doi:10.4149/neo_2017_305

Microarray expression analysis of MYCN-amplified neuroblastoma cells after inhibition of CDK2

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Received July 18, 2016 / Accepted October 28, 2016

The study aimed to explore the underlying molecular mechanisms of CDK2 inhibition in neuroblastoma by bioinformatics analysis. Gene expression profile GSE16480 was downloaded from the Gene Expression Omnibus. The differentially expressed genes (DEGs) were identified from IMR32 between each time point and average expression of all time points. Gene significance was calculated using dSVDsig algorithm of dnet package. Protein-protein interaction (PPI) network was built. Then, integrated with gene significance, a core PPI network was detected by dNetPipeline algorithm in dnet package. Finally, pathway enrichment analysis was performed for genes in network. Totally, 1524 DEGs were identified. *CCNA2* (cyclin A2), *EXO1* (exonuclease 1), *RAD51AP1* (RAD51 associated protein 1), *TOP2A* (topoisomerase (DNA) II alpha) and *CDK1* (cyclin-dependent kinase 1) were selected as DEGs with higher connectivity after PPI network analysis. In the network, *CCNA2*, *CDK1*, *BUB1B* (BUB1 mitotic checkpoint serine/threonine kinase B) and *CCNB1* (cyclin B1) were involved in cell cycle pathway. Additionally, *CCNB1*, *CDK1*, *CCNE2* (Cyclin E2), and *RRM2B* (ribonucleotide reductase subunit M2B) were involved in p53 signaling pathway. Cell cycle and p53 signaling pathway were closely associated with neuroblastoma after CDK2 inhibition. The DEGs, such as *CCNA2*, *CCNB1, CDK1* and *RRM2B* may be the potential targets for neuroblastoma.

Key words: neuroblastoma, CDK2 inhibition, gene significance, bioinformatics

Neuroblastoma is the most common malignant pediatric tumor accounting for about 15% of cancer deaths in children [1]. Neuroblastomas are embryonic tumors originating from the transformation of neural crest progenitor cells [2]. More than half of patients subjected to neuroblastoma have metastases, leading to 5-year survival rate of high-risk neuroblastoma patients below 35% [3].

MYCN is the strongest genetic marker for treatment failure, being amplified in 25% of neuroblastoma tumors [4, 5]. The MYCN proto-oncogene has been shown to regulate the expression of genes involved in many processes, including cell cycle and proliferation [6]. A variety of cell cycle regulating proteins in tumorigenesis has been identified, including cyclin-dependent kinases (CDK). A recent study has illustrated the importance of p21 in inhibiting CDK2 and restraining cell proliferation during development and differentiation [7]. Moreover, p21 is an vital target of p53 and regulates DNA-damage-induced cell-cycle arrest in G1 and G2 phases [8]. In consequence, CDK2 inhibition may induce apoptosis of neuroblastoma cells and be an effective therapy for childhood with MYCN-amplified neuroblastoma [9]. However, the specific underlying molecular mechanism associated with CDK2 inhibition and neuroblastoma is still largely unknown.

The microarray data GSE16480 [7] was generated and analyzed by Molenaar et al. Six most strongly regulated genes were identified by them and the study indicated that P53 pathway was involved in the CDK2 silencing-induced cell apoptosis. While, large information still remain in the mRNA expression profiles. In this study, we downloaded GSE16480 and identified the differentially expressed genes (DEGs) between each time point and average expression of all time points. Then, gene significance of DEGs were calculated and protein-protein interaction (PPI) network analysis were detected, followed by pathway enrichment analysis.

Materials and methods

Microarray data. The microarray data GSE16480 [7] were obtained from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database based on the platform of Affymetrix Human Genome U133 Plus 2.0 Array. The dataset consisted of MYCN-amplified neuroblastoma cell IMR32 induced by CDK2 shRNA at 5 time points (0, 8, 24, 48 and 72 h after transfection) in biological triplicate experiments.

Data preprocessing. The raw data in CEL files were preprocessed using AFFY package [10] in R software, following 4 steps including background correction by robust multi-array average (RMA), quantile normalization, probe summarization and log 2 transformation of expression intensity. Then the expression value of probe level was obtained. The profiles in the probe set were averaged to get the expression value of each gene.

Identification of DEGs. DEGs from IMR32 between each time point and average expression of all time points were identified by Limma package in R [11]. Multiple testing was corrected by the Benjamini and Hochberg (BH) [12] to obtain the adjusted p-value. The genes with adjusted P-value <0.05 and |log 2 FC (fold change)| ≥ 0.5 were considered to be statistically significant. Then, those significant genes were analyzed using hierarchical clustering of the log 2 values and displayed in a heatmap. Significant kyoto encyclopedia of genes and genomes (KEGG) pathway was plotted with plugin CyKEGGParser [13] in cytoscape.

Calculation of gene significance of DEGs. Singular value decomposition (SVD) is a factorization of a real or complex matrix, with many useful applications in statistics and signal processing. Gene significance was calculated using dSVDsig algorithm in dnet package [14] as follows: $A_{mxn} = S_{mxm}V_{mxn}D_{nxn}$·$S_{mxm}$ and $D_{nxn}$are unitary matrixes, V_{mxn} is an augmented diagonal matrix, and the value on the diagonal of V_{mxn} is the singular value of A_{mxn}.

Detection of modules. Search Tool for the Retrieval of Interacting Genes (STRING) [15] is an online database which has been designed as a global perspective to evaluate PPI information. In this study, STRING was used to analyze the PPI network and only those with the threshold of combined score more than 0.9 screened from each time point were selected as significant. Integrated with gene significance and PPI network, module with the maximum score was detected by dNetPipeline algorithm in dnet package [14].

Pathway enrichment analysis. Database for annotation, visualization, and integrated discovery (DAVID) [16] online tool was used in the present study to perform KEGG and Biocarta pathway enrichment analysis of DEGs in network. A P-value < 0.05 was considered as statistically significant. In addition, significant pathways using plugin KEGGParser in cytoscape [17] were plotted to explore the important genes in neuroblastoma.

Table 1. Pathways enriched by differentially expressed genes (DEGs) in the protein-protein interaction (PPI) network.

Term	Count	P Value
hsa04110:Cell cycle	14	3.10E-14
hsa04114:Oocyte meiosis	7	2.83E-05
hsa04914:Progesterone-mediated oocyte maturation	6	1.06E-04
hsa04115:p53 signaling pathway	5	5.40E-04
hsa03030:DNA replication	4	1.05E-03
hsa00240:Pyrimidine metabolism	5	1.90E-03
hsa03410:Base excision repair	3	1.66E-02

Count: number of genes enriched in the term.

Results

Identification of DEGs. Totally, 1524 DEGs were screened from IMR32 between each time point and average expression of all time points. Specifically, 649 DEGs (411 up-regulated and 238 down-regulated) at T0, 499 DEGs (331 up-regulated and 168 down-regulated) at T8, 220 DEGs (192 up-regulated and 28 down-regulated) at T24, 398 DEGs (152 up-regulated and 246 down-regulated) at T48, and 1392 DEGs (552 up-regulated and 840 down-regulated) at T72 were identified. Hierarchical clustering revealed systematic variations in the expression of genes between DEGs at each time point group compared to the average expression of all time points group (Figure 1). The results demonstrated that there were two cases of these DEGs: the expression of genes was decreased or increased with increasing time. In addition, pathways enriched by DEGs in these two cases were shown in figure 2. Especially, both of DEGs in two modules were enriched in p53 signaling pathway. Thus, the distribution of DEGs in p53 signaling pathway was further studied, and function annotation revealed that down-regulated DEGs were mainly involved in cell cycle and up-regulated DEGs were mainly involved in apoptosis (Figure 3).

PPI network analysis. Based on the provided networks of gene significance and STRING, a new core PPI network of DEGs which included 103 nodes and 4393 edges with connectivity of 0.84 was constructed (Figure 4A). In this network, *CCNA2* (cyclin A2), *EXO1* (exonuclease 1), *RAD51AP1* (RAD51 associated protein 1), *TOP2A* (topoisomerase (DNA) II alpha) and *CDK1* (cyclin-dependent kinase 1) were selected as DEGs with higher connectivity. In addition, the time-changing expression trend of genes in network was plotted, and the results showed that time-changing of the majority of genes was relatively flat in the first 24 hours but decreased after 24 hours (Figure 4B). However, time-changing expression of 5 genes was different. They were *RRM2B* (ribonucleotide reductase M2 B), *MSH4* (mutS homolog 4), *HIST1H2BD* (histone cluster 1, H2bd), *RET* (ret proto-oncogene) and *LUM* (lumican).

Pathway enrichment analysis of DEGs in the network. KEGG and Biocarta pathway enrichment analysis showed that genes in PPI network were significantly enriched in cell cycle-related pathways, including Cell cycle, Oocyte meiosis, Cell Cycle: G2/M Checkpoint and p53 signaling pathways (Table 1 and Figure 5). In the network, *CCNA2*, *CDK1*, *BUB1B*

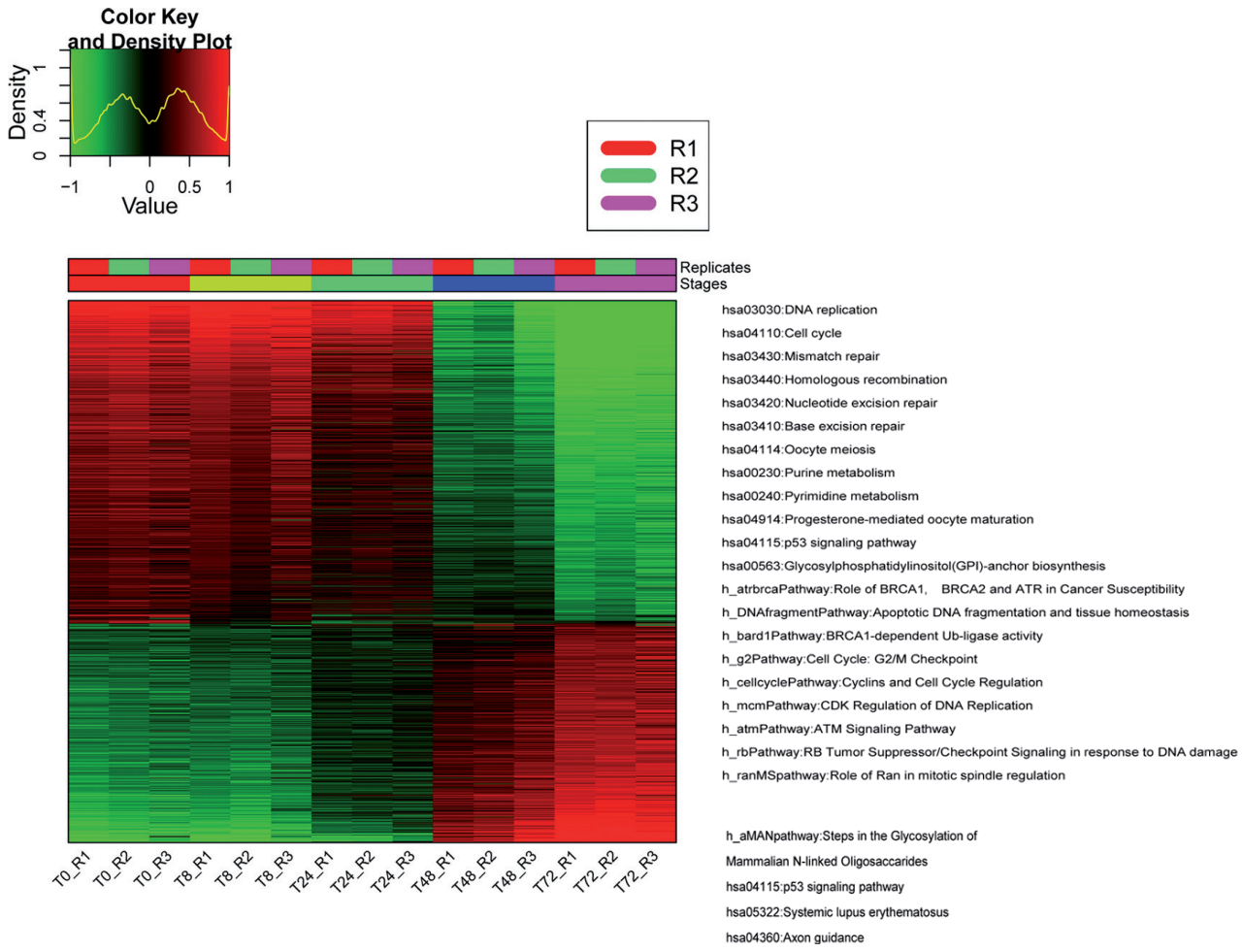

Figure 1. Hierarchical clustering heatmap of differentially expressed genes (DEGs) from neuroblatoma cells after inactivation of CDK2. Expression data are depicted as a data matrix where each row represents a gene and each column represents a sample. Expression levels are depicted according to the color scale shown at the top. Red and green indicate expression levels above and below the median, respectively. R1, R2 and R3 represent the time of replicates.

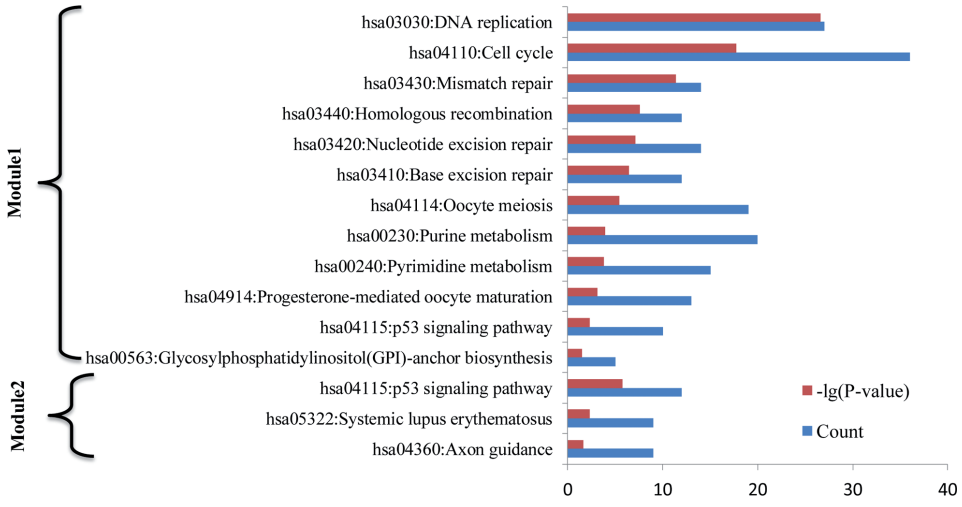

Figure 2. Pathways enriched by differentially expressed genes (DEGs) in two modules. Pathway terms were ranked by -lg(P-value) from top to bottom. Count indicates the number of genes enriched in the term.

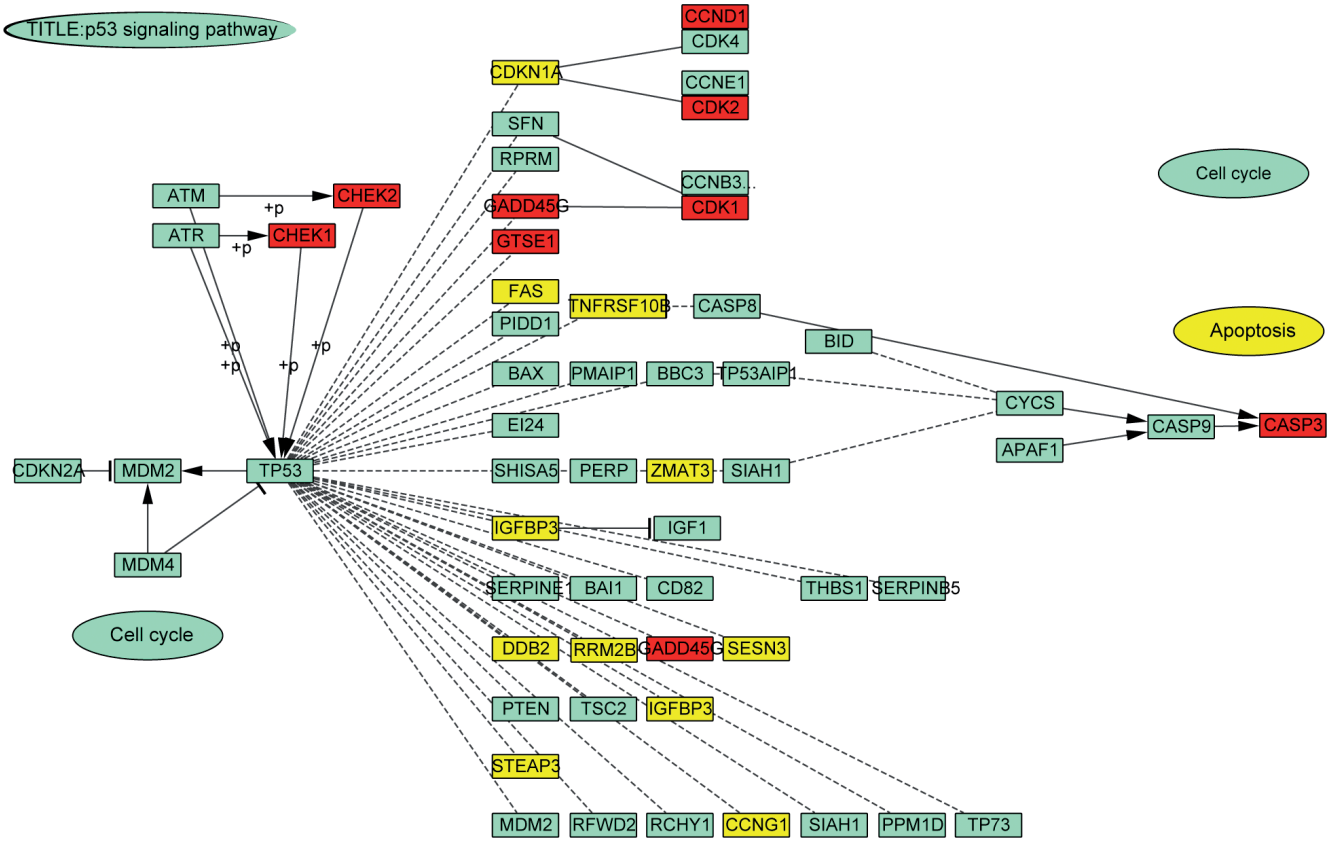

Figure 3. Distribution of differentially expressed genes (DEGs) in p53 signaling pathway. Red node represents down-regulated gene and yellow node represents up-regulated gene with increasing time. Green node represents genes without differential expression.

(BUB1 mitotic checkpoint serine/threonine kinase B) and *CCNB1* (cyclin B1) were involved in pathway of cell cycle. Additionally, *CCNB1*, *CDK1*, *CCNE2* (Cyclin E2), and *RRM2B* (ribonucleotide reductase subunit M2B) were involved in p53 signaling pathway.

Discussion

In this study, we found that after transfection with anti-CDK2 shRNA, a total of 1524 DEGs were screened from IMR32 cell line. In addition, in the PPI network analysis, *CCNA2* and *CCNB1* were involved in cell cycle pathway. *CCNB1*, *CDK1*, *CCNE2* and *RRM2B* were involved in p53 signaling pathway.

CDK2 inhibitor has been found synthetically lethal to MYNC over-expressing neuroblastoma cells [7]. In human cancer cells, CDK2 is an essential component of the cell cycle and with key function in tumorigenesis [18]. CDK2 in complex with Cyclin E is given a special importance since the complex sequentially phosphorylates retinoblastoma proteins (Rb) on different threonine and serine residues which results in Rb inactivation required for transcriptional activation of genes in G1/S phase [19]. Once the cell passes restriction point of G1, CDK2 forms a complex with Cyclin A to carry out S phase events [20]. In parallel, one level of regulation of CDK2-Cyclin complexes is provided by their bindings to CDK inhibitors.

CCNA2 and *CCNB1* both belong to the highly conserved cyclin family that functions in cell proliferation and differentiation. The two cyclin members are essential for the control of the cell cycle at the checkpoint of G1/S and G2/M [21]. Recently, *CCNA2* and *CCNB1* have been found to interact with *CDK1* that is important to drive the mammalian cell cycle [22]. Previous study has shown that *CCNA2* has the ability to bind and activate *CDK2* [23]. Taken together, all of these may hint that *CCNA2*, and *CCNB1* might be implicated in neuroblastoma through cell cycle pathway.

In addition, we constructed the PPI network with DEGs, performed pathway enrichment analysis of DEGs in the network, and found that the selected *CDK1* and *RRM2B* were involved in p53 signaling pathway. *CDK1* is a member of the serine/threonine kinase family. The activity of *CDK1* plays a key role in the control of the eukaryotic cell cycle by modulating the centrosome cycle as well as mitotic onset; promoting G2-M transition, and regulating G1 progress and G1-S transition via association with multiple interphase cyclins [24]. In addition, *CDK1* inhibition has been found to induce cell death of neuroblastoma cells through the miR-34a-MYCN-survivin

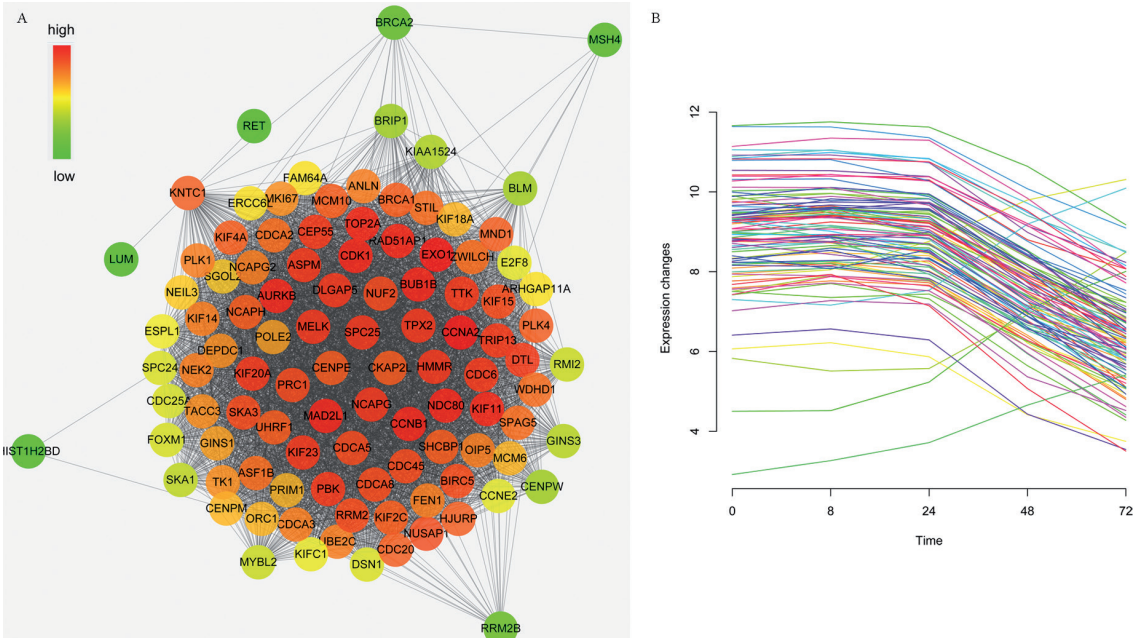

Figure 4. Protein interaction analysis of differentially expressed genes (DEGs). (A) A core protein-protein interaction network is detected. Color of node represents the size of the degree. (B) Time-changing expression trend of genes in network. The horizontal axis represents the time after transfection of small hairpin RNA. The vertical axis represents gene expression change.

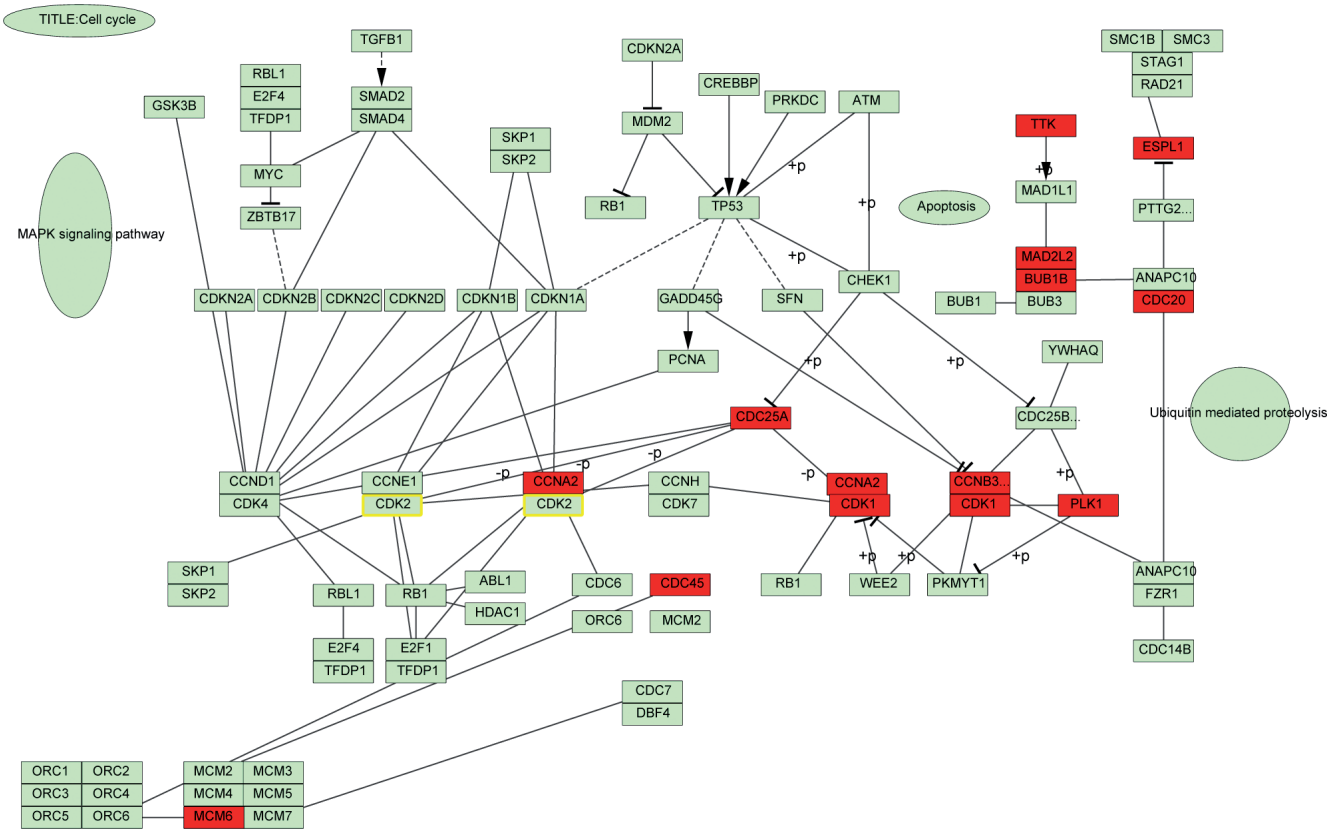

Figure 5. Distribution of genes of protein-protein interaction network in cell cycle pathway. The red nodes represent genes in network and highlight box node represents *CDK2*.

pathway [25], suggesting that *CDK1* might play an important role in inhibiting neuroblastoma.

RRM2B, also known as p53R2, encodes a homologue of the R2 subunit of ribonucleotide reductase that forms a competent enzyme complex with the catalytic R1 subunit in nonproliferative cells [26]. p53R2 has recently been shown to function in nuclear DNA repair and to provide dNTPs for mitochondria DNA replication [27, 28]. After DNA damage in response to irradiation or chemotherapeutic agents, p53 enhances the expression of p53R2 and induces cell cycle arrest in G1/G2 phases [29]. Additionally, p53R2 has been shown to up-regulate p21 and down-regulate cyclin D contributing to cell cycle arrest [30]. Moreover, p53R2 has dual opposite roles in before cancer development and after cancer formation [31]. Interestingly, p53R2 and Akt put each other in line with cancer development and progression [32]. Notably, the Akt pathway is activated in neuroblastoma cells and inhibition could decrease neuroblastoma tumor mass [33, 34]. As mentioned above, up-regulated *RRM2B* after CDK2 silencing may inhibit neuroblastoma progression.

In conclusion, we found that cell cycle and p53 signaling pathway were closely associated with neuroblastoma after CDK2 inhibition. Our data provide a comprehensive bioinformatics analysis of DEGs and pathways which may be involved in neuroblastoma after CDK2 inhibition. The findings in current study may contribute to our understanding of the underlying molecular mechanisms of neuroblastoma and the DEGs such as *CCNA2*, *CCNB1, CDK1* and *RRM2B* have the potential to be used as targets for neuroblastoma diagnosis and treatment. However, more studies on improving diagnosis of neuroblastoma through regulating DEGs are still needed.

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