Investigation of the molecular mechanisms underlying myotonic dystrophy types 1 and 2 cataracts using microRNA-target gene networks

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Abstract. The purpose of the present study was to investigate the molecular mechanisms of myotonic dystrophy (DM) 1 and 2 cataracts using bioinformatics methods. A microarray dataset (E-MEXP-3365) downloaded from the Array Express database included lens epithelial samples of DM1 and DM2 cataract patients (n=3/group) and non-DM lens epithelial samples as a control (n=4). Differentially expressed genes (DEGs) were identified between DM1 and control samples, and between DM2 and control samples. Pathway enrichment analyses were performed for the DEGs. Potential micro (mi)RNAs regulating these DEGs were predicted. An miRNA-target gene network was constructed for DM1 and DM2. The study identified 223 DEGs in DM1, and 303 DEGs in DM2. DM1 and DM2 shared 172 DEGs. The DEGs in DM1 were enriched with calcium, Wnt and axon guidance signaling pathways. The DEGs in DM2 were linked by adherens junction signaling pathways. miRNA (miR)-197, miR-29b and miR-29c were included in the network modules of DM1. miR-197, miR-29c and miR-29a were involved in the network modules of DM2. It is therefore hypothesized that these signaling pathways and miRNAs underlie DM1 and DM2 cataracts, and may represent potential therapeutic targets for the treatment of this disorder.

Introduction

Myotonic dystrophy (DM) is a progressive multi-systemic disease consisting of highly heterogeneous clinical syndromes, affecting ~1 in 8,000 people globally (1). It has two subtypes:

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DM1 and DM2. DM1 is primarily attributed to the mutation of a CTG expansion residing in the 3'-untranslated region of the dystrophia myotonica-protein kinase (DMPK) gene, whereas DM2 is ascribed to an untranslated CCTG expansion in the cellular retroviral nucleic acid binding protein 1/zinc finger 9 (CNBP) gene (2). Several body components are affected by the disease, including skeletal muscles, the central nervous system, the heart and the eyes (3). DM causes various ocular syndromes, such as early onset cataracts, external ophthalmoplegia and pigmentary retinopathy (4,5).

DM1 and DM2 share many clinical features, and their underlying mechanisms are often investigated jointly. It has been reported that the stability of mutant RNAs (CUG and CCUG) in DM1 and DM2 is increased as a consequence of deficiency of RNA helicase p68 (6). Dysregulation of intracellular calcium homeostasis in DM1 and DM2 myotubes has been identified to be associated with the aberrant splicing of Ca^{2+} handling genes (7). Bachinski *et al* (8) suggested that DM1 and DM2 are largely identical, as they are associated with dysregulated expression of genes in skeletal muscles. Previous studies have yielded significant findings concerning the molecular mechanisms of DM1 and DM2 (9,10). However, little attention has been paid to the molecular mechanisms of DM1 and DM2 cataracts. DM1 and DM2 cataracts are almost identical in appearance and age of onset (11,12). Rhodes *et al* (13) demonstrated that upregulated interferon-regulated genes and the type 1 interferon signaling pathway may be involved in the common mechanism of DM1 and DM2 cataracts. Further efforts are required to unveil the underlying molecular mechanisms.

With the aid of high output microarray analysis, the present study identified differentially expressed genes (DEGs) between DM1 and non-DM cataracts samples, and between DM2 and non-DM cataracts samples. Gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to investigate the biological functions and pathways that may involve the DEGs. Furthermore, the present study examined potential micro (mi)RNAs that may be involved in DM1 and DM2 by constructing a microRNA (miRNA)-gene network for DM1 and DM2, respectively.

Materials and methods

Microarray dataset. The present study is a secondary study using the microarray dataset (E-MEXP-3365) (13) downloaded from the Array Express database (http://www.ebi.ac.uk/arrayexpress) (14), including 3 lens epithelial samples from DM1 cataract patients, 3 lens epithelial samples from DM2 cataract patients and 4 non-DM lens epithelial samples (control).

Data preprocessing and identification of DEGs. The microarray data were preprocessed using the lumi Bioconductor package version 2.3 (15,16). Each probe was mapped to its corresponding gene, followed by background correction and quartile data normalization. If a probe corresponded to >1 gene, the probe would be deleted. When several probes mapped to one gene, the mean expression values of these probes were calculated as the expression value of the gene.

Differentially expressed genes were identified between DM1 cataract samples and non-DM cataract samples, and between DM2 cataract and non-DM cataract samples, using a t-test. The strict cutoff was set at fold change (|logFC|)>2 and false discovery rate (FDR)<0.05.

GO and KEGG pathway enrichment analysis. In order to investigate the biological function involving the DEGs identified in the DM1 and DM2 cataracts, GO (17) and KEGG (18) pathway enrichment analysis was performed using Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/tools.jsp) (19). Significant GO terms and KEGG pathways (adjusted P≤0.05, gene number ≥2) were identified. There were 3 types of GO terms: Biological process (BP), cellular component (CC) and molecular function (MF).

Construction of miRNA-DEG networks and analysis of network modules. Potential miRNAs that may be involved in regulating the DEGs identified in MD1 and MD2 cataract S were predicted using 7 algorithms, including PicTar (20), DIANA-microT (21), miRanda (22), miRBase (23), RNAhybrid (24), RNA22 (25) and TargetScan (26). Only the miRNAs that were validated using at least 3 out of the 7 algorithms were selected. With the predicted miRNAs and DEGs, a miRNA-gene network was constructed for DM1 and DM2, respectively, using Cytoscape software version 3.2.0 (27). In the network, a node denoted a gene or a miRNA; an undirected link represented an interaction between two genes, or between a gene and a miRNA. The degree of a node referred to the number of interactions of a gene or a miRNA. Furthermore, significant network modules were extracted from the network using Mcode plugin in Cytoscape (27) (cutoff value: Degree ≥2) and analyzed.

Results

Identification of DEGs. A total of 223 DEGs were screened between DM1 cataract and non-DM cataract samples. A total of 303 DEGs were screened between DM2 cataract and non-MD cataract samples. Furthermore, 172 DEGs were shared by DM1 and DM2 cataracts.

GO and KEGG pathway enrichment analysis. GO enrichment analysis revealed that most of significant CC and MF terms were common to DM1 and DM2 cataracts (Tables I and II). The overlapped MF terms were related to nucleotide binding and ribonucleotide binding. With regards to BP terms, DEGs in DM1 cataracts were significantly enriched with intracellular transport, cell cycle, mRNA metabolic process, RNA processing and vesicle-mediated transport (Table I), whereas the DEGs in DM2 cataract were significantly associated with regulation of apoptosis, regulation of programmed cell death, regulation of cell death, intracellular transport and vesicle-mediated transport (Table II).

The KEGG pathway enrichment analysis revealed that the amyotrophic lateral sclerosis and Alzheimer's disease signaling pathways were shared by DM1 and D2 cataracts. Furthermore, DEGs in DM1 cataracts were significantly enriched with calcium signaling, and markers from the Wnt and axon guidance signaling pathways (Table III), whereas DEGs in DM2 cataracts were markedly associated with the adherens junction signaling pathway (Table IV).

Analysis of miRNA-gene networks. In the current study, a total of 345 miRNAs were predicted to be associated with the DEGs identified in DM1 cataracts, which constituted a total of 1,022 miRNA-target gene pairs. The DEGs in DM1 cataracts were predicted to be regulated by 371 miRNAs, and a total of 1,357 miRNA-target gene pairs were obtained.

For the purpose of deciphering the associations between DEGs, and between DEGs and the predicted miRNAs, an miRNA-gene network was constructed for DM1 (Fig. 1) and DM2 (Fig. 2). A total of three modules were extracted from the miRNA-gene network for DM1 cataracts. As presented in Fig. 3, miR-197, miR-29b, miR-29c, miR-130a and miR-130b were included in the network modules. A total of seven modules were extracted from the miRNA-gene network for DM2 cataracts (Fig. 4). miR-197, miR-29c, miR-29c, miR-152, miR-302d, miR-302d, miR-372, miR-20a, miR-20b, miR-133a, miR-133b, miR-148a, miR-148b and miR-515-3p were involved in these network modules. miR-197 and miR-29c were shared by the network modules of DM1 and DM2.

Discussion

There are limited studies specifically focused on the molecular mechanisms of DM1 and DM2 cataracts. The present study identified 223 DEGs in DM1 cataracts and 303 DEGs in DM2 cataracts. Notably, a total of 172 overlapped DEGs were identified in DM1 and in DM2 cataracts. They shared several identical significant GO terms, but there were additionally some GO terms specific to DM1 or DM2 cataracts. DEGs in DM1 cataracts were significantly enriched in the calcium, Wnt and axon guidance signaling pathways, whereas DEGs in DM2 cataracts were significantly associated with the adherens junction signaling pathway. One highlight of the current study was the construction of an miRNA-gene network for DM1 and DM2 cataracts. miR-197, miR-29b, miR-29c, miR-130a and miR-130b were included in the three network modules extracted from the miRNA-gene network for DM1 cataracts. miR-197, miR-29c, miR-29c, miR-152, miR-302d, miR-372, miR-20a, miR-20b, miR-133a, miR-133b, miR-148a, miR-148b

Table I. Significant GO terms for DEGs in DM1 cataracts.

GO terms	Description	Count of genes	Q-value
BP	Intracellular transport	18	0.011184278
	Cell cycle	18	0.045910811
	mRNA metabolic process	15	7.41E-04
	RNA processing	15	0.022266862
	Vesicle-mediated transport	15	0.03260547
	mRNA processing	14	6.10E-04
	Protein folding	13	7.45E-06
	Cytoskeleton organization	13	0.020266359
	Regulation of cellular protein metabolic process	13	0.035471916
	RNA splicing	12	0.002271744
CC	Non-membrane-bounded organelle	51	0.00607
	Intracellular non-membrane-bounded organelle	51	0.00607
	Membrane-enclosed lumen	48	1.60E-05
	Organelle lumen	46	4.69E-05
	Intracellular organelle lumen	45	5.82E-05
	Cytosol	32	0.002344
	Nuclear lumen	32	0.008374
	Vesicle	22	3.57E-04
	Membrane-bounded vesicle	21	1.08E-04
	Cytoplasmic vesicle	21	5.36E-04
MF	Nucleotide binding	39	0.075474
	Purine ribonucleotide binding	34	0.049174
	Ribonucleotide binding	34	0.049174
	Purine nucleotide binding	34	0.08014
	Nucleoside binding	30	0.063175
	ATP binding	29	0.039048
	Adenyl ribonucleotide binding	29	0.045135
	Adenyl nucleotide binding	29	0.076671
	Purine nucleoside binding	29	0.088577
	RNA binding	20	0.003752

GO, gene ontology; DEG, differentially expressed genes; DM, myotonic dystrophy; BP, biological process; CC, cellular component; MF, molecular function.

and miR-515-3p were involved in the seven network modules extracted from the miRNA-gene network for DM2 cataract. Notably, miR-197 and miR-29c were shared between DM1 and DM2 cataracts.

It has been demonstrated that DM1 and DM2 share several clinical and genetic features (2). Both of them are toxic RNA diseases. Rhodes *et al* (13) identified a high proportion of overlapped dysregulated genes in DM1 and DM2 cataracts, and suggested the involvement of the innate immune response and interferon signaling in their common underlying mechanisms. The present study demonstrated that DM1 and DM2 cataracts shared 172 DEGs. Furthermore, the two forms of DM cataracts were associated with identical CC and MF terms associated with nucleotide binding and ribonucleotide binding in the present study. In addition, they were associated intracellular transport and vesicle-mediated transport. These findings indicated that nucleotide binding, ribonucleotide binding, intracellular transport and vesicle-mediated transport may be

implicated in the common molecular mechanisms underlying DM1 and DM2 cataracts.

DM1 and DM2 are two genetically distinct entities. DM1 is due to a mutation of the CTG expansion in DMPK, whereas DM2 is caused by a CCTG expansion in the CNBP gene (9). DM2 is rarer and has a milder phenotype and later onset of symptoms, when compared with DM1 (28). The DM1 repeat number increases over the generations. Conversely, the DM2 repeat number may decrease over the generations (12). In addition to the common molecular mechanisms, differences do exist between DM1 and DM2 (29). Franc *et al* (30) demonstrated that the gray matter volumes are significantly decreased in DM1 patients, rather than in DM2 patients. Passeri *et al* (31) identified that the phosphate levels are decreased in DM1 patients, but not in DM2 patients. In the present study, results of GO functional enrichment analysis indicated that the DEGs in DM1 cataracts may affect cell cycle, mRNA metabolic process and RNA processing, whereas

Table II. Significant GO terms for DEGs in DM2 cataracts.

GO term	Description	Gene count	Q-value
BP	Regulation of apoptosis	25	0.015950693
	Regulation of programmed cell death	25	0.017739841
	Regulation of cell death	25	0.018408079
	Protein transport	23	0.028778834
	Establishment of protein localization	23	0.031394209
	Intracellular transport	22	0.011776008
	Vesicle-mediated transport	19	0.023259474
	Membrane organization	18	8.39E-04
	Protein complex assembly	17	0.028082861
	Protein complex biogenesis	17	0.028082861
CC	Intracellular non-membrane-bounded Organelle	59	0.039736
	Non-membrane-bounded organelle	59	0.039736
	Membrane-enclosed lumen	57	4.79E-05
	Organelle lumen	53	3.82E-04
	Intracellular organelle lumen	52	4.07E-04
	Cytosol	48	4.18E-06
	Nuclear lumen	38	0.017793
	Organelle membrane	34	0.002298
	Mitochondrion	30	0.020663
	Endomembrane system	29	3.82E-04
MF	Nucleotide binding	53	0.037027
	Purine nucleotide binding	44	0.085847
	Ribonucleotide binding	42	0.096712
	Purine ribonucleotide binding	42	0.096712
	Nucleoside binding	39	0.057925
	Adenyl nucleotide binding	38	0.064446
	Purine nucleoside binding	38	0.076716
	ATP binding	36	0.063612
	Adenyl ribonucleotide binding	36	0.073955
	RNA binding	26	0.001454

KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed genes; DM, myotonic dystrophy; BP, biological process; CC, cellular component; MF, molecular function; ATP, adenosine triphosphate.

Table III. KEGG pathways for DEGs in DM1 cataracts.

KEGG	Gene count	Adjusted P-value	Description
KEGG_PATHWAY	5	0.020744	Long-term potentiation
KEGG_PATHWAY	4	0.048553	Amyotrophic lateral sclerosis
KEGG_PATHWAY	6	0.10831	Alzheimer's disease
KEGG_PATHWAY	5	0.131481	Spliceosome
KEGG_PATHWAY	6	0.137285	Calcium signaling pathway
KEGG_PATHWAY	4	0.171196	Dilated cardiomyopathy
KEGG_PATHWAY	5	0.206866	Wnt signaling pathway
KEGG_PATHWAY	3	0.221622	Pathogenic *Escherichia coli* infection
KEGG_PATHWAY	4	0.243621	Oocyte meiosis
KEGG_PATHWAY	4	0.32439	Axon guidance

KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed genes; DM, myotonic dystrophy.

Table IV. KEGG pathways for DEGs in DM2 cataracts.

KEGG	Gene count	Q-value	Description
KEGG_PATHWAY	10	0.013726	Huntington's disease
KEGG_PATHWAY	4	0.037286	Basal transcription factors
KEGG_PATHWAY	7	0.053893	Parkinson's disease
KEGG_PATHWAY	5	0.080184	Adherens junction
KEGG_PATHWAY	4	0.101893	Amyotrophic lateral sclerosis
KEGG_PATHWAY	4	0.106299	Non-small cell lung cancer
KEGG_PATHWAY	4	0.119952	Pathogenic *Escherichia coli* infection
KEGG_PATHWAY	2	0.120912	Methane metabolism
KEGG_PATHWAY	7	0.131916	Alzheimer's disease
KEGG_PATHWAY	4	0.14904	Glioma

KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed genes; DM, myotonic dystrophy.
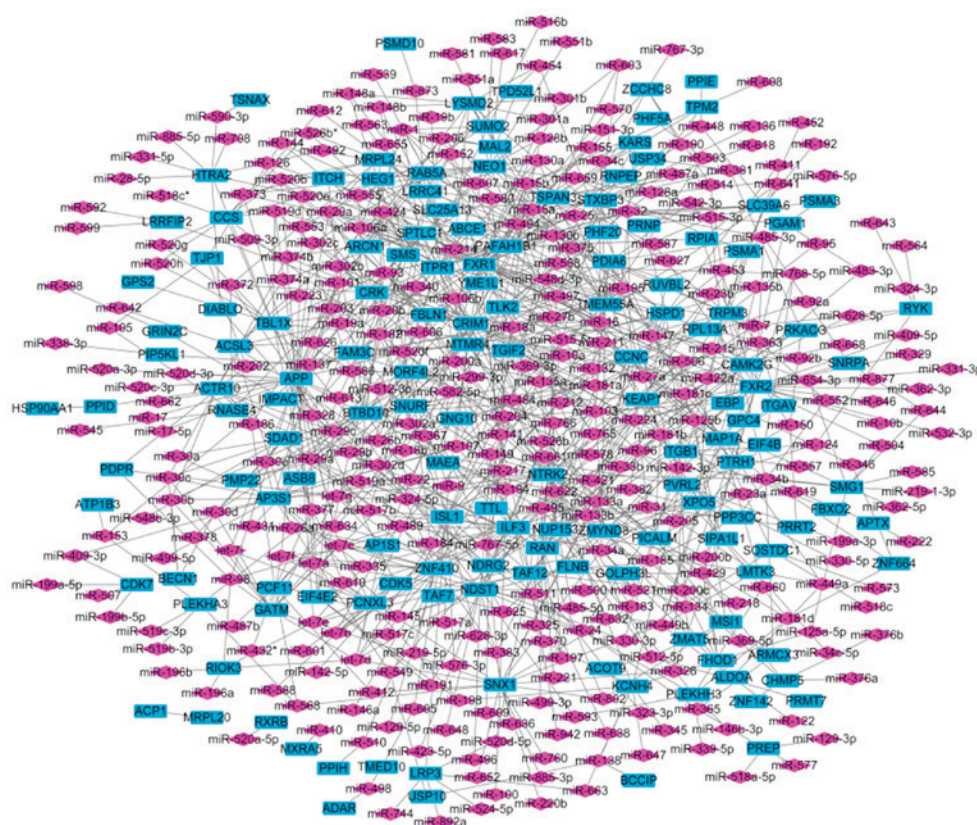

Figure 1. An miRNA-gene network for DM1 cataracts. Square nodes denote genes and diamond-shaped nodes denote miRNAs. An undirected link represents an interaction between two genes, or between a gene and an miRNA. miRNA, microRNA; DM, myotonic dystrophy.

the DEGs in DM2 cataract may be involved in regulation of apoptosis, regulation of programmed cell death and regulation of cell death. These results provide evidences in favor of the discrepancy in the underlying molecular mechanisms between DM1 and DM2 cataracts.

It has been presented that, in DM1, unstable CTG repeats in the DMPK gene induce mis-splicing to fetal/neonatal isoforms of many transcripts associated with cellular Ca2+ homeostasis, and several calcium-associated proteins are abnormally expressed in DM1 and DM2, including Ca^{2+}

release channel ryanodine receptor and calsequestrin 2 (32). Botta *et al* (33) suggested that unbalanced Ca^{2+} homeostasis causes muscle degeneration in DM1 muscle cells by activating endoplasmic reticulum stress. In support of these findings, the present study indicated that DEGs in DM1 cataracts were significantly enriched in the calcium signaling pathway, indicating that this may serve a role in the pathogenesis of DM1 cataracts. Furthermore, results of the KEGG pathway enrichment analysis suggested that the Wnt axon guidance signaling pathways may be involved in DM1 cataract, whereas
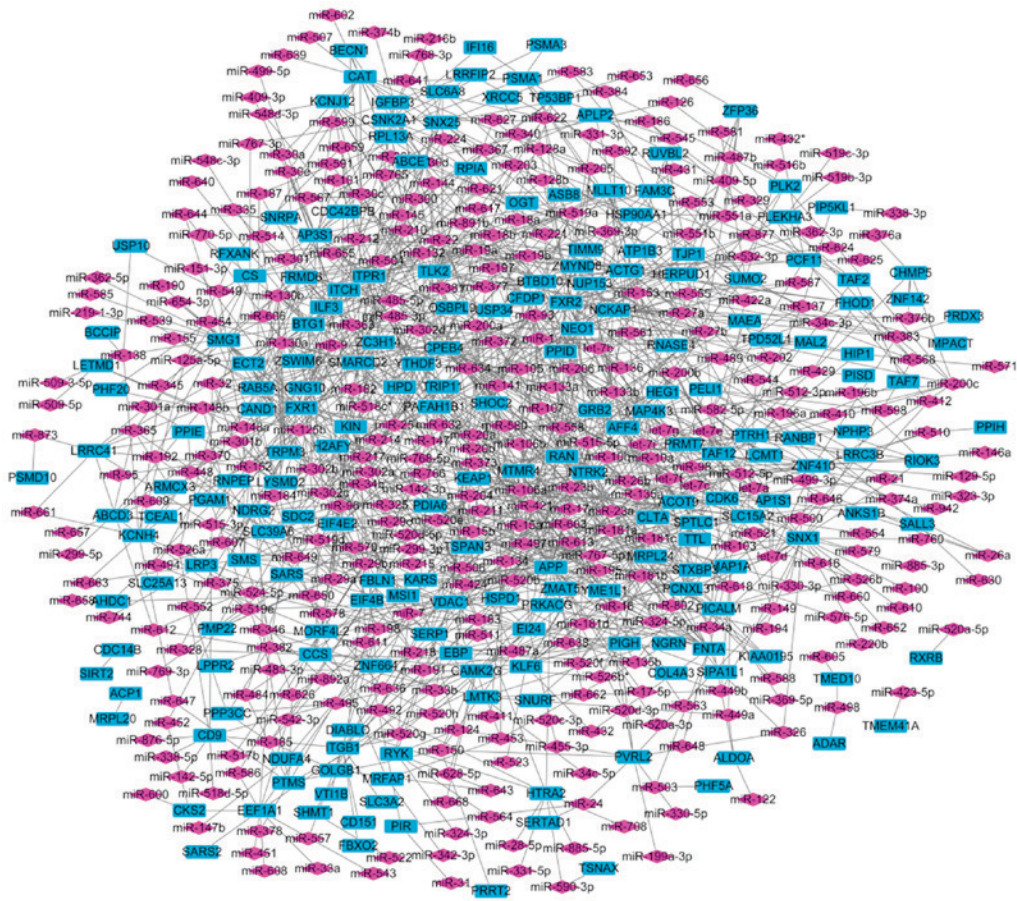
Figure 2. miRNA-gene network for DM2 cataracts. Square nodes denote genes and diamond-shaped nodes denote miRNAs. An undirected link represents an interaction between two genes, or between a gene and an miRNA. miRNA, microRNA; DM, myotonic dystrophy.
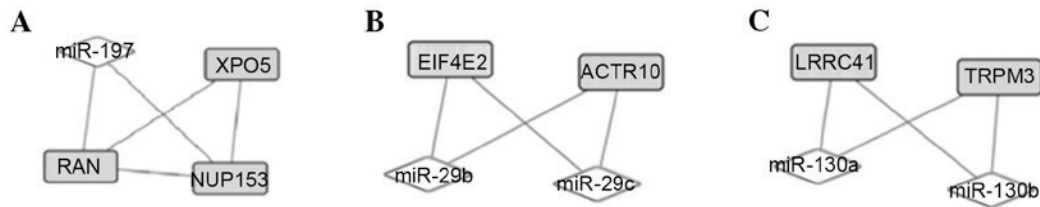
Figure 3. (A-C) A total of three modules extracted from the miRNA-gene network for DM1 cataract. Square nodes denote genes and diamond-shaped nodes denote miRNAs. An undirected link stands for an interaction between two genes, or between a gene and an miRNA. miRNA, microRNA; DM, myotonic dystrophy.
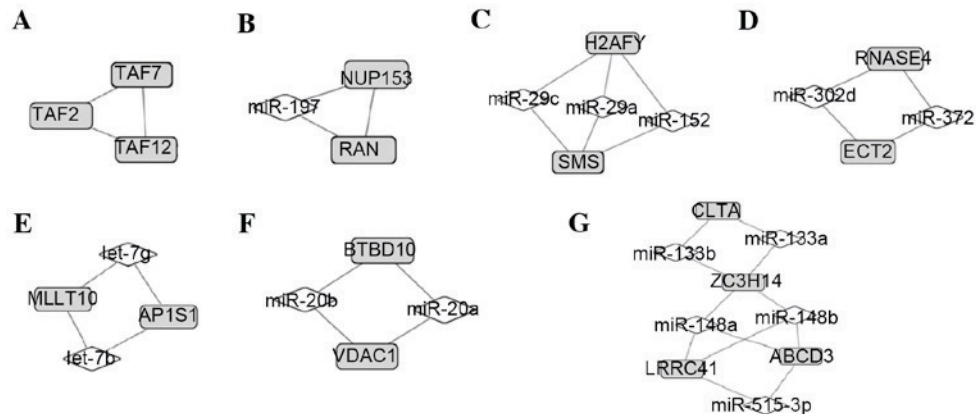
Figure 4. (A-G) A total of seven modules extracted from the miRNA-gene network for DM2 cataracts. Square nodes represent genes and diamond-shaped nodes represent miRNAs. An undirected link refers to an interaction between two genes, or between a gene and an miRNA.

the adherens junction signaling pathway may be involved in DM2 cataracts. To the best of our knowledge, these findings have not been reported before.

miRNAs are highly conserved, noncoding RNAs that are involved in a wide range of biological processes, including myogenesis and muscle regeneration. The deregulation of miRNAs in the pathogenesis of MD has been an area of intense investigation. Perbellini *et al* (34) discovered that miR-29b, miR-29c and miR-33 are downregulated in skeletal muscle biopsies of DM1 patients relative to controls. Greco *et al* (35) created a list of dysregulated miRNAs in skeletal muscle biopsies of DM2 patients compared with controls, including miR-34a-5p, miR-34b-3p and miR-34c-5p. Furthermore, miR-206 is over-expressed in the skeletal muscle of DM1 patients (36), and miR-1 is associated with heart defects in DM1 and DM2 (37). However, the dysregulated miRNAs in the pathogenesis of DM1 and DM2 cataracts remain to be clearly defined. The present study suggested that miR-197, miR-29b, miR-29c, miR-130a and miR-130b are potentially involved in DM1 cataracts, while miR-197, miR-29c, miR-29a, miR-152, miR-302d, miR-372, miR-20a, miR-20b, miR-133a, miR-133b, miR-148a, miR-148b and miR-515-3p may be implicated in DM2 cataracts. miR-197 and miR-29c were involved in DM1 and DM2 cataracts. The present study added more insights concerning miRNA deregulation in the pathogenesis of DM.

The results of the current study advanced understanding of the molecular mechanisms underlying DM1 or DM2 cataracts. However, it had a limited sample size. More importantly, further experimental studies are warranted to verify these findings derived from the microarray data analysis.

In conclusion, the present study identified potential genes, pathways and upstream miRNAs, which may be involved in the pathogenesis of DM1 or DM2 cataracts. The calcium, Wnt and axon guidance signaling pathways, and miR-197, miR-29b and miR-29c, may be involved in DM1 cataracts. The adherens junction signaling pathway, miR-197, miR-29c and miR-29a may be involved in DM2 cataracts. Further studies are required to elucidate the different underlying molecular mechanisms between DM1 and DM2 cataracts, and to develop potential treatments for this disorder.

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