Systematic analysis of the p53-related microRNAs in breast cancer revealing their essential roles in the cell cycle

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Abstract. Numerous miRNAs have been found to be involved in the regulation of the p53 signaling pathway. Conversely, p53 regulates the transcription or processing of microRNAs (miRNAs). Given that complexities in the association between p53 and miRNAs exist, and due to the rapidly increasing amount of literature regarding the interactions between p53 and miRNAs, the present study systematically analyzed the associations between miRNAs and p53 in breast cancer using a literature-based discovery approach, natural language processing. A total of 22 miRNAs were found to be associated with p53. Next, three popular online tools (PicTar, miRanda and TargetScan) were used to predict the targets of each miRNA, and certain targets were validated by experiments. Gene Ontology annotation and network analysis demonstrated that the majority of the targets of the p53-related miRNAs were enriched in the cell cycle process. These results suggest that, in addition to regulating the transcription of cell cycle-related genes, p53 also indirectly modulates the cell cycle via miRNAs.

Introduction

Tumor suppressor p53 plays a central role in protecting cells against carcinogenesis, mainly functioning as a transcription factor. In response to stress signals, such as DNA damage, oncogenic stimuli and hypoxia, the p53 protein regulates the transcription of numerous different genes, leading to cell cycle arrest, apoptosis, DNA repair or senescence (1). The inactivation of p53 by mutation is a frequent event in carcinogenesis. Mutations in the TP53 gene, which encodes the p53 protein, occur in around half of all tumor specimens, but the overall frequency of p53 mutations in breast cancer is only 20-30% (2,3). It is believed that, in breast cancer harboring the wild-type p53 gene, p53 function is compromised by other genetic or epigenetic alterations (4,5). A number of studies have demonstrated that changes in interactome components or the target genes of p53 could contribute to reduce the roles of p53 during stress [reviewed in (4,5)].

Recently, a number of microRNAs (miRNAs) have been found to be involved in the p53 signaling pathway and breast carcinogenesis (6). Certain miRNAs directly target the mRNA of p53 and negatively regulate p53 expression, such as miR-125b (7), miR-375 (8) and miR-504 (9). A study in murine models of postmenopausal breast cancer suggested that miR-504 expression induced by obesity contributes to the reduced p53 protein expression and mammary tumor progression (9). Another class of miRNAs indirectly affected p53 signaling through regulating genes associated with p53. For example, miR-21 antagonizes the p53 pathway in breast cancer by inhibiting the expression of p53-regulated genes (10); oncomiRs miR-221/222 promoted proliferation in breast cancer by inhibiting p53 upregulated modulator of apoptosis expression (10). Conversely, p53 can regulate miRNA transcription, for example, that of miR-10b (11), miR-22 (12), miR-26a (13), miR-34a (14), miR-148a (15), miR-200b (16), miR-200c (16) and miR-205 (17), or miRNA processing, such as that of miR-16 (13,18), miR-145 (18,19) and miR-203 (20).

Given the rapidly increasing amount of literature regarding the interaction between p53 and miRNAs, and as complexities in the association between p53 and miRNAs exist, the present study systematically analyzed p53-related miRNAs and their targets in breast cancer using a literature-based discovery approach, natural language processing (NLP).

Materials and methods

NLP analysis of miRNAs associated with p53 and breast cancer. NLP analysis was performed as described by Gao *et al* (21) and Tang *et al* (22). Briefly, a PubMed search was conducted with the following combination of query terms:

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('mammary cancer' OR 'mammary tumour' OR 'mammary tumor' OR 'mammary neoplasm' OR 'mammary carcinoma' OR 'breast cancer' OR 'breast tumour' OR 'breast tumor' OR 'breast neoplasm' OR 'breast carcinoma') AND ('p53' OR 'TP53' OR 'TRP53'). All the miRNAs reported in each of the studies were compiled in a list, and then subjected to gene mention tagging using A Biomedical Named Entity Recognizer, an open source tool for automatically tagging genes, proteins and other entity names in text (23). For conjugated terms, conjunction resolution was performed to obtain individual terms, for example, 'miR-200b/c' was resolved into 'miR-200b' and 'miR-200c'. In the present study, all the genes and miRNAs were named using the official symbol in the Entrez and miRBase databases, respectively. Finally, the co-citation frequency of each miRNA with p53 and breast cancer in the PubMed abstracts was calculated as described by Gao *et al* (21). The higher the co-citation frequency of a miRNA with p53 and breast cancer, the closer it is associated with p53 and breast cancer.

Prediction of miRNA targets. The targets of the miRNAs were predicted using the following computational programs: PicTar 2005 (24) (http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi), miRanda v5 (25) (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5) and TargetScan 5.1 (26) (http://www.targetscan.org).

Analysis of gene ontology (GO), pathways and networks. Go analysis was performed with GSEABase package from R statistical platform (http://www.r-project.org/). Genes were categorized based on biological process (BP), molecular function (MF) and cellular component (CC). GenMAPP v2.1 was used to map genes to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, and calculate the enrichment P-value for each pathway (27).

Network analysis of miRNA targets. To construct gene interaction networks, the following three different interaction associations were integrated: i) Protein interaction, gene regulation and protein modification in the KEGG database; ii) high-throughout protein interaction experiments such as yeast two-hybrid experiments; and iii) gene interaction associations that have previously been reported. Pathway data were downloaded from the KEGG database and used to analyze the interaction associations of genes [including enzyme-enzyme associations, protein-protein interactions (PPIs) and gene expression interactions] with the KEGGSOAP package (http://www.bioconductor.org/packages/2.4/bioc/html/KEGGSOAP.html). The PPI data were downloaded from the MIPS database (http://mips.helmholtz-muenchen.de/proj/ppi/) (28). For the interactions that have been reported, the co-citation frequency of each gene pair in the PubMed abstracts was calculated as described by Gao *et al* (21). Finally, all three types of data were integrated and mapped in a network structure using Medusa (29).

Construction of miRNA target expression plasmid. Each pair of complementary oligonucleotides containing the predicted miRNA target region were synthesized, annealed and ligated into pmirGLO Dual-Luciferase miRNA target expression vectors (Promega, Madison, WI, USA) at *Nhe*I/*Sal*I sites. To ensure that the overhangs created by oligonucleotide annealing were complementary to the two ends of linearized vector, CTAG (protruding sequence of *Nhe*I digestion) and TCGA (protruding sequence of *Sal*I digestion) were added to the 5' ends of the forward and reverse oligonucleotides, respectively. For clone confirmation, a *Kpn*I restriction site was added to each pair of oligonucleotides. When digested with *Kpn*I, the correct construct releases an ~500-bp insert due to a *Kpn*I site at position 478 in the vector. All the plasmids were further confirmed by DNA sequencing.

Cell culture, transfection and luciferase assay. Cell culture, transfection and luciferase assays were performed as previously described (30). Human embryonic kidney 293 (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were cultured on 24-well plates, and co-transfected with 10 pmol miRNA mimics (GenePharma, Shanghai, China) and 0.4 μg miRNA target expression plasmids. At 24-h post-transfection, the cells were harvested and assayed for luciferase activity using the Dual-Glo luciferase assay system (Promega). The Firefly luciferase activities were normalized to *Renilla* luciferase activity. The relative Firefly luciferase activity of the cells transfected with miRNA mimics was represented as the percentage of activity relative to that of the cells transfected with negative control miRNA mimics. For each transfection, the luciferase activity was averaged from three replicates.

Statistical analysis. The Student's t-test was used to evaluate statistical significance and all statistical analyses were performed using R project statistical software (http://www.r-project.org/). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of miRNAs associated with p53 and breast cancer by NLP analysis. NLP has been successfully used to identify molecular interactions. To find the miRNA interacting with p53 in breast cancer, the present study searched PubMed with the following combination of query terms: ('mammary cancer' OR 'mammary tumour' OR 'mammary tumor' OR 'mammary neoplasm' OR 'mammary carcinoma' OR 'breast cancer' OR 'breast tumour' OR 'breast tumor' OR 'breast neoplasm' OR 'breast carcinoma') AND ('p53' OR 'TP53' OR 'TRP53'), and obtained 5,525 studies reporting on p53 and breast cancer. Further analysis, as described in the Materials and methods section, identified 22 miRNAs that are reported to interact with p53 in breast cancer (Table I). Among these miRNAs, the three most frequently cited were miR-34a, miR-21 and miR-200c, which were cited by 8, 6 and 5 studies, respectively.

Computational prediction and experimental investigation of miRNA targets. To make a reliable prediction, three popular online tools (PicTar, miRanda and TargetScan) were used to predict the targets of each p53- and breast cancer-related miRNA. These tools make predictions based on different features of miRNA-mRNA interactions (31). Therefore, for a certain miRNA, the three tools provide different lists of predicted targets. The common targets predicted by these

Table I. p53- and breast cancer-related miRNAs and their predicted targets.

miRNA	PubMed count	Predicted targets
miR-34a	8	**ZNF281**, **RPS6KA4**, PNOC, SYVN1, MYRIP, CRHR1, TAF5, MPP2, CACNB3, DPYSL4, **EVI5L**, STRN3, UHRF2, AXL, **COPS7B**, ACSL4, ASB1, SNX15, ALDOA
miR-21	6	WWP1, NFIB, CCL1, C17ORF39, NTF3, **ASPN**, **CNTFR**, PELI1, SOX2, JAG1, RECK, TGFBI, MATN2, SPRY2
miR-200c	5	DGKA, BAP1, **NDST1**
miR-200b	4	**HS3ST1**
miR-200a	3	SPAG9, DIXDC1, GATA6, TCERG1, HMG20A, **TP53INP1**, SOX5, PCDH9
miR-203	3	**COPS7B**, PHLDA3, CCNG1, **DLG5**, DGKZ, CITED2, GLI3, DUSP5, DLX5, ACO2, ARNTL, FOXK2, CUL1, C18ORF34, CSN2, OVOL1, **ZNF281**, GABRB2
miR-205	3	DLG2, E2F1, C21ORF63, LRP1, **HS3ST1**, **ERBB3**, PHC2, FRK, ADAMTS9, INHBA, INPPL1, **IPO7**
miR-145	2	GLIS1, SEMA3A, FKBP3, NEDD9, **ATXN2**, C11ORF9, SLITRK4, CCNL1, ZBTB10, PLCL2, RGS7, RTKN, CTNNBIP1, LENG8, SEMA6A, ZNF423, ACTG1, ARPC5, SRGAP1
miR-155	2	**SALL1**, IKBKE, SDCBP, HIVEP2, BOC, H3F3A, FBXO11, ACTA1, BRD1, LRP1B, CARHSP1, SOCS1, **TP53INP1**, WEE1, RNF123, MYO10, DNAJB7, AICDA, ASTN2, CSNK1G2, **CHD7**, MAP3K10, **CSF1R**, HBP1, CEBPB
miR-10b	1	DOCK11, HS6ST2, CECR6, **NCOR2**, FXR2, ARIH2, DAZAP1
miR-133a	1	SOLH, PTHR1, CTBP2, **ATP6AP2**, RAPH1, CSNK1G3, RCE1, CLTA, EVI1, ELF2, TFAP2D, MLLT3, VPS54, NRIP3, PTPRD, LRRC7, NDRG1, ABCA2, GDI2
miR-148a	1	**ATP6AP2**, ITSN2, ROBO1, GTF2H1, YPEL3, USP47, KLF4, AKAP1, ABCB7, **RAB34**, CNTN4, WNT10B, **ALS2CR2**, SFRS11, NOG, **PRKAG2**, MTF1, **GAP43**, CCKBR, SYNJ1, MAF1, GPR116, C1GALT1, GADD45A, **DYRK1B**, TRPS1, DNMT1, PLAA, SFRS2IP, UCP3, MLLT10, USP48, LBR, **CHD7**, COL2A1, RNF38
miR-16	1	CHRNE, CCNE1, WBP11, LPHN2, SH3GL2, ZSWIM3, RSBN1, CCNT2, KCTD8, DLL4, **ATXN2**, ADRB2, OMG, COPS2, SCOC, ADAMTS18, YWHAQ, TGFBR3, SEMA6D, TAF15, EPHA1, **KIF21A**, CHEK1, STXBP3, G0S2
miR-191	1	RNF139, **GAP43**, PLCD1, **NDST1**
miR-210	1	EFNA3
miR-22	1	JMJD1A, PURB, **ERBB3**, ODF1, **CSF1R**, MAX, EMILIN3, SATB2, **IPO7**, PRR6, RFXANK, SV2A, EPC1, STAG2, TRUB1, FAM49B, MTHFD2, IL13RA1, DNAJB5, TAGLN, CAV3, CDKN1A, MECP2, ZFYVE9, NFYA, BCL9L, MAT2A
miR-222	1	RBM24, CDKN1C, GNAI2, IRX5, KHDRBS2, RSBN1L, CDKN1B, MESDC1
miR-26a	1	RCN2, NFE2L3, USP15, ABHD2, ZDHHC18, ADM, DEPDC1B, EPHA2, PITPNC1, ULK1, CDK2AP1, HOXA5, COX5A, RLF, PRKCD, **ASPN**, MTX2, **SALL1**, HAO1, **DLG5**, SMAD1, PTPN13, HIPK1, **PRKAG2**, ZNF238, CAMSAP1, PTER, ZDHHC6, PDHX, NAP1L5, PAPD4, COL1A2, KCNQ4, **ALS2CR2**, SENP5, RPS6KA6, EPC2, PAN3, **SACS**, MGAT4A
miR-9	1	CCNE2, ENTPD5, FOXP4, NOX4, ONECUT1, RNF111, RBM9, **RPS6KA4**, **DYRK1B**, ITPKC, **CNTFR**, PYGO2, GAD1, **RAB34**, ARPC1A, SLC35B3, ODZ1, PARG, **SACS**, FBXW2, FBN1, AUH, ARMCX2, LEPRE1, PLSCR3, LRCH4, MUM1L1, **KIF21A**, ERBB2IP, CALB2, TNFRSF21, CTHRC1, TBPL1, **EVI5L**, **NCOR2**, TESK2, SLC30A3, HDAC5, ARID1A, SLC31A2, RANBP2, SLC27A4, DHX40, AP2M1, PCSK6, LAMP1, PALMD, NID2, CSDA, DBNL, DIAPH1, SLC10A3, SNX7, LMNA, TGOLN2, P4HA2, TRIM2, AP3B1, LHFP
miR-342	1	-
miR-497	1	-
miR-504	1	-

Common targets of two or more miRNAs are shown in bold. The underlined genes belong to GO term 'cell cycle and proliferation'. GO, Gene Ontology; miRNA, microRNA.

three prediction tools were chosen for further analysis in the present study. With the exception of miR-342, miR-497 and miR-504, each miRNA exhibited a different number of predicted targets. The miRNA with the most targets was miR-9, with 59 targets, while miR-200b and miR-210 only had one target. A total of 320 genes were predicted to be targeted

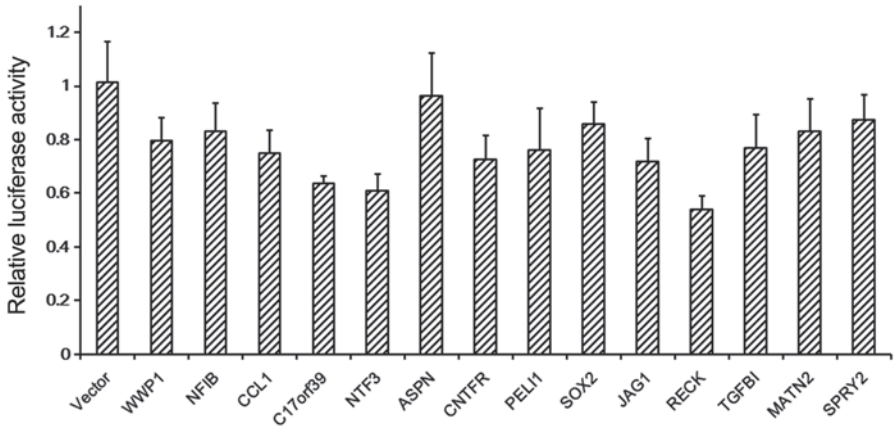

Figure 1. Repression of firefly luciferase by the interaction between miR-21 and its predicted binding site. HEK293 cells were co-transfected with miRNA mimics and miRNA target expression plasmids. At 24-h post-transfection, the cells were harvested and assayed for luciferase activity. The firefly luciferase activities were normalized to *Renilla* luciferase activity. The relative firefly luciferase activity of the cells transfected with miRNA mimics is represented as the percentage of activity relative to that of the cells transfected with negative control miRNA mimics. Data are shown as the mean ± standrad deviation of three independent experiments. miR/miRNA, microRNA.

Table II. Top 5 significantly enriched Gene Ontology terms in the microRNA targets.

Category	Term	n	P-value
CC	Nucleus	108	1.67×10^{-7}
	Extracellular matrix	14	2.98×10^{-4}
	ER/golgi	23	0.347901
	Plasma membrane	47	0.351261
	Cytosol	6	0.530201
MF	Transcription regulatory activity	40	9.53×10^{-7}
	Kinase activity	30	3.15×10^{-5}
	Extracellular structural activity	3	0.007524
	Enzyme regulator activity	19	0.009502
	Nucleic acid binding activity	56	0.010738
BP	Cell cycle and proliferation	42	5.36×10^{-6}
	RNA metabolism	70	3.13×10^{-5}
	Cell organization and biogenesis	50	4.74×10^{-4}
	Protein metabolism	57	0.027053
	Cell death	21	0.031232

MF, molecular function; CC, cellular component; BP, biological process.

Table III. Kyoto Encyclopedia of Genes and Genomes pathways overrepresented in the lists of microRNA targets.

Pathway	n	P-value
Cell cycle	11	3.83×10^{-5}
Axon guidance	9	0.002147584
p53 signaling pathway	6	0.003804563
Notch signaling pathway	4	0.019950756
Phosphatidylinositol signaling system	5	0.026477865
Hedgehog signaling pathway	4	0.037347722
TGF-β signaling pathway	5	0.041980514
Basal transcription factors	3	0.042039395

by these 19 miRNAs (Table I). Among these, 25 genes were able to be targeted by two miRNAs.

To validate the above prediction, the pmirGLO Dual-Luciferase miRNA target expression vector was used to investigate whether miR-21 could bind to its targets as predicted. Each of the 14 predicted miR-21 binding sites was cloned downstream of the Firefly luciferase of the pmirGLO vector, and co-transfected with miR-21 or scramble mimics into HEK293 cells. Luciferase assay showed that 13/14 of the predicted targets of miR-21 (not the ASPN gene) could be regulated by miR-21 (Fig. 1). Moreover, 8/14 of the predicted miR-21 targets have been validated by other studies [WWP1 (32), NFIB (33), PELI1 (34), SOX2 (35), JAG1 (36), RECK (37), TGFBI (36) and SPRY2 (38)]. These results suggested that the present miRNA target prediction is reliable.

GO annotation analysis of miRNA targets. These 320 miRNA target genes were subjected to GO enrichment analysis. All these genes were categorized based on BP, MF and CC (Table II). In the CC category, the nucleus term was the most significant term (with the lowest P-value) and contained the largest number of genes. In the MF category, the term with the lowest P-value was transcription regulatory activity. In the BP category, the most significantly enriched genes belonged to the cell cycle and proliferation process, and a total of 42 genes were categorized to this process. These 42 genes belonged to the targets of 16 miRNAs (Table I). KEGG pathway analysis also showed similar results, with the number of genes involved in the cell cycle being the largest (Table III). This suggested that the targets of p53-related miRNAs mainly play roles in the cell cycle and proliferation process.

Network analysis of miRNA targets. To understand the association between these miRNA targets, the KEGG dataset, PPI and Pubmed datasets were integrated to construct a network

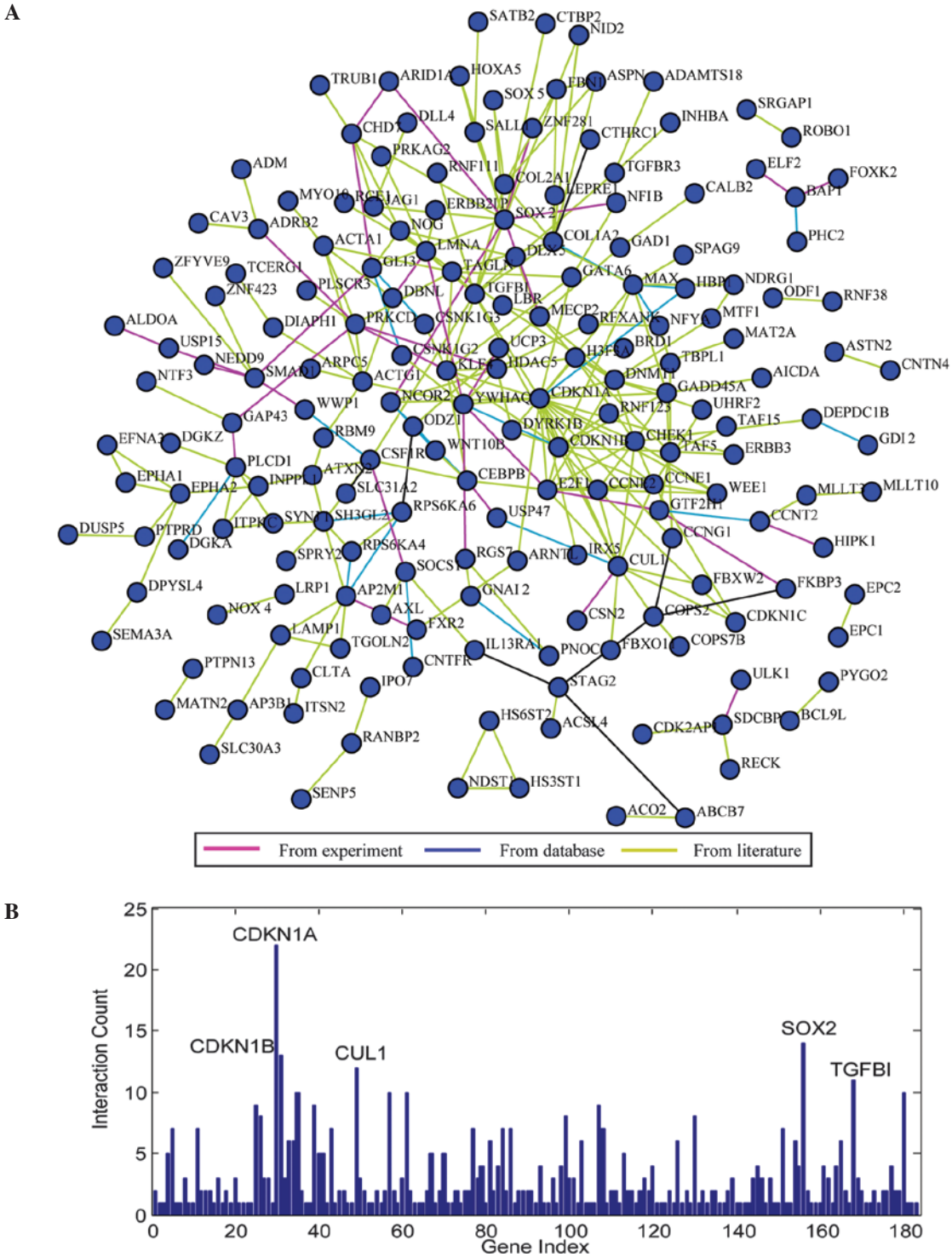

Figure 2. Network analysis of miRNA targets. (A) A network was constructed by integrating three types of data (Kyoto Encyclopedia of Genes and Genomes database, high-throughout protein interaction experiments and the literature data), and mapped in a network structure using Medusa. Each node corresponds to a gene and an edge indicates a direct interaction. (B) Interaction gene counts for microRNA target genes.

of miRNA targets (Fig. 2A). The resulting network was composed of nodes (genes) and edges (interactions). Fig. 2B shows the degree (i.e., the number of edges emanating from a node) of each node. In the present network, the nodes with degree >10 were defined as hubs, including CDKN1A, SOX2, CDKN1B, CUL1 and TGFBI. According to the aforementioned GO annotation, these hub genes, with the exception of TGFBI, were annotated to the term 'cell cycle and proliferation' (Table I). In a molecular interaction network, hubs are more essential for the global network structure than non-hubs (39). Therefore, it indicates the roles of targets of p53-related miRNAs in the cell cycle and proliferation, in accordance with the aforementioned pathway analysis.

Discussion

In the present study, for the first time, the interactions of miRNAs and p53 were systematically analyzed in breast

cancer using NLP analysis, and 22 miRNAs associated with p53 in breast cancer were identified. Among these miRNAs, 11 are transcriptionally or post-transcriptionally upregulated by p53 [miR-10b (11), miR-16 (13,18), miR-22 (12), miR-26a (13), miR-34a (14), miR-145(18,19), miR-148a (15), miR-200b (16), miR-200c (16), miR-203 (20) and miR-205 (17)], one directly targets p53 [miR-504 (9)], and others do not directly interact with p53, but indirectly play roles in the p53 signaling pathway [e.g., miR-9 (40), miR-21 (41) and miR-222 (10)]. Bioinformatics analysis identified 320 targets of p53-related miRNAs.

Although these 22 p53-related miRNAs have different sets of targets, GO annotation revealed that the majority of the miRNA targets were significantly enriched in the cell cycle and proliferation process. In the network of miRNA targets, the five hub genes, with the exception of TGFBI, were annotated to cell cycle processes. TGFBI has been recently reported to affect the cell cycle via the regulation of p21 and p53 expression (42). Cyclin-dependent kinase inhibitor 1A (CDKN1A; also known as p21, Cip1 or WAF1) was identified as the most highly connected hub gene. CDKN1A has been proven to be a direct target of the p53 tumor suppressor and to play key roles in mediating p53-dependent cell cycle arrest in response to DNA damage. In a molecular interaction network, hubs are more essential for the global network structure than non-hubs (39). The results suggest that p53-related miRNAs play roles in the cell cycle. A number of studies have demonstrated that p53 acts as a key regulator of the cell cycle, mainly by transcriptional regulation of certain key genes in the cell cycle, such as CDKN1A. The present study suggested that, in addition to transcriptionally regulating cell cycle-related genes, p53 also indirectly regulates them through miRNAs. These results also suggest a previously unknown mechanism for p53 function, and thus provide an important contribution to our knowledge of p53. Furthermore, the results of the present study were consistent with those of Otsuka *et al* (43) which revealed that p53-induced miRNAs control the cell cycle and cell survival via the repression of cell-cycle regulators and/or antiapoptotic proteins. Additionally, Rokavec *et al* (44) summarized previously published data regarding the interaction between p53 and miRNAs in gastrointestinal cancer, and found that a total of 32 p53-related miRNAs exhibit differential expression between normal and tumor tissue and are associated with clinical and pathological parameters of gastrointestinal cancer . Among the 32 miRNAs, only 9 miRNAs (miR-34a, miR-200a, miR-200b, miR-200c, miR-205, miR-145, miR-16, miR-22, miR-504) are common in both gastrointestinal cancer and breast cancer (Table I). Thus, we hypothesize that p53 regulates different sets of miRNAs in various types of cancer.

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