hsa-miR-301a- and SOX10-dependent miRNA-TF-mRNA regulatory circuits in breast cancer

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Abstract: Breast cancer is the most common cancer among women and the molecular pathways that play main roles in breast cancer regulation are still not completely understood. MicroRNAs (miRNAs) and transcription factors (TFs) are important regulators of gene expression. It is important to unravel the relation of TFs, miRNAs, and their targets within regulatory networks to clarify the processes that cause breast cancer and the progression of it. In this study, mRNA and miRNA expression studies including breast tumors and normal samples were extracted from the GEO microarray database. Two independent mRNA studies and a miRNA study were selected and reanalyzed. Differentially expressed (DE) mRNAs and miRNAs between breast tumor and normal samples were listed by using BRB-Array Tools. CircuitsDB2 analysis conducted with DE miRNAs and mRNAs resulted in 3 significant circuits that are SOX10- and hsa-miR-301a-dependent. The following significant circuits were characterized and validated bioinformatically by using web-based tools: SOX10→hsa-miR-301a→HOXA3, SOX10→hsa-miR-301a→KIT, and SOX10→hsa-miR-301a→NFIB. It can be concluded that regulatory motifs involving miRNAs and TFs may be useful for understanding breast cancer regulation and for predicting new biomarkers.

Key words: Breast cancer, miRNA, mRNA, transcription factor, regulatory circuits

1. Introduction

Breast cancer is a complex genetic disorder that is not controlled by a single factor but is rather controlled by many factors (<http://www.cancer.org/>). Although many factors (genes, microRNAs (miRNAs), transcription factors, etc.) that cause breast cancer and play a role in its development have been identified with the advancement of high-throughput technologies, the molecular mechanisms playing a role in disease regulation have still not been revealed. For this reason, it is important and inevitable to identify the regulatory circuits and networks that will explain the development and progression of breast cancer.

Gene expression regulation is an important mechanism for controlling biological processes in the cell. Transcriptional factors (TFs) are the regulators that function at the transcriptional level, while miRNAs work at the posttranscriptional level. In view of the fact that the transcription of mRNA and miRNAs is controlled by TFs and the expression of TFs is regulated by miRNAs, these two important mechanisms cannot be separated from each other. Therefore, characterization of these combinatorial regulatory mechanisms is important to reveal the biological processes that take part in breast cancer in detail by constructing networks and circuits.

miRNAs are small, noncoding RNA molecules that regulate gene expression in posttranscriptional stages (Lagos-Quintana et al., 2001; Lee and Ambros, 2001). miRNAs play important roles in biological processes such as development, cell division, cell differentiation, and programmed cell death. In this context, it is possible that altered miRNA expression may contribute to the development and progression of diseases such as cancer. The presence of abnormal miRNA expression profiling in many diseases, including breast cancer, has been demonstrated and validated by intensifying miRNA studies (Iorio et al., 2005; Lowery et al., 2009; Enerly et al., 2011; Romero-Cordoba et al., 2012). The finding that miRNA expressions are often dysregulated in cancers has made these molecules important candidates for cancer markers (Calin et al., 2002; Lu et al., 2005). Therefore, the search for the relationship of miRNAs with target genes (this may be an mRNA or a TF) has great importance for the diagnosis and treatment of breast cancer.

TFs are essential regulatory elements in the transcriptional pathway, which work by binding to target genes that have specific DNA sequences, usually in the promoter region (Latchman, 1997). TFs regulate their targets at the transcription level by inhibiting or enhancing

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their expression while miRNAs regulate target mRNAs at the posttranscriptional level in the form of inhibition. miRNAs also undergo the transcription process and they regulate their target genes like TFs. When all these reasons are taken into consideration:

- Expression of a miRNA may be regulated by a transcription factor;
- Expression of a TF may be regulated by a miRNA;
- Similarly, the transcription factor and miRNA may regulate the expression of target genes together.

In recent years, many researchers have been working on TFs, miRNAs, and their regulation mechanism on posttranscriptional and transcriptional levels. With the help of system biology approaches, transcription factor-miRNA-target gene relationships were started to be explored by an increasing number of studies. These studies have mostly used and/or developed bioinformatics and statistical methods and they have reviewed the existing information. The tools that analyze miRNA-TF relationships can be grouped into three subgroups. The tools in the first group are intended to analyze statistically the gene clusters organized by cooperating miRNAs and/or miRNAs using matched miRNA and mRNA expression profiles (Yoon and De Micheli, 2005; Joung et al., 2007; Tran et al., 2008; Joung and Fei, 2009; Nam et al., 2009). The tools in the second group again predict gene expression relationships using gene expression data (Naeem et al., 2011). The tools in the third group provide static circuits, combining statistical data obtained from literature (e.g., CircuitDB) (Friard et al., 2010b).

In light of the above mentioned information, this work aims to identify breast cancer-specific miRNA-TF-mRNA circuits bioinformatically that could be important in breast cancer development. We have identified regulatory circuits and molecule associations that differ between the normal group and breast cancer patients by using previously performed expression studies.

2. Materials and methods

2.1. Selection of miRNA and mRNA microarray studies

It was aimed to obtain miRNA and mRNA microarray data related to breast cancer in the literature. GEO, a publicly

available database at <http://www.ncbi.nlm.nih.gov/geo/> (Edgar et al., 2002), was used for this purpose. “miRNA / mRNA”, “microarray”, and “breast cancer” terms were used together when searching in GEO. The datasets were selected from among the ones that have freshly frozen tumor and normal tissue information. Microarray data obtained from blood samples, formalin-fixed paraffin-embedded tissues, and cell lines were excluded from the study to prevent biased expression profiles.

As a result, 2 independent mRNA studies and 1 miRNA study including breast tumors and normal samples were selected to be analyzed (Table 1).

2.2. Class comparison analysis

The raw data were obtained from the microarray database (GEO) and normalized by using quantile normalization in BRB-Array Tools, which is an Excel-integrated package for the visualization and statistical analysis of microarray gene expression data. Quantile normalization, one of the most widely adopted methods for analyzing microarray data, was used as the normalization method. Normalization is important to remove any differences that may arise from technical problems and to make the data comparable. Class comparison tests were performed to find out differentially expressed miRNAs and mRNAs between tumor samples and normal samples ($P \leq 0.05$, 2-fold change). This test provides powerful methods for finding differentially expressed genes when controlling the ratio of false positives. Differentially expressed and common genes (by Venny 2.1.0) were found for tumor vs. normal comparison in GSE3744 and GSE5764 (http://bioinfogp.cnb.csic.es/tools/venny_old/venny.php). Lastly, differentially expressed miRNAs were found in GSE45666.

2.3. Identification of TFs in DE mRNA lists

Transcription factor lists that have been identified in the human body were accessed by using the Animal Transcription Factor DataBase 2.0 (<http://www.bioguo.org/AnimalTFDB/>) (Zhang et al., 2015) and the TFs that were in the DE genes were determined by comparison with these lists.

2.4. Circuit analysis

The CircuitsDB2 analysis tool was used to identify the breast cancer-specific circuits as regulatory loops among

Table 1. The characteristics of the datasets.

GEO ID	Platform	Normal samples	Tumor samples
GSE3744	Affymetrix Human Genome U133 Plus 2.0 Array	7	40
GSE5764	Affymetrix Human Genome U133 Plus 2.0 Array	15	15
GSE45666	Agilent-021827 Human miRNA Microarray G4470C	15	80
GSE17907 (Test data)	Affymetrix Human Genome U133 Plus 2.0 Array	4	51

the members of DE miRNAs, mRNAs, and TFs. This user-friendly tool provides static circuits by combining statistical data obtained from the literature. It contains precompiled transcriptional and posttranscriptional networks, the sets of network motifs, and series of functional and biological information, which are based on JASPAR DB and the ENCODE project. Members of the DE miRNAs, mRNAs, and TFs were searched in microRNA-mediated FFLs in which a TF is the master regulator in CircuitsDB2 (Friard et al., 2010b).

2.5. Circuit member analysis in diseases, biological processes, and pathways

For the characterization of the members of the significant circuits three different tools, PhenomiR, WebGeshtalt, and DIANA, were used.

The PhenomiR 2.0 database, completely generated by manual curation of experienced annotators, was used to obtain information about differentially regulated miRNA (circuit member miRNA, hsa-miR-301a here) expression in diseases (breast cancer here) (Ruepp et al., 2010).

WebGestalt is the “WEB-based GENE SeT AnaLysis Toolkit”, in which the functional genomic, proteomic, and large-scale genetic studies from large numbers of gene lists (e.g., DE gene list) are continuously generated. Function analysis of the circuit member genes and TFs was performed with the WebGestalt Protein Interaction Network Module (Wang et al., 2013).

DIANA-mirPath v.3 was used to search the pathways of circuit member miRNA. It is a miRNA analysis tool that enables users to find targets of mRNAs by using different algorithms and to find pathways in which these genes are significantly enriched (Maragkakis et al., 2009; Vlachos et al., 2015).

2.6. Validation analysis

For advanced bioinformatics analysis for in silico validation of significant circuits, the mirExTra 2.0 under DIANA was used. Our miRNA and mRNA lists were analyzed using the Central microRNA Discovery Module (CmD) and miRNA-mRNA relationships in the circuits were tried to be validated. This module combines microRNA and mRNA expression data results (names, P-values, fold changes) in order to identify functional microRNAs responsible for changes in mRNA expression by utilizing in silico interactions from DIANA-microT-CDS. In this study, DE miRNA and mRNA lists were used for mirExtra analysis. It enabled us to determine the central regulator miRNAs and their targets and provided us the opportunity to validate our results (Maragkakis et al., 2009; Vlachos et al., 2016).

3. Results

3.1. The miRNA and mRNA datasets

The miRNA microarray studies and mRNA microarray studies were searched to find differentially expressed

miRNAs and mRNAs between breast cancer and normal tissues. Three independent microarray datasets were obtained from GEO (<http://www.ncbi.nlm.nih.gov/geo/>) (Edgar et al., 2002).

One of the datasets was a miRNA microarray study performed with 80 breast cancer samples and 15 normal samples (GSE45666 (Lee et al., 2013)). Two of the datasets were mRNA microarray studies (GSE3744 (Richardson et al., 2006), GSE5764 (Turashvili et al., 2007)). The total number of breast cancer samples and normal samples were 55 and 22, respectively. The details of the platforms and the sample numbers are given in Table 1.

3.2. Differentially expressed miRNA and mRNA lists

The miRNA microarray study and two mRNA studies (Table 1) were independently analyzed by BRB-Array Tools, which is an integrated package for the visualization and statistical analysis of gene expression data (Simon et al., 2007). Quantile normalization was used as the normalization method. All studies consisted of 2 groups, which are tumor and normal. Class comparison tests were performed to find out differentially expressed miRNAs and mRNAs between tumor and normal samples. This test provides powerful methods for finding differentially expressed genes when controlling the ratio of false positives. This method is similar to the significance analysis of microarrays method (Tusher et al., 2001) but provides more control of the false discovery rate (Simon et al., 2007).

As a result of class comparison analysis, miRNA and mRNA lists were obtained. Twenty-three miRNAs (11 upregulated and 12 downregulated; Table S1) were found to be differentially expressed between tumor and normal samples. Since two mRNA studies were performed, the DE mRNAs were listed as common DE genes in both of the studies. A total of 264 mRNAs (187 downregulated and 77 upregulated; Table S1) were found to be differentially expressed between tumor and normal samples and were common for each mRNA study.

3.3. Differentially expressed transcription factors list

To obtain the TFs among DE mRNA lists, which are given in Table S1, AnimalTFDB 2.0 was used (Zhang et al., 2015). This tool has a dataset that consists of 1691 transcription factors in 68 families in humans. When we compared it to the DE list, 18 of the genes in the DE gene list were found to encode proteins functioning as TFs: *ATF3*, *BHLHE41*, *EHF*, *FOSB*, *HOXA3*, *ID4*, *IRX1*, *NFIB*, *SOX10*, *MAFF*, *FOS*, *NR3C2*, *STAT1*, *EGR1*, *JUN*, *ZNF662*, *THRB*, and *ZBTB16*.

3.4. Significant breast cancer-specific circuits

CircuitsDB2 was used to identify important and regulatory circuits that consist of the members of DE miRNA, mRNA, and TF lists. CircuitsDB2 is a web service that includes

data as regulatory loops stored in a relational database that can be accessed through a dynamic web interface (Friard et al., 2010b). With use of this interface it is possible to find different kinds of circuits like feedforward loops (FFLs) in which a TF regulates a miRNA and they both regulate a target gene (Friard et al., 2010a). Members of the DE miRNA, mRNA, and TF lists were searched in microRNA-mediated FFLs in which a TF is the master regulator in CircuitsDB2.

Three significant circuits that are SOX10- and hsa-miR-301a-dependent were identified (Figure 1a) by CircuitsDB2 [5] analysis; in each circuit, hsa-miR-301a was found to be upregulated and its TF target SOX-10 was downregulated. Additionally, the three target genes that were under regulation of both miR-301a and SOX10 were downregulated as expected (Figure 1b).

3.5. Circuit members in diseases, biological processes, and pathways

To characterize the significant circuits in detail, their presence in diseases, biological processes, and signaling pathways were identified in detail by using related web-based tools.

The first step was to search the expression information of miRNA hsa-miR-301a. When breast cancer association data were extracted, concordant with our finding, it was found to be overexpressed in breast tumors. Since the data in PhenomiR (Ruepp et al., 2010) were obtained from independent studies and contain results from both cell lines and tumor samples, our results related to hsa-miR-301a could be accepted as robust for breast cancer (Table 2). Given the pathological, histological, and molecular differences (e.g., MCF-7: ER+; SK-BR-3: HER2+; MDA-MB-231: triple-negative) of the cell lines in Table 2, hsa-miR-301a, which is upregulated in the different cell lines and tumor specimens, may be accepted as a generalized and important marker for breast cancer. Additionally the expression profiles of other members of the circuits, SOX10, HOXA3, KIT, and NFIB, were searched in an independent microarray dataset (GSE17907; 51 tumor and 4 normal breast samples) and compatible to our results found to be significantly downregulated in tumor samples compared to normal ones (SOX10, P-value 1.00E-07, 11-fold downregulation; HOXA3, P-value 0.0004616, 4-fold downregulation; KIT, P-value, 3.00E-07, 11-fold

Figure 1. Significant circuits, which were results of CircuitsDB2 analysis. a) The triangular shapes are circuit diagrams taken from CircuitsDB2. The dark blue circles represent the TF, the green circles represent the miRNA, and the light blue circles represent the targets. b) Downregulation and upregulation terms indicate the differentiation of expression according to tumor vs. normal.

Table 2. Expression changes of hsa-miR-301a in previous studies in the literature. The table was created with the information obtained from PhenomiR 2.0 (Ruepp et al., 2010).

No.	ID	miRNA name	Disease	Tissue/cell line	PubMed ID	Regulation	Study design
1	447	hsa-mir-301a	Breast cancer	Breast epithelium	16754881	Up	Patient study, phenotype-control
2	451	hsa-mir-301a	Breast cancer	MCF-7 cell	16192569	Up	Cell culture study
3	366	hsa-mir-301a	Breast cancer	T-47D cell	16192569	Up	Cell culture study
4	481	hsa-mir-301a	Breast cancer	MDA-MB-231 cell	16192569	Up	Cell culture study
5	377	hsa-mir-301a	Breast cancer	SK-BR-3 cell	16192569	Up	Cell culture study
6	482	hsa-mir-301a	Breast cancer	MDA-MB-361 cell	16192569	Up	Cell culture study

downregulation; and NFIB, P-value 0.0012966, 3.7-fold downregulation).

The second step was to find out the pathways in which the targets of hsa-miR-301a were taking part. DIANA-mirPath v.3 was used for this purpose (Maragkakis et al., 2009; Vlachos et al., 2015). This tool aims to find the targets of microRNAs by using 3 different target algorithms (TargetScan, microT-CDS, and Tarbase) and determining the pathways in which these genes are significantly enriched. The members of our circuits, KIT, NFIB, and HOXA3, were among the identified targets (by mirPath) of hsa-miR-301a (data not shown). It was also seen in the HeatMap plotted after the pathway analysis that the targets of this microRNA function in pathways that are known to be important in cancer (Figure 2).

The third step was function analysis of the genes. For the characterization of the members of the significant circuits, which are SOX10, KIT, NFIB, and HOXA3, function analysis was performed for each of them using WebGestalt protein interaction network module-related function analysis (Table 3) (Wang et al., 2013).

3.6. Advanced bioinformatics analysis for in silico validation

To validate the significant circuits, the DIANA-mirExTra 2.0 tool was used (Maragkakis et al., 2009; Vlachos et al., 2016). We used DE miRNA and mRNA lists with fold change and P-value information as an input and the algorithm identified which miRNAs were central regulators (interactions based on microT-CDS and threshold type based on fold change with 2-fold change). Analysis showed that the most regulatory upregulated (tumor vs. normal) miRNA was hsa-miR-301a ($P = 7.3e-$

03), while the most regulatory downregulated (tumor vs. normal) miRNA was hsa-miR-376c ($P = 6.4e-03$), which is also the most downregulated DE miRNA in our list. As can be seen in Figure 3, hsa-miR-301a, which is the regulatory miRNA of the 3 circuits (Figure 1), was expressed as one of the central miRNAs, and as can be seen in the interaction details, KIT, NFIB, and HOXA3 are among the regulated target genes (Figure 3). These advanced and independent analysis results confirm and validate our CircuitsDB2 circuit analysis results.

4. Discussion

Breast cancer is a complex genetic disorder that is not controlled by a single factor but is controlled by many variables and is still the most common type of cancer in women today (<http://www.cancer.org/>). Although many scientific studies have been carried out on breast cancer, it is still unclear in many respects. Since breast cancer is a complex disease, to understand the development of breast cancer new biomarkers need to be discovered. In addition to the discovery of new biomarkers, it is also of great importance to investigate their relationships with each other (e.g., miRNA-TF-mRNA circuits). In recent years, systems biology approaches have become more important in the understanding of diseases. The development of the methods that help to understand large molecular data and the molecular mechanisms of breast cancer development is inevitable.

The aim of this study was to analyze the TF-miRNA-target gene relationships in a global perspective in breast cancer samples and to determine miRNA-TF-mRNA circuits that can play a role in breast cancer.

Figure 2. Pathway enrichment results of hsa-miR-301a targets. Targets of the miRNA and KEGG pathway results were used to construct the heat map, which was obtained from DIANA (Maragkakis et al., 2009; Vlachos et al., 2015). The intensity of color represents the logarithmic P-values.

Table 3. Function analysis results for the members of the significant circuits. The table was created with the information obtained from the WebGestalt protein interaction network module and related function analysis (Wang et al., 2013).

Gene name	Biological process	Cellular component	Molecular function
<i>SOX10</i>	Positive regulation of	Extrinsic to mitochondrial	HMG box domain
	gliogenesis	outer membrane	binding
	P = 4.84e-07	P = 9.81e-04	P = 1.91e-11
<i>KIT</i>	Intracellular signal	Cytosol	Signaling adaptor
	transduction		activity
	P = 4.67e-10	P = 3.22e-06	P = 1.07e-09
<i>NFIB</i>	Anterior/posterior pattern	Nucleus	Sequence-specific DNA
	specification		binding
	P = 0e+00	P = 0e+00	P = 0e+00
<i>HOXA3</i>	Negative regulation of	Perinuclear theca	Biotin-[acetyl-CoA-carboxylase]
	DNA replication		ligase activity
	P = 1.54e-03	P = 3.36e-03	P = 2.03e-03

Figure 3. Validation results of the circuits members. hsa-miR-301a (upregulated) and hsa-miR-376c (downregulated) are the central regulator miRNAs for the tumor vs. normal comparison. KIT and NFIB are targeted by hsa-miR-301a and hsa-miR-376c while HOXA3 is targeted by only hsa-miR-301a. The figure was obtained from DIANA-mirExTra 2.0 (Maragkakis et al., 2009; Vlachos et al., 2016).

In this study we focused on finding out regulatory miRNA-TF-mRNA circuits specific to breast cancer with the help of various web-based tools and databases. Through the workflow designed in the study and the bioinformatics methods used, three independent microarray studies (Table 1) were reanalyzed and were converted to circuits that are important in breast cancer development. The whole process enabled us to make more use of the data in the literature, which was achieved by using a microarray that is a high-throughput and expensive method. The tools used in the workflow are not only used to cross-check the data but also to validate the robustness of the results of this work. Such a work flow can also be applied for other diseases (Figure 4).

Circuit analysis revealed 3 significant miRNA-TF-mRNA circuits that are SOX10- and hsa-miR-301a-dependent: $SOX10 \rightarrow hsa-miR-301a \rightarrow HOXA3$, $SOX10 \rightarrow hsa-miR-301a \rightarrow KIT$, and $SOX10 \rightarrow hsa-miR-301a \rightarrow NFIB$ (Figure 1a). hsa-miR-301a, the common member of the circuits, is known to be an important miRNA in breast

cancer. It was found to be upregulated in breast tumors for our circuits (Figure 1b). In 2011, it was reported as a novel oncogene in lymph node-negative breast cancer and it was shown to mediate proliferation, migration, and invasion (Shi et al., 2011). Since then, other studies have been performed regarding the role of miR-301a upregulation in breast cancer (Ma et al., 2014; McDermott et al., 2014). In 2014, upregulation of that miRNA was found to be associated with poor prognosis in triple-negative breast cancers (Yu et al., 2014). When we searched the expression information of miRNA in PhenomiR (Ruepp et al., 2010), it was found to be overexpressed in breast tumors in independent studies (Table 2). Considering the concordance of the literature with our results and the pathological, histological, and molecular variability (e.g., ER+, HER2+, triple-negative) of the samples in Table 2 and in the literature, hsa-miR-301a, which is consistently upregulated in different breast cancer cell lines and tumors, may be accepted as a generalized marker for breast cancer development.

Figure 4. General study design and workflow. Purple boxes represent bioinformatics tools used for the processing of the data, green boxes represent main work steps, and orange ellipses represent input and outputs.

The results of DIANA-mirPath together with WebGestalt may strengthen the role of these significant circuits in breast cancer. The pathway enrichment analysis results showed that the target genes and the target TF SOX10 were found to be members of cancer-specific pathways like TGF-beta, mTOR, and p53 signaling pathways. SOX10, which is the common TF in our circuits, was reported to be one of the regulated TFs in breast cancer by Zang et al. Those authors combined seven studies that conducted next-generation sequencing for breast tumors and normal samples and, concordant with our in silico results, SOX10 was found to be downregulated in breast cancers (Zang et al., 2017).

The two common members of the three circuits in this study are two regulative elements of gene regulation, a miRNA and a TF. The balance of the reciprocal regulation among these two important elements may determine the fate of the cell, which is in our situation cancer progression. In the suggested circuits in this study the regulative effect of miRNA is seen to be dominant and it

is consistently upregulated and negatively regulates the TF (SOX10) expression. This tight regulation among miRNA-TF affected the cancer-related target genes *HOXA3*, *KIT*, and *NFIB*. The well-known regulation of these target genes in different cancers (Moon et al., 2011; Jachetti et al., 2017; Zhang et al., 2017) and validation of them to be downregulated in breast tumor samples by an independent microarray dataset support the idea of the systems biology approach in cancers originating from different tissues.

In summary, the results of this study show that regulatory motifs involving miRNAs and TFs may be useful for understanding breast cancer regulation and for predicting new biomarkers. Characterization of these combinatorial regulatory mechanisms is important to reveal the biological processes that take part in breast cancer in detail by constructing networks and circuits. Finding out new circuits combining miRNAs, TFs, and mRNAs may provide further insight to improve our knowledge of breast cancer development.

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