RESEARCH ARTICLE

Transcription Regulation Network Analysis of MCF7 Breast Cancer Cells Exposed to Estradiol

Jun-Zhao Wu[1], Peng Lu[2]*, Rong Liu[3], Tie-Jian Yang[2]

Abstract

Background: In breast cancer, estrogen receptors have been demonstrated to interact with transcription factors to regulate target gene expression. However, high-throughput identification of the transcription regulation relationship between transcription factors and their target genes in response to estradiol is still in its infancy. Purpose: Thus, the objective of our study was to interpret the transcription regulation network of MCF7 breast cancer cells exposed to estradiol. Methods: In this work, GSE11352 microarray data were used to identify differentially expressed genes (DEGs). Results: Our results showed that the MYB (v-myb myeloblastosis viral oncogene homolog [avian]), PGR (progesterone receptor), and MYC (v-myc myelocytomatosis viral oncogene homolog [avian]) were hub nodes in our transcriptome network, which may interact with ER and, in turn, regulate target gene expression. MYB can up-regulate MCM3 (minichromosome maintenance 3) and MCM7 expression; PGR can suppress BCL2 (B-cell lymphoma 2) expression; MYC can inhibit TGFB2 (transforming growth factor, beta 2) expression. These genes are associated with breast cancer progression via cell cycling and the TGFβ signaling pathway. Conclusion: Analysis of transcriptional regulation may provide a better understanding of molecular mechanisms and clues to potential therapeutic targets in the treatment of breast cancer.

Keywords: Regulation network - network motif analysis - breast cancer - estradiol - microarray - transcriptome network

Asian Pacific J Cancer Prev, **13**, 3681-3685

Introduction

Breast cancer is by far the most common and important malignancy in women. Despite surgery and adjuvant systemic therapy, many patients will eventually relapse and die of metastatic breast cancer (Müller et al., 2011). Therefore, it is urgent to explore new therapeutic strategies.

Lifetime exposure of breast tissue to female sex steroid estrogens is widely considered as a major risk factor for the development of breast cancer (Yager and Davidson, 2006). Estrogens promote cell replication by binding to the estrogen receptors alpha and beta (ERα and ERβ, respectively). Ligand-activated ER can regulate target gene expression via directly binding to estrogen response elements (EREs), non-consensus EREs and ERE half sites of these genes (Klinge, 2000). In addition, ER can tether to other transcription factors, such as Sp1 (Zhao et al., 2005) and then subsequently modulate corresponding targets gene expression. Example of the tethering mechanism of ER transactivation include ERα interaction with SP1 in conferring estrogen responsiveness on transforming growth factor α (Vyhlidal et al., 2000), LDL receptor genes (Li et al., 2001), and α5 integrin subunit gene (Sisci et al., 2010).

However, high-throughput screening of multiple transcription factors and their target genes in response to estradiol is still rare. Therefore, the objective of this study was to identify potential transcription factor in response to estradiol and explore the transcription regulation relationships between transcription factors and differentially expressed target genes in breast cancer by using the microarray data and transcriptional network analysis. Moreover, we characterized their underlying molecular mechanisms by KEGG pathway enrichment analysis.

Materials and Methods

Affymetrix microarray data

The transcription profile of GSE11352 was obtained from NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) which is based on the Affymetrix Human Genome U133 Plus 2.0 Array. Total 18 chips, purchased from Microarray and Expression Genomics in Genome Institute of Singapore, were used for analysis. We used oligonucleotide expression microarrays (Affymetrix GeneChip U133 Plus 2.0) to identify estradiol (E2)-responsive genes in the estrogen-receptor positive breast cancer cell line, MCF7. MCF7 cells were grown to 30-50% confluency and exposed to 10nM E2 (or vehicle only) at 12, 24, and 48 hours. Each time point was performed

*[1]Breast Cancer Diagnosis and Treatment Center, Henan Cancer Hospital, Henan, [2]Department of Oncological Surgery, [3]Academician Experts Workstation of Henan Province, People's Hospital of Zhengzhou, Zhengzhou, China *For correspondence: lupeng.surgeon@gmail.com*

in triplicate (ie, biological replicates). Total RNA was isolated from cells using the Qiagen RNeasy kit, and 5 micrograms of total RNA was amplified, labeled and hybridized to the array according to the manufacturer's protocols. E2 treated are used as controls.

Regulation data

A total of 774 pairs of regulatory relationship between 219 transcription factors (TFs) and 265 target genes were collected from TRANSFAC (Matys et al., 2003) (http://www.gene-regulation.com/pub/databases.html). A total of 5722 pairs of regulatory relationship between 102 TFs and 2920 target genes were collected from TRED (Jiang et al., 2007) (http://rulai.cshl.edu/TRED/). By combining the above two regulation datasets, total 6328 regulatory relationships between 276 TFs and 3002 target genes were collected.

Differentially expressed genes (DEGs) analysis

For the GSE11352 dataset, the limma method(Smyth, 2004) was used to identify Differentially expressed genes(DEGs). The original expression datasets from all conditions were extracted into expression estimates, and the linear model was constructed. The DEGs only with the fold change value larger than 2 and p-value less than 0.05 were selected.

Co-expression analysis

For demonstrating the potential regulatory relationship, the Pearson Correlation Coefficient (PCC) was calculated for all pair-wise comparisons of gene-expression values between TFs and the DEGs. The regulatory relationships whose absolute PCC are larger than 0.75 were considered as significant.

Regulation network construction

Using the regulation data that have been collected from TRANSFAC (Matys et al., 2003) database and TRED (Jiang et al., 2007) database, we matched the relationships between differentially expressed TFs and their differentially expressed target genes.

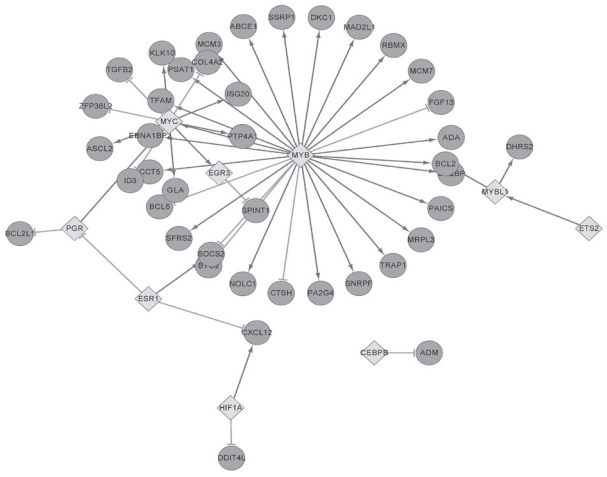

Figure 1. Regulation Network. The diamond nodes indicate TFs and the circle node stand for target genes. The black lines indicate up-regulation and the grey lines indicate down-regulation

Based on the above two regulation datasets, the significant relationships (PCC > 0.75 or PCC < -0.75) between TFs and their target genes, we constructed the regulation networks by Cytoscape (Shannon et al., 2003).

Network motif

Fanmod (Wernicke and Rasche, 2006) is a tool for finding so-called networks motifs in a network, that is, it finds small vertex-induced subgraphs that occur significantly more often than in random networks.

We applied enumeration algorithm to search for 5 Size of subgraphs, which were found more than 5 times, |Z-Score| >5, and p-value < 0.05. The Z-Score is the original frequency minus the random frequency divided by the standard deviation. The higher the Z-Score, the more significant is a motif. The p-value of a motif is the number of random networks in which it occurred more often than in the original network, divided by the total number of random networks. Therefore, p-values range from 0 to 1; the smaller the p-value, the more significant is the motif.

KEGG pathway analysis

DAVID (Huang da et al., 2009), a high-throughput and integrated data-mining environment, analyzes gene lists derived from high-throughput genomic experiments. Use the DAVID to identify KEGG pathway analysis.

Results

Microarray data analysis

Publicly available microarray data set GSE11352 was obtained from GEO. Total 1699 DEGs with the fold change value > 2 and p-value < 0.05 were selected using the limma method. All of these genes are positive expression genes.

Regulation network analysis

To obtain regulation network, based on the significant relationships (PCC > 0.75 or PCC < -0.75) between TFs and their target genes, 49 expression relationships including 9 TFs and 41 target genes were selected. By integrating expression relationships above, a regulation network was built between its TFs and target genes (Figure 1). MYB (v-myb myeloblastosis viral oncogene homolog [avian]), PGR (Progesterone receptor), and MYC (v-myc myelocytomatosis viral oncogene homolog [avian]) were hub nodes in our transcriptome network. MYB can up-

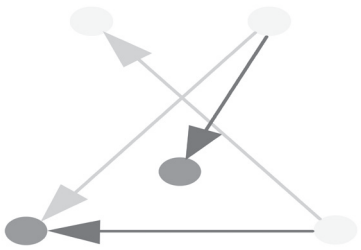

Figure 2. Network Motif Subgraph. The light grey circle nodes indicate TFs and the black circle nodes indicate target genes. The black lines indicate up-regulation and the grey lines indicate down-regulation

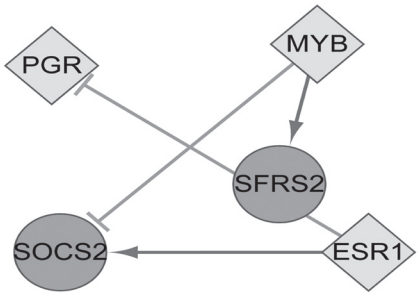

Figure 3. 5 Size Network Motif Contrast Between Network Motif Subgraph and Regulation Network. The diamond nodes indicate TFs and the circle node indicate target genes. The black lines indicate up-regulation and the grey lines indicate down-regulation

Table 1. KEGG Enrichment Pathways

Term	Description	Count	p-value	FDR
hsa04110	Cell cycle	5	0.002145	2.037548
hsa05200	Pathways in cancer	7	0.002717	2.574967
hsa05222	Small cell lung cancer	4	0.006067	5.667882
hsa05220	Chronic myeloid leukemia	3	0.044476	35.35193

regulate MCM3 (minichromosome maintenance 3) and MCM7 expression; PGR can suppress BCL2 (B-cell lymphoma 2) expression; MYC can inhibit TGFB2 (transforming growth factor, beta 2) expression.

Network motif analysis

To search for 5 Size of subgraphs, which were found more than 5 times, |Z-Score| > 5, p-value < 0.05 between TFs and its target genes, 49 expression relationships including 9 TFs and 41 target genes were selected, network motif subgraph were built between TFs and their target genes (Figure 2 and 3).

Function analysis of the network

Using the KEGG pathways to descript the function of the Regulation network, several KEGG pathways were enriched among these pathways in the regulation network, including Cell cycle (hsa04110), Pathways in cancer (hsa05200) and TGF-beta signaling pathway (hsa04350) and so on (Table 1). Table 1 only listed the enriched KEGG pathways with p-value<0.05.

Discussion

In this study, we systemically investigated the regulation network of breast cancer between TFs and target genes and their underlying molecular pathways. We have shown that the genes MYB, PGR, and MYC were hub nodes in our transcriptome network. Among them, MYB may be involved in Cell cycle pathway via up-regulation of MCM3 and MCM7 expression; PGR can suppress BCL2 expression to involve in Pathway in cancer; MYC can be associated with TGFβ signaling via inhibition of TGFB2 expression.

With regard to the cell cycle, MCM3 and MCM7 are members of the highly conserved mini-chromosome maintenance proteins that are involved in the initiation of eukaryotic chromosome replication (Pacek and Walter, 2004). The acetylation of MCM3 inhibits the initiation of DNA replication and cell cycle progression (Takei et al., 2002). MCM3 gene and protein are identified overexpression in various human cancers, including breast cancer. More importantly, MCM3 protein is stable in MCF-7 breast cancer cells even up to 96 hours after serum starvation (Ha et al., 2004). Approximately 60% of the aggressive prostate cancer specimens had increased MCM7 protein expression. Amplification or overexpression of MCM7 is significantly associated with relapse, local invasion and a worse tumor grade (Ren et al., 2006).

MYB encodes a transcription factor that predominantly been associated with the regulation of hematopoiesis and tumorigenesis. MYB is overexpressed in a high proportion of human breast tumors and this expression strongly correlates with ER positivity (Kauraniemi et al., 2000). Further study suggest estrogen/ER acts directly to relieve transcriptional attenuation due to sequences within the first intron, specifically, the SL-poly (dT) motif (Drabsch et al., 2007). Our study also indicated MYB can promote MCM3 and MCM7 expression, which is in accordance with previous reports because five (MCM3-7) out of the six MCM genes (80%) are experimentally shown down-regulated after MYB silencing (Lefebvre et al., 2010).

Regarding pathways in cancer, BCL2 is an anti-apoptotic protein that is overexpressed in more than 60% of breast cancer patients. BCL2 protein expression in breast cancer is associated with an indolent phenotype of low-grade, slowly proliferating, ER+ breast tumors (Dawson et al., 2010). BCL2 exerts its oncogenic role by preventing cells from undergoing apoptosis or autophagic cell death(Akar et al., 2008). Overexpression of BCL2 contributes to the development of resistance to chemotherapy and radiation and for its aggressive tumor phenotype in patients with a variety of cancers (Real et al., 2002).

PGRs are ligand-activated transcription factor members of the steroid hormone family of nuclear receptors. PGR activates the signal-transducing Src/p21ras/Erk pathway in human breast cancer cells depended on an interaction of ER (Migliaccio et al., 1998). Low PGR levels were significantly associated with worse outcome in all breast cancer patients (Pathiraja et al., 2011), with a greater risk of locoregional recurrence (Albert et al., 2010). Upon progestin binding, PGR translocates to the nucleus and binds to specific progesterone response elements (PREs) in the promoter of target genes, such as BCL2 (Yin et al., 2007). BCL2 expression is down-regulated in progesterone receptor positive T47-D cancer cells, thus inhibiting growth and inducing apoptosis of breast cancer cells (Formby and Wiley, 1998).

There is accumulating evidence that transforming growth factor (TGF)-β signaling pathway plays a dichotomous role in tumor progression, acting as a tumor suppressor early and as a pro-metastatic pathway in late-stages (Wiercinska et al., 2011). TGFB2 mRNA expression is detected in breast cancer samples and the TGFB2 mRNA level changed in 8 cases, increasing seven times and decreasing once after tamoxifen treatment (BRANDT et al., 2003).

MYC is a transcription factor with basic region/

helix–loop–helix/leucine zipper domain. It has been implicated to participate in many physiological events, such as cellular proliferation, growth, metabolism, apoptosis, and differentiation (Dang, 1999). c-MYC is a well-characterized ERα target gene that plays a critical role in the ability of estrogen to enhance the proliferation of breast cancer cells (Cheng et al., 2006). MYC proto-oncogene is shown frequently up-regulated expression in breast cancers (Chrzan et al., 2001). Knockdown of c-Myc expression by RNAi could significantly inhibit MCF-7 breast tumor cells growth both in vitro and in vivo (Wang et al., 2005). Up-regulation of c-MYC by ERα results in further recruitment of this transcription factor partner to other ERα-responsive promoters and led to influence target gene expression.

c-Myc induce a marked down-regulation of TGFB2 (3.5-fold down regulated) in a human epithelial cell line, hT-RPE/MycER cells and then inhibit tumor growth (Pocsfalvi et al., 2011). The ability of c-Myc to bind Miz1 is shown to be important for the repression of TGFB2 and to antagonize growth suppression and induction of senescence by TGFβ during lymphomagenesis. Therefore, repression of TGFB2 by c-Myc may be mediated by Miz1 and could play an important role in cancer progression (Van Riggelen et al., 2010). Our result also found a down-regulation of TGFB2 by MYC in our regulation network of breast cancer.

In conclusion, our present findings shed new light on the ER-response regulation network of breast cancer. We showed MYB, PGR, and MYC TFs could interact with ER and, in turn, regulate the expression of MCM3, MCM7, BCL2, and TGFB2 genes which are all associated with breast cancer progression.

Acknowledgements

The author(s) declare that they have no competing interests.

References

Akar U, Chaves-Reyez A, Barria M, et al (2008). Silencing of Bcl-2 expression by small interfering RNA induces autophagic cell death in MCF-7 breast cancer cells. *Autophagy,* **4**, 669.

Albert JM, Gonzalez-Angulo AM, Guray, M, et al (2010). Estrogen/progesterone receptor negativity and HER2 positivity predict locoregional recurrence in patients with T1a, bN0 breast cancer. *Int J Radiation Oncol Biol Physics,* **77**, 1296-302.

Brandt S, Kopp A, Grage B, Knabbe C (2003). Effects of tamoxifen on transcriptional level of transforming growth factor beta (TGF-β) isoforms 1 and 2 in tumor tissue during primary treatment of patients with breast cancer. *Anticancer Res,* **23**, 223-9.

Cheng ASL, Jin VX, Fan M, et al (2006). Combinatorial analysis of transcription factor partners reveals recruitment of c-MYC to estrogen receptor-α responsive promoters. *Molecular Cell,* **21**, 393-404.

Chrzan P, Skokowski J, Karmolinski A, et al (2001). Amplification of c-myc gene and overexpression of c-Myc protein in breast cancer and adjacent non-neoplastic tissue. *Clin Biochem,* **34**, 557-62.

Dang CV (1999). c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol Cell Biol,* **19**, 1-11.

Dawson S, Makretsov N, Blows F, et al (2010). BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. *Br J Cancer,* **103**, 668-75.

Drabsch Y, Hugo H, Zhang R, et al (2007). Mechanism of and requirement for estrogen-regulated MYB expression in estrogen-receptor-positive breast cancer cells. *Proc Natl Acad Sci U S A,* **104**, 13762-7.

Formby B, Wiley T, et al (1998). Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53. *Ann Clin Lab Sci,* **28**, 360-9.

Ha SA, Shin SM, Namkoong H, et al (2004). Cancer-associated expression of minichromosome maintenance 3 gene in several human cancers and its involvement in tumorigenesis. *Clin Cancer Res,* **10**, 8386-95.

Huang da W, Sherman BT, Lempicki RA (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc,* **4**, 44-57.

Jiang C, Xuan Z, Zhao F, Zhang MQ (2007). TRED: a transcriptional regulatory element database, new entries and other development. *Nucleic Acids Res* **35**, D137-40.

Kauraniemi P, Hedenfalk I, Persson K, et al (2000). MYB oncogene amplification in hereditary BRCA1 breast cancer. *Cancer Res,* **60**, 5323-8.

Klinge CM (2000). Estrogen receptor interaction with co-activators and co-repressors. *Steroids,* **65**, 227-51.

Lefebvre C, Rajbhandari P, Alvarez MJ, et al (2010). A human B-cell interactome identifies MYB and FOXM1 as master regulators of proliferation in germinal centers. *Mol Syst Biol,* **6**, 377.

Li C, Briggs MR, Ahlborn TE, et al (2001). Requirement of Sp1 and estrogen receptor α interaction in 17β-estradiol-mediated transcriptional activation of the low density lipoprotein receptor gene expression. *Endocrinology,* **142**, 1546-53.

Matys V, Fricke E, Geffers R, et al (2003). TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res,* **31**, 374-8.

Migliaccio A, Piccolo D, Castoria G, et al (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO J,* **17**, 2008-18.

Müller A, Kohrt HEK, Cha S, et al (2011). Long-term outcome of patients with metastatic breast cancer treated with high-dose chemotherapy and transplantation of purified autologous hematopoietic stem cells. *Biol Blood Marrow Transplant,* **18**, 125-33.

Pacek M, Walter JC (2004). A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. *EMBO J,* **23**, 3667-76.

Pathiraja TN, Shetty PB, Jelinek J, et al (2011). Progesterone receptor isoform-specific promoter methylation: association of PRA promoter methylation with worse outcome in breast cancer patients. *Clin Cancer Res,* **17**, 4177-86.

Pocsfalvi G, Votta G, De Vincenzo A, et al (2011). Analysis of secretome changes uncovers an autocrine/paracrine component in the modulation of cell proliferation and motility by c-Myc. *J Proteome Res,* **10**, 5326-37.

Real PJ, Sierra A, De Juan A, et al (2002). Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. *Oncogene,* **21**, 7611-8.

Ren B, Yu G, Tseng GC, et al (2006). MCM7 amplification and overexpression are associated with prostate cancer progression. *Oncogene,* **25**, 1090-8.

Shannon P, Markiel A, Ozier O, et al (2003). Cytoscape: a software environment for integrated models of biomolecular

interaction networks. *Genome Res,* **13**, 2498-504.
Sisci D, Middea E, Morelli C, et al (2010). 17β-Estradiol enhances α 5 integrin subunit gene expression through ERα–Sp1 interaction and reduces cell motility and invasion of ERα-positive breast cancer cells. *Breast Cancer Res Treat,* **124**, 63-77.
Smyth GK (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol,* **3**, Article3.
Takei Y, Assenberg M, Tsujimoto G, Laskey R (2002). The MCM3 acetylase MCM3AP inhibits initiation, but not elongation, of DNA replication via interaction with MCM3. *J Biol Chem,* **277**, 43121-5.
Van Riggelen J, Müller J, Otto T, et al (2010). The interaction between Myc and Miz1 is required to antagonize TGFβ-dependent autocrine signaling during lymphoma formation and maintenance. *Genes Dev*, **24**, 1281-94.
Vyhlidal C, Samudio I, Kladde M , Safe S (2000). Transcriptional activation of transforming growth factor alpha by estradiol: requirement for both a GC-rich site and an estrogen response element half-site. *J Mol Endocrinol,* **24**, 329-38.
Wang Y, Liu S, Zhang G, et al (2005). Knockdown of c-Myc expression by RNAi inhibits MCF-7 breast tumor cells growth in vitro and in vivo. *Breast Cancer Res,* **7**, R220-8.
Wernicke S, Rasche F (2006). FANMOD: a tool for fast network motif detection. *Bioinformatics,* **22**, 1152-3.
Wiercinska E, Naber HPH, Pardali E, et al (2011). The TGF-β/Smad pathway induces breast cancer cell invasion through the up-regulation of matrix metalloproteinase 2 and 9 in a spheroid invasion model system. *Breast Cancer Res Treat,* **128**, 657-66.
Yager JD, Davidson NE (2006). Estrogen carcinogenesis in breast cancer. *New Engl J Med,* **354**, 270-82.
Yin P, Lin Z, Cheng YH, et al (2007). Progesterone receptor regulates Bcl-2 gene expression through direct binding to its promoter region in uterine leiomyoma cells. *J Clin Endocrinol Metab,* **92**, 4459-66.
Zhao Y, Han W, Li Q, et al (2005). Mechanism of transcriptional regulation of LRP16 gene expression by 17-β estradiol in MCF-7 human breast cancer cells. *J Mol Endocrinol,* **34**, 77-89.