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Effects of β-catenin on Differentially Expressed Genes in Multiple Myeloma*

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Summary: This study aimed to identify the differentially expressed genes after silencing of β-catenin in multiple myeloma transduced with β-catenin shRNA. The DNA microarray dataset GSE17385 was downloaded from Gene Expression Omnibus, including 3 samples of MM1.S (human multiple myeloma cell lines) cells transduced with control shRNA and 3 samples of MM1.S cells transduced with β-catenin shRNA. Then the differentially expressed genes (DEGs) were screened by using Limma. Their underlying functions were analyzed by employing Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses. Moreover, DEGs annotation was conducted based on the databases of tumor associated genes, tumor suppressed genes and the transcriptional regulation from patterns to profiles. Furthermore, the protein-protein interaction (PPI) relationship was obtained from STRING and the protein-protein interaction network and the functional modules were visualized by Cytoscape. Then, the pathway enrichment for the DEGs in the functional module was performed. A total of 301 DEGs, including 124 up-regulated and 117 down-regulated DEGs, were screened. Functional enrichment showed that CCNB1 and CDK1 were significantly related to the function of cell proliferation. FOS and JUN were related to innate immune response-activating signal transduction. Pathway enrichment analysis indicated that CCNB1 and CDK1 were most significantly enriched in the pathway of cell cycle. Besides, FOS and JUN were significantly enriched in the Toll-like receptor signaling pathway. FOXM1 was identified as a transcription factor. Moreover, there existed interactions among CCNB1, FOXM1 and CDK1 in PPI network. The expression of FOS, JUN, CCNB1, FOXM1 and CDK1 may be affected by β-catenin in multiple myeloma.
Key words: multiple myeloma; β-catenin; differentially gene expression; functional enrichment

Multiple myeloma (MM) is yet reckoned as an incurable B cell malignancy characterized by the accumulation of malignant plasma cells in the bone marrow leading to the bone pain, hypogammaglobulinemia and failure in normal immune responses[1, 2]. In 2012, a total of approximately 21 700 new cases of MM were diagnosed, among which 10 710 died[3].

Previous report showed that the canonical Wnt signaling pathway was activated in MM cells by constitutively active β-catenin, a messenger molecule associated with growth, survival and migration of MM cells[4]. In 2009, in order to further understand the function of β-catenin in MM, Dutta-Simmons *et al* used the microarray expression profile to identify the differentially expressed genes (DEGs) between MM1.S (human MM cell lines) cells transduced with control shRNA and MM1.S cells transduced with β-catenin shRNA by using a linear model for microarray data (Limma) moderated *t*-test (adjusted $P<0.01$) and finally suggested that Aurora kinase A was a target of Wnt/β-catenin involved in the progression of MM[5]. However, recent years, multiple studies demonstrated various roles of β-catenin in MM. For example, c-myc was reduced while the expression of cleaved caspase-3 was increased in β-catenin-silenced MM cells, thus leading to the inhibition of MM growth *in vivo*[6]. Moreover, β-catenin signaling pathway could regulate the expression of fibroblast activation protein to avoid the MM cells apoptosis[7]. Despite extensive research, so far, no specific treatment is available for MM. β-catenin, as a central mediator of Wnt signaling pathway, binds to members of the lymphoid enhancer factor (LEF)/T cell factor (TCF) family of transcription factors to modulate hundreds of genes[8]. Thus, it is of great significance to further study the role of β-catenin in MM.

In this study, we, by using the microarray data of Dutta-Simmons *et al*[5], to further screen the DEGs between MM1.S cells transduced with control shRNA and MM1.S cells transduced with β-catenin shRNA, with Limma package based on a relatively wide range of threshold ($P<0.05$ and $|\log2FC|\geq1.5$) and identified the specific DEGs in MM cells after silencing β-catenin, and Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, protein-protein interaction (PPI) network. A previous study proposed that analyses based on different statistical

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tests might result in different outcomes[9]. Therefore, we believed some different results may come from our further processing of data reported by Dutta-Simmons *et al*[5].

1 MATERIALS AND METHODS

1.1 Microarray Dataset

The microarray dataset of GSE17385[5] was downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), which was based on the platform of Affymetrix Human Genome U133 Plus 2.0 Array [(Affymetrix Inc., Santa Clara, California, USA)]. A total of 6 samples, including 3 samples of MM1.S (human MM cell lines) cells transduced with control shRNA and 3 samples of MM1.S cells transduced with β-catenin shRNA, were studied.

1.2 Pre-processing of Dataset

Affymetrix (Affy) package of Bioconductor, in combination with the Affymetrix (Affy) chip annotation files supplied by Brain Array Lab were used for the data pre-processing. The background adjustment, normalization and summarization of data were conducted by the Robust Multichip Averaging (RMA)[10]. Finally, the gene expression matrices of samples were obtained.

1.3 DEGs Screening

The Limma package (available at http://www.bioconductor.org/packages/release/bioc/html/limma.html)[11] was employed to identify the DEGs between the MM1.S cells transduced with control shRNA and MM1.S cells transduced with β-catenin shRNA. Then the Benjamini-Hochberg method in multtest package[12] was applied to correct the raw *P* values into false discovery rate (FDR). The corrected *P* (FDR)<0.05 and |log2Fold Change (FC)|≥1.5 were used as the cut-off values.

1.4 Functional and Pathway Enrichment Analysis

GO analysis is commonly used for functional studies of large-scale genomic or transcriptomic data[13]. The KEGG pathway (http://www.genome.jp/ kegg/ pathway.html) contains higher order of functions in terms of the network of the interacting molecules[14]. GO and KEGG pathway enrichment analyses were performed for the DEGs between MM1.S cells transduced with control shRNA and MM1.S cells transduced with β-catenin shRNA.

1.5 DEGs Annotation

The transcriptional regulation from patterns to profiles (TRANSFAC) database (http://www.gene-regulation.com) comprising data on transcription factors, their target genes and regulatory binding sites were used to discover the transcription factors[15]. Additionally, tumor suppressor gene (TSGene) database (http://bioinfo.mc.vanderbilt.edu/TSGene/), a collection of detailed annotations for each TSG, such as cancer mutations, gene expressions, methylation sites, transcription factor (TF) regulations and protein-protein interactions, was employed to screen the TSGs[16]. Besides, tumor-associated gene (TAG) database (http://www.binfo.ncku.edu.tw/TAG/), containing information about commonly shared functional domains in well-characterized oncogenes and TSGs, was applied for screening the TAGs[17]. The *P*<0.05 was used as the cut-off value.

1.6 PPI Network Construction and Sub-network Mining

The interaction relationships of the proteins were analyzed by using the online tool (Search Tool for the Retrieval of Interacting Genes, http://string-db.org/) STRING[18] and the required confidence (combined score) ≥0.4 was used as the cut-off criterion. Subsequently, the Cytoscape was used to visualize the network[19]. Furthermore, the sub-network was mined by using BioNet package (http://bionet.bioapps.biozentrum.uni-wuerzburg.de)[20] with the FDR of 0.00001.

2 RESULTS

2.1 DEGs Identification

Compared with the MMS.1 cells transduced with the control shRNA, a total of 935 transcripts (table 1) corresponding to 301 DEGs including 124 up-regulated (such as FOS and JUN) and 177 down-regulated (such as FOXM1, CCNB1 and CDK1) DEGs were screened in MMS.1 cells transduced with the β-catenin shRNA. The top ten up- and down-regulated DEGs are listed in table 2.

Table 1 The numbers of DE-transcripts and DEG in MM1.S cells transduced with β-catenin shRNA compared with MM1.S cells transduced with control shRNA

	DE-transcript counts	DEG counts
Down-regulated	546	177
Up-regulated	389	124
Total	935	301

DE-transcripts: differentially expressed transcripts; DEG: differentially expressed gene; shRNA: short hairpin RNA

2.2 Functional Enrichment Analysis

For the down-regulated DEGs in the MMS.1 cells transduced with the β-catenin shRNA, CCNB1 and CDK1 were significantly related to the cell proliferation (*P*=2.18E–4). Besides, FOXM1 was significantly associated with GO term of regulation of cell cycle (*P*=0.00). For the up-regulated DEGs, FOS and JUN were related to innate immune response-activating signal transduction (*P*=1.19E–05). The top ten enriched GO terms for the up- and down-regulated DEGs are listed in table 3.

The KEGG pathway enrichment analysis displayed that the down-regulated DEGs of CCNB1 and CDK1 were most significantly enriched in the pathway of cell cycle (*P*=9.37E–14). Moreover, the up-regulated DEGs of FOS and JUN were significantly enriched in the Toll-like receptor signaling pathway (*P*=8.60E–05). The enriched KEGG pathways for both the up- and down-regulated DEGs are listed in table 4.

Table 2 The top ten up- and down-regulated DEGs in MM1.S cells transduced with β-catenin shRNA compared with MM1.S cells transduced with control shRNA

DEGs	Gene symbols	log_2FC	FDR
Up-regulated	*FOS*	4.782756461	3.17E–08
	DUSP1	3.58616604	1.51E–08
	CCL4	3.487710965	2.68E–08
	ATF3	3.431819113	1.51E–08
	KLF6	3.307144515	1.51E–08
	NA	3.297761934	2.41E–08
	KLF6	3.269829491	1.51E–08
	TRIB1	3.151910578	2.45E–08
	ZFP36L1	3.070750259	1.79E–07
	KLF4	3.035927636	1.82E–07
Down-regulated	*SPC25*	–3.998349698	3.17E–08
	E2F8	–3.573136394	2.83E–07
	CENPW	–3.186276025	1.11E–07
	NA	–3.186276025	1.11E–07
	CCNA2	–3.153122495	2.45E–08
	DLGAP5	–3.029647741	5.75E–07
	CHORDC1	–3.012927298	3.79E–07
	SLIRP	–2.99482457	2.83E–07
	CCNB1	–2.978736669	3.04E–07
	C1orf112	–2.9265198	1.35E–07

DEGs: differentially expressed genes; FC: fold change; FDR: false discovery rate; shRNA: short hairpin RNA

Table 3 The top ten enriched GO terms for both the up- and down-regulated DEGs in MM1.S cells transduced with β-catenin shRNA compared with MM1.S cells transduced with control shRNA

DEGs	ID	Terms	Counts	*P*
Up-regulated	GO:0010033	Response to organic substance	42	3.78E–12
	GO:0034976	Response to endoplasmic reticulum stress	11	3.19E–10
	GO:0006950	Response to stress	49	3.68E–10
	GO:0042221	Response to chemical stimulus	47	7.23E–10
	GO:0071310	Cellular response to organic substance	32	2.08E–09
	GO:0070887	Cellular response to chemical stimulus	36	2.80E–09
	GO:0030968	Endoplasmic reticulum unfolded protein response	8	1.11E–07
	GO:0014070	Response to organic cyclic compound	17	1.14E–07
	GO:0034620	Cellular response to unfolded protein	8	1.22E–07
	GO:0033993	Response to lipid	17	1.30E–07
Down-regulated	GO:0000070	Mitotic sister chromatid segregation	16	0
	GO:0000226	Microtubule cytoskeleton organization	35	0
	GO:0000278	Mitotic cell cycle	89	0
	GO:0000280	Nuclear division	59	0
	GO:0000819	Sister chromatid segregation	17	0
	GO:0006259	DNA metabolic process	48	0
	GO:0006996	Organelle organization	89	0
	GO:0007017	Microtubule-based process	46	0
	GO:0007049	Cell cycle	102	0
	GO:0007051	Spindle organization	23	0

DEGs: differentially expressed genes; GO: gene ontology; shRNA: short hairpin RNA

Table 4 The pathway enrichment for both the up- and down-regulated DEGs in MM1.S cells transduced with β-catenin shRNA compared with MM1.S cells transduced with control shRNA

DEGs	ID	Terms	Counts	*P* value
Up-regulated	4620	Toll-like receptor signaling pathway	6	8.60E–05
	4623	Cytosolic DNA-sensing pathway	4	0.000695
	4010	MAPK signaling pathway	7	0.002998
	4142	Lysosome	4	0.011407
	4380	Osteoclast differentiation	4	0.013806
	5140	Leishmaniasis	3	0.015377
	4662	B cell receptor signaling pathway	3	0.017149
	20	Citrate cycle (TCA cycle)	2	0.020026
	250	Alanine, aspartate and glutamate metabolism	2	0.022624
	260	Glycine, serine and threonine metabolism	2	0.022624
	4012	ErbB signaling pathway	3	0.02534
	5323	Rheumatoid arthritis	3	0.028466
	4141	Protein processing in endoplasmic reticulum	4	0.031728
	4960	Aldosterone-regulated sodium reabsorption	2	0.037511
	5142	Chagas disease (American trypanosomiasis)	3	0.039977
	4062	Chemokine signaling pathway	4	0.048407
Down-regulated	4110	Cell cycle	16	9.37E–14
	4114	Oocyte meiosis	10	2.34E–07
	4914	Progesterone-mediated oocyte maturation	9	2.53E–07
	5130	Pathogenic Escherichia coli infection	6	2.56E–05
	4540	Gap junction	6	0.00037
	3040	Spliceosome	7	0.00038
	3030	DNA replication	4	0.000549
	4115	p53 signaling pathway	5	0.000752
	240	Pyrimidine metabolism	4	0.021231
	3013	RNA transport	5	0.022393
	4145	Phagosome	5	0.023552
	3430	Mismatch repair	2	0.024815
	3410	Base excision repair	2	0.048374

DEGs: differentially expressed genes; shRNA: short hairpin RNA

2.3 DEGs Annotation Analysis

According to the TRANSFAC database, a total of 15 TFs were screened, including 11 up-regulated (such as JUND and FOSB) and 4 down-regulated (such as ID1 and FOXM1) ones. Additionally, a total of 34 DEGs were identified as TAGs, including 18 up-regulated and 16 down-regulated DEGs based on the TAG and TSG databases (table 5). Among them, JUN and FOS were identified to be oncogenes.

Table 5 The identified TFs, TAGs and TSGs among DEGs

DEGs	TF counts	TFs	TAG counts	TSGs	Oncogenes	Others
Up-regulated	11	*STAT4*, *NR1D2*, *MAFF*, *MAF*, *KLF4*, *KLF2*, *JUND*, *IRF7*, *FOSB*, *DDIT3*, *ATF3*	18	*SLC22A18*, *PLK2*, *KLF6*, *IDH1*, *EIF1*, *DUSP6*, *CDKN1A*, *BEX2*, *ANXA1*	*JUND*, *JUN*, *FOS*, *DDIT3*	*RGS2*, *PRKCB*, *MAF*, *KLF4*, *EVI2B*
Down-regulated	4	*MYBL2*, *ID1*, *FOXM1*,*CTNNB1*	16	*BUB1B*	*NET1*, *MYBL2*, *MYB*, *CTNNB1*, *CSE1L*, *CEP55*, *CDC25B*, *CCNA2*, *AURKA*	*TACC3*, *PLK1*, *NUP98*, *BUB1*, *BIRC5*, *ANP32B*

DEGs: differentially expressed genes; TF: transcription factor; TAG: tumor associated gene; TSG: tumor suppressed gene

2.4 PPI Network Analysis

In the PPI network (fig. 1), the top ten degrees in the PPI network were obtained (table 6), such as CDK1 (degree=33) and CCNB1 (degree=19). Besides, there existed interaction among these genes, such as CCNB1-CDK1, CCNB1-FOXM1, CDK1-FOXM1 and FOS-JUN. Furthermore, the functional module was also mined (fig. 2). In this functional module, CDK1 possessed the highest degree of 32. And it could interact with CCNB1 and FOXM1. Moreover, FOS could interact with JUN. The KEGG pathway enrichment for the genes in the functional module showed that CCNB1 and CDK1 were most significantly enriched in the cell cycle pathway (P=0.00) (table 7).

Table 6 The top ten degrees in the protein-protein interaction network

Gene symbols	Degrees
CDK1	33
PLK1	24
CDC20	23
BUB1	23
NDC80	22
BUB1B	21
BIRC5	21
AURKB	19
CCNB1	19
CENPF	18

Table 7 The pathway enrichment for the DEGs in the functional module

Term	Gene counts	*P*	Gene symbols
Cell cycle	16	0.00	*BUB1*, *BUB1B*, *CCNA2*,*CCNB1*, *CCNB2*, *CDC20*,*CDC25A*, *CDC25B*, *CDC45*, *CDK1*, *CDKN1A*, *MAD2L1*, *MCM2*, *ORC1*, *PLK1*, *TTK*
Progesterone-mediated oocyte maturation	9	1.12E–10	*BUB1*, *CCNA2*, *CCNB1*, *CCNB2*, *CDC25A*, *CDC25B*, *CDK1*, *MAD2L1*, *PLK1*
Oocyte meiosis	9	1.24E–09	*AURKA*, *BUB1*, *CCNB1*, *CCNB2*, *CDC20*, *CDK1*, *FBXO5*, *MAD2L1*, *PLK1*
p53 signaling pathway	6	5.96E–07	*CCNB1*, *CCNB2*, *CDK1*, *CDKN1A*, *GTSE1*, *RRM2*
Osteoclast differentiation	4	2.94E–03	*FOS*, *FOSB*, *JUN*, *JUND*
Colorectal cancer	3	3.04 E–03	*BIRC5*, *FOS*, *JUN*

DEGs: differentially expressed gene

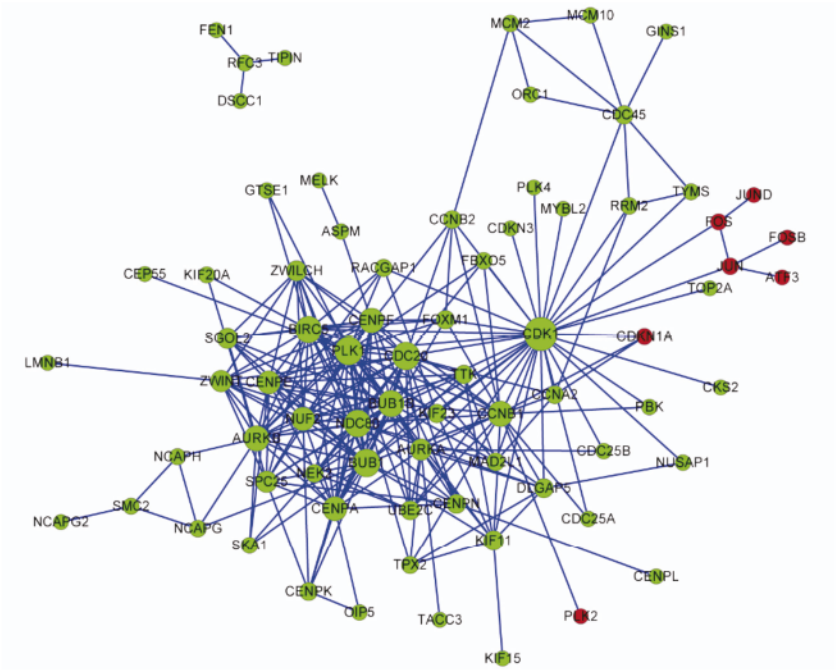

Fig. 1 The PPI network of DEGs between MM1.S cells transduced with control shRNA and MM1.S cells transduced with β-catenin shRNA

Green spots represent the down-regulated DEGs; red spots represent the up-regulated DEGs.

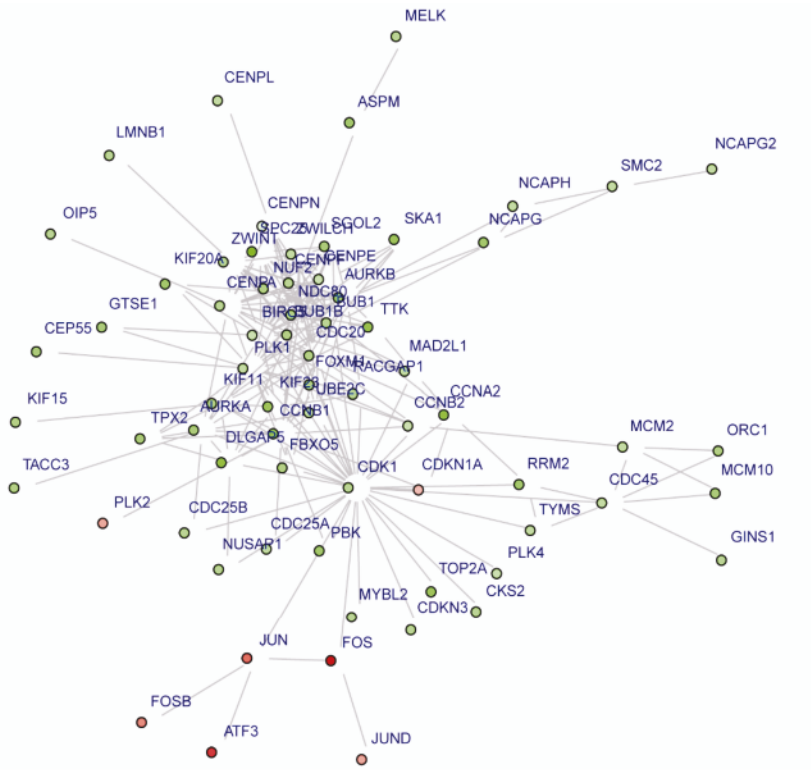

Fig. 2 The functional module

Green spots represents the down-regulated DEGs and red spots the up-regulated DEGs. The scale of color shade denotes the logFold Change.

3 DISCUSSION

In this study, we investigated the gene expression profile between the MM1.S cells transduced with control shRNA and MM1.S cells transduced with β-catenin shRNA, and obtained a total of 935 differentially expressed transcripts corresponding to 301 DEGs, including 124 up-regulated DEGs and 177 down-regulated DEGs. The results suggested that alternative splicing occurred. Alternative splicing has been proposed as an important post-translational change that alters specificity of gene function and dysregulated alternative splicing has been reported in myeloma, the event exerting an effect on overall clinical outcome[21, 22]. A previous study demonstrated that, in MM cells, the bone marrow microenvironment could affect the pathogenesis of MM by down-regulating alternative splicing factor Fox2, which was correlated with the frequency of RNA splicing and disease prognosis in MM[23]. Therefore, the post-transcriptional alternative splicing may play a pivotal role in specificity of gene function related to MM.

Additionally, our study exhibited that the down-regulated DEGs, such as CCNB1 and CDK1, were most significantly enriched in the pathway of cell cycle. Moreover, CCNB1 was found to interact with CDK1 in the PPI network. It was previously demonstrated that CCNB1 and CDK1 encoding G_2/mitosis-specific and cyclin-dependent kinase 1 respectively, could interact with each other and participate in cell cycle regulation[24]. Dai *et al* illustrated that the MM cells proliferation could be inhibited through the regulation of cell cycle arrest[25]. In addition, FOXM1, which was found to be a TF according to the TRANSFAC database, could interact with CCNB1 and CDK1. Functional enrichment showed that FOXM1 was significantly related to the regulation of cell cycle. It was proven that FOXM1 could preferentially bind to the CDK1/CCNB1 complex in G_2 to regulate the cell cycle[26]. Consequently, we are led to speculate that the down-regulated FOXM1 could lead to a decreased expression of CCNB1 and CDK1 in the β-catenin-silenced MM1.S cells, which could be involved in the regulation of MM cell proliferation via the cell cycle pathway.

Moreover, CCNB1 and CDK1 were also significantly enriched in p53 signaling pathway. p53 has been known as a transcriptional factor that could activate multiple genes involved in cell cycle regulation, DNA repair, and programmed cell death[27]. What's more, the induction of p53 signaling pathway could promote the MM cell apoptosis[28]. Therefore, we were led to hypothesize that CCNB1 and CDK1 might be involved in the regulation of MM cell apoptosis via the p53 signaling pathway.

As for the up-regulated DEGs, FOS and JUN were significantly enriched in the Toll-like receptor signaling pathway. TLRs could not only regulate cell proliferation and survival which serves to expand useful immune cells and integrate inflammatory responses but also drive inflammatory responses[29]. Previous report demonstrated that TLR activation could promote MM cell growth and survival by inhibiting endoplasmic reticulum stress factor CHOP[30]. Moreover, triggering of TLR-4 in human multiple myeloma cells was found to promote proliferation and alter cell responses to immunity[31]. Functional analysis displayed that FOS and JUN were associated with innate immune response-activating signal transduc-

tion. Innate immunity component natural killer T cells play a role in the control of the malignant growth of this incurable B cell tumor in patients[32, 33]. In addition, FOS could interact with JUN. As a result, we were led to assume that up-regulated FOS and JUN in the β-catenin-silenced MM1.S cells may take part in the modulation of innate immunity on MM tumor cells growth via TLR signaling pathway.

In conclusion, our comprehensive bioinformatical analysis suggested that FOS, JUN, CCNB1, FOXM1 and CDK1 might play key important roles in MM by regulating β-catenin. However, further researches are warranted to better understand the underlying mechanism.

Conflict of Interest Statement

The authors declared no potential conflicts of interest.

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