Signal transduction by M3 muscarinic acetylcholine receptor in prostate cancer

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Abstract. The present study aimed to investigate the potential mechanisms used during signal transduction by M3 muscarinic acetylcholine receptor (CHRM3) in prostate cancer. The microarray datasets of GSE3325, including 5 clinically localized primary prostate cancers and 4 benign prostate tissues, were downloaded from the Gene Expression Omnibus database. The differentially-expressed genes (DEGs) in primary prostate cancer tissues compared with benign controls were screened using the Limma package. Gene Ontology and pathway enrichment analyses were performed using the Database for Annotation Visualization and Integrated Discovery. Next, a protein-protein interaction (PPI) network was constructed. Additionally, microRNAs (miRNAs) associated with DEGs were predicted and miRNA-target DEG analysis was performed using a Web-based Gene Set Analysis Toolkit. Finally, the PPI network and the miRNA-target DEG network were integrated using Cytoscape. In total, 224 DEGs were screened in the prostate cancer tissues, including 113 upregulated and 111 downregulated genes. CHRM3 and epidermal growth factor (EGF) were enriched in the regulation of the actin cytoskeleton. EGF and v-myc avian myelocytomatosis viral oncogene homolog (Myc) were enriched in the mitogen-activated protein kinase (MAPK) signaling pathway. EGF with the highest degree of connectivity was the hub node in the PPI network, and miR-34b could interact with Myc directly in the miRNA-target DEG network. EGF and Myc may exhibit significant roles in the progression of prostate cancer via regulation of the actin cytoskeleton and the MAPK signaling pathway. CHRM3 may activate these two pathways in prostate cancer progression. Thus, these two key factors and pathways may be crucial mechanisms during signal transduction by CHRM3 in prostate cancer.

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Introduction

Prostate cancer is one of the leading causes of cancer-associated mortality in males, accounting for >240,000 new cancer cases and 28,000 fatalities annually in males in the United States (1,2). Although effective surgical and radiation treatments exist for clinically localized prostate cancer, the majority of patients with metastatic prostate cancer eventually succumb to the disease (3). Therefore, in order to develop more effective outcomes for the diagnosis and treatment of prostate cancer, articulation of the genetic underpinning and novel therapeutic targets are critically required.

Recently, the presence and function of muscarinic acetylcholine receptors (mAChRs) in the human prostate have aroused wide concern (4,5). mAChR and its ligands have been found to play key roles in regulating cellular proliferation and cancer progression (6). mAChRs are preferentially localized to the glandular epithelium of the prostate and promote the paracrine/autocrine actions within the prostate gland, which are critical for cancer cell survival, proliferation and migration (4,6,7). mAChRs consist of five distinct subtypes (M1-M5), and the M3 mAChR (also known as CHRM3) has been found to be a key member involved in prostate cancer. The stimulation of CHRM3 is strongly associated with the tumor growth of prostate carcinomas (4). Additionally, CHRM3 can effectively mediate the contractions of the mouse prostate elicited by acetylcholine (8). A great deal of attention has been focused on the roles of CHRM3 in prostate cancer, however, the signal transduction by CHRM3 in the pathophysiology of prostate cancer is not well understood. Therefore, investigation of the signal transduction by CHRM3 will herald a prominent expansion in our understanding of the molecular mechanisms of prostate cancer.

The microarray data of GSE3325 has been used to reveal critical genomic regions in the prostate tumor microenvironment for investigating novel biomarkers (9) or to reveal signatures of the metastatic progression of prostate

cancer (10). In contrast to previous findings, the present study downloaded the GSE3325 microarray data and utilized comprehensive bioinformatics methods to identify the differentially-expressed genes (DEGs) associated with prostate cancer. Additionally, functional enrichment analysis of the DEGs was performed, and a protein-protein interaction (PPI) network and microRNA (miRNA)-target DEG network was constructed. The study aimed to elucidate the potential mechanisms used during signal transduction by CHRM3 in prostate cancer.

Materials and methods

Affymetrix microarray data. The microarray data of GSE3325 were downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), based on the platform of GPL570 (Affymetrix Human Genome U133 Plus 2.0 Array; Affymetrix Inc., Santa Clara, CA, USA). The gene expression files were deposited by Varambally *et al* (10). A total of 19 samples were applied to the development of the array data, including 6 benign, 7 clinically localized primary and 6 metastatic prostate cancer tissues. Each category contained 2 pooled samples. In order to better investigate the molecular pathogenesis of prostate cancer, pooled samples were discarded, and the expression profiles analyzed in this study were derived from 9 samples, including 5 clinically localized primary prostate cancer and 4 benign prostate tissues.

Data preprocessing and DEG screening. The raw data were first preprocessed by the robust multiarray average algorithm (11) with application of Affy package (12) in R language. The gene expression matrix of the samples was then acquired.

The DEGs in the primary prostate cancer tissues compared with the benign controls were screened by Limma package (13,14) in R language. The P-value was adjusted using Student's t-test (13) in Limma. Fold-change (FC) of the gene expression was also observed for differential-expression test. The DEGs with adjusted P-values <0.05 and $|\log_2 FC| >1$ were considered to be significant.

Gene Ontology (GO) and pathway enrichment analysis. The GO database (15) is a large-scale collection of gene annotation terms. The Kyoto Encyclopedia of Genes and Genomes (KEGG) (16) is a pathway-related database for classification of correlating gene sets into their respective pathways. The Database for Annotation Visualization and Integrated Discovery (DAVID) (17) is an online tool used for systematically associating the functional terms with large gene or protein lists.

In order to analyze the function of DEGs, GO annotation associated with biological process (BP) and KEGG pathway enrichment analysis of DEGs by DAVID online tool were performed. P<0.05 and gene counts >2 were set as the threshold.

PPI network construction. The Search Tool for the Retrieval of Interacting Genes (STRING) (18) is an online database that provides comprehensive information on predicted and experimental interactions of proteins in a given cell. The interactions of protein pairs are displayed with a combined score. In the present study, the DEGs were mapped into the STRING database to construct a PPI network with a combined score of protein pairs of >0.4 as the cut-off value. In addition, the connectivity degree of each node in the PPI network was calculated and the hub node was then identified (19).

Prediction of miRNAs associated with DEGs. The Web-based Gene Set Analysis Toolkit (WebGestalt) (20) is a web-based integrated system for analyzing large-scale gene sets in various functional categories, such as transcription factor and miRNA targets. In order to screen the potential miRNAs associated with DEGs, the DEG lists were uploaded to the WebGestalt system, and a miRNA-target DEG analysis was performed by WebGestalt. Enriched gene counts of ≥2 and rawP<0.05 were defined as the cut-off value.

Then miRNA-target DEG network composed of miRNAs and their target DEGs was visualized in the application of Cytoscape (21) software.

Integration of PPI network and miRNA-target DEG network. In the present study, the miRNA-target DEGs and PPI networks were further integrated, and the PPI pair-miRNA network composed of miRNAs and their target protein pairs was established with Cytoscape (21) software.

Results

DEG screening. Using the cut-off value of adjusted P-values <0.05 and $|\log_2 FC| >1$, a total of 224 DEGs in the primary prostate cancer tissues compared with the benign controls were screened, including 113 upregulated and 111 downregulated genes.

GO and pathway enrichment analysis. GO and pathway analyses were performed for the upregulated and downregulated genes, respectively. The overrepresented GO-BP terms of the upregulated DEGs were mainly associated with amine transport, amino acid transport and the enzyme-linked receptor protein signaling pathway (Table I). The downregulated DEGs were mainly involved in the regulation of cell proliferation, the response to organic substances and the negative regulation of the nitrogen compound metabolic process (Table II).

The upregulated DEGs were significantly enriched in the ErbB signaling pathway, thyroid cancer and O-glycan biosynthesis (Table I), while downregulated DEGs were significantly enriched in drug metabolism, axon guidance and metabolism of xenobiotics by cytochrome P450 (Table II). Notably, CHRM3 and epidermal growth factor (EGF) were significantly enriched in regulation of the actin cytoskeleton (Fig. 1). EGF and v-myc avian myelocytomatosis viral oncogene homolog (Myc) were enriched in the mitogen-activated protein kinase (MAPK) signaling pathway.

PPI network analysis. Based on the STRING database, a total of 27 upregulated PPIs (Fig. 2A) and 15 downregulated

Table I. GO-BP terms and KEGG pathways enriched by upregulated genes.

A, GO-BP terms

Term	Description	Count	P-value
GO:0015837	Amine transport	5	$5.95x10^{-3}$
GO:0006865	Amino acid transport	4	$1.78x10^{-2}$
GO:0007167	Enzyme-linked receptor protein signaling pathway	7	$1.85x10^{-2}$
GO:0051674	Localization of cell	6	$4.03x10^{-2}$
GO:0048870	Cell motility	6	$4.03x10^{-2}$
GO:0035239	Tube morphogenesis	4	$4.32x10^{-2}$
GO:0001501	Skeletal system development	6	$4.62x10^{-2}$
GO:0035295	Tube development	5	$4.63x10^{-2}$

B, Enriched KEGG pathways

Term	Description	Count	P-value
hsa04012	ErbB signaling pathway	3	$8.21x10^{-2}$
hsa05216	Thyroid cancer	2	$1.48x10^{-1}$
hsa00512	O-glycan biosynthesis	2	$1.53x10^{-1}$
hsa04514	CAMs	3	$1.64x10^{-1}$
hsa00071	Fatty acid metabolism	2	$1.99x10^{-1}$
hsa05219	Bladder cancer	2	$2.08x10^{-1}$
hsa05213	Endometrial cancer	2	$2.51x10^{-1}$
hsa05221	Acute myeloid leukemia	2	$2.75x10^{-1}$
hsa03320	PPAR signaling pathway	2	$3.19x10^{-1}$
hsa04810	Regulation of actin cytoskeleton	3	$3.33x10^{-1}$
hsa04350	TGF-β signaling pathway	2	$3.84x10^{-1}$
hsa04060	Cytokine-cytokine receptor interaction	3	$4.27x10^{-1}$
hsa05200	Pathways in cancer	3	$5.48x10^{-1}$
hsa04310	Wnt signaling pathway	2	$5.71x10^{-1}$
hsa04144	Endocytosis	2	$6.45x10^{-1}$
hsa04510	Focal adhesion	2	$6.78x10^{-1}$
hsa04080	Neuroactive ligand-receptor interaction	2	$7.66x10^{-1}$
hsa04010	MAPK signaling pathway	2	$7.80x10^{-1}$
hsa04740	Olfactory transduction	2	$8.86x10^{-1}$

Term, identification number of GO term or KEGG pathway; Description, name of GO term or KEGG pathway; Count, number of genes enriched in GO term or KEGG pathway; GO, Gene Oncology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CAMs, cell adhesion molecules; MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor-β; PPAR, peroxisome proliferator-activated receptor.

PPIs (Fig. 2B) with a combined score of >0.4 were obtained. The upregulated nodes with a connectivity degree of >2 were EGF (4), myoglobin (MB) (3), α-methylacyl-CoA racemase (3), early growth response 2 (3), fibromodulin (3) and activating transcription factor 3 (3). The downregulated node with a connectivity degree of >2 was peroxisome proliferator-activated receptor γ.

Prediction of miRNAs associated with DEGs. The miRNAs associated with DEGs were predicted by WebGestalt. A total of 11 upregulated miRNAs, including miR-23A, miR-23B, miR-219 and miR-143, and 4 downregulated miRNAs, including miR-193a, miR-193b, miR-124a and miR-302c, were selected (Table III). Notably, miR-34b could interact with Myc directly.

Integration of PPI and miRNA-target DEG networks. With Cytoscape software, the PPI and miRNA-target DEG networks were successfully integrated together. The upregulated and downregulated PPI pair-miRNA networks with significant protein pairs and their corresponding miRNAs were also established (Fig. 3).

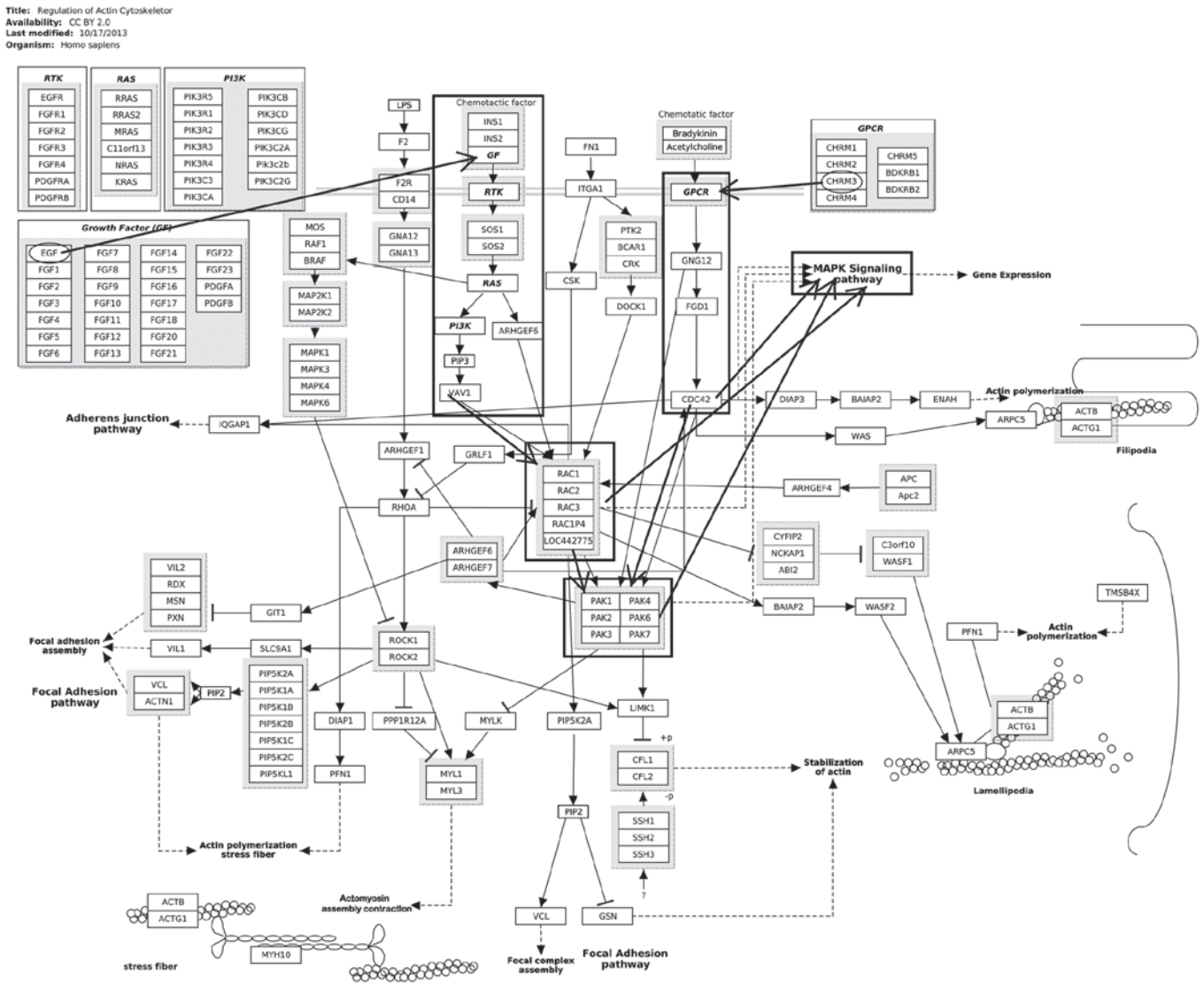

Figure 1. Regulation of actin cytoskeleton pathway.

Discussion

In the present study, the bioinformatics approach was used to investigate the potential mechanisms used during signal transduction by CHRM3 in prostate cancer. The results showed that CHRM3 and EGF were significantly enriched in regulation of actin cytoskeleton. EGF and Myc were enriched in the MAPK signaling pathway. Notably, as shown in Fig. 1, the activation of the regulation of the actin cytoskeleton may induce the activation of MAPK signaling pathway to a certain extent, thus promoting the progression of prostate cancer. Therefore, these DEGs and the aforementioned two pathways may be potential mechanisms during signal transduction by CHRM3 in prostate progression.

EGF, with the highest degree of connectivity, was the hub protein in the PPI network. Growth factors (GFs), including EGF, and their transmembrane receptor tyrosine kinases (RTKs) play important roles in the growth, proliferation, migration and differentiation of various human tumor cells (22,23). The EGF receptor (EGFR) family of RTKs is formed by four members: EGFR/ErbB1, human epidermal growth factor (HER)2/ErbB2, HER3/ErbB3 and HER4/ErbB4 (24). Increased expression and/or amplification of EGFR and HER2 have been recorded in a range of human cancer types (24). Additionally, stimulation with EGF results in the activation of the lipoprotein kinase, phosphatidylinositol 3-kinase (PI3K), which phosphorylates phosphatidylinositol 4,5-bisphosphate, generating the second messenger phosphatidylinositol (3,4,5)-trisphosphate (25). PI3K-Akt signaling pathway activation promotes prostate cancer cell metastasis and invasion (26,27). In the present study, the PI3K-Akt signaling pathway activated by EGF was a part of the regulation of the actin cytoskeleton. Therefore, the present results are in line with previous findings and suggest that EGF may play roles in the metastasis and invasion of prostate cancer via regulation of the actin cytoskeleton.

Furthermore, EGFR and HER2 have been identified to be essential pathway elements in the signaling from G protein-coupled receptors (GPCRs) (24). CHRM3 belongs to the GPCRs and is coupled to MAPK via EGFR (28). GPCRs may interact with Rho guanosine triphosphatases, including RhoA and Cdc42, which play roles in regulating

Table II. Top 10 GO-BP terms and KEGG pathways enriched by downregulated genes.

A, GO-BP terms

Term	Description	Count	P-value
GO:0042127	Regulation of cell proliferation	11	$9.36x10^{-3}$
GO:0010033	Response to organic substance	9	$3.99x10^{-2}$
GO:0051172	Negative regulation of nitrogen compound metabolic process	8	$2.16x10^{-2}$
GO:0031327	Negative regulation of cellular biosynthetic process	8	$3.13x10^{-2}$
GO:0009890	Negative regulation of biosynthetic process	8	$3.45x10^{-2}$
GO:0044092	Negative regulation of molecular function	7	$9.21x10^{-3}$
GO:0008015	Blood circulation	6	$3.29x10^{-3}$
GO:0003013	Circulatory system process	6	$3.29x10^{-3}$
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	6	$1.44x10^{-2}$
GO:0045892	Negative regulation of transcription, DNA-dependent	6	$4.31x10^{-2}$

B, The enriched KEGG pathways

Term	Description	Count	P-value
hsa00982	Drug metabolism	3	$3.14x10^{-2}$
hsa04360	Axon guidance	3	$1.14x10^{-1}$
hsa00980	Metabolism of xenobiotics by cytochrome P450	2	$2.39x10^{-1}$
hsa04512	ECM-receptor interaction	2	$3.19x10^{-1}$
hsa04350	TGF-β signaling pathway	2	$3.28x10^{-1}$
hsa04916	Melanogenesis	2	$3.64x10^{-1}$
hsa04630	Jak-STAT signaling pathway	2	$5.10x10^{-1}$
hsa04020	Calcium signaling pathway	2	$5.56x10^{-1}$
hsa04510	Focal adhesion	2	$6.05x10^{-1}$
hsa05200	Pathways in cancer	2	$7.85x10^{-1}$

Term, identification number of GO term or KEGG pathway; Description, name of GO term or KEGG pathway; Count, number of genes enriched in GO term or KEGG pathway. BP, biological process; GO, Gene Oncology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TGF-β, transforming growth factor-β; ECM, extracellular matrix; Jak-STAT, Janus kinase-signal transducer and activator of transcription.

cell motility (29). Moreover, GPCRs can induce EGFR transactivation, thus generating signals defining the required biological response (24). The roles of EGFR are as aforementioned. In addition, previous findings have strongly linked the excessive activation of the GPCR and RTK pathways to prostate cancer metastasis (26). Therefore, as shown in Fig. 1, CHRM3 may activate the regulation of the actin cytoskeleton via EGFR or GPCRs for the promotion of the metastasis of prostate cancer.

Notably, the highly-conserved MAPK signaling pathway can be activated by EGFR (22,24,30). Previous studies have also suggested that the activation of the MAPK cascade can be mediated by GPCRs via several distinct pathways (31). Moreover, the MAPK signaling pathway may be activated by means of a PI3K-dependent feedback loop in human cancer (32). The PI3K/Akt/mTOR and MAPK signaling pathways are often observed in prostate tumors (33). Therefore, CHRM3 may be further involved in the MAPK signaling pathway via the roles of EGFR or the activation of the PI3K-Akt signaling pathway. Additionally, MAPK signaling pathway activation is necessary for inhibitor of DNA binding 1-induced serum-independent prostate cancer cell growth (34). Targeting MAPK signaling pathway activated by AKT/mTOR and MEK/ERK can inhibit the progression of prostate cancer in humans (35). Taken together, these results and the present study results suggest that CHRM3 may play important roles in prostate cancer progression via activating the MAPK signaling pathway.

In addition to EGF, Myc was found to be significantly enriched in the MAPK signaling pathway in the present study. Myc is a basic helix-loop-helix leucine zipper transcriptional factor that functions in cell proliferation, differentiation and death (36). An increased Myc gene copy number is found in human prostate cancer, and Pim-1 kinase can cooperate with Myc in tumorigenesis (37). c-Myc may link v-ets avian erythroblastosis virus E26 oncogene homolog to a major oncogenic pathway in prostate cancer (38). In addition, Myc could interact with miR-34b directly in the present study. miR-34 mediates androgen receptor-dependent p53-induced apoptosis in prostate cancer (39). The study by Corney *et al* confirmed that miR-34b

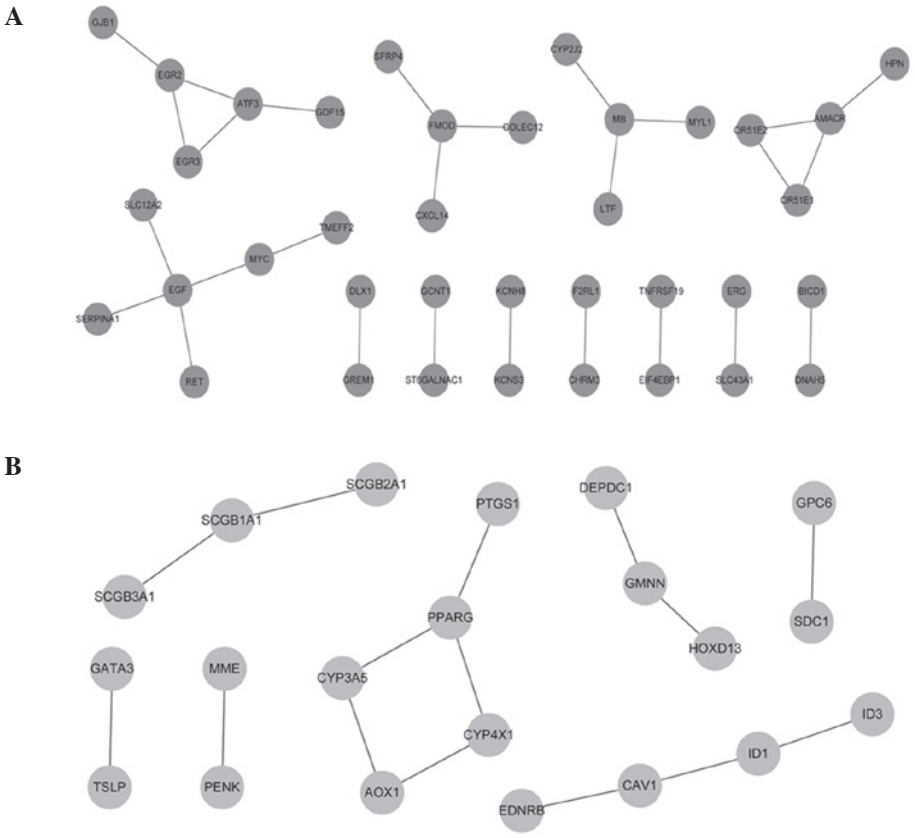

Figure 2. Protein-protein interaction networks of differentially-expressed genes. (A) The dark gray nodes indicate upregulated genes and (B) the light gray nodes indicate downregulated genes. The lines indicate interactions between these genes.

Table III. Predicted miRNAs associated with DEGs.

A, Upregulated

miRNA	Count	P-value
hsa_GACAATC, miR-219	5	1.10×10^{-3}
hsa_TCATCTC, miR-143	5	1.20×10^{-3}
hsa_AATGTGA, miR-23a, miR-23b	8	2.00×10^{-3}
hsa_ACTGCCT, miR-34b	5	6.20×10^{-3}
hsa_AAGCAAT, miR-137	5	6.30×10^{-3}
hsa_GAGCCAG, miR-149	4	7.60×10^{-3}
hsa_TCTGATA, miR-361	3	1.34×10^{-2}
hsa_CATGTAA, miR-496	4	1.41×10^{-2}
hsa_GCAAGAC, miR-431	2	2.33×10^{-2}
hsa_TACTTGA, miR-26a, miR-26b	5	2.33×10^{-2}
hsa_CAGTGTT, miR-141, miR-200a	5	2.61×10^{-2}

B, Downregulated

miRNA	Count	P-value
hsa_GGCCAGT, miR-193a, miR-193b	3	1.11×10^{-2}
hsa_TGCCTTA, miR-124a	7	2.22×10^{-2}
hsa_ATGTTAA, miR-302c	4	3.31×10^{-2}

P-values are calculated from hypergeometric tests (rawP) and adjusted by the multiple test adjustment. Count, number of genes that interact with miRNAs; miRNAs, microRNAs; DEGs, differentially-expressed genes.

is a target of p53 and plays important roles in the control of cell proliferation (40). Furthermore, the MAPK/heterogeneous nuclear ribonucleoprotein K pathway controls the oncogenic potential of breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1 (BCR/ABL) oncoprotein via the regulation of Myc mRNA translation (41). Therefore, Myc may play an important role in prostate cancer via the MAPK signaling pathway, and the present results are consistent with the findings that the MAPK signaling pathway may be the key mechanism used during signal transduction by CHRM3 in prostate cancer.

In conclusion, EGF and Myc may play significant roles in the progression of prostate cancer via regulation of the actin cytoskeleton and the MAPK signaling pathway. CHRM3 may activate these two pathways via EGFR or GPCRs in prostate cancer progression. Thus, regulation of the actin cytoskeleton, the MAPK signaling pathway and the two key factors of EGF and Myc may be crucial mechanisms during signal transduction by CHRM3 in prostate cancer. The present findings shed new light on the molecular mechanism of prostate cancer and have implications for future research. However, a relatively small sample size and no experimental validation are limitations to the present study, and further genetic studies are required to confirm these observations.

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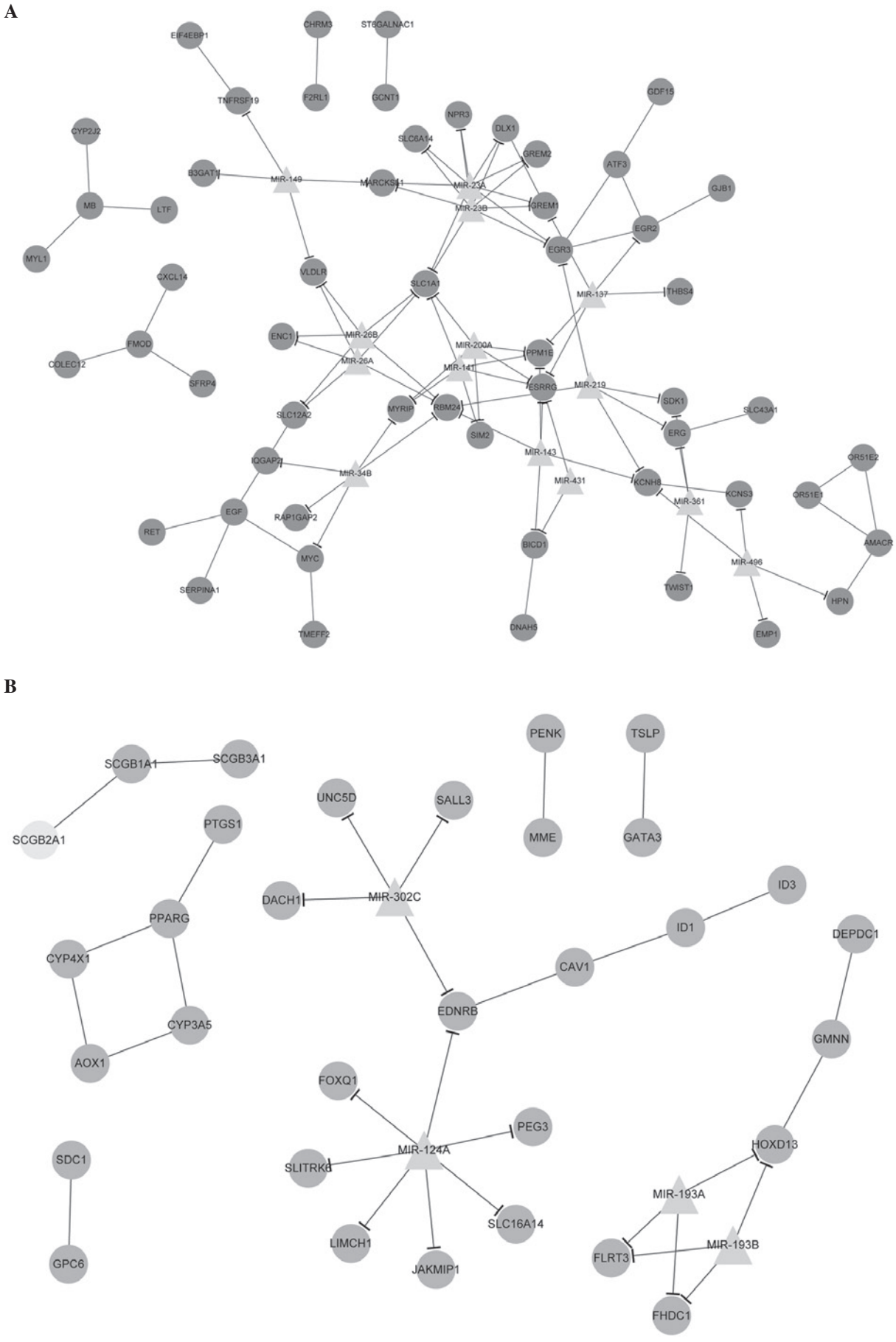

Figure 3. Integration of protein-protein interaction and miRNA-target differentially-expressed gene networks. (A) The dark gray circular nodes indicate upregulated genes and (B) the light gray circular nodes indicate downregulated genes. Gray triangular nodes indicate microRNAs. The lines indicate interactions between these nodes.

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