miRNAs Associated with Nasopharyngeal Carcinoma: New Prognostic Biomarkers and Therapeutic Targets

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Abstract

Background: Most of the studies included in this analysis highlighted miRNAsrole in the prognosis of nasopharyngeal carcinoma (NPC) patients. Our aim in this bioinformatics study was to synthesize relevant data associated with NPC to identify new prognostic markers.

Methods: miRNA and mRNA data related to NPC were downloaded from the Gene Expression Omnibus (GEO) database. A variety of online analytical tools (starBase, TargetScanHuman7.2, Metascape, Cytoscape_v3.6.0, and GEPIA) were used to analyze the downloaded data to identify biomarkers with extremely high sensitivity and further research value.

Results: Changes in the expression levels of 13 miRNAs played crucial roles in the overall survival of NPC patients (miR-375 was down-regulated, miR-96-5p, miR-155-5p, miR-320a, miR-378a-3p, miR-15b-5p, miR-21-5p, miR-25-3p, miR-93-5p, miR-493-3p, miR-493-5p, miR-494-3p, and let-7i-5p were up-regulated). Additionally, eight central genes (CDK1, CCNB1, CCNA2, TOP2A, AURKA, MAD2L1, CDC6, and CHEK1) were identified as target genes for further NPC therapy.

Conclusions: Our findings further elucidate the underlying relationship between miRNAs and prognosis in NPC. The identified miRNAs are closely related to NPC prognosis and have extremely high research value for future medical treatment.

1. Introduction

Nasopharyngeal carcinoma (NPC), which has a high mortality rate, remains a medical challenge in Southwest China. In 2018, there were 129,000 new cases of NPC worldwide. However, due to insufficient reliable evidence in the literature, several issues regarding the pathogenesis and clinical management of NPC remain unresolved [1]. Approximately one-quarter of patients with NPC are in the advanced stage [2]. Although radiotherapy remains the most essential treatment for NPC, the recurrence rate of the disease is as high as 82% due to radiotherapy resistance [3, 4]. In recent years, there has been abundant research on radioresistance, drug resistance, and angiogenesis to uncover the underlying mechanisms for miRNA involvement in NPC progression[5]. Changes in radiotherapy sensitivity and the prognosis of NPC patients can also occur due to changes in miRNA expression. By influencing signal pathways, MiRNAs can participate in radiotherapy resistance in NPC cells. For example, NPC cell proliferation and tumor progression are regulated by miR-375, which is up-regulated through the promotion of the pyruvate dehydrogenase kinase 1 (PDK1) / phosphatidylinositol (3,4,5)-trisphosphate (PIP3) / protein kinase B (Akt) axis [6]. In contrast, the down-regulation of miR-429 functions as a tumor suppressor[7]. For these reasons, new strategies to treat NPC need to be explored. There is sufficient evidence closely relating miRNAs to the prognosis of NPC patients.

Gene chip is a highly reliable technology that can quickly detect differentially expressed genes (DEGs), as demonstrated by more than 10 years of research [8]. The technology simplifies the identification of specific

items in a database and provides various possibilities for further research. Some authors conducted in-depth research on miRNAs related to NPC prognosis using a bioinformatics approach[9]. However, the research outcomes were limited. Therefore, we aimed to conduct more detailed research with broader applications.

The Gene Expression Omnibus (GEO) can provide us with various data required for our research, including the respective expression data for genes and miRNAs. The expression data for patients with NPC were downloaded from this database for further research, which yielded substantial results. We found miRNAs that could serve as prognostic biomarkers for NPC, the key genes they target, and the network of connections between them. We subsequently visualized these connections. The results may help us establish a clearer regulatory network of miRNAs that target mRNAs and understand the role of the above networks in NPC prognosis.

Based on the GSE70970 and GSE12452 datasets from GEO, the GEO2R online tool and Venn diagram software were used to identify DEGs, which were collectively overexpressed. Afterward, we identified highly sensitive prognostic biomarkers by using starBase (http://starbase.sysu.edu.cn/), Targetscan (http://www.targetscan.org/), Metascape (http://metascape.org/gp/), ONCOMIR (http://www.oncomir.org/), and Cytoscape_v3.6.0 software to analyze the two GEO datasets. Finally, we identified 13 miRNAs (miR-96-5p, miR-155-5p, miR-320a, miR-378a-3p, miR-15b-5p, miR-21-5p, miR-25-3p, miR-93-5p, miR-493-3p, miR-493-5p, miR-494-3p, let-7i-5p, and miR-375) and eight central genes (cyclin-dependent kinase 1 (CDK1), cyclin B (CCNB1), cyclin A2 (CCNA2), DNA topoisomerase II alpha (TOP2A), aurora kinase A (AURKA), MAD2LI, cell division cycle 6 (CDC6), and checkpoint kinase 1 (CHEK1)).

2. Methods

2.1 Microarray data information

We obtained two public datasets (GSE70970 and GSE12452) on NPC and NPC tissues from GEO. The microarray data for GSE70970 and GSE12452 consisted of 246 NPC tissues with 17 normal tissues and 31 NPC tissues with 10 normal tissues, respectively. The experimental platforms used for GSE70970 and GSE12452 were as follows.

GPL20699 nCounter® Human miRNA Assay (v1.0, Nanostring) and GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array

2.2 Data processing of DEGs

DEGs between NPC specimens and normal NPC specimens were selected via the GEO2R online tool[10] based on the following standard: |log fold change (FC)| > 1.5 and an adjusted P-value of < 0.05. The raw data were processed in Venn software to detect commonly expressed genes present in both GSE70970 and GSE12452. The criterion for the down-regulation of miRNA was logFC < 0, and the criterion for the up-regulation of miRNA was logFC > 0.

2.3 miRNA target prediction

In order to verify the target genes of miRNAs and the accuracy of the results, we combined starBase (http://starbase.sysu.edu.cn/) [11] with TargetScanHuman7.2 (http://www.targetscan.org/) [12] for accurate identification of the target genes. We then performed miRNA target prediction and analyzed the mRNA gene variance. The results of these procedures jointly revealed that the target genes were related to the prognosis of NPC. To better understand the organic capabilities of the selected genes, we used an online tool to perform Gene Ontology (GO) and pathway enrichment analyses. GO analysis is a generally accepted method for defining genes and their RNA or protein products to identify distinct molecular biological functions through high-throughput screening for tumor-specific transcripts [13]. Metascape (https://metascape.org) is an online tool that produces results with high credibility. Metascape has a wealth of functions, including providing a comprehensive gene list annotation, analyzing resources, and visual enrichment analysis [14]. Metascape provides a biologist-oriented resource for the analysis of system-level datasets, and we used these functions to perform enrichment and pathway analyses of DEGs ($P < 0.05$).

2.4 Protein interaction network

We imported the predicted target genes from the STRING search tool (http://string-db.org) to evaluate the reciprocity within the protein-protein interaction (PPI) network for the genes. There were 589 nodes and 5,095 edges in the PPI network generated from the results. We then selected the top 50 nodes (ranked by degree) from the above results and visualized them with Cytoscape software[15] using a threshold value of 0.4 for the intermediate fiducial interval.

2.5 Survival analysis and central gene expression

To identify the central genes among the predicted target genes, we used CentiScaPe (an application in Cytoscape_v3.6.0 software) and prudently selected central genes with a rigid threshold of ≥110 as high-connectivity nodes for subsequent analysis[16]. We established a network of targeting miRNAs and regulating mRNAs with Cytoscape software and performed a visual analysis. Subsequently, the online analysis tool ONCOMIR (http://www.oncomir.org/)[17] was used to evaluate the prognostic value of the central genes. The identified genes accorded with the $P < 0.05$ criterion significantly correlated with survival and were estimated to be prognostic genes. We obtained the expression data for the central genes in normal tissues and tumor tissues through the Oncomine database. Immunohistochemistry staining results obtained from the Human Protein Atlas confirmed that the expression levels of the central genes were closely related to NPC prognosis.

3. Results

3.1 Identification of DEGs in NPC

In our study, the selected GSE70790 dataset had 246 NPC tissues and 17 normal tissues. We extracted 40 DEGs from 735 genes through applying the GEO2R online tool for analysis. We explored 13 common DEGs in total, comprising one up-regulated miRNA (miR-375; logFC < 0) and 12 down-regulated miRNAs (miR-96-5p, miR-155-5p, miR-320a, miR-378a-3p, miR-15b-5p, miR-21-5p, miR-25-3p, miR-93-5p, miR-493-3p, miR-493-5p, miR-494-3p, and let-7p-5i in the NPC organization; logFC > 0; Fig. 1).

3.2 Identification of differentially expressed miRNAs in NPC

We downloaded the GSE12452 dataset from the GEO database as described above. This dataset contains data for 41 NPC-related mRNA samples (10 normal and 31 tumors). The dataset was confirmed and studied by using the GEO2R online tool to identify differentially expressed mRNAs. $P < 0.05$ and $|logFC| > 1.5$ were specified as the standard (Fig. 2).

3.3 MiRNA target prediction and study of the functions

The online target gene prediction tools starBase and TargetScanHuman 7.2 were employed to complete our target gene prediction, resulting in a total of 1976 genes. We employed a Venn diagram taking into consideration the accuracy of the biological analysis to evaluate genes closely related to the miRNAs and mRNAs identified and finally selected 589 target genes. To investigate the effects of these genes at the molecular level, we used Metascape to perform GO enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the genes to predict their biological functions and the related pathways among distinct DEGs. Figure 3A shows that the functions of the DEGs were related to cellular processes, regulation of biological processes, and cellular component organization or biogenesis. The DEGs were significantly enriched in the cell cycle, non-integrin membrane–extracellular matrix (ECM) interactions, transcriptional regulation of E2F6, interleukin-4, and interleukin-13 signaling, as well as signaling by the KIT stem cell factor (KIT-SCF), as shown in Fig. 3B. Furthermore, to perform PPI enrichment analysis and confirm the connection of the network modules, we employed MCODE. Each MCODE module pathway and process enrichment analysis was applied independently. The results provided evidence of a close correlation between the biological functions of the selected genes and the “cell cycle,” “non-integrin membrane–ECM interaction,” “cilium organization,” “regulation of DNA recombination,” “DNA replication,” “response to radiation,” “organelle fission,” and “cell division” (Figs. 4, Fig. 5 and Fig. 6).

3.4 PPI network and verification of central genes

The STRING online tool was utilized to further validate the interactions between our target genes (Fig. 7A). We also used Cytoscape software to visualize the top 50 results, providing a direct view of the most critical results from the study as a PPI network ranked by degree (Fig. 7B). There were 583 nodes and 5,095 edges between proteins in the network. We calculated the connectivity between the nodes using CentiScaPe software by following highly connected genes with regard to the disease. Drawing conclusions from the results, eight nodes (with degree ≥ 110) were selected as central genes (CDK1,

CCNB1, CCNA2, TOP2A, AURKA, MAD2L1, CDCC, and CHEK1). During the retrospective analysis of the miRNA-mRNA regulatory network, most of the genes mentioned above were found to be regulated by the differentially expressed miRNAs related to NPC. The eight central genes were regulated by nine miRNAs (miR-96-5p and miR-375 regulated the expression of CDK1, miR-493-3p regulated the expression of CCNB1; miR-320a regulated the expression of CCNA2; miR-21-5p and miR-96-5p regulated the expression of TOP2A; miR-25-3p and let-7i-5p regulated the expression of CDC6; miR-493-3p regulated the expression of MAD2L1, miR-493-3p and miR-493-5p regulated the expression of AURKA; miR-378a-3p and let-7i-5p regulated the expression of CHEK1).

3.5 Survival analysis for central genes and miRNAs

We used the online tool ONCOMIR to further assess the clinical characteristics of the 13 miRNAs and their correlations with survival, based on previous research. The results demonstrated that the expression of the 13 miRNAs was crucial for the survival of patients with NPC (Fig. 8). Moreover, the P values for the central eight genes were far less than 0.05 in the survival analysis. Significantly elevated expression of the central genes was observed in NPC as compared to normal tissues during the analysis using the Oncomine database (Fig. 9). This indicates that the significant increase in the 10-year survival rate of NPC patients is related to the low-level expression of the eight central genes (Fig. 10). Moreover, immunohistochemistry staining data acquired from the Human Protein Atlas database also confirmed the findings regarding the expression of central genes (Fig. 9).

4. Discussion

Some uncertainties remain in the research and exploration of NPC treatment, especially oncogenic mutation patterns in NPC [1, 2, 18]. This study was performed using tumor-related data from GEO to identify NPC-related miRNAs and mRNA regulatory molecules, with the aim of achieving a breakthrough in the research process for NPC gene therapy. In recent years, a growing number of studies have demonstrated the potential of miRNAs in NPC research. This study was conducted using bioinformatical methods based on two datasets (GSE70790 and GSE12452) from the GEO database. Through a tool named GEO2R, with | logFC | > 1.5, and adjusted P-value < 0.05 as the research standard, we demonstrated that one miRNA (miR-375) was up-regulated and 12 were down-regulated (miR-96-5p, miR-155-5p, miR-320a, miR-378a -3p, miR-15b-5p, miR-21-5p, miR-25-3p, miR-93-5p, miR-493-3p, miR-493-5p, miR- 494-3p, and let-7 i-5p) in NPC. Identification of these miRNAs and mRNA targets that are closely related to the prognosis of NPC lays a solid foundation for future research. The abovementioned molecules may be related to the prognosis of NPC, as suggested in previous related studies. In NPC, high expression of miR-375 was reported to regulate PDK1 and modulate the progression of NPC cells. MiR-96-5p is a target of CDK1 and serves as an inhibitor of NPC. MiR-494-3p promotes NPC cell growth, migration, and invasion by targeting Sox7. Although there are no reports about miR-155, miR-320a, miR-15b-5p, miR-21-5p, miR-23-3p, miR-93-5p, miR-493-3p, miR-494-3p, and let-7i-5p related to NPC, they have been reported as tumor

inhibitors in other carcinomas such as lung cancer and hepatocellular carcinoma [19–24]. This indicates that they also have considerable potential in further research of NPC.

To gain a deeper understanding of the uncharted territory of miRNAs, we constructed a PPI network using Metascape and performed target gene prediction and functional analysis of 344 generated target genes. These target genes were found to possess various functions related to tumor progression in previous studies, such as the cell cycle, non-integrin membrane–ECM interactions, cilium organization, DNA recombination, DNA replication, and response to radiation. The main pathological type of NPC is squamous cell carcinoma, but cell cycle genes have been reported to affect the differentiation of squamous cells[25, 26]. ECM-related genes play an indispensable role in the tumor microenvironment, and their potential as target genes and biomarkers has been described in many studies [27]. The susceptibility to NPC in the Chinese population is closely related to the interleukin (IL)-13 gene [28], while IL-4 has been shown to affect the progression of NPC by affecting the signal transducer and activator of transcription (STAT) pathway [29]. There is much evidence to support the indispensable role of KIT-SCF in other cancers [30, 31].

An analysis was conducted in the form of a PPI network for the in-depth detection and study of target genes. We identified eight genes as central genes based on connectivity scores ≥ 110 and conducted follow-up survival analysis on these genes. The results showed that the central genes (CDK1, CCNB1, CCNA2, TOP2A, AURKA, MAD2L1, CDC6, and CHEK1) played a remarkable role in the 10-year survival rates of patients with NPC.

miRNAs are non-coding small RNA molecules that can inhibit transcription and translation and cleave target mRNAs to accelerate their degradation. In his review, Syafirah suggested that a single miRNA marker is not adequate for NPC prognosis. Therefore, a study on manifold miRNA biomarker profiles can help improve the reliability of prognostic research on NPC [32]. Our study has advanced the existing knowledge on combined applications for miRNAs and mRNAs, and the results of our research support the hypothesis we put forward. MiR-96-5p and miR-375 regulate the expression of CDK1, miR-493-3p regulates the expression of CCNB1, miR-320a regulates the expression of CCNA2, miR-21-5p and miR-96-5p regulate the expression of TOP2A, miR-25-3p and let-7i-5p regulate the expression of CDC6, miR-493-3p regulates the expression of MAD2L1, miR-493-3p and miR-493-5p regulate the expression of AURKA, and miR-378a-3p and let-7i-5p regulate the expression of CHEK1. There have been quite a few studies on the abovementioned central genes in the past. CDK1 can be integrated with CCNB1 to actuate the G2-M transition and bind to other cyclins to further adjust and control the G1 process and G1-S transition [33]. In a previous study, CDK1 was confirmed as a direct regulated site of miR-96-5p and reportedly served as a tumor inhibitor for NPC[34]. As shown in some studies, CDK1 may be related to radiosensitivity in tumor cells [35]. CCNA2 was reported to be a downstream target of miR-29c-3p and mainly enriched in the cell cycle [36]. A study on promoter methylation revealed that CCNA2 might be a tumor suppressor gene for NPC [37]. TOP2A is a subtype of TOP2, which plays an important role in DNA synthesis and transcription[38]. CCNB1 is an important component of the cell cycle pathway and one of the hub genes that substantially

influences cancer development. Some studies have revealed the potential role of CHK1-related pathways and CDC6 in reversing radioresistance[39, 40]. Some previous bioinformatics studies related to NPC showed that AURKA, CCNB1, and MAD2LI were genes with considerable value for further research [41]. However, to our knowledge, there have been no reports about the abovementioned genes (CCNB1, CDC6, TOP2A, CHK1, and AURKA) in the field of NPC research to date. These genes are expected to become new research targets in the future.

5. Conclusion

Drawing conclusions from the results of the present study, we methodically analyzed mRNAs and miRNAs related to NPC based on data from GEO and the systematic bioinformatics prediction approach. Our research deepens the comprehension of NPC at the molecular biology level and lays the foundation for future studies regarding the prognosis of patients with NPC. This study is expected to become a cornerstone for future studies on NPC.

Abbreviations

nasopharyngeal carcinoma (NPC)

Gene Expression Omnibus (GEO)

pyruvate dehydrogenase kinase 1 (PDK1)

differentiate expressed genes (DEGs)

cyclin-dependent kinase 1 (CDK1)

cyclin B (CCNB1)

cyclin A2 (CCNA2)

phosphatidylinositol-trisphosphate (3,4,5-PIP3)

protein kinase B (Akt)

DNA topoisomerase II alpha (TOP2A)

aurora kinase A (AURKA)

cell division cycle 6 (CDC6)

checkpoint kinase 1 (CHEK1)

fold change (FC)

Gene Ontology (GO)

protein-protein interaction (PPI)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

non-integrin membrane–extracellular matrix (ECM)

KIT stem cell factor (KIT-SCF)

interleukin (IL)

signal transducer and activator of transcription (STAT)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Data availability

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

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Author contributions

JYX and SL designed/performed most of the investigation, XHL data analysis and wrote the manuscript; CSZ provided pathological assistance; WW and LCF contributed to interpretation of the data and analyses. All of the authors have read and approved the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

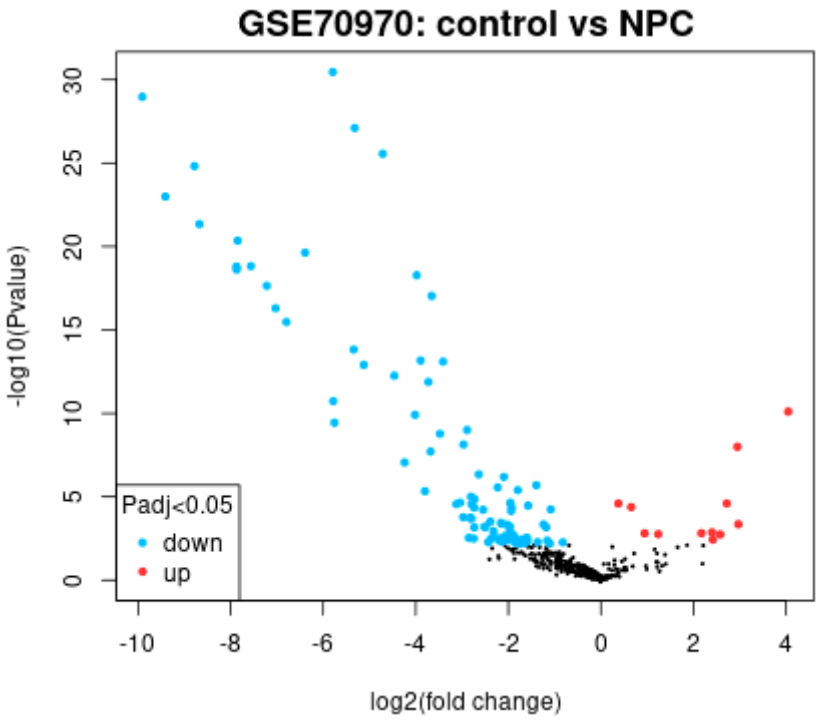

Figure 1

Volcano plot of differentially expressed mRNAs. The red dots represent up-regulated miRNAs, and the blue dots represent down-regulated mRNAs.

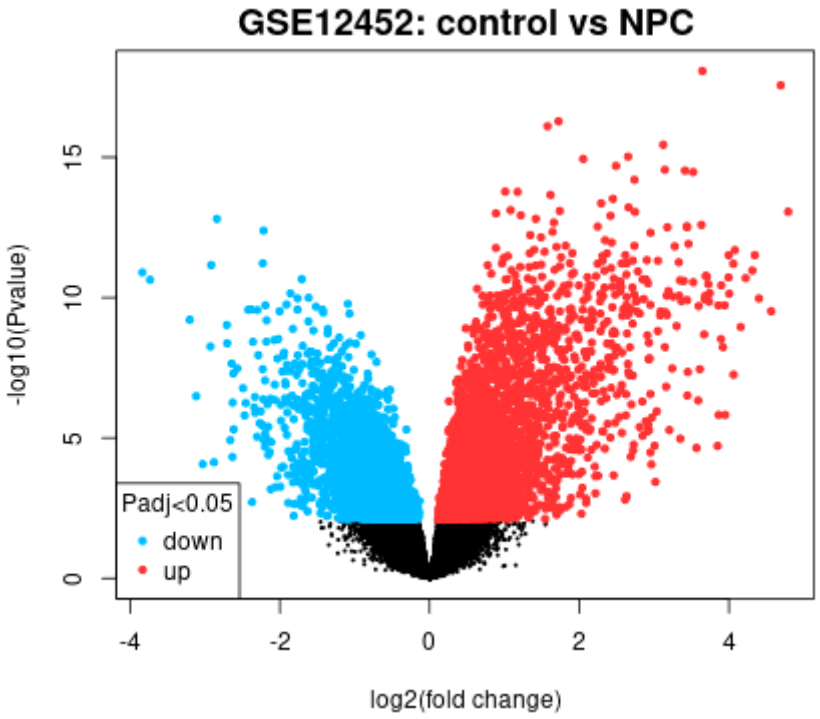

Figure 2

Volcano plot of differentially expressed miRNAs. The red dots represent up-regulated miRNAs, and the green dots represent down-regulated miRNAs.

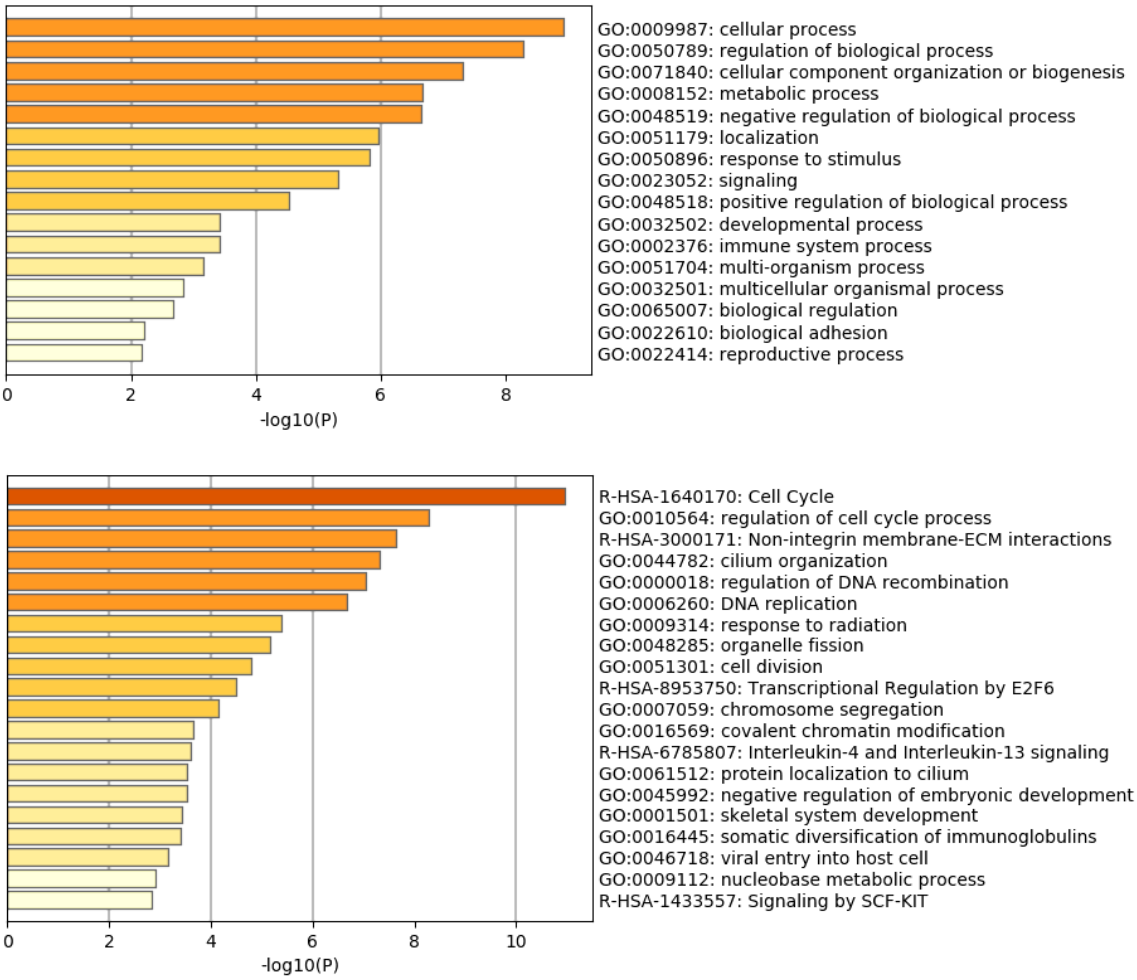

Figure 3

Enriched ontology clusters. GO and KEGG pathway enrichment analyses of primary key DEGs (A and B).

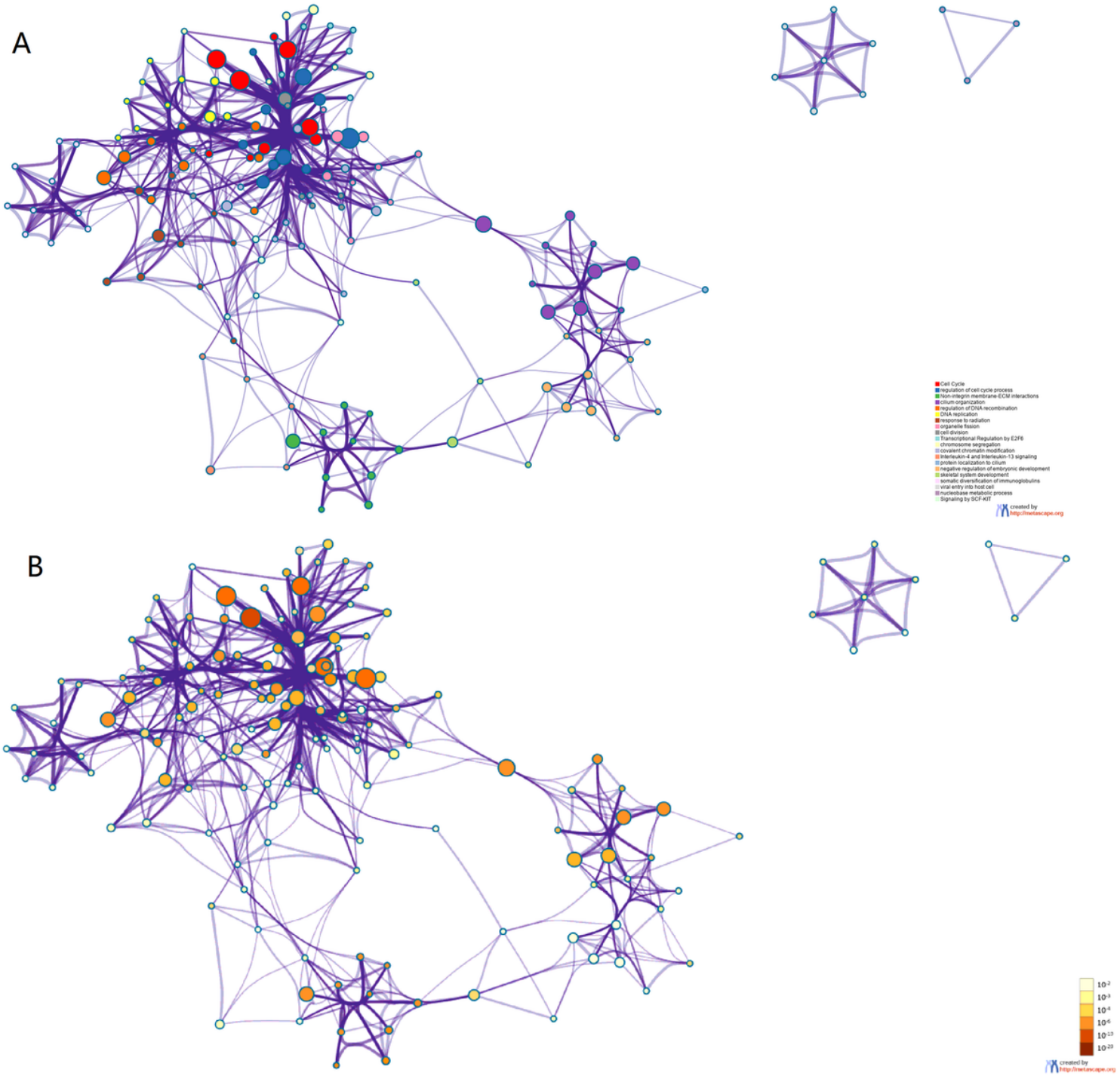

Figure 4

Enriched ontology clusters.

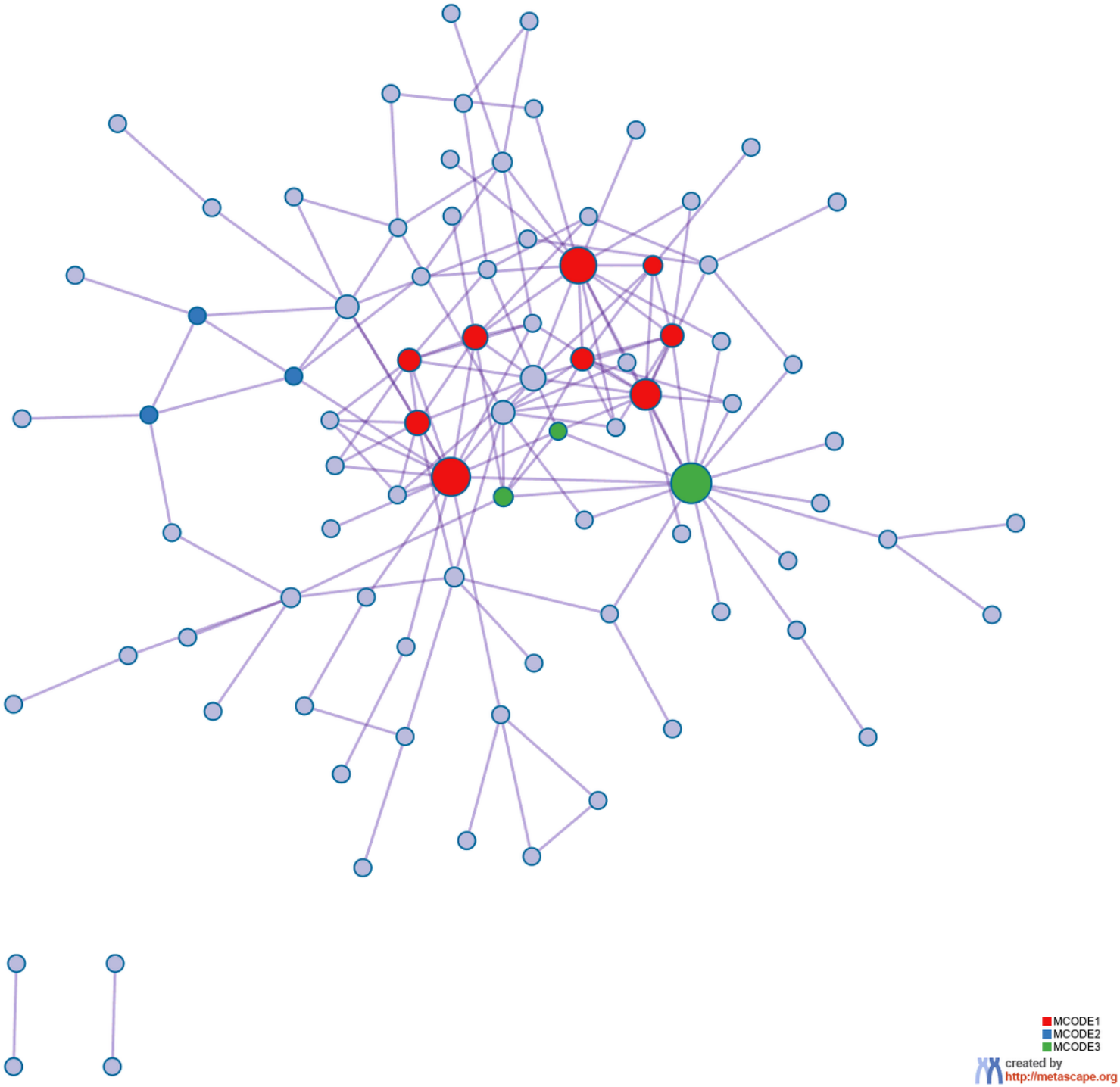

Figure 5

Protein-protein interaction network.

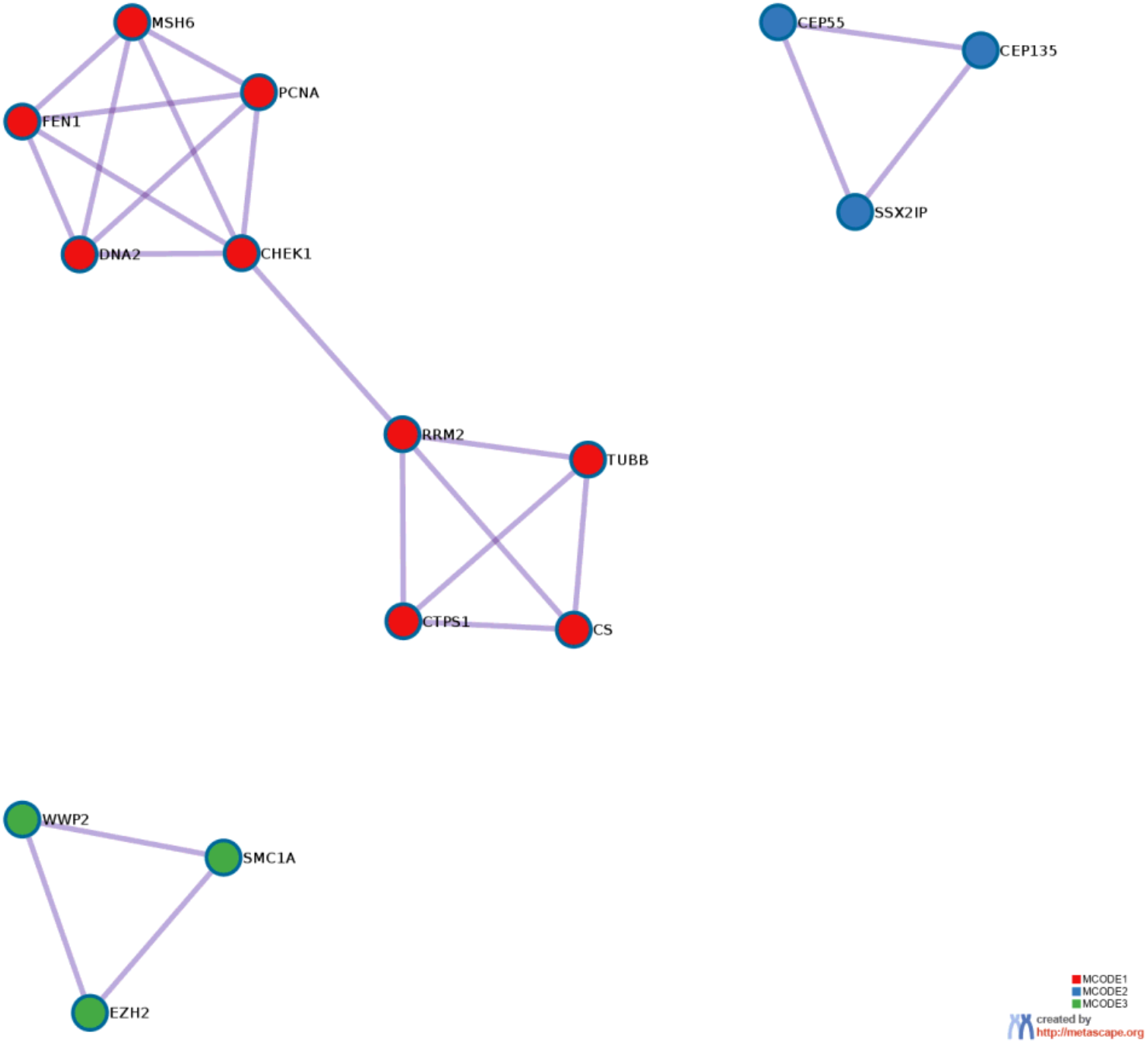

Figure 6

PPI MCODE components.

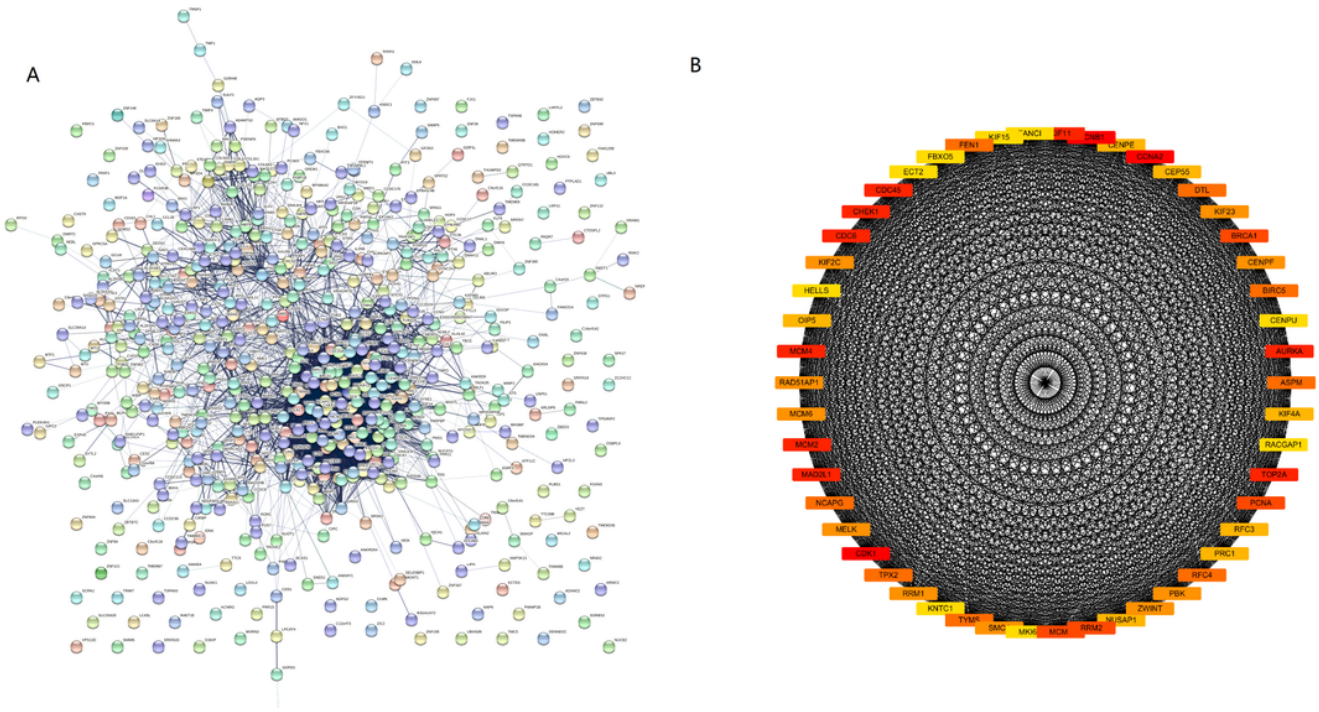

Figure 7

PPI network of NPC-related target genes and related genes.

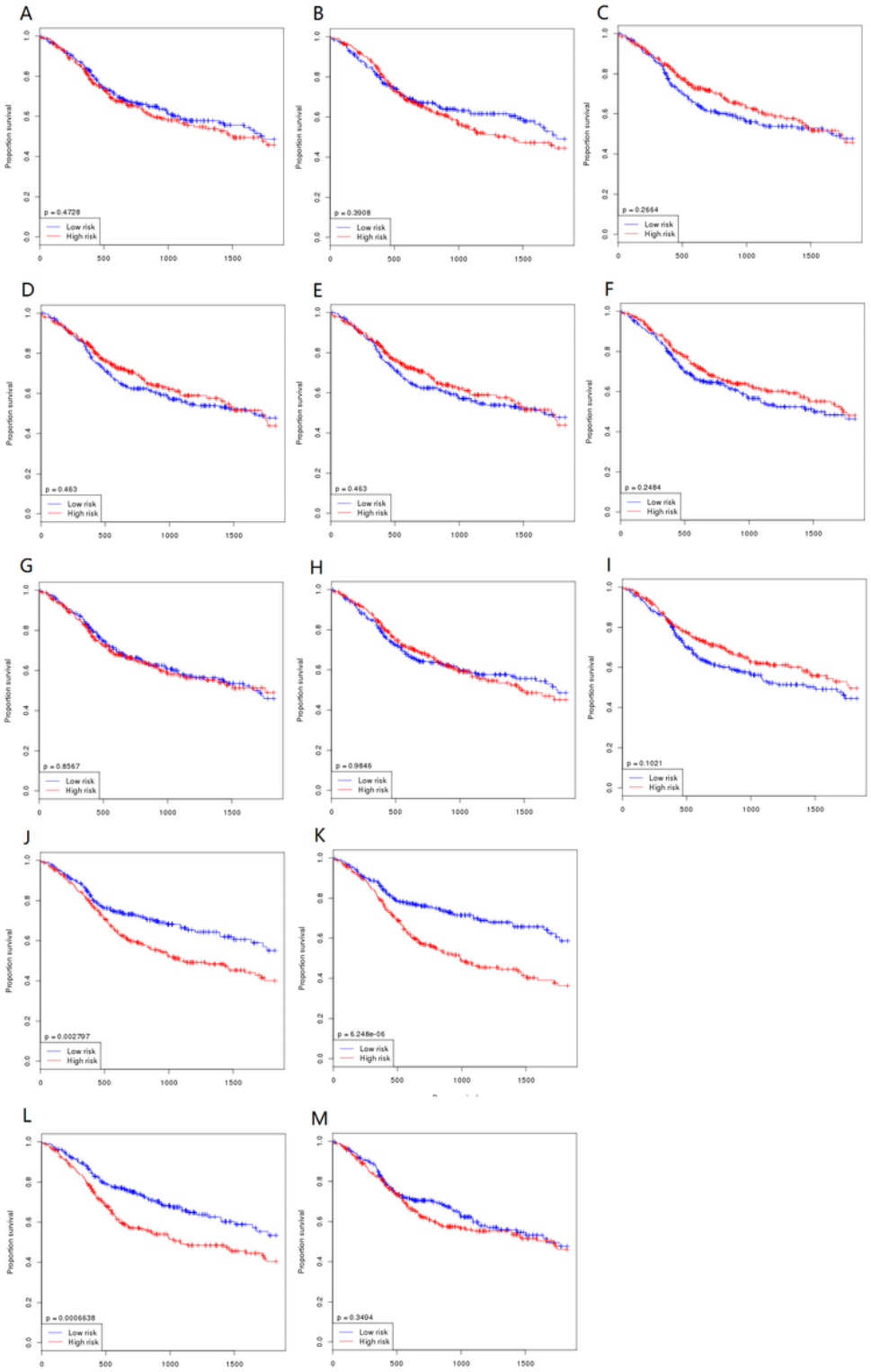

Figure 8

Thirteen differentially expressed miRNAs were associated with survival in NPC patients using ONCOMIR. (A) miR-15b-5p; (B) miR-21-5p; (C) miR-25-3p; (D) miR-93-5p; (E) miR-96-5p; (F) miR-155-5p; (G) miR-320a; (H) miR-375; (I) miR-378a-3p; (J) miR-493-3p; (K) miR-493-5p; (L) miR-494-3p; (M) let-7i-5p.

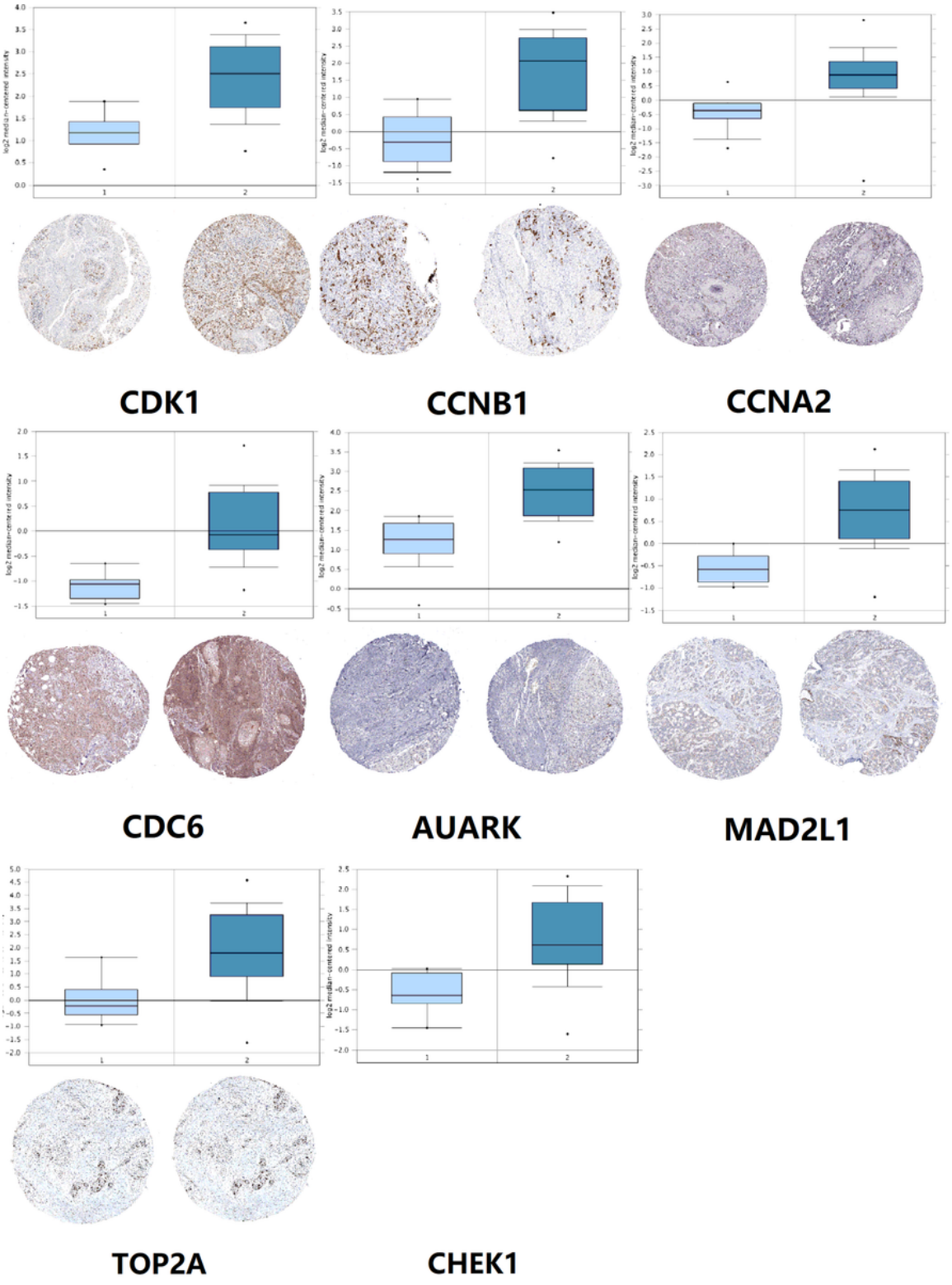

Figure 9

Validation of the expression of central genes at the transcriptional and translational levels using the Oncomine database and the Human Protein Atlas database (immunohistochemistry).

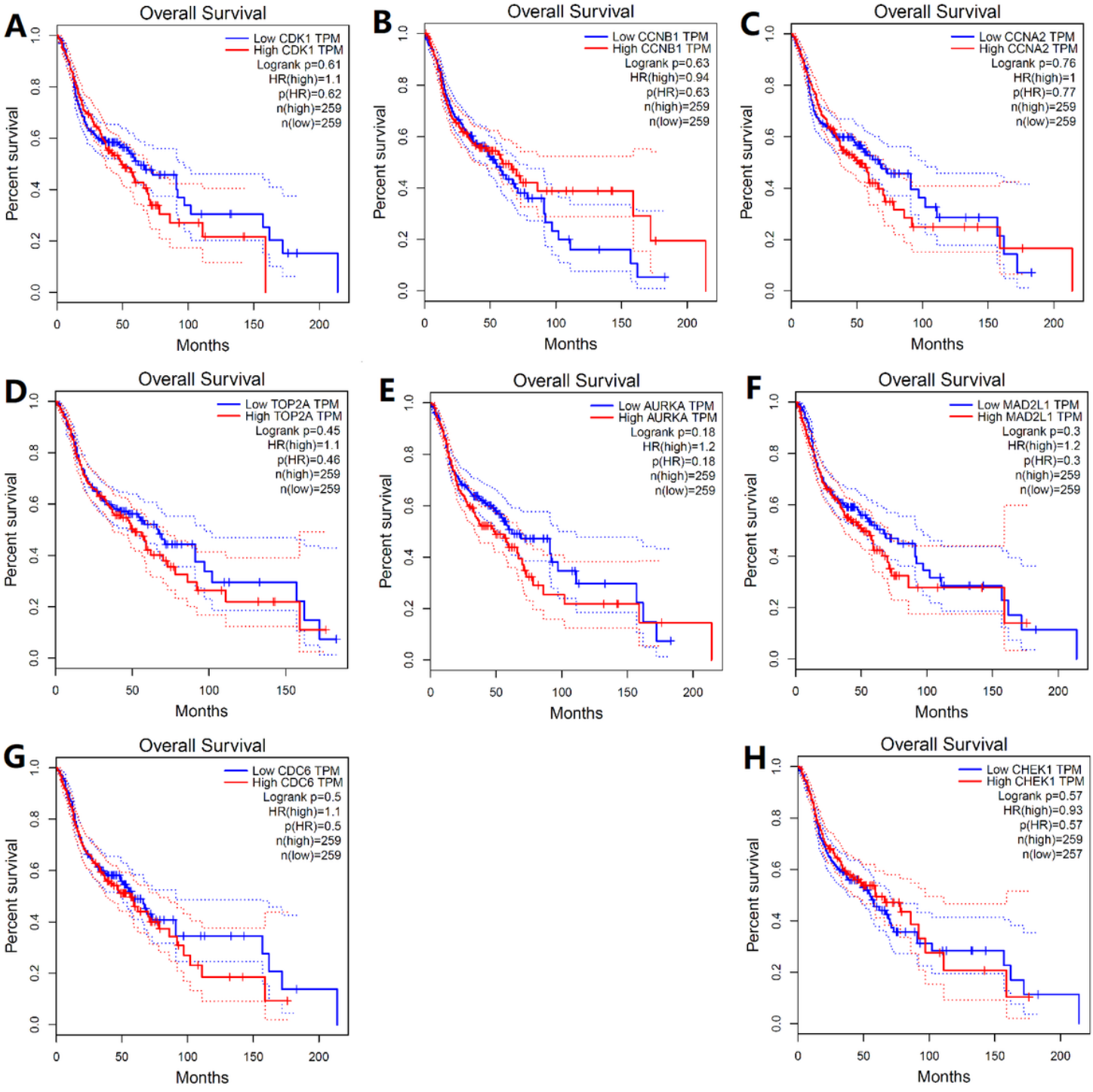

Figure 10

Eight central mRNAs were associated with survival in NPC patients by using GEPIA. (A) CDK1; (B) CCNB1; (C) CCNA2; (D) TOP2A; (E) AURKA; (F) MAD2L1; (G) CDC6; (H) CHEK1.