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Network-based analysis of the molecular mechanisms of multiple myeloma and monoclonal gammopathy of undetermined significance

ZHI LIU[1*], JING HUANG[1,2*], QI ZHONG[1], YANLING SHE[3], RUIMIN OU[1], CHENG LI[3], RUI CHEN[3], MENGDONG YAO[1], QING ZHANG[1] and SHUANG LIU[1]

[1]Department of Hematology, Guangdong Second Provincial General Hospital, Guangzhou, Guangdong 510317; [2]Department of Hematology, The First Hospital of Kashgar District of Xinjiang, Kashgar, Xinjiang 844000; [3]Guangdong Traditional Medical and Sports Injury Rehabilitation Research Institute, Guangdong Second Provincial General Hospital, Guangzhou, Guangdong 510317, P.R. China

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Abstract. The present study aimed to reveal the molecular mechanisms of multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS). This was a secondary study on microarray dataset GSE80608, downloaded from the Gene Expression Omnibus database, which included 10 control samples, 10 MGUS samples and 10 MM samples. Differentially expressed genes (DEGs) were identified between control and MGUS samples, and between control and MM samples. A protein-protein interaction (PPI) network was built for studying the interactions between the DEGs. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was performed for the genes in a gene co-expression network. A microRNA (miRNA/miR)-gene network was built to the evaluate possible the miRNAs and genes involved in the diseases. The present study identified 136 common upregulated DEGs and 165 common downregulated DEGs between MM and MGUS. Pathway enrichment analysis of the genes in the gene co-expression network revealed that the complement and coagulation cascades pathway was significantly enriched for certain complement and coagulation-associated genes. Endothelin-1 (EDN1) was significantly enriched in the hypoxia inducible factor-1 (HIF-1) and tumor necrosis factor signaling pathways. EDN1 was an important node in the PPI network, and a target gene of let-7e, let-7b and miR-19a in the miRNA-gene network. The results of the present study indicate that complement and coagulation-associated genes, the complement and coagulation cascades pathway, EDN1, let-7e, let-7b-5p, miR-19a, and the tumor necrosis factor and HIF-1 signaling pathways may all be implicated in MM and MGUS.

Correspondence to: Dr Shuang Liu or Dr Qing Zhang, Department of Hematology, Guangdong Second Provincial General Hospital, 466 Xin Gang Zhong Road, Guangzhou, Guangdong 510317, P.R. China
E-mail: luuiS789@163.com
E-mail: zhqing@vip.163.com

*Contributed equally

Key words: multiple myeloma, monoclonal gammopathy of undetermined significance, pathway, gene co-expression network, microRNA

Introduction

Multiple myeloma (MM) is a cancer of the plasma cells within the bone marrow (BM), which caused ~79,000 mortalities in 2013 (1). It has been widely accepted that MM arises from monoclonal gammopathy of undetermined significance (MGUS), which is a pre-malignant condition that can be present for several years prior to the development of MM (2). As MM is incurable in the vast majority of patients (3), there is an urgent requirement for novel therapies. Intervening at the MGUS stage may aid to prevent the progression of MGUS to MM (4,5). Therefore, a thorough understanding of the molecular pathogenesis of MM and MGUS is urgently required to guide interventions that target the precursor state (MGUS) and MM itself.

Kubiczkova *et al* (6) revealed the identity of five microRNAs (miRNAs/miRs) that were deregulated in the circulating serum of patients with MM and MGUS, compared with that of healthy subjects. Shvartsur *et al* (7) suggested that Raf-1 kinase inhibitor protein-associated genes could be implicated in MM and MGUS using a bioinformatics approach. Amend *et al* (8) provided *in vivo* evidence that the absence of SAM domain, SH3 domain and nuclear localization signals 1 may be associated with genetic susceptibility to MGUS in mice. Furthermore, there is evidence that a dysregulated cyclin D/retinoblastoma signaling pathway is involved in the molecular pathogenesis of MM and MGUS (9,10). The nuclear factor-κB (NF-κB) signaling pathway serves an important role in the mechanism of MM pathogenesis (11). Despite these advances, the molecular mechanisms behind the transformation of MUGS into MM have not been fully elucidated.

McNee *et al* (12) observed that the citrullination of histone H3 promotes interleukin-6 (IL-6) production by BM mesenchymal stem cells, resulting in pro-malignancy signaling in patients with MGUS and MM. However, the genes and pathways involved in MM and MGUS have not been fully delineated. Therefore, the present study reports a secondary analysis of the microarray dataset GSE80608 (12). Differentially expressed genes (DEGs) in MM, MGUS and control subjects were identified, and a protein-protein interaction (PPI) network was built to analyze the interactions between genes. A gene co-expression network was then built to analyze the co-expression of these DEGs in MGUS and MM. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway-enrichment analysis was performed to reveal the signaling pathways that may be involved in MGUS and MM. The data may provide indications of the genes and pathways that determine the progression of MGUS to MM.

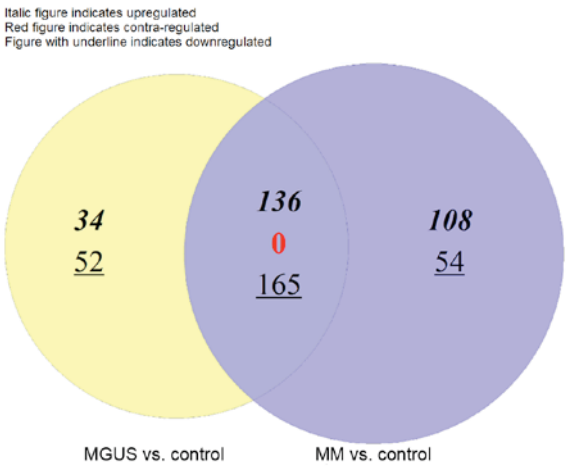

Figure 1. Venn diagram of differently expressed genes in MM and MGUS. MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance.

Materials and methods

Microarray dataset preprocessing. The present study was a secondary study on the GSE80608 microarray dataset, obtained from the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) (13) and based on the Affymetrix Human Exon 1.0 ST Array platform (Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The dataset consisted of 10 control BM samples, 10 BM samples from the iliac crest of 10 patients with MGUS and 10 BM samples from the iliac crest of 10 patients with MM.

With the aid of oligo software (version 5.1; National Biosciences, Inc., Plymouth, Minn., USA), the raw data were subjected to data preprocessing, including background correction, data normalization and calculation of probe expression values. By using huex10sttranscriptcluster.db (14) and annotate (15) software in R (16), each probe was mapped to its corresponding gene symbol.

DEGs screening. DEGs were screened between MGUS and control, and between MM and control samples, using the LIMMA package in R (17). A strict cutoff was set at a false discovery rate <0.05 and fold change ($|\log_2 FC|$) ≥1.5.

Venn diagram analysis and KEGG pathway enrichment analysis. DEGs in MGUS or MM were analyzed using a Venn diagram (18), which revealed the number of the common upregulated DEGs, common downregulated DEGs and contra-regulated DEGs between MGUS and MM in a Venn diagram.

KEGG pathway enrichment (19) analysis was performed for the identified DEGs using DAVID software (20). KEGG pathways with a gene count ≥2 and P<0.05 were considered to indicate a statistically significant difference.

Analysis of a PPI network. A PPI network (PPI score=0.7) was constructed with the identified DEGs using the STRING online tool (21) to analyze the interactions between the DEGs. In the PPI network, a node denoted a gene and a link between two nodes denoted the interaction between the two genes. The number of interactions between one gene and other genes in the network was represented by the 'degree' value. The topological parameters of the nodes in the PPI network were analyzed using the Cytoscape CytoNCA application (22), and included degree, betweenness (23), closeness (24) and subgraph (25).

Gene co-expression analysis. A gene co-expression network was built to analyze the co-expression of the DEGs in MGUS and MM. The co-expression coefficient between two genes was calculated based on the Pearson's correlation coefficient. The P-value was calculated using the Z-score (26). Significant co-expressed gene pairs were determined to have a co-expression coefficient >0.85 and P<0.05. KEGG pathway enrichment analysis was then conducted for the co-expressed genes.

Analysis of disease-related miRNA and target genes. MM-associated miRNAs were downloaded from the miR2Disease database (27), which included 17 miRNAs that were associated with MM. On the basis of these miRNAs, target genes were predicted using miRwalk2.0 (28). The miRNA-target gene interaction pairs were obtained from the validated target module in miRwalk2.0, which had been validated previously (28). Finally, the differentially expressed target genes were selected.

Results

Identification and Venn diagram analysis of DEGs. The present study identified 387 DEGs between MGUS and control samples, and 463 DEGs between MM and control samples. Venn diagram analysis revealed that there were 136 common upregulated DEGs, 165 common downregulated DEGs and no contra-regulated DEGs between MGUS and MM (Fig. 1). MGUS had 34 specific upregulated DEGs and 52 specific downregulated DEGs, whereas MM had 108 specific upregulated DEGs and 54 specific downregulated DEGs. A heat map revealed that DEG expression in the control samples was markedly different from that in MM and MGUS samples (Fig. 2).

KEGG pathway enrichment analysis. KEGG pathway enrichment analysis (Table I) revealed that the common upregulated DEGs were significantly enriched in five pathways, including

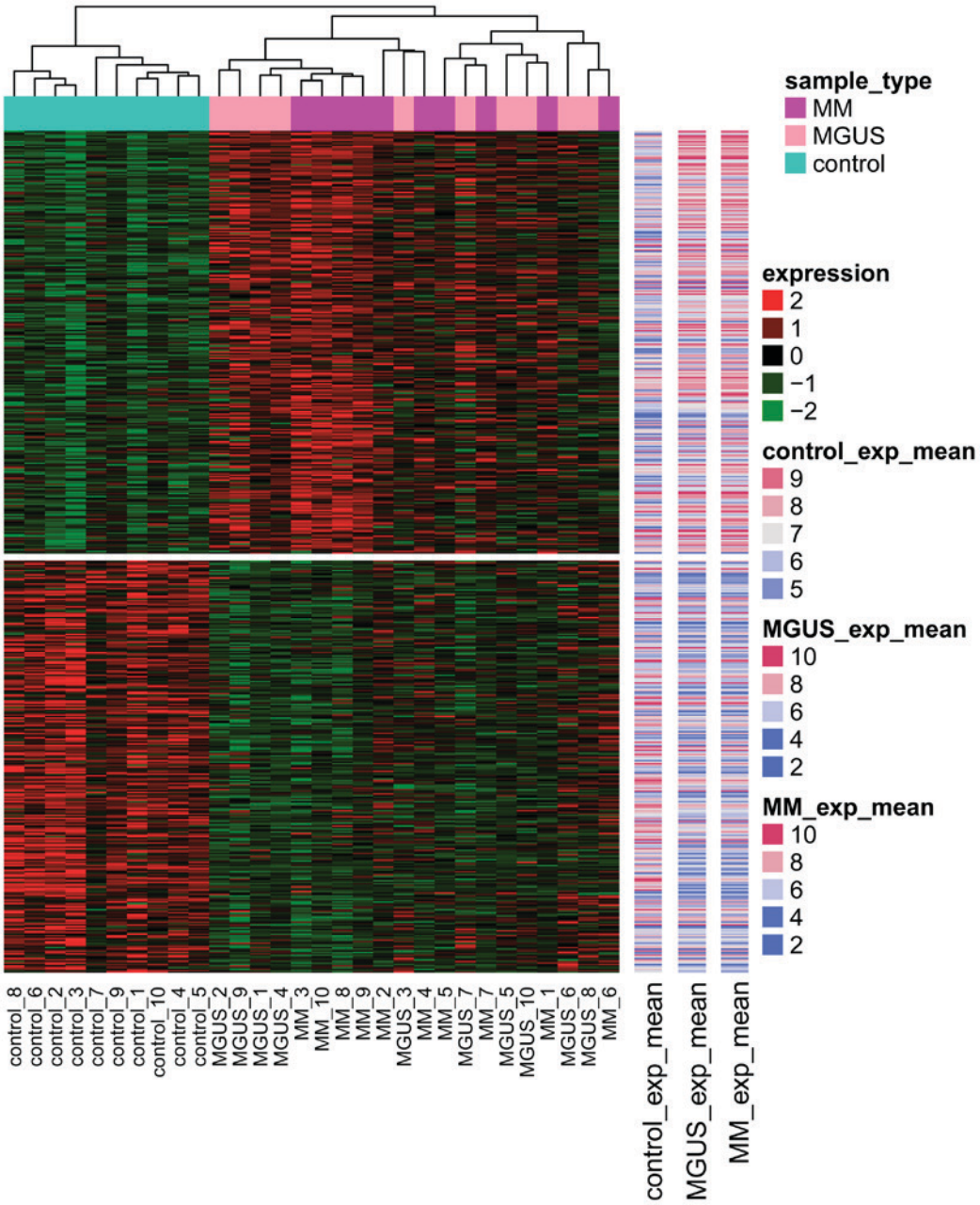

Figure 2. Heat map of differently expressed genes. MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; exp, expression.

the Rap1 signaling pathway and the regulation of actin cytoskeleton pathway. The common downregulated DEGs were significantly associated with the complement and coagulation cascades, including cluster of differentiation 55 (CD55), thrombomodulin (THBD), mannan-binding lectin serine protease 1 (MASP1), complement factor B (CFB), coagulation factor III (F3), complement component 1 (C1)R, C1S and coagulation factor II receptor (F2R). Furthermore, common downregulated DEGs were significantly associated with axon guidance, ATP-binding cassette transporters, *Staphylococcus aureus* infection response and steroid hormone biosynthesis pathways. MM-specific upregulated DEGs were significantly associated with the regulation of actin cytoskeleton pathway.

PPI network. A PPI network was built with the DEGs in MM and MGUS (Fig. 3). The network included 233 nodes and 435 gene pairs. Of these genes, 39 were MGUS-specific, 59 were MM-specific and 125 were common between MGUS and MM.

In the PPI network, the top 20 genes (nodes) were selected, based on their values of degree, betweenness, closeness and subgraph, to be the important nodes in the PPI network (Table II). Of the top 20 genes, endothelin 1 (EDN1), C-X-C motif chemokine ligand 12, adenylate cyclase 4, matrix metallopeptidase 1, insulin like growth factor 1 and Finkel-Biskis-Jinkins osteosarcoma oncogene were common between MGUS and MM.

Analysis of a gene co-expression network. The gene co-expression network included 312 co-expressed gene pairs, 186 MM- or MGUS-specific genes, and 141 genes common to MM and MGUS. The co-expressed genes exhibited consistent changes in MM and MGUS (Fig. 4). These co-expressed genes

Table I. Significant Kyoto Encyclopedia of Genes and Genomes pathways of DEGs.

DEGs	Pathway	P-value	Genes
Common upregulated DEGs	hsa04360: Axon guidance	8.43×10^{-4}	PLXNA3, SEMA7A, MET, NTN4, SEMA3A, CXCL12, EPHA3
	hsa04514: Cell adhesion molecules	8.17×10^{-3}	NRXN3, ICAM2, CD4, ITGB2, NECTIN3, PDCD1LG2
	hsa05200: Pathways in cancer	2.16×10^{-2}	FGF5, PLCB4, PGF, SLC2A1, MET, BDKRB1, ITGA3, FGF1, CXCL12
	hsa04015: Rap1 signaling pathway	3.75×10^{-2}	FGF5, PLCB4, PGF, MET, ITGB2, FGF1
	hsa04810: Regulation of actin cytoskeleton	3.82×10^{-2}	FGF5, SCIN, BDKRB1, ITGB2, ITGA3, FGF1
Common downregulated DEGs	hsa04610: Complement and coagulation cascades	7.15×10^{-6}	CD55, THBD, MASP1, CFB, F3, C1R, C1S, F2R
	hsa04360: Axon guidance	1.09×10^{-2}	EPHA4, SEMA6D, NTNG1, EFNA5, UNC5D, SLIT3
	hsa02010: ABC transporters	1.11×10^{-2}	ABCA8, ABCC9, ABCA9, ABCA6
	hsa05150: *Staphylococcus aureus* infection	1.65×10^{-2}	MASP1, CFB, C1R, C1S
	hsa00140: Steroid hormone biosynthesis	2.32×10^{-2}	AKR1C3, AKR1C2, HSD11B1, AKR1C1
MM-specific upregulated DEGs	hsa04810: Regulation of actin cytoskeleton	4.79×10^{-2}	RAC2, MRAS, ITGA8, DIAPH3, ITGA7
MGUS-specific downregulated DEGs	hsa05410: Hypertrophic cardiomyopathy	1.91×10^{-2}	SGCD, IGF1, CACNA2D3
	hsa05414: Dilated cardiomyopathy	2.20×10^{-2}	SGCD, IGF1, CACNA2D3

DEG, differently expressed gene; hsa, *Homo sapiens*; ABC, ATP-binding cassette.

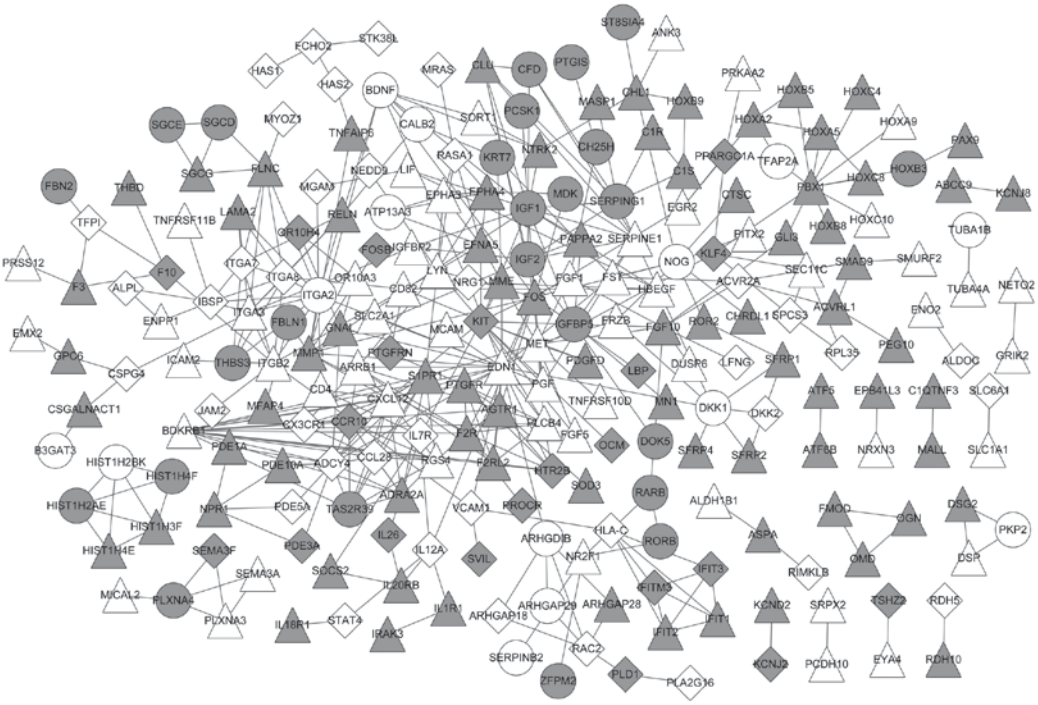

Figure 3. Protein-protein interaction network. Grey nodes, downregulated genes; white nodes, upregulated genes; round nodes, MGUS-specific genes; diamond nodes, MM-specific genes; triangular nodes, common genes between MGUS and MM. A link between two nodes represents an interaction between two genes. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma.

Table II. Degree, betweenness, closeness and subgraph of the top 20 nodes in the protein-protein interaction network.

Node, gene	Subgraph	Degree	Betweenness	Closeness
BDKRB1	1,515.014801	15		$2.18x10^{-2}$
EDN1	1,337.5355	24	14,298.21	$2.20x10^{-2}$
CXCL12	1,014.57958	14	1,799.90	$2.18x10^{-2}$
ADCY4	691.12317			
F2R	687.05615	11		$2.18x10^{-2}$
CCR10	641.9886599	9		
S1PR1	633.8678	10		
CCL28	597.2848528	9		
AGTR1	580.683	10		$2.18x10^{-2}$
ADRA2A	559.71972			
TAS2R39	559.718772			
RGS4	542.425323			
F2RL2	511.2666327			
PLCB4	510.37097	9		$2.18x10^{-2}$
PTGFR	486.30338			
HTR2B	432.8887689			
ARRB1	363.97263			
MMP1	248.22731	11	4,609.06	$2.19x10^{-2}$
IGF1	233.1976220	17	4,473.53	
FOS	213.7347773	13	6,338.76	$2.19x10^{-2}$
ADCY4		13	2,133.74	$2.18x10^{-2}$
KIT		13	4,026.67	$2.18x10^{-2}$
ITGA2		12	5,403.44	$2.19x10^{-2}$
MET		12	3,152.53	$2.18x10^{-2}$
PBX1		11	3,496.13	
ITGA8		10		
LYN		10	3,282.67	$2.18x10^{-2}$
ITGA3		10	2,372.76	
KLF4			4,272.19	
ITGB2			3,974.00	
FGF10			3,904.25	
VCAM1			2,679.60	
NOG			2,237.87	
ARHGDIB			2,199.00	
IL12A			1,979.33	
F10			1,848.00	
FST				$2.17x10^{-2}$
BDNF				$2.18x10^{-2}$
ARRB1				$2.18x10^{-2}$
MME				$2.18x10^{-2}$
DKK1				$2.18x10^{-2}$
PGF				$2.19x10^{-2}$
IGF1				$2.19x10^{-2}$

were significantly associated with the complement and coagulation cascades pathway, tumor necrosis factor (TNF) signaling pathway, *S. aureus* infection pathway, hypoxia-inducible factor-1 (HIF-1) signaling pathway and pathways in cancer (Table III).

Analysis of a miRNAs-gene network. A total of 16 MM-associated miRNAs were obtained from the miR2Disease database: miR-99b, miR-93, miR-561, miR-342, miR-335, miR-32, miR-25, miR-19b, miR-19a, miR-181b, miR-181a, miR-140-3p, miR-125a-5p, miR-106b, let-7e and let-7b. Fig. 5 shows a miRNA-gene network, featuring the associations between the 16 miRNAs and their target genes.

Discussion

MGUS is premalignant plasma-cell proliferative condition that consistently precedes MM (29). The current study identified that common downregulated DEGs, including CD55, THBD, MASP1, CFB, F3, C1R, C1S and F2R, were significantly

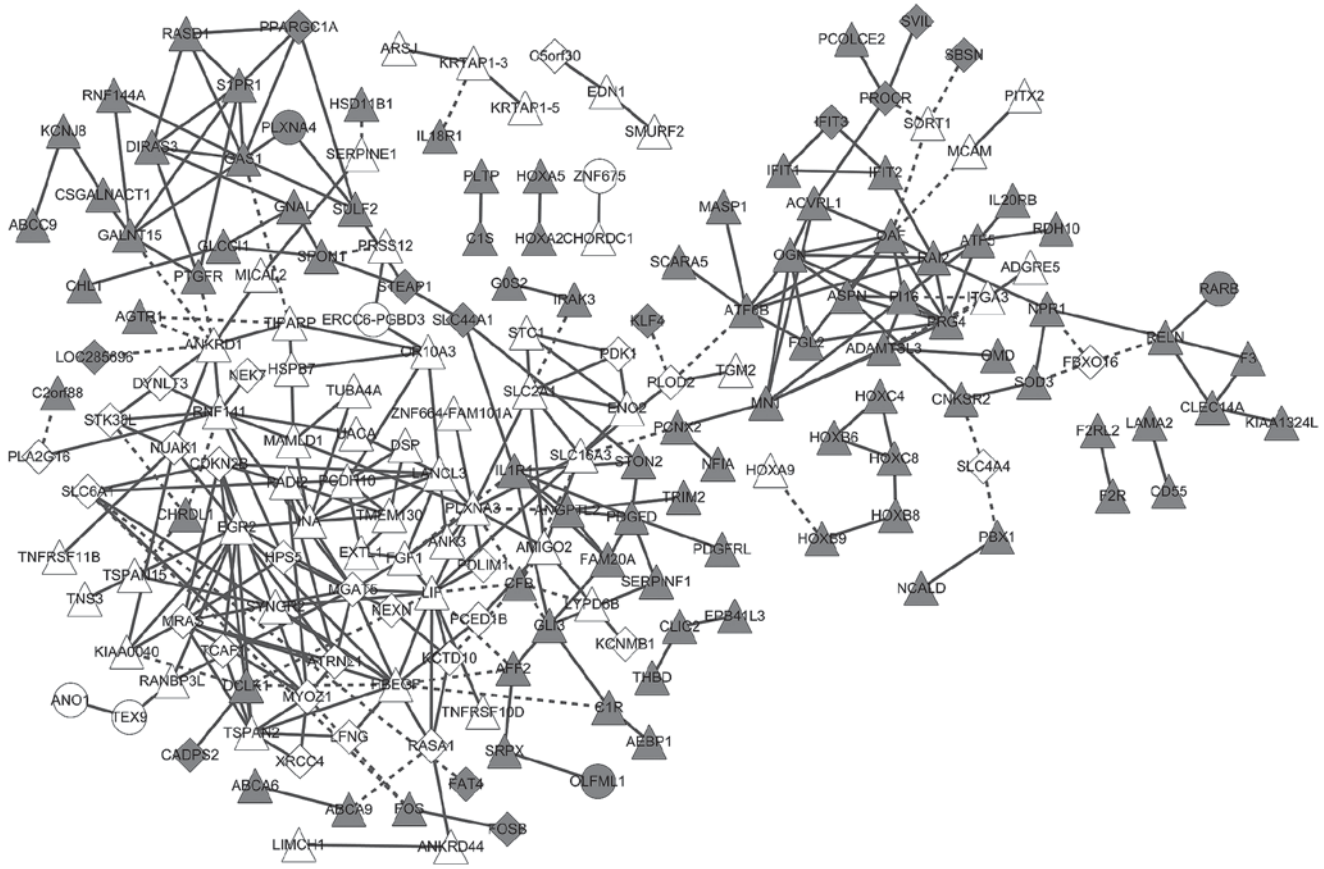

Figure 4. Gene co-expression network. Grey nodes, downregulated genes; white nodes, upregulated genes; round nodes, MGUS-specific genes; diamond nodes, MM-specific genes; triangular nodes, common genes between MGUS and MM. Solid lines indicate positive correlations, dotted lines indicate negative correlations. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma.

enriched in complement and coagulation cascades pathway. CD55 encodes complement decay-accelerating factor, which regulates the complement system on the cell surface (30). THBD encodes thrombomodulin, which participates in the anticoagulant pathway. MASP1 is an enzyme involved in the lectin pathway of the complement system (31). CFB, F3, C1R, C1S and F2R are components of the complement or coagulation system (32). Crowely *et al* (33) suggested that patients with MGUS have an intermediate coagulation profile, between that of patients with myeloma and that of healthy controls. There is evidence that MM is associated with an increased risk of venous thromboembolism (34). These findings indicate that these downregulated complement and coagulation genes may be involved in the development of MM and MGUS through the regulation of the complement and coagulation cascade pathways.

HIFs are transcription factors that respond to hypoxia; the HIF signaling pathway mediates the effects of hypoxia on the cell (35). It has been suggested that a hypoxic BM environment serves a role in the pathogenesis of MM (36,37). Azab *et al* (38) provided evidence that hypoxia promotes the dissemination of MM. In concordance with these findings, the current study noticed that the HIF-1 signaling pathway was significantly enriched for co-expressed genes. This enrichment indicates that the HIF-1 signaling pathway may have a role in the development of MM and MGUS. Furthermore, suppression of HIF-1 decreases the expression of the anti-apoptotic protein survivin, strengthening the sensitivity of MM cells to melphalan (39). HIF-1 inhibition may be a promising therapeutic strategy for treating MM and premalignant MGUS.

Lee *et al* (40) reported that TNF expression is significantly increased in patients with active MM and regulates IL-6 production. Previous studies have demonstrated that the NF-κB pathway, which is downstream of TNF, is involved in MM (11,41). In the present study, the TNF signaling pathway was significantly enriched by co-expressed genes. EDN1, a common DEG between MGUS and MM and an important node in the PPI network, was significantly enriched in the TNF and HIF-1 signaling pathways. EDN1 is a potent vasoconstrictor produced by vascular endothelial cells, whose expression is stimulated by hypoxia (42). These findings indicate that END1 could serve an important role in premalignant MGUS and MM by influencing the TNF and HIF-1 signaling pathways.

The present study additionally investigated a miRNA-gene regulatory network. In the miRNA-gene regulatory network, END1 was regulated by let-7e-5p, let-7b-5p, and miR-19a-5p, suggesting that the role of EDN1 in premalignant MGUS and MM may be affected by these miRNAs. Similarly, Kubiczkova *et al* (6) revealed that circulating serum levels of let-7e are deregulated in MM and MGUS compared with those in healthy subjects.

The present study has certain limitations. Firstly, the study samples are limited. It is a secondary study on the microarray dataset GSE80608, meaning that the number of samples in

Table III. Significantly enriched pathways of genes in the gene co-expression network.

Pathway	P-value	Genes
hsa04610: Complement and coagulation cascades	8.11×10^{-7}	CD55, THBD, MASP1, CFB, F3, SERPINE1, C1R, C1S, F2R
hsa05150: *Staphylococcus aureus* infection	0.019029	MASP1, CFB, C1R, C1S
hsa04066: HIF-1 signaling pathway	0.023618	PDK1, EDN1, SLC2A1, SERPINE1, ENO2
hsa04668: TNF signaling pathway	0.029493	LIF, IL18R1, FOS, EDN1, ATF6B
hsa05200: Pathways in cancer	0.030231	LAMA2, AGTR1, FOS, CDKN2B, SLC2A1, ITGA3, RARB, FGF1, GLI3, F2R

hsa, *Homo sapiens;* HIF, hypoxia inducible factor; TNF, tumor necrosis factor.

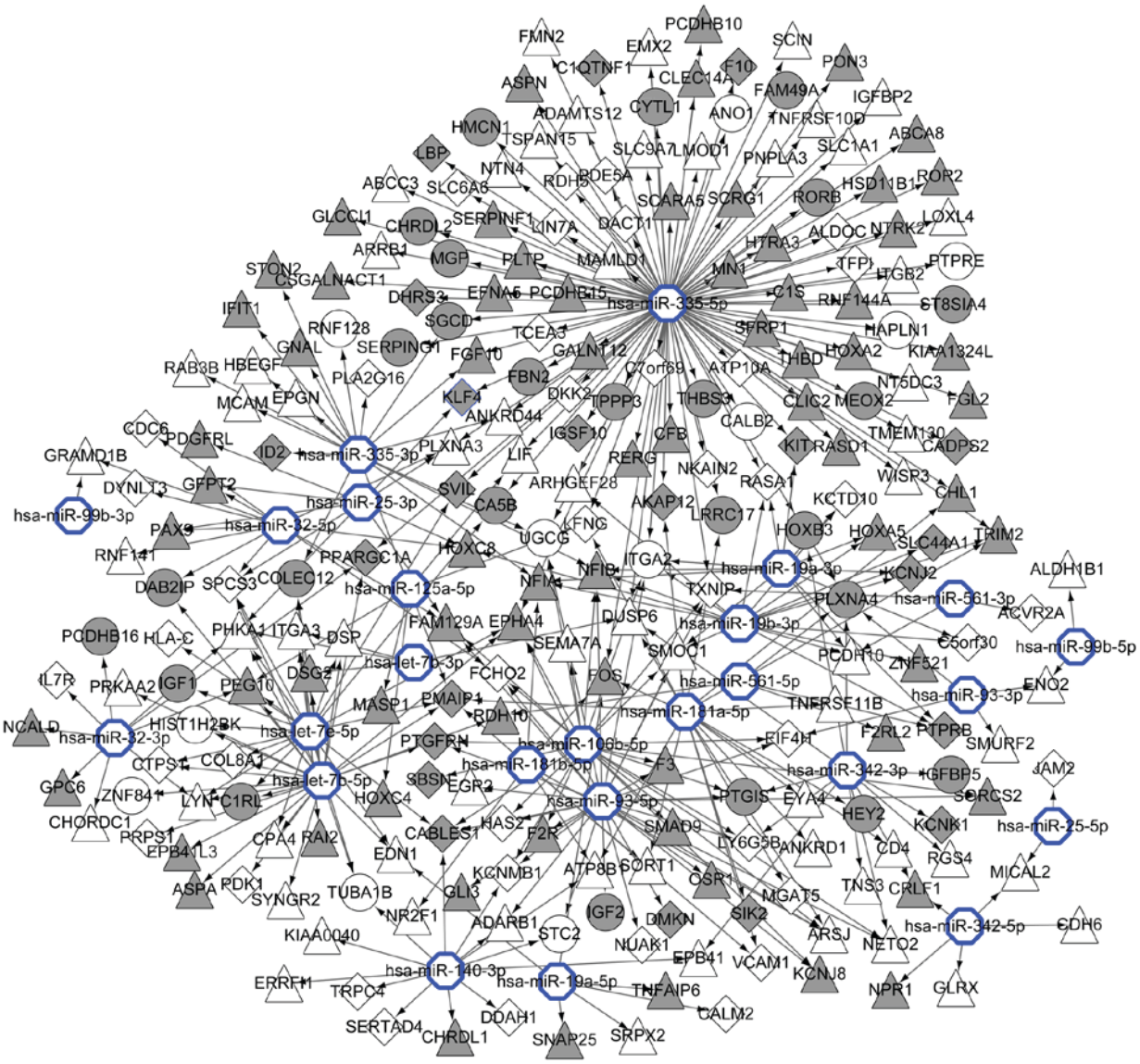

Figure 5. miRNA-gene network. Blue hexagonal nodes represent miRNAs; grey nodes, downregulated genes; white nodes, upregulated genes; round nodes, MGUS-specific genes; diamond nodes, MM-specific genes; triangular nodes, common genes between MGUS and MM. Connecting arrows indicate regulatory interactions between miRNAs and target genes. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; miRNA, microRNA.

the study cannot be increased. Secondly, the study only used bioinformatics approaches, meaning that experimental data are necessary to validate these findings in further studies.

The present study suggests that the complement and coagulation-associated genes EDN1, let-7e, let-7b, miR-19a, along with the complement and coagulation, and the TNF and

HIF-1 signaling pathways, may be involved in the molecular mechanism of MM and MGUS pathogenesis. Further experiments are warranted to verify the findings of the current study.

Acknowledgements

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