Research Article

Differential Expression of EGFR, MAP2K4 and E2F3 Genes as Targets of miR-141 and Its Association with Immune System Pathway

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Abstract

MicroRNAs by their structural complementarity capabilities have canonical roles in gene regulation. In this paper; we investigate expression of EGFR, MAP2K4 and E2F3 genes targeted by miR-141, a member of miR-200 family. EGFR, MAP2K4 and E2F3 were predicted as the potential targets of mir-141 by using online miRNA bioinformatics tools. MCF-7 cells were transfected with mir-141-precursor and inhibitor vectors. Expression of miR-141 and target genes was determined by using qRT-PCR. To see the most relevant pathways regulated by miR-141, we constructed two separate networks by NetworkAnalyst and enriched list of underlying genes by Enrichment analysis tools. The expression changes of all three predicted targets were higher in transfected cells with anti-mir-141 vector, compared with the control untransfected cells. By contrast, in transfected cells with pre-mir 141, we did not see significant expression changes in EGFR, E2F3 and MAP2K4. List of genes in total networks as well as explored functional modules were enriched separately. Enrichment analysis shows that immune system pathway has the strongest relationship with the proteins potentially targeted by miR-141. The present study demonstrated potential role of miR-141 in regulation of EGFR, MAP2K4 and E2F3 expression and suggested innate immunity pathways as the key pathway through which this regulatory network contributes to breast cancer development.

***Keywords*:** Breast cancer, MiR-141, EGFR, MAP2K4, E2F3

Introduction

Ranked as the second leading cause of cancer death worldwide, breast cancer is a major health concern. The current trend underlines the need for a greater understanding of the molecular biology of breast cancer. With regard to breast cancer development, microRNA regulation of cancer-related pathways plays an important role.

MicroRNAs are short non-coding RNAs with 16-22 nucleotides. MiRNA seeds can make imperfect matches either on 3'-UTR or 5' UTR of targeted mRNAs.

Following this imperfect match, numerous biological events can occur, including translational inhibition, mRNA complete and incomplete degradation (Xu et al., 2013) . Since miRNAs regulatory mechanisms through seed matching endowed it with a master regulatory role, the targets could be as numerous as possible. miRNAs control many biological processes and molecular functions including; tumorigenesis, metastasis, drug resistance, invasion, self-renewal and proliferation.

Since the first reports that demonstrated relationship of pathological Epithelial to Mesenchymal Transition and cancer stem cell with miR-200 family in breast cancer, many researchers choose members of miR-200 family as a vital potential candidate in cancer (Park et al., 2008; Shimono et al., 2009). miR-200 family includes five members, miR 200a/b/c, miR 429 and miR-141. miR 200a/b and miR 429 located on human chromosome 1, miR-200c and miR-141 located on human chromosome 12. MiR-141 is short structured with low GC content(Kim et al., 2012) . Many studies showed that members of miR-200 family act as a tumor suppressor in cancer.

To address some of those important studies, cell growth, cell proliferation and metastasis in tissue samples and cell lines of hepatocellular carcinoma have been shown to be strongly regulated by miR-141 (Xue et al., 2014). Analysis of miRNA-mRNA interactions in MDA-MB-231 cells revealed down regulation of miR-141 in invasive cell lines (Luo et

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al., 2013). In addition, bioinformatics analysis in miRNA profiling of bladder cancer patients indicated miR-141 as a hub-miRNA and E2F3 as a hub target gene (Canturk et al., 2014)

To investigate targets of miR-141 in MCF-7 breast cancer cell line, we applied online databases and software. By looking in overlapping results from different software, we predicted E2F3, MAP2K4 and EGFR as the potential targets of miR-141.

E2F3, among other members, causes transition of cells from G1 to S phase by interacting with pocket protein Retinoblastoma. Through this interaction, Rb is phosphorylated and inactivated, resulting in release of E2F3 molecule. E2F3 employs cycline E and to initiates transcription of cascades of genes (Shimono et al., 2009). E2F3 causes the transition of cells from G1 to S phase by employing cycline E and CDK2. Transcription of E2F3 initiates transcription of cascades of genes, leading to cell cycle progression. In breast cancer, studies in four different cell lines have shown that responsible receptors for proliferation pathways is highly regulated by E2F3, especially in ER positive cell lines (Nguyen-Vu et al., 2013). MAP2K4 belongs to the protein kinase superfamily. Activation of protein kinase MAP2K4 initiates phosphorylation of p38 and JNK that results in phosphorylation of Stress Activated Protein Kinase (SAPK) pathway (Marasa et al., 2009). To date, many discrepancies have been found about role of MAP2K4 as a tumor suppressor or pro-oncogene in cancer. Even in a same type cancer, many controversies are raised about this major signaling molecule. In some studies, expression of MAP2K4 is increased by downregulation of mir-141(Marasa et al., 2009).

EGFR ligand binding switches on a cascade of downstream signaling pathways including RAS-RAF-MEK-ERK, PI3 kinase-AKT, PLC gamma-PKC, STATs. Increased expression of EGFR has been proved to result in Erk-induced EMT, increased tumor size and chemotherapy resistance in triple Negative Breast Cancer (TNBC) and Inflammatory Breast Cancer (IBC) (Masuda et al., 2012).

Strong evidence of correlation of EGFR and miR-200 family member are provided by Uhlmann et.al in 2010. MiR-200bc/429 and miR-200a/141 regulate cell cycle progression through p27/Kip1 (Uhlmann et al., 2010).

In this study, expression changes of E2F3, MAP2K4 and EGFR following ectopic transfection of pre-mir-141 and anti-mir-141 is investigated. To suggest the most probable associated signal pathways with mir-141- target genes circuit, two different PPI (Protein-Protein Interaction) network regulated with miR-141 is constructed and functional modules of each PPI networks are detected. PPI networks are then enriched, using Enrichr.

Materials and Methods

Cell Line and Culture Condition

MCF-7 cells (human breast adenocarcinoma cell line) were purchased from Iranian Biological Resource Center (IBRC). MCF-7 cells have been cultured in DMEM/F12 medium (Caisson Labs, USA) supplemented with 10% Fetal Bovin Serum (Atocel, Austria), with 1% Penicillin/Streptomycin (Biowest, Canada) antibiotic. Cells were grown in 5% CO2 at 37-degree incubator, humidified 90%.

Construction of MiR-141 Precursor (Pre-miR-141) and MiR-141 Inhibitor (Anti-MiR-141) Vector

Genomic sequence of mir-141 precursor, extracted from human normal White Blood Cells was amplified by Pfu polymerase (GeneAll, South Korea), using CCCTGTAGCAACTGGTGAGC as forward primer and CCCTGAAGGTTACTGCCGAG as reverse primer. The PCR product is then ligated into pCR®2.1 (Invitrogen, USA) and transformed into DH5α competent cells (TaKaRa, Japan). Ligated sequence was directly cloned into pTracer™-SV40 vector (Invitrogen, USA) and transformed to DH5α competent cells (TaKaRa, Japan). Zeocin™ antibiotic (Life Technologies, USA) was used as the selective marker. mir-141- pTracer™-SV40 vector was further purified by Plasmid Midiprep Kit (Qiagene, USA) according to the protocol.

PLenti-III-mir141-off vector and mock vector were chemically synthesized by ABM (Canada) and then transformed into DH5α competent cells (TaKaRa, Japan). Positive selected clones were chosen and purified by Plasmid Midiprep Kit (Qiagene, USA) according to the protocol.

Transient Transfection

MCF-7 cells were transfected with mir-141-pTracer™-SV40 vector and pLenti-III-mir141-off vector in 2 different 24-well plates. A day prior to transfection, 110000 cells were seeded into a 24-well plate. Cells were seeded into plates with complete DMEM/F12 medium supplemented with 10% FBS cells and were kept in antibiotic free media. Cells were transfected with P-tracer SV40- mir141, PLenti-III-mir141-off vector and mock vector as well. Fluorescent microscope (Olympus IX2-RFACA) was used to monitor the transfected cells

for GFP-positive signals 24h and 48h after transfection.

MTT Assay

Cell viability was determined 48 hours' post-transfection by MTT method. 20 * 10^4 cells were seeded in 96-well plates and cultured for 24 hours before transfection. Cells were then transfected with 1µg of pre-mir 141 as well as anti-mir 141 vectors for 48 hours. 1X MTT working solution (Atocel, Austria) was prepared using Phosphate Buffer Saline. 10µl of 1X MTT working solution was added to each well and incubated for 4 hours in co2 incubator 100 µl of DMSO was added as solubilization buffer. Absorbance was measured at 590 nm using a microtiter plate reader (BioRad, USA). Viability of untreated cells was set at 100%, and absorbance of wells with medium and without cells was set as zero.

RNA Isolation, Reverse-Transcription and Real-Time qRT-PCR for Mir-141 and Target Genes

Total RNA was extracted by Tripure (Roche Applied Science, Germany), then was used as the template for Reverse transcriptase reaction. Reverse transcription was performed using stem-loop RT primers providing in the kit (Biorbyte, England). Real-time quantitative PCR analysis was performed using hsa-miR-141 Real-time Detection kit and U6 Calibration (Biorbyte, England). RT Random hexamer primers were used for revers transcription according to the manufacturer (Roche Applied Science, Germany). Real-time quantitative PCR analysis was performed using QuantiTect SYBR® Green PCR Kits (Qiagene, USA). Primer Sequences of predicted target genes are given (Table 1).

Table 1. Primer sequences of EGFR, E2F3 and MAP2K4 genes

1- EGFR	*Forward primer* *5'* TGCCACCTGCGTGAAGAAG *3'*
	Reverse primer *5'* ACCTATTCCGTTACACACTTTGC *3'*
2- E2F3	*Forward primer* *5'*GCCTGACTCAATAGAGAGCCTAC *3'*
	Reverse primer *5'* AGTCTTTGGAAGCGGGTTTAGG *3*'
3- MAP2K4	*Forward primer* *5'* ACTTCGGCATCAGTGGACAG *3'*
	Reverse primer *5'* GACATCAGAGCGGACATCATATC *3'*

Bioinformatics and Statistical Analysis:

Target Prediction

Online bioinformatics tools are applied to predict targets of miRNA-141. Targets with the highest score matching to miR-141 which were also overlapping in Target Scan, Mir Walk, mirBASE, mir Map and miRANDA were predicted.

PPI Network Construction

Two discrete PPI networks are differently seeded and constructed. For the first network, that is Network A, EGFR, MAP2K4 and E2F3 proteins are considered as seeds. The second PPI network, Network B, is seeded by target proteins predicted by miRmap. 60 proteins with the probability of >99% to be targeted by miR-141 is retrieved from miRmap. To find the corresponding proteins, target genes were mapped on PPI network which is used by NetworkAnalyst, including high-quality protein-protein interaction (PPI) database based on InnateDB that also cover other PPI databases like IntAct, MINT, DIP, BIND, and BioGRID. Of these targets, 51 proteins are selected as seeds of the network. Networkanalyst is used for network construction and visualization.

Functional Module Analysis

In the next step, NetworkAnalyst module detector is employed to find functional modules of these two constructed networks. Top three functional modules of each network are screened and Functional Pathway Enrichment Analysis is done by Enrichr. Pathways are then studied using Reactome 2016.

Results

Target Prediction

Table 2 to 4 show predicted consequential pairing of target region and miRNA. The type of seed matching is also indicated. Position of the predicted target genes that will be complemented to has-miR-141-3p or has-miR-141-5p has been indicated in the first right column in table 2.

Seed match sequence is also represented graphically in the second column. Description of types of seed matching that is shown in the last columns. Table 3 shows possible complementarity of has-miR-141-3p or has-miR-141-5p with EGFR, MAP2K4 and E2F3 based on miRmap online target prediction tool. Structural properties as well as miRmap score are also represented. Table 4 shows miRDB results of predicted targets for has-miR-141-3p. Location of seed sequences, target ranks and scores are shown.

Table 2. List of predicted targets by Target scan showing the type of seed matching

	Predicted Consequential Pairing of Target Region (top) and miRNA (bottom)	Seed Match
Position 69-75 of E2F3 3' UTR hsa-miR-141	5' …AGAACAUCUGUCAUGCAGUGUUG… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8
Position 2603-2610 of E2F3 3' UTR hsa-miR-141	5' …ACAUGAGCUGUCAAACAGUGUUA… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	8mer
Position 2771-2777 of E2F3 3' UTR hsa-miR-141	5' ...UUGUAAUUUUUUAAGAGUGUUAU... |||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-1A
Position 28-34 of EGFR 3' UTR hsa-miR-141-3p	5' …AUAUAAAUGGGAAAUCAGUGUUU… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8
Position 75-81 of MAP2K4 3' UTR hsa-miR-141	5' …UUUCAUCCCGUAUCACAGUGUUU… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8
Position 192-199 of MAP2K4 3' UTR hsa-miR-141	5' …ACCUGAUUGAUCACACAGUGUUA… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	8mer

* 7mer-m8: An exact match to positions 2-8 of the mature miRNA (the seed + position 8)
** 8 mer: An exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'

Table 3. List of predicted targets by miRmap

miRNA	**Gene**	**ΔG Open**	**Probability Exact**	**miRmap Score**
hsa-miR-141-5p	EGFR	95.42	95.13	98.54
hsa-miR-141-3p	MAP2K4	76.45	97.05	98.29
hsa-miR-141-3p	E2F3	77.88	77.15	97.55
hsa-miR-141-3p	EGFR	71.78	66.87	87.11
hsa-miR-141-5p	E2F3	55.93	48.53	49.72

Table 4. List of predicted targets by miRDB

MiRNA Name	**Gene Symbol**	**Target Rank**	**Target Score**	**Seed Location**	**3'UTR Length**
hsa-miR-141-3p	EGFR	10	81	1488	1737
hsa-miR-141-3p	E2F3	3	68	69, 2603	3302
hsa-miR-141-3p	MAP2K4	23	77	75, 192	2571

Viability of MCF 7 Cells

In this study, MTT assay was done to ascertain whether viability of MCF-7 cells has declined after transfection and the transfection reagent caused any toxicity to cells. As seen in figure 1, viability of cells transfected with mir-141 precursor and inhibitory vector compared to control untransfected cells, has shown 0.02 and 0.09 differences respectfully. Since this is not a significant change, we conclude that transfection process itself, have not changed viability of MCF-7 cells.

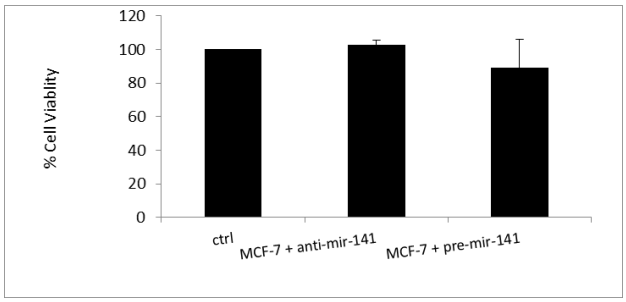


Figure 1. Cell viability (MTT assay) 48 hours after transfection.

Figure 1 shows MTT assay in 3 different groups of cells 48 hour after transfection. No significant changes in viability of cells are seen after transfection.

GFP Expression and Death of Transfected Cells

GFP expression was confirmed by fluorescent microscopy 48 hour following ectopic expression of mir-141 inhibitory as well as overexpression vector. All transfected cells with mock vector were also checked to make sure that cell death is not resulted from plasmid transfection (figure 2).

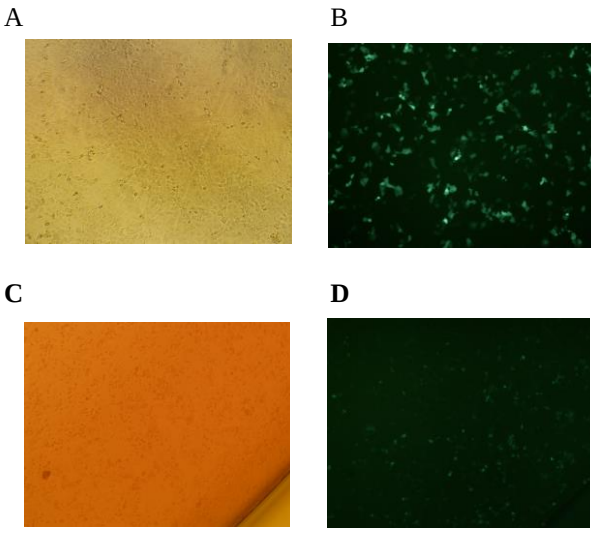


Figure 2. GFP signal of MCF-7 cells 48 h after transfecting by mir-141-Tracer and inhibitory vector. a) Optical microscopy of transfected MCF-7 cells with anti-mir-141 b) Fluorescent microscopy of MCF-7 transfected with anti-mir-141 vector after 48-hour for the same slide, c) Optical microscopy of transfected MCF-7 cells with pre-mir-141 vector, d) Fluorescent microscopy of MCF-7 transfected with pre-mir-141 vector.

Expression Level of MiR-141 in MCF-7 Cells

Decreased expression level of miR-141 was determined in cells transfected with mir-141 inhibitory vector, using real-time PCR. Data was normalized against U6 RNA. Using student t-test, the discrepancy between these groups was statistically significant ($p < 0.05$). By contrast, in cells transfected with pre-mir 141, increased expression of miR-141 is not statistically significant (figure 3).

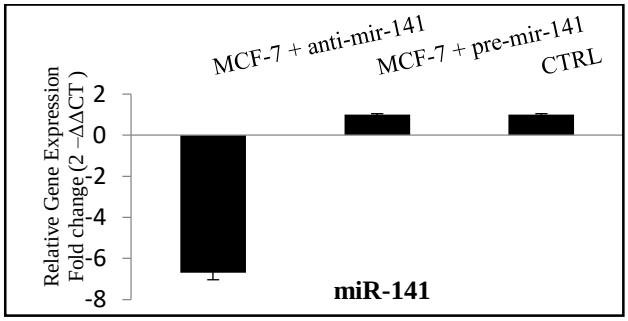


Figure 3. Expression level of miR-141 in transfected cells by real time-PCR.

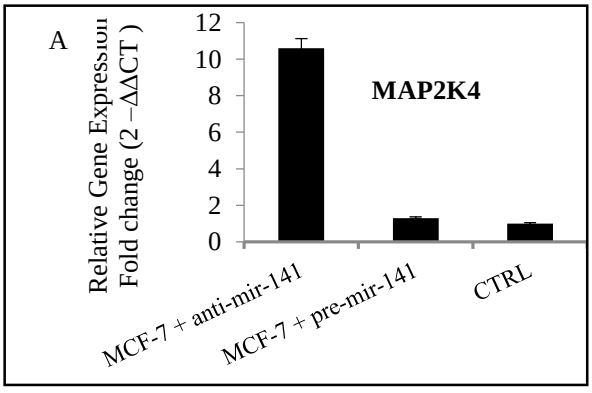
Figure 3 shows the relative expression changes of miR-141 in untreated cells as control group as well as transfected cells with pre-mir-141 and anti-mir-141 vector. Data is normalized with U6 as housekeeping gene. For relative quantification of gene expression, real time-PCR test has been repeated for 3 times (n=3).

Downregulation of MiR-141 Increased EGFR, MAP2K4 and E2F3 Expression

Our study demonstrated that down-regulation of mir-141 increased expression level of EGFR (5.7fold), MAP2K4 (10.6 fold) and E2F3 (6.7 fold) in cells transfected with mir-141 inhibitory vector; however, MAP2K4 expression fold change is greater. Data was normalized against GAPDH and was statistically significant ($p < 0.05$).

Effect of MiR-141 Ectopic Overexpression on EGFR, MAP2K4 and E2F3 Expression Level

In contrast to the preceding group of cells, expression level of EGFR, MAP2K4 and E2F3 was not statistically significant in miR-141 overexpressed cells or control group. However, E2F3 expression showed a greater fold change.



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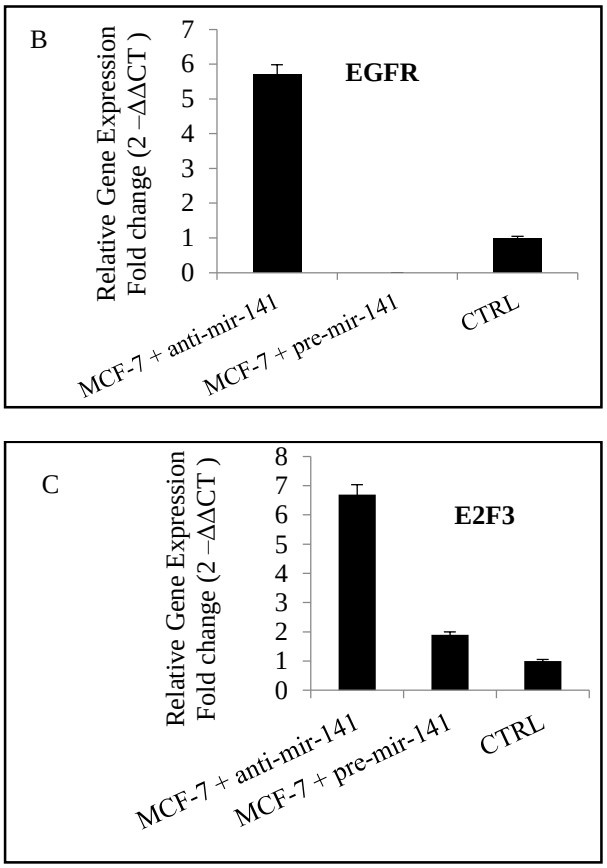

Figure 4. Expression level of predicted target genes in transfected cells by real time-PCR. A) E2F3, B) EGFR, C) MAP2K4

Figure 4 shows the relative expression changes of MAP2K4, EGFR and E2F3 genes in untreated cells as control group as well as transfected cells with pre-mir-141 and anti-mir-141 vector.
Data is normalized with GAPDH as housekeeping gene. For relative quantification of gene expression each test has been repeated for 3 times (n=3).

Network Construction, Module Finding and Enrichment Analysis

PPI Network Construction

PPI Network A encompasses 460 nodes and 464 edges (Figure 5), compared with 803 nodes and 969 edges in PPI network B (seed protein =47) (Figure 7).
A subnetwork containing 10 nodes of degree of 2 or higher and 14 edges is derived from network A (Figure 6). Sub network B contains 152 nodes and 318 edges. Proteins with the highest degree nodes are shown in figure 8.

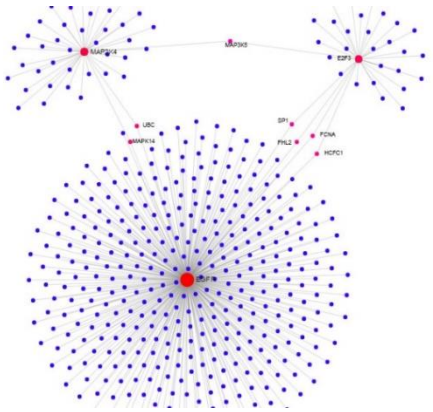

Figure 5. PPI total network A

Figure 5 shows nodes and edges in network A. EGFR is a hub protein with highest degree node.

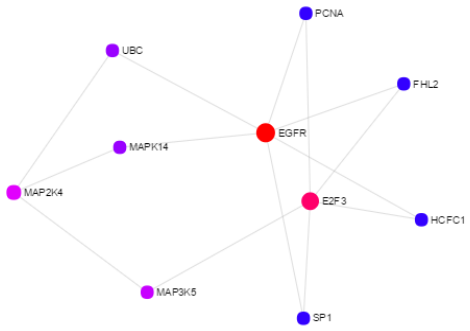

Figure 6. Subnetwork A

From subnetwork A that is retrieved from network Aproteins with the highest betweenness and degree are represented in figure 6. PPI network B contains 803 nodes and 969 edges. Red circles are hub proteins.

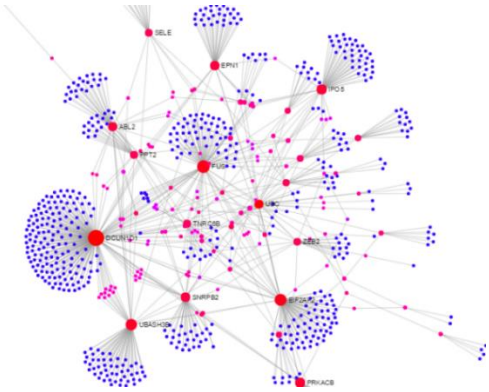

Figure 7. PPI total network B

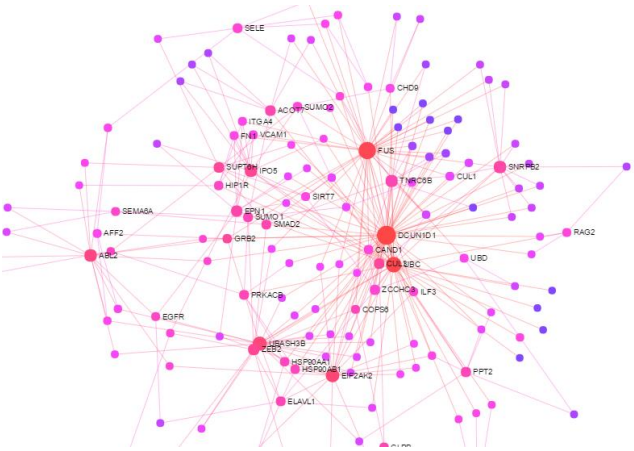

Figure 8. Subnetwork B, derived from network B

Proteins with the highest degree nodes are shown as red color circles in figure 8. Sub network B contains 152 nodes and 318 edges.

Module Analysis in the Network

Three functional modules are explored for each network with connection-first approach algorithm of networkanalyst. When Functional Pathway Enrichment Analysis is sorted by p-value, total network A shows the lowest p-value in immune system (P-value = 0), signaling by interleukins (P=7.19E-28) and innate immune system pathways (P-value = 1.11E-27).

The largest module in Network A was enriched in response to immune system (p-value = 1.19E-28), signaling by EGFR in cancer pathways (P-value = 1.13E-29). Pathways with the highest hits in this module include immune system (P-value = 1.13E-29, 113 hits), signal transduction (P-value =7.35E-10, 101 hits) and adaptive immune system pathways (P-value = 1.59E-17, 68 hits). Interestingly, in both cases when data are sorted by p-value and number of hits, the second detected module in network A shows its most relationship with immune system pathways such as Toll Like Receptor 10 (TLR10) Cascade (P-value =6.06E-17, 12 hits)., Toll Like Receptor 5 (TLR5), Cascade (P-value= 6.06E-17, 12hits), MyD88 cascade initiated on plasma membrane (P-value= 6.06E-17, 12hits). In spite of the two previous modules, the third one was enriched in response to cell cycle pathway (P-value= 6.68E-09, 9hits).

Total network B with function first approach of networkanalyst module explorer enriched to Processing of Capped Intron-Containing Pre-mRNA (P-value= 4.04E-23, 54 hits) and immune system (P-value = 1.94E-20, 155 hits) respectively. In Subnetwork B, proteins with highest degree nodes are enriched in response to Developmental Biology (P-value= 1.01E-07), Immune System (P-value=5.64E-07), Adaptive Immune System (P-value=6.47E-07), Cytokine Signaling in Immune system (P-value= 1.28E-06) respectfully. Moreover, sorted Pathways with the highest hits in this network are similar to pathways sorted by P-value; Immune System (P-value=5.64E-07, 27 hits), Signal Transduction (P-value= 0.00222, 26 hits), Adaptive Immune System (P-value=6.47E-07, 20 hits), Developmental Biology (P-value=1.01E-07, 17 hits).

The largest detected module in Network B with connection-first approach is enriched in response to mRNA Splicing (*P-value*= 7.63E-15), mRNA Splicing - Major Pathway (*P-value*= 7.63E-15), Processing of Capped Intron-Containing Pre-mRNA (*P-value* = 1.42E-14), mRNA Processing (*P-value*= 1.89E-14); however, when enriched pathways are sorted with hits, proteins in Gene Expression (*P-value*=1.01E-07, 17 hits), disease (*P-value*=1.01E-07, 17 hits) and Immune System pathways (*P-value*=1.01E-07, 17 hits) are targeted significantly. Top hits pathways in the second module of the network B are Gene Expression (*P-value*= 7.43E-11, 29 hits), Immune System (*P-value* = 0.00162, 18 hits), Adaptive Immune System (*P-value*= 8.86E-05,15hits), Signaling by NGF (*P-value*= 1.05E-05, 11 hits) and downstream signaling with B cell receptor (*P-value*= 6.49E-07, 11hits). Top hits pathways of the third module are also related to immunity comparatively. These pathways are as follows: Immune System *(P-value* = 0.000133, *17hits)*, Cytokine Signaling in Immune system (*P-value*= 5.60E-07, 11 hits), Disease (*P-value*= 0.0199, 11 hits), Innate Immune System (*P-value* = 0.00306, 9 hits) and Signaling by Interleukins (*P-value*= 4.62E-06, 7 hits).

Discussion

Most of miRNAs in cancer research have been categorized according to the mechanism they are involved, i.e. metastatic, oncogenic, apoptotic. MiR-200 family members are usually categorized as metastamirs. They are responsible for tumor progression, metastasis, invasion and treatment responses (Taylor and Schiemann, 2014). In different types of cancer, intracellular miRNAs are underlying cause of regulation of many caretaker and gatekeeper genes. Adding this to the mechanisms controlled by exosomal miRNA, thousands of cellular and extracellular components is under miRNA's charge.

Among members of miR-200 family, fewer studies have been attributed to miR-141. There have been some well researches documented with regard to the importance of miR-141 in breast cancer. As it has been pointed out in Neves et al, not only MiR200c/141- ZEB1/2 feedback loop, but also methylation of miR-141 promoters is responsible for EMT phenotype of breast cancer (Neves et al., 2010). On the other hand, circulating miR-141 in blood of breast cancer patients has been reported to inform us of many critical features including survival, responses to drugs and metastatic stages (Antolin et al., 2015).

To draw a comparison between our study and other researches that investigate targeting of Map2K4 by miR-141, we found similar evidences in Marasa et al study in ovarian cancer. Since our results is consistent with in this study, it can be concluded that in loss of miR-141, cancer cells are tend to proliferate by a signaling pathway that is triggered by MAPK (Marasa et al., 2009). Moreover, a same mechanism is reported in Wang et all study in pancreatic cells; considering that the mechanism was attributed to another MAPK signaling molecule, MAP4K4, which is functionally the same as MAP2K4 (Wang et al., 2004).

Given that mutation or any gene expression changes in EGFR will lead to resistance to tyrosine kinase inhibitors and undesirable therapeutic consequences (Gazdar, 0000), increased expression of EGFR followed by down-regulation of miR-141 should be taken into account as a notable finding that have to be investigated more in pharmacogenomics studies.

In the current study apart from investigating EGFR, MAP2K4 and E2F3 genes as potential targets for miR-141, which is the first study that suggests targeting of these three genes in MCF-7 cell line, we construct PPI networks to hypothesize other pathways in breast cancer. Accordingly, we find out that in either of networks, although seeded by different targets, enriched pathways of the two PPI networks and detected modules has shown the greatest relationships with innate immunity responses. Experimental studies in different cancer cell lines and tissues also revealed evidence of targeting of immune system by miRNAs; interfering with immunological synopsis in tumor microenvironment, downregulation of MHC1, deregulation of CTL activities as well as differentiation of tumor associated, to name but a few (Rusek et al., 2015).

In glioma cells, miR-29 has proved to downregulate ICAM1.Therefore ICAM can no longer attach to LFA1, hence dysregulation of cytotoxic T cells activities (Ueda et al., 2009). Moreover, in breast cancer PD-L1 is indirectly targeted by miR-200 by TGFβ. PD-L1 by providing a negative feedback causes CTL exhaustion. Chen et.al has shown PD-L1 suppression by miR-200 upregulation. Interestingly, PD-L1 is a mesenchymal marker that is increased in EMT (Chen et al., 2014).

Increased expression of Th17 and decreased expression of regulatory T cells by miR-141 and miR-200 in Multiple- sclerosis patients in relapsing phase has been also reported (Naghavian et al., 2015). Nevertheless, this is the first study that specifically suggests relationships of immune system mechanism with miR-141 in a systematic way. On the other hand, when retrieving high degree nodes proteins in subnetworks retrieved from PPI network A and B, we detect UBC protein from subnetwork A and subnetwork B. These common nodes are related to ubiquitin systems. Interestingly, based on biological process gene ontology term, UBC or Polyubiquitin-C is responsible for common pathways to the pathways associated with target genes we have selected in our study.

These include, MAPK activity, cell surface receptor signaling pathway and antigen presenting antigen processing and presentation of peptide antigen via MHC class I.

Considering the analogy of these pathways, the correlation of breast cancer development by employing components of innate immune system can be hypothesized.

It has to be considered that this in-vitro study has confirmed EGFR, MAP2K4 and E2F3 as the exact target of miR-141 in transcriptome level; however, further research is needed to corroborate the mechanism by proteomics study.

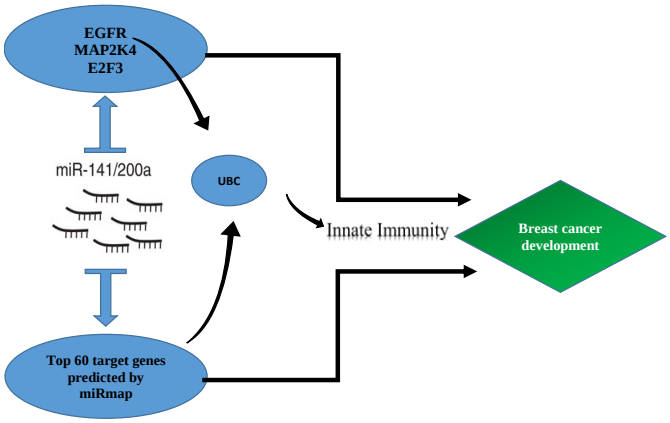

Figure 9. Proposed regulatory mechanisms by miR-141 in breast cancer development through immune system pathway.

Enrichment analysis showed that Both Network A and B take innate immunity signaling molecules as chief molecules by which they control breast cancer development. Note that flashes from UBC to innate immunity do not infer that the only related protein with immune pathway is UBC. UBC is the common protein of the two network, although there are many components that regulate this core pathway. As described previously, UBC protein is one of the highest degree nodes which is retrieved from both of networks. On the other hand, UBC itself have shown to be strongly related to cancer development through immune system pathway and it is structurally interacted with EGFR.

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Conflict of Interest

Author Arshad Hosseini has received research grant number 25802 from Iran University of Medical Sciences. Author Soheila Shokrollahzade has no conflict of interest. Author Shamim Sarhadi has no conflict of interest. Author Majid Safa has no conflict of interest.This article does not contain any studies with human participants or animals performed by any of the authors.

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