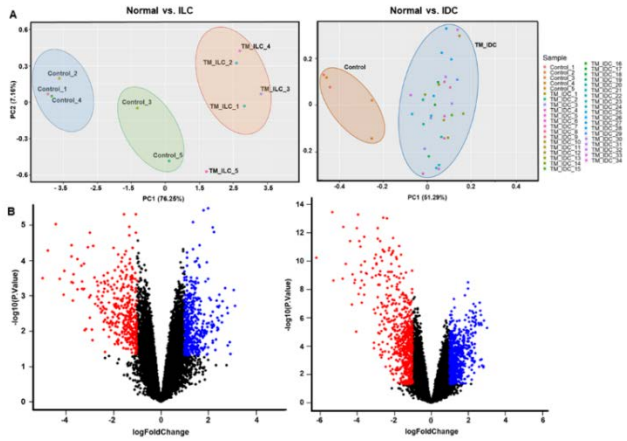

Fig. 1. (A). Principal Components Analysis of IDC and ILC Samples with Normal Samples. (B). Volcano Plots for DEGs where Red Dots and Blue Dots Represent up and Downregulated Genes Respectively.

B. *The Structure of GGIN and Identification of Target Genes and Enrichment Analysis*

From GeneMANIA studies, the DEGs were found to have a well-established role in the pathogenesis of cancer based on co-expression, co-localization, and pathways. The GGIN obtained from STRING consisted of 1509 nodes and 21993 edges, with average local clustering coefficient of 0.381 and average node degree of 29. CytoHubba plugin of Cytoscape 3.7.1 identified central elements of the GGIN. Hubs are nodes with maximum interactions and occupy a central position in the interaction networks. Maximum Clique Centrality measure identified the hub genes including BUB1B, CDCA8, CDK1, TOP2A, NUSAP1, MAD2L1, SPAG5, KIF2C, ASPM, CCNB2 (Fig. 2A). In cBioPortal, we examined genetic alterations in the top ten hub genes and identified that for CDCA8, CDK1, NUSAP1, TOP2A, MAD2L1 and CCNB2 the frequency of mutations and copy number variants were less than one. Since these genes showed the least genetic alterations, therefore, were selected as target genes for further study. The enrichment analysis of target genes revealed that they were associated with metabolic processes of amino acids, lipids, drugs and nucleoside bisphosphates (Fig. 2B). Furthermore, they were enriched in Progesterone mediated Oocyte mutation; Cell Cycle; p53 signaling and cellular senescence pathways (Fig. 2C). The identified biological processes and signaling pathways have a well-established role in cancer pathogenesis.

C. *Identification of Validated miRNAs and Transcriptions Factors for Target Genes*

The regulatory elements of the gene i.e. validated miRNAs and Transcription Factors were obtained from miRtarBase and RegNetwork (Table I). The interactions between gene-miRNA in and the interactions between gene-miRNA-cooperative miRNA in forming a triplex were given in Table II. The experimentally validated miRNAs and Transcription Factors (Table I) were identified for the target genes MAD2L1 and CDK1; for other target genes, no validated regulators were found.

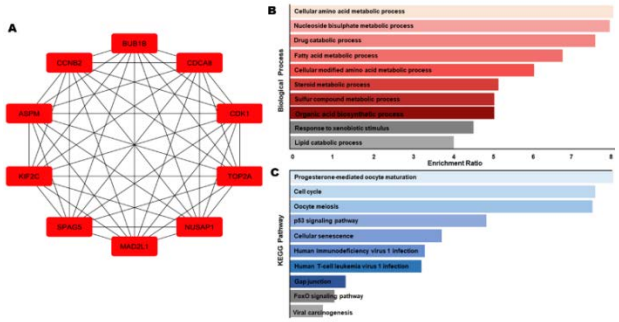

Fig. 2. (A) Top 10 Hub Genes of GGIN as Identified from CytoHubb. Functional Enrichment Analysis of Target Genes (B) Biological Processes (C) Signaling Pathways.

TABLE I. VALIDATED MIRNAS FOR CDK1 AND MAD2L1

Gene	Regulatory Elements	
	Transcription Factor	***miRNA***
MAD2L1	E2F4, ESR2 MYB, MYC	hsa-miR-28-5p hsa-miR-192-5p
CDK1	RB1, SP1, ATF1, E2F1, E2F4, ETS2, MYB, MYC	hsa-miR-31-5p hsa-miR-663a hsa-miR-24-3p hsa-miR-302a-3p

D. *Seed Pairing and Construction of Gene Regulatory Network*

In case of seed pairing CDK1-hsa-miR-31-5p form a complementarily pairing with a binding free energy of -34.33 Kcal/mol (Table II) and with triplex formation, CDK1-hsa-miR-31-5p-hsa-miR-543 forms a canonical triplex to initiate the process of regulation. Similarly, MAD2L1-hsa-miR-28-5p-hsa-miR-203 forms a canonical triplex. The interaction between the genes: CDK1, miRNAs: hsa-miR-28-5p, hsa-miR-31-5p and transcription factors (TFs): RB1, SP1, ATF1, E2F1, E2F4, ETS2, MYB and MYC is given in Fig. 3.

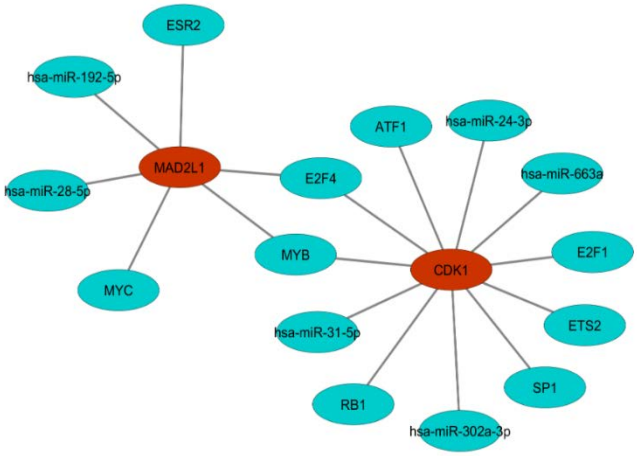

Fig. 3. Network Representing Regulation of CDK1 and MAD2L1 by miRNAs and TFs, where the Target Genes are represented in Red Color and Regulatory Elements in Blue Color.

TABLE II. INTERACTION OF GENE-MIRNA (SEED PAIRING) AND GENE-MIRNA-COOPERATIVE MIRNA (TRIPLEX)

Gene	Regulatory Element (miRNA)	Binding Free Energy (Kcal/mol)	Triplex Form-ation (cooperative miRNA)	Binding Free Enery of Triplex (Kcal/mol)	Nature of Complementarily (Self/Duplex/Triplex)
MAD2L1	hsa-miR-28-5p	-92.58	hsa-miR-203	-28.86	Canonical Triplex
CDK1	hsa-miR-31-5p	-34.33	hsa-miR-543	-33.36	Canonical Triplex
			hsa-miR-181a	-32.96	miRNA Self Complementarily
			hsa-miR-181b	-31.96	Canonical Triplex
			hsa-miR-181c	-31.76	miRNA Self Complementarily
			hsa-miR-146a	-31.16	Canonical Triplex
			hsa-miR-146b	-31.16	Canonical Triplex
			hsa-miR-494	-30.76	miRNA Self Complementarily
			hsa-miR-181d	-30.56	target self complementarity
			hsa-miR-329	-29.26	miRNA self-complementarity
			hsa-miR-362-3p	-28.16	miRNA self-complementarity

E. Analysis of miRNA-Mediated Mechanisms of Translation Repression and Gene Regulation via Transcription Factors

The gene MAD2L1 was regulated by transcription factors E2F4, ESR2, MYB and MYC and repressed by hsa-miR-192-5p and hsa-miR-28-5p. Similarly, the regulation of the gene CDK1 is a combination of activation by transcription factors RB1, SP1, ATF1, E2F1, E2F4, ETS2, MYB and repression by miRNAs hsa-miR-31-5p, hsa-miR-63a, hsa-miR-24-3p and hsa-miR-302a-3p. However, it was observed that the binding free energy of MAD2L1-hsa-miR-28-5p is less than CDK1-hsa-miR-31-5p (Table II). Therefore, the stability of MAD2L1-hsa-miR-28-5p was more than CDK1-hsa-miR-31-5p. Therefore, MAD2L1-hsa-miR-28-5p was taken forward for further understanding its role in driving the regulatory pathogenesis of Invasive Ductal and Invasive Lobular Breast Carcinoma.

F. Analytical Modeling of Gene Regulation to Understand the Regulatory Pathogenesis of Breast Cancer

In the mathematical modeling of the miRNA mediated transcriptional regulatory network which was associated with the pathogenesis of breast cancer, the involved factors are (i) gene: MAD2L1, miRNA: hsa-miR-28-5p, hsa-miR-203 and TFs: E2F4, ESR2, MYB and MYC. Hence, the regulatory pathogenesis of Breast cancer is initiated by the regulation of the gene: MAD2L1, activated by the TFs: E2F4, ESR2, MYB and MYC and repressed by the miRNAs: miRNA: hsa-miR-28-5p with the cooperation of hsa-miR-203. Since the cooperative miRNA, hsa-miR-203 act as a catalyst in driving the pathogenesis, the core miRNA hsa-miR-28-5p is preferred in the modeling and the ordinary differential equation of the mathematical model that initiates the regulatory pathogenesis in Breast Cancer is illustrated as:

[d (MAD2L1) / dt = k (Synthesis of E2F4, ESR2, MYB and MYC) + MAD2L1 + k (Up-regulation of E2F4, ESR2, MYB and MYC) (hsa-miR-28-5p) - k (Degradation of E2F4, ESR2, MYB and MYC) (E2F4, ESR2, MYB and MYC)] where k represents the rate of synthesis/degradation of TFs.

Additionally, the mRNA binding sites of the corresponding miRNA hsa-miR-28-5p and hsa-miR-203a of breast cancer-associated gene MAD2L1 were obtained by gene-miRNA mapping pair. The binding sites are shown in Fig. 4. We found that hsa-miR-28-5p has one binding site on MAD2L1 while ha-miR-203a has two binding sites on MAD2L1. For hsa-miR-192-5p, there was no binding site found on MAD2L1.

G. mRNA-miRNA-TF Regulatory Analysis through Feed-Forward Loops

Although no specific protocol has been studied for the mechanism of miRNAs, comparative studies have identified that genes regulated by miRNAs might have considerable contribution in cellular processes [25]. The available experimental shreds of evidence justify the regulation of genes due to miRNA through translational repression with or without mRNA decay. However, the variation in these contributions over time remains undefined which is also went unnoticed in case of TFs [26]. Therefore, we design a transcription network utilizing one of the most significant motifs i.e. Feed Forward Loop (FFL) motif [27]. In this study, the FFL is composed of a transcription factor (E2F4, ESR2, MYB, MYC) which regulates other or the miRNA (hsa-miR-28-5p), then TFs and miRNA both bind at the regulatory region of the target gene (MAD2L1) and jointly modulate its transcription rate. This FFL collectively has three transcription interactions, which could be either activation or repression. A recent computational analysis demonstrated that FFL, containing TF and miRNAs, are overrepresented in gene regulatory networks, assuming that they confer useful regulatory opportunities [28].

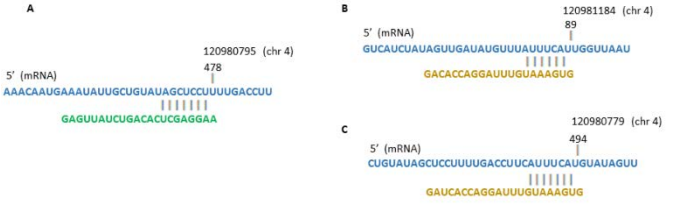

Fig. 4. Target Binding Site of miRNA (A) hsa-miR-28-5p and (B) hsa-miR-203a with MALD21.

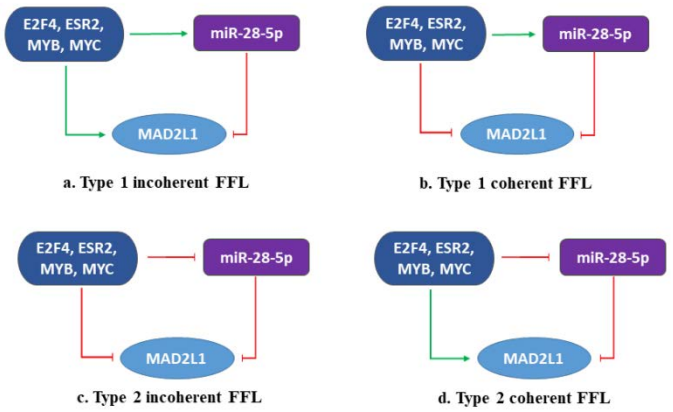

Fig. 5. The Incoherent and Coherent Feed forward Loops between the Target Gene and its Regulatory Factors is Represented; where Arrows (Green) Indicate Activation, the T-bars Indicate Repression. The Dark Blue Shape with Circular Ends Represent Transcription Factors.

The FFL has two possible structure configurations in both coherent and incoherent FFL specified as type 1 or 2 coherent FFLs and type 1 or 2 incoherent FFLs respectively as shown in Fig. 5(a, b, c, d) where miR-28-5p is miRNA and MAD2L1 is the target protein. In 5(a) represents Type 1 incoherent FFL where TF activates both target mRNA and miRNA synthesis. 5(b) Type 1 coherent FFL, miRNA and TF represses target mRNA but activates miRNA synthesis. 5(c) Type 2 incoherent FFL, TF represses both target mRNA and miRNA synthesis. 5(d) Type 2 coherent FFL, TF activates target mRNA and represses miRNA synthesis. Additionally, we emphasize that this study is carried out utilizing secondary data repositories which encompass a limited number of genes/miRNAs; hence these may provide bounded results. In this study, we performed a comprehensive analysis using various bioinformatics approaches to understand the molecular interaction between genes and miRNA and their involvement in the pathogenesis of breast cancer. The findings from this study strongly suggest that hsa-miR-28-5p might play a critical role by regulating MAD2L1 which subsequently inhibits cell cycle and p53 signaling pathway in breast cancer patients. The potential role of hsa-miR-28-5p mediating breast cancer pathogenesis on binding with MAD2L1 gene may act as a significant therapeutic target for the diagnosed subjects.

V. Conclusion

The systematic analysis for identification of target genes majorly contributing in the primary stage of development of invasive breast carcinoma, MAD2L1 was found to have experimentally validated miRNA and transcription factor targets from multiple databases. The regulation of MAD2L1 is a combination of activation by transcription factors E2F4, ESR2, MYB and MYC and repression by hsa-miR-28-5p and hsa-miR-192-5p. In case of seed pairing, MAD2L1-hsa-miR-28-5p forms a complementarily pairing with a binding free energy of -92.58 Kcal/mol and a canonical triplex MAD2L1-hsa-miR-28-5p-hsa-miR-203. Since, the binding free energy of MAD2L1-hsa-miR-28-5p seed pairing is 3-folds lesser than CDK1, the stability of MAD2L1-hsa-miR-28-5p will be much greater than CDK1-hsa-miR-31-5p. Therefore, the mRNA-miRNA-TF complex MAD2L1 - hsa-miR-28-5p and associated transcription factors: E2F4, ESR2, MYB and MYC might play a vital target in driving the regulatory pathogenesis of breast cancer. The regulatory pathogenesis of breast cancer is initiated by the regulation of the gene: MAD2L1, which is activated by the TFs: E2F4, ESR2, MYB and MYC and repressed by the miRNA: hsa-miR-28-5p with the cooperation of miRNA: hsa-miR-203. Since the cooperative miRNA hsa-miR-203 act as a catalyst in driving the pathogenesis, the mathematical model that initiates the regulatory pathogenesis in Breast Cancer is illustrated as:

[d(MAD2L1)/dt = k(Synthesis of E2F4, ESR2, MYB and MYC) + MAD2L1 + k(Up regulation of E2F4, ESR2, MYB and MYC)(hsa-miR-28-5p) – k(Degradation of E2F4, ESR2, MYB and MYC) (E2F4, ESR2, MYB and MYC)].

Additionally, the type 1 and type 2 coherent and incoherent feed-forward loops help to determine the regulatory mechanism of TFs and miRNA associated with MAD2L1, which further need invitro validation to determine the exact conditional mechanism of these regulators in breast cancer pathogenesis.

Since limited studies have been focused on the regulatory role of MAD2L1 associated miRNAs and their impact on tumor progression in breast cancer, this study will help in understanding the pathogenesis of MAD2L1 regulation in estimating the cascade of processes associated with breast cancer initiation and progression throughout invasive ductal carcinoma and invasive lobular carcinoma. The constructed model requires simulation with experimentally determined values in a larger time scale.

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