



Molecular mechanism and candidate biomarkers of morphine for analgesia and addiction effects

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Background: Morphine and its substitutes are frequently used in the clinical treatment of acute severe pain and advanced cancer patients. Long-term irregular use of morphine will lead to severe dependence. However, the genes behind the analgesic and addictive effects of morphine still need to be revealed.

Methods: We retrieved and downloaded RNA expression data sets related to morphine pain and addiction effects from the Gene Expression Omnibus (GEO) database to identify differentially expressed genes. Functional enrichment analysis was performed to analyze relevant pathways. Gene expression trends was used to screen key genes associated with addiction effects. miRNAs and PPIs were used to explore the functional mechanisms of genes.

Results: A total of 163 up-regulated and 277 down-regulated genes were obtained in the dataset for analgesic effects. A total of 1,482 up-regulated and 1,754 down-regulated differentially expressed genes (DEGs) were obtained in the dataset for addictive effects. By taking the intersection, 8 up-regulated and 22 down-regulated mRNAs which showed high correlations with both analgesic and addictive effects were identified. Based on the DEGs, a comprehensive network combining the mRNA-miRNA network and protein-protein interaction (PPI) network was established. Among the networks, 1 up-regulated miRNA (miR-129) and 3 down-regulated miRNAs (miR-714, miR-2135, and miR-2145) were identified. Gene expression trends and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms indicated that Fos may be a biomarker for morphine addiction.

Conclusions: Our findings will provide a valuable foundation for future genetic mechanism studies of the analgesic and addictive effects of morphine and provide inspiration for finding analgesic substitutes and relieving the addiction of analgesic drugs.

Keywords: Morphine; analgesic effect; addictive effect

Submitted Nov 23, 2021. Accepted for publication Jan 21, 2022.

doi: 10.21037/atm-21-7037

View this article at: <https://dx.doi.org/10.21037/atm-21-7037>

Introduction

Morphine is an opioid drug, originally extracted and purified from opium poppy (1). Morphine is also the main psychoactive chemical substance in opium. With the deepening understanding of the structure of morphine,

researchers began to synthesize morphine hydrochloride solution with higher purity and more convenient preparation and use (2). Because of its good sedative effect, it is widely used to relieve pain, cough, and diarrhea in patients undergoing surgery and those with advanced cancer (3).

However, with the inappropriate use of morphine, its side effects are also becoming apparent. For example, excessive dosage of morphine may lead to hypotension, bradycardia, and respiratory failure (4). Patients with advanced cancer require increased frequency and dose of medication to achieve pain relief (4). Long-term irregular use of morphine can result in serious dependence, which can lead to addiction (5). After entering the body, morphine first binds to opioid receptors and triggers a cascade of downstream reactions through signal transduction, thus exerting its effect (6). The opioid receptor is a G protein-coupled receptor and therefore has a basic structure consisting of an external amino terminus region, 7 transmembrane domains in the middle, and an internal carboxy-terminus region (7).

Neuropathic pain afflicts many people worldwide. It can be caused by nerve damage, by diseases that affect peripheral nerve function, or as a manifestation of diseases such as cancer (8). Today, with some exceptions, clinical treatment for patients with acute severe pain or advanced cancer relies on the oral opioid morphine and its substitutes (9). Although morphine has high analgesic activity, it is also accompanied by certain side effects, including poor stability of enzymatic hydrolysis, difficulty in crossing the blood-brain barrier, incurable analgesic effect, and lack of oral activity (10). Morphine also has certain effects on the endocrine system of animals, including lowering the testosterone concentration and affecting the production of gonadotropin (11). In addition to the above effects, morphine also has a certain regulatory effect on the cardiovascular system (12). Exogenous opioid peptides can inhibit the activity of cultured osteoblasts, and some opioid peptides and their modified analogues can have indirect effects on bone metabolism (13). However, the molecular mechanism of morphine analgesia still needs to be explored.

The mechanism of drug addiction involves various complex neural adaptive changes. Under the stimulus of addictive drugs, the brain's reward system is triggered, involving a variety of neurotransmitters and receptors (14). Drug addiction can be considered a disease of learning and memory, and many experiments have confirmed that synaptic plasticity is the neurological basis of learning and memory (15). At present, many studies have been conducted to explore the molecular mechanism of morphine addiction (16). Dopamine is a monoamine neurotransmitter that plays an important role in the communication between neurons (17). A growing number of studies have found that dopamine receptors are involved in the regulation of drug addiction (18). In addition, dopamine receptors also play

an important regulatory role in morphine addiction (19). Glutamate is a major excitatory neurotransmitter in the brain, regulating up to 70% of synaptic neurotransmission in the central nervous system (20). Drug-induced synaptic plasticity is associated with adaptive changes in the limbic midbrain circuits resulting from chronic drug exposure, ultimately leading to addictive behavior, and glutamate receptors are involved in this process (21). Long-term abuse of morphine can lead immune dysfunction, an increase in the susceptibility of the body to infection including viruses, liver injury, and the susceptibility of offspring (22). However, the key genes for the addictive effects of morphine remain unknown.

The aim of this study is to identify the genetic mechanisms of the analgesic effects and addictive effects of morphine. Using transcriptional expression datasets, the mRNA-miRNA network and protein-protein interaction (PPI) network were constructed. Several differentially expressed miRNAs and mRNAs were obtained which showed high correlations with the phenotypes. Our results may highlight new causal candidate genes or miRNAs which underlie both the addictive and analgesic effects of morphine. We present the following article in accordance with the STREGA reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-21-7037/rc>).

Methods

Data sources

The Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) database was used to download the expression matrix associated with morphine. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The GSE62346 dataset contained 9 miRNA samples and 15 mRNA samples which were used to explore the analgesic effect of morphine. Among the 9 miRNA samples, 3 samples belonged to control groups with 0 mg/kg morphine treatment. The other 6 samples were treated with 2 mg/kg morphine and 5 mg/kg morphine. The 15 mRNA samples were also treated with 0, 2, and 5 mg/kg morphine. The other mRNA dataset for the analgesic effect of morphine was GSE50382. This dataset contained 2 groups. The control group contained 3 samples with saline treatment while the analgesic group received morphine treatment.

Three mRNA datasets for studying the addictive effects of morphine were downloaded, namely GSE9525, GSE7762, and GSE78280. The GSE9525 dataset contained

8 hypothalamus samples and 10 pituitary samples. The GSE7762 dataset contained 12 control samples treated with saline and 12 samples treated with chronic morphine. The GSE78280 dataset contained 6 samples with saline treatment and 6 samples with protracted morphine treatment. GSE15774 was selected from an mRNA time series dataset for morphine addiction studies.

Data pre-processing

The mRNA and miRNA combined dataset GSE62346 for analgesia was extracted from the Series Matrix File. The probe ID was converted into gene symbol or miRNA ID through the platform annotation information table. The same gene symbol or miRNA ID was incorporated. Expression matrixes of mRNA and miRNA samples were screened out according to ID. Other datasets were preprocessed in the same way.

Correlation and principal component analysis

Principal component analysis (PCA) is an unsupervised feature learning data dimensionality reduction algorithm, which shows the classification of data through the expression data of samples. The R packages ggplot2 (23), plyr (24), ggbiplot (25), and scales (26) were used to perform PCA based on the genes with significant mean differences (ANOVA, $P \leq 0.05$) in the sample.

The correlation of gene expression levels between samples is an important reference index for experimental reliability and reasonable selection of samples. The correlation coefficient can represent the similarity between samples, and the closer the correlation coefficient is to 1, the higher the similarity between 2 samples. The R packages hmisc (27) and corrplot (28) were used to perform correlation analysis between 2 samples.

Differential expression analysis

The R package limma (29) was used to perform differential expression analysis of genes in the mRNA and miRNA datasets. Differentially expressed genes (DEGs) were defined by P value < 0.05 , fold change > 1.2 , or fold change $< 1/1.2$. Datasets for morphine addiction were also analyzed by the R package limma. The screening criteria for differential genes were fold change > 1.5 , fold change < 1 , and P value < 0.05 .

Prediction of the miRNA-mRNA regulatory relationship

TargetScan, miRTarBase, miRDB, miRanda, and miRMap databases were used to predict the regulatory relationships between up-regulated miRNAs and down-regulated mRNAs, and between down-regulated miRNAs and up-regulated mRNAs.

PPI network prediction

The DEGs in the miRNA-mRNA regulatory network were extracted and input into the STRING database (<https://string-db.org/>) for protein-protein interaction (PPI) network prediction. Interaction score > 0.7 (highest confidence) was selected as the threshold of PPI. After the PPI relation pairs were obtained, Cytoscape (30) software was used to analyze the topology structure of the PPI relation network.

Functional enrichment analysis

Based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases, the functions of the genes in the miRNA-mRNA networks were analyzed. Fisher's exact test was used to calculate the correlations between DEGs and functional terms. On the other hand, Gene Set Enrichment Analysis (GSEA) were performed using the R package clusterProfiler. P values were used to evaluate significance, and smaller P values represented a more significant relationship.

Analysis of gene expression trends

The expression trends of the expression values at 1, 2, 4, and 8 h in the mRNA time series dataset used for addiction research were clustered, and those with similar expression patterns were grouped into 1 group. The genes in the classes whose overall expression trend was increasing or decreasing were selected, and the genes in the addiction-related pathways in the functional enrichment analysis of DEGs in the mRNA dataset used for addiction research were intersected.

Statistical analysis

Student's *t*-test was used for assessing differences between groups. P value less than 0.05 was considered to be

statistically significant. R software (version: 4.0.2) was used to perform all statistical analyses.

Results

PCA and differential expression analysis

To verify the gene expression differences between groups, PCA was performed based on the gene expression matrixes. As shown in *Figure 1*, gene expression was significantly different between each group in the 8 datasets.

Using the R package limma, DEGs were obtained. The mRNA data of the GSE62346 dataset used to study analgesia contained 440 DEGs, among which 163 were up-regulated and 277 were down-regulated (*Table 1*). This dataset also contained 23 differentially expressed miRNAs which contained 12 up-regulated miRNAs and 11 down-regulated miRNAs. Using the same method, a total of 192 DEGs were obtained from the validation dataset GSE50382, containing 79 up-regulated genes and 192 down-regulated genes. Then, the DEGs of the 2 datasets were combined and 627 DEGs were finally obtained for further analysis. Four mRNA datasets were downloaded and analyzed to study morphine addiction. Dataset GSE9525 contained 796 up-regulated mRNAs and 993 down-regulated mRNAs among the 1,789 DEGs. Another 3 datasets contained 1,738, 136, and 27 DEGs. A total of 1,482 up-regulated and 1,754 down-regulated DEGs were obtained by combining the 4 datasets. In addition, heatmaps were generated based on the DEGs among the 6 datasets. As shown in *Figure 2*, the DEGs of different groups could be well clustered together.

The common DEGs for the analgesic and addictive effects of morphine

Based on the 1,482 up-regulated and 1,754 down-regulated mRNAs for addictive effects and the 230 up-regulated and 367 down-regulated mRNAs for analgesic effects, the intersection which contained 8 common up-regulated mRNAs and 22 common down-regulated mRNAs was obtained (*Figure 3*). The 8 common up-regulated genes were *Aloxe3*, *Chrm2*, *Epha8*, *Folh1*, *Has1*, *Hes3*, *Palm2*, and *Rgs17*. The 22 common down-regulated genes were *1700028P14Rik*, *1700047G07Rik*, *Anxr2*, *Celsr3*, *Col8a1*, *Crhr2*, *Ctf1*, *Cxcr4*, *Glb11*, *Ins2*, *Lass4*, *Lect1*, *Lrrc17*, *Mfap4*, *Myo5a*, *Plxn3*, *Postn*, *Prrx2*, *Six5*, *Slc28a3*, *Tcn2*, and *Thbs1*.

MiRNA-mRNA regulatory networks

Combining the differently expressed mRNAs and miRNAs, an mRNA-miRNA regulatory network was generated (*Figure 4*). The network contained 101 regulatory relation pairs, among which 54 up-regulated miRNA and 47 down-regulated miRNA regulatory relation pairs were obtained. As shown in *Figure 4*, up-regulated miR-129-5p showed regulatory pairs with 54 down-regulated mRNAs. Three down-regulated miRNAs, namely miR-714, miR-2145, and miR-2135, showed regulatory relations with 47 up-regulated mRNAs. Among the up-regulated mRNAs, *Tcf712* showed common regulatory relationships with miR-714 and miR-2145. Meanwhile, miR-2145 indirectly correlated with miR-2135 by regulating 3 mRNAs, namely *Vwc2*, *Upk1a*, and *Gad1*.

PPI network prediction

The STRING database was used to predict the PPI network with the highest confidence (interaction score >0.7). A total of 12 relation pairs were obtained with 17 DEGs, comprising 7 up-regulated mRNAs and 10 down-regulated mRNAs (*Figure S1*). There was no significant difference in the degree of connectivity between the protein interaction network nodes, which ranged from 1 to 2. Then, Cytoscape was used to integrate the PPI and mRNA-miRNA networks. As shown in *Figure 5*, the separated subnetworks were integrated into whole networks by several mRNA-mRNA regulatory pairs, such as *Ins1-Sorcs1*, *Dst-Lamc2*, *Reln-Gad1*, *Kalm-Trh*, and *Ryr2-Camk2d*.

GO and KEGG analysis of the genes in the mRNA-miRNA network

By blasting genes with the GO and KEGG pathway databases, genes in the mRNA-miRNA network were annotated. As for biological processes (BP) in the GO analysis (*Figure 6A*), most genes were enriched in anatomical structure development, animal organ development, cell differentiation, and multicellular organism development. KEGG results (*Figure 6B*) showed that the DEGs were enriched in insulin secretion, circadian entrainment, Cushing syndrome, and alanine aspartate and glutamate metabolism pathways.

As for the DEGs for addiction function, the enriched BP terms were cell communication, signal transduction, regulation of cellular metabolic process, and regulation

Table 1 The differentially expressed genes (DEGs) in the 6 datasets

Type	Analgesia			Addiction			
	GSE62346 (mRNA)	GSE62346 (miRNA)	GSE50382	GSE9525	GSE9525	GSE7762	GSE78280
Up-regulated	163	12	79	796	805	45	16
Down-regulated	277	11	113	993	933	91	11
Total	440	23	192	1,789	1,738	136	27

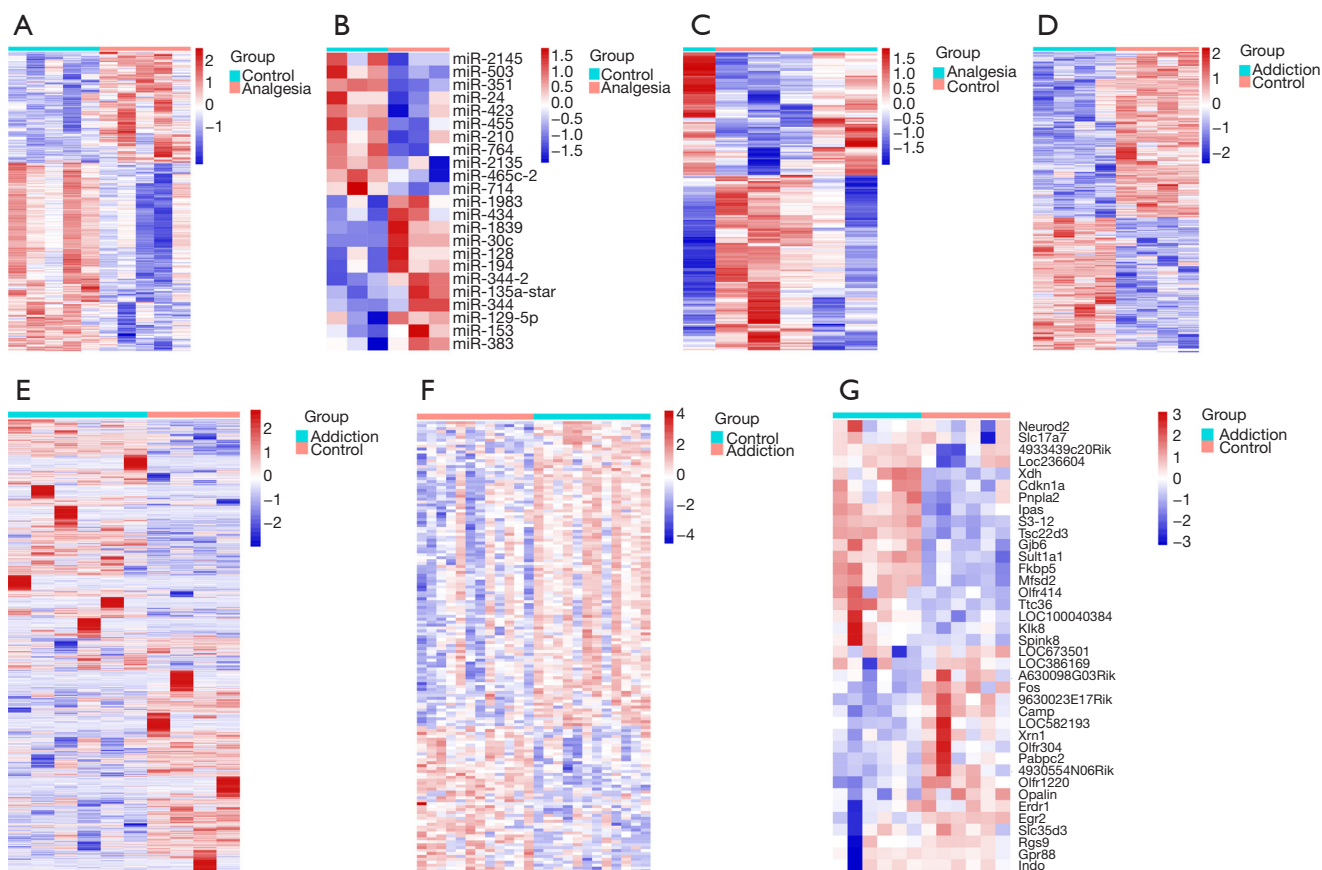


Figure 2 Heatmaps of the DEGs of the 6 datasets. Heatmaps of the DEGs of mRNA samples in GSE62346 (A), and miRNA samples in GSE62346 (B), and GSE50382 (C), and GSE9525 (D), and GSE9525 (E), and GSE7762 (F), and GSE78280 (G). DEGs, differentially expressed genes.

of nitrogen compound metabolic process (Figure 6C). The KEGG results (Figure 6D) showed that olfactory transduction, calcium signaling, neuroactive ligand-receptor interaction, and cancer pathways were enriched.

Cluster analysis of gene expression trends

The expression trends of mRNA time series datasets used

for addiction research at 1, 2, 4, and 8 h were clustered by the WKNN (weighted K-Nearest neighbor) method, and those with similar expression patterns were grouped into 1 class. The results showed that the expression trends could be divided into 4 categories including down-up trend (cluster 1), continuous-down trend (cluster 2), up-down trend (cluster 3), and continuous-up trend (cluster 4) (Figure 7). Then, genes with the continuous up (cluster 3) or down (cluster

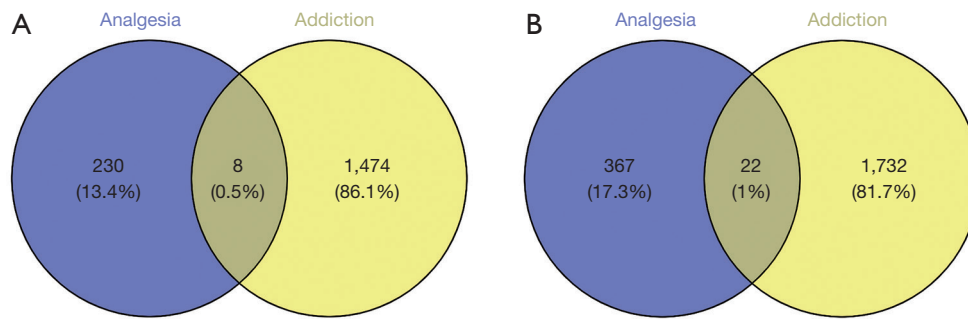


Figure 3 Venn diagrams of the DEGs related to the analgesic and addictive effects of morphine. (A) Up-regulated genes; (B) down-regulated genes. DEGs, differentially expressed genes.

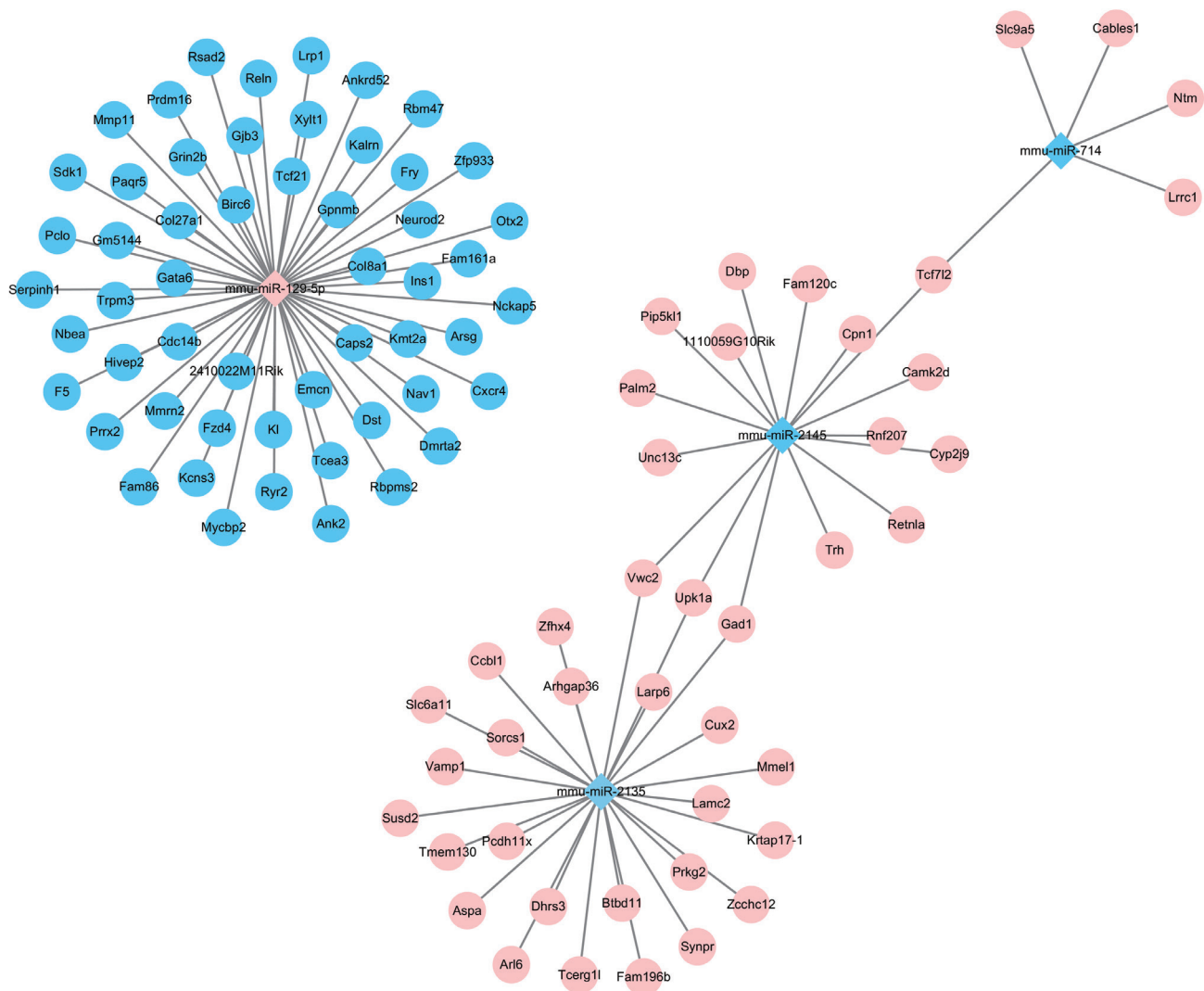


Figure 4 Networks of differentially expressed mRNAs and miRNAs. The red circles represent the up-regulated mRNAs. The blue circles represent the down-regulated mRNAs. The red rhombuses represent the up-regulated miRNAs. The blue rhombuses represent the down-regulated miRNAs.

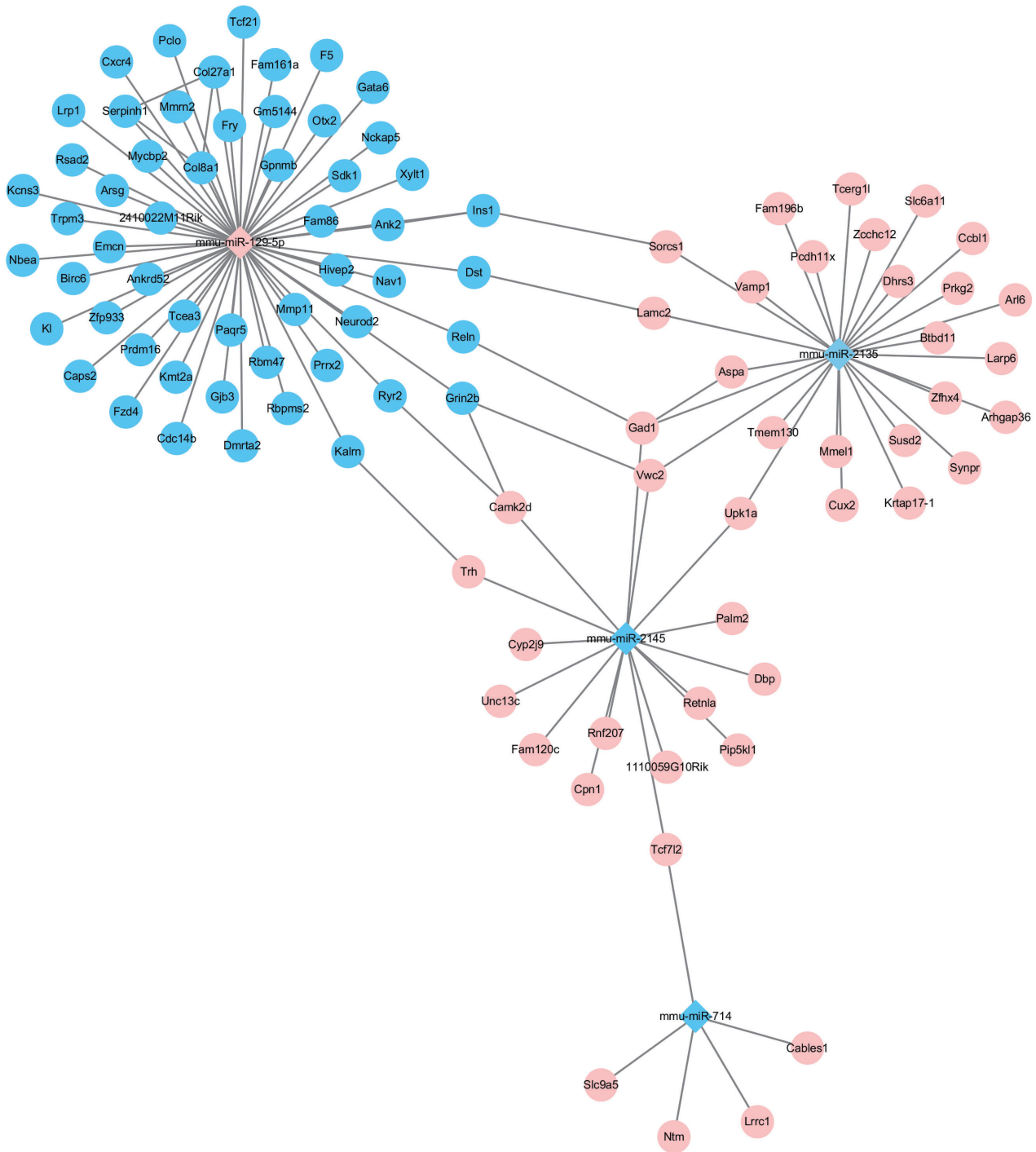


Figure 5 Integrated network combining the PPI and mRNA-miRNA networks. The red circles represent the up-regulated mRNAs. The blue circles represent the down-regulated mRNAs. The red rhombuses represent the up-regulated miRNAs. The blue rhombuses represent the down-regulated miRNAs. PPI, protein-protein interaction.

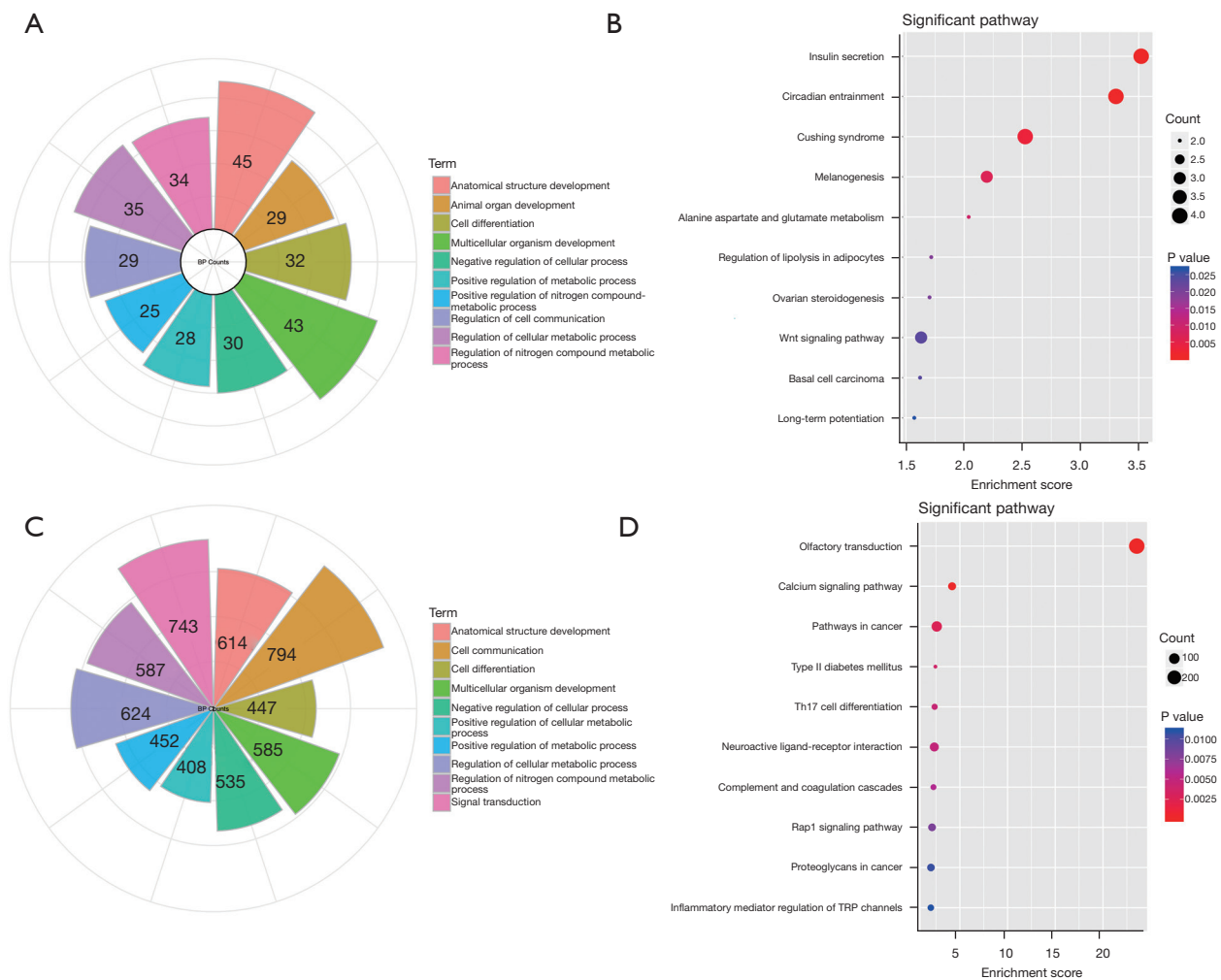


Figure 6 GO and KEGG analysis of the genes in the mRNA-miRNA network. (A,B) GO and KEGG terms of genes in the datasets for analgesia studies; (C,D) GO and KEGG terms of genes in the datasets for addictive studies. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

2) expression trends were selected for intersection analysis with the genes involved in the GO or KEGG terms. The results showed that Fos was the only gene in cluster 2, which intersected with the cAMP signaling pathway. In order to further explore the function of Fos gene, we performed GSEA analysis after grouping based on the expression level of Fos gene (divide by median). According to GSEA analysis, we found that high expression group is more enriched in the microtubule anchoring AND mRNA metabolism related pathway (Figure 8).

Discussion

Compared with other analgesic drugs, morphine has strong

analgesic effect and strong addiction, and is mainly used for the treatment of cancer in clinic. However, the molecular mechanisms of morphine's analgesic and addictive effects still need to be uncovered. In this study, we used expression data on the analgesic and addictive effects of morphine to identify the mRNAs and miRNAs involved in transcriptome alteration. A comprehensive network combining an mRNA-miRNA network which contained 101 regulatory relation pairs and a PPI network was built. From the network, 4 miRNAs including 1 up-regulated miRNA (miR-129-5p) and 3 down-regulated miRNAs (miR-714, miR-2135, and miR-2145) were found.

MiR-129 has long been studied as a biomarker for cancers such as gastric, rectal, and bladder cancer. In this

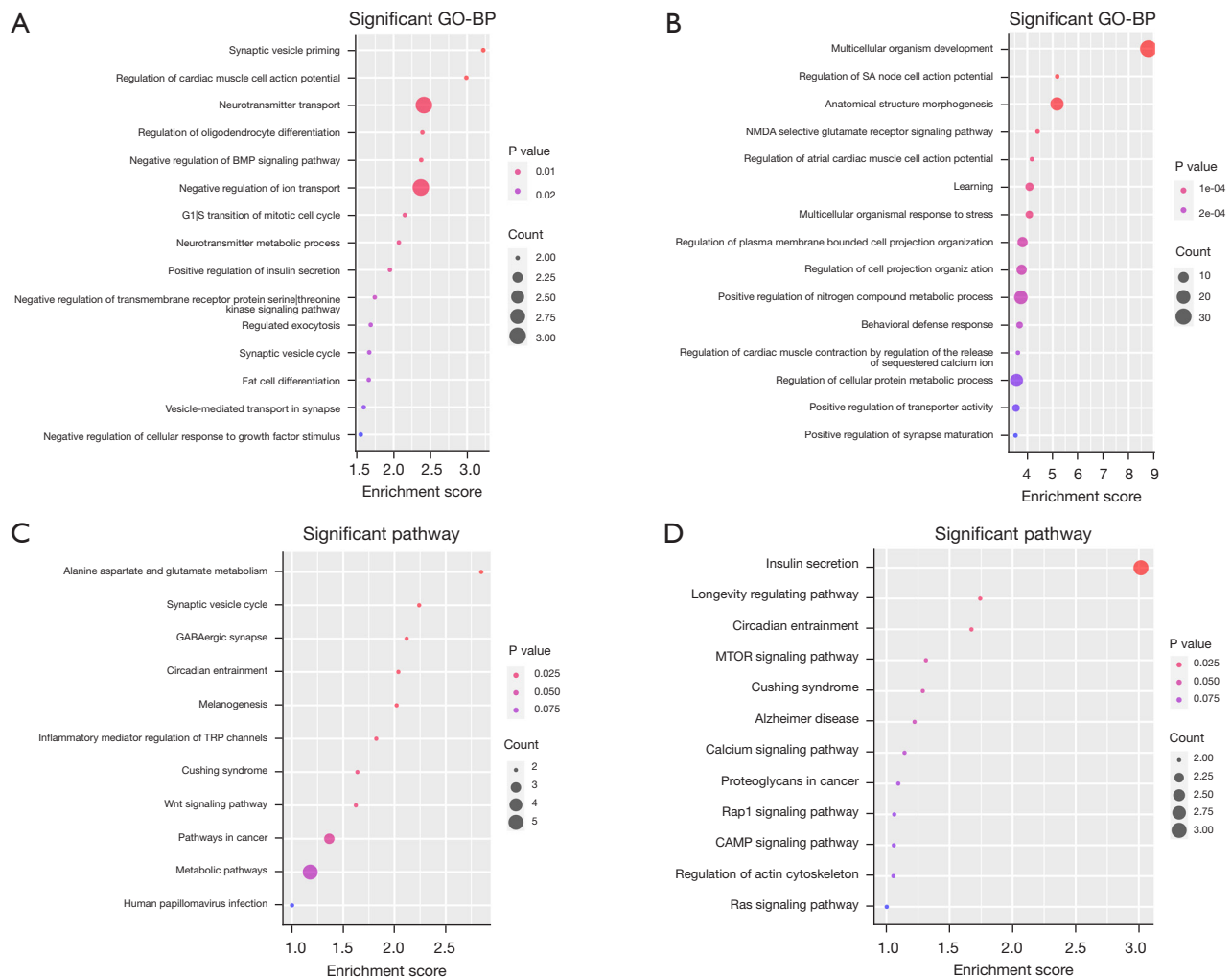


Figure 7 Cluster analysis of gene expression trends. GO, Gene Ontology; BP, biological processes.

study, the up-regulated miR-129-5c was recognized to respond to treatment with morphine in the transcriptome datasets for analgesia. These results were consistent with Tian *et al.*'s study which indicated that miR-129-5p alleviates neuropathic pain through regulating HMGB1 expression in chronic constriction injury (CCI) rat models (31). Peregud *et al.* also demonstrated that miR-129 that may regulate the content of brain-derived neurotrophic factor in the frontal cortex of rats after spontaneous morphine withdrawal (32). Yu *et al.* discovered the analgesic effects of miR-129-5p against bone cancer pain through the EphB1/EphrinB2 signaling pathway in mice (33). Our results confirmed that miR-129 may be closely related to the analgesic effect of morphine. Another 3 differently expressed miRNAs, namely miR-714, miR-2135, and miR-2145, also showed significant

regulatory effects after treatment with morphine. However, no research has associated morphine or its analgesic effect with these 3 miRNAs. Further research needs to be conducted to demonstrate the effects of miR-714, miR-2135, and miR-2145 on morphine analgesia.

The insulin I (Ins1) gene encodes insulin to regulate the metabolism of sugars and fats. In our study, up-regulated miR-129 could directly regulate the expression of Ins1. However, there is still no evidence that Ins1 is associated with analgesia. By taking the intersection of genes, 8 common up-regulated and 22 down-regulated genes were obtained, which respond to both the analgesic and addictive effects of morphine. Lipoxygenase-3 (ALOXE3) encodes a hydroperoxide isomerase and was implicated in skin differentiation (34). In previous studies, ALOXE3 was shown

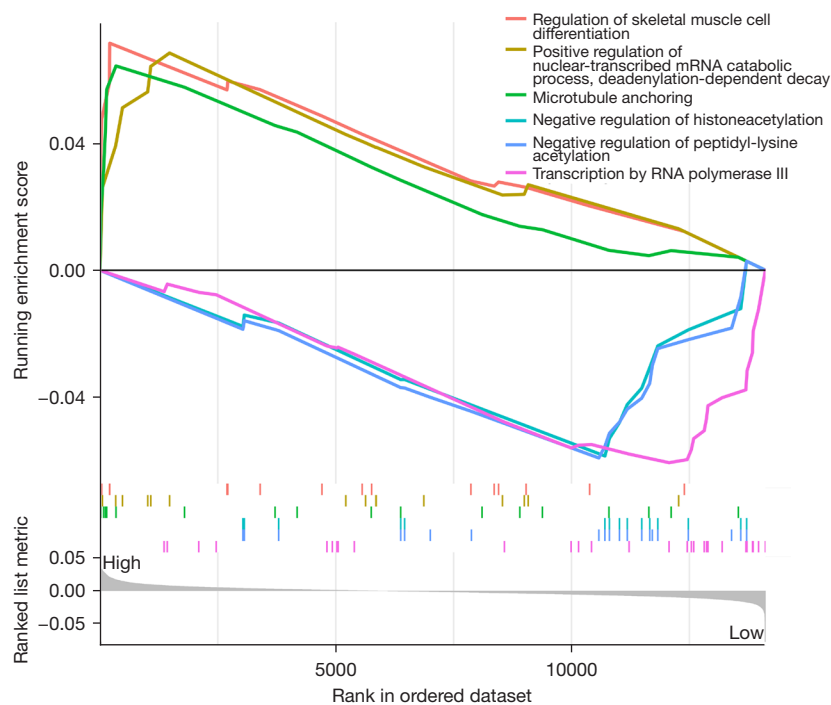


Figure 8 GSEA analysis between high and low expression groups of Fos gene. GSEA, Gene Set Enrichment Analysis.

to play a role in analgesia by inhibiting the expression of IL-6 (35-37). The corticotropin-releasing hormone receptor 2 (CRHR2) gene has been linked to mood and depression (38). In this study, CRHR2 was associated with both the analgesic and addictive effects of morphine. Pirnik *et al.* also found the expression of CRHR2 changed with the treatment of morphine (39). Mousa *et al.* also proved that CRHR2 could mediate the inhibition of inflammatory pain (40). Drug addiction and heroin addiction were discovered and evaluated in an African American population and in rats, respectively (41,42). CXCR4 is the receptor for SDF1, which has important functions in T lymphocyte trafficking (43). Wilson *et al.* revealed that CXCR4 signaling could mediate morphine-induced tactile hyperalgesia (44). In addition, numerous studies have linked this gene to the analgesic effects of morphine (45-47). Activation of CXCR4 by CXCL12 triggered the release of glutamate, which played a crucial role in alcohol and drug addiction (48).

Combining the results of genes in the continuously down-regulated cluster and KEGG analysis results, the Fos gene was obtained. Fos encodes a nuclear phosphoprotein to detect nerve cells that have recently been activated (49). Chang *et al.* demonstrated that morphine activated c-fos expression in the rat brain (50). In addition, Fos expression induced

by electroacupuncture in the periaqueductal gray led to higher levels of analgesia (51). Takeda *et al.* revealed that Fos expression showed a high correlation with analgesia in the anterior cingulate cortex in a rat neuropathic pain model (52). For addiction effects, Cruz *et al.* used Fos to investigate neuronal ensembles in corticostriatal circuitry in addiction and found correlative evidence supporting a role for neuronal ensembles in addiction (53). Interestingly, alcohol, nicotine, and cocaine addiction were related to Fos mRNA expression (54-56). In summary, Fos is a key gene involved in pain relief and addiction, the development of inhibitors targeting the Fos gene may be beneficial to reduce the adverse effects of morphine addiction in clinical treatment.

Acknowledgments

Funding: This work was supported by Changning District Medical and Health Research Special Project (CNKW2020Y28).

Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at <https://atm.>

amegroups.com/article/view/10.21037/atm-21-7037/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-21-7037/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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(English Language Editor: C. Betlazar-Maseh)

Cite this article as: Wang T, Chen X, Zeng K. Molecular mechanism and candidate biomarkers of morphine for analgesia and addiction effects. *Ann Transl Med* 2022;10(2):89. doi: 10.21037/atm-21-7037

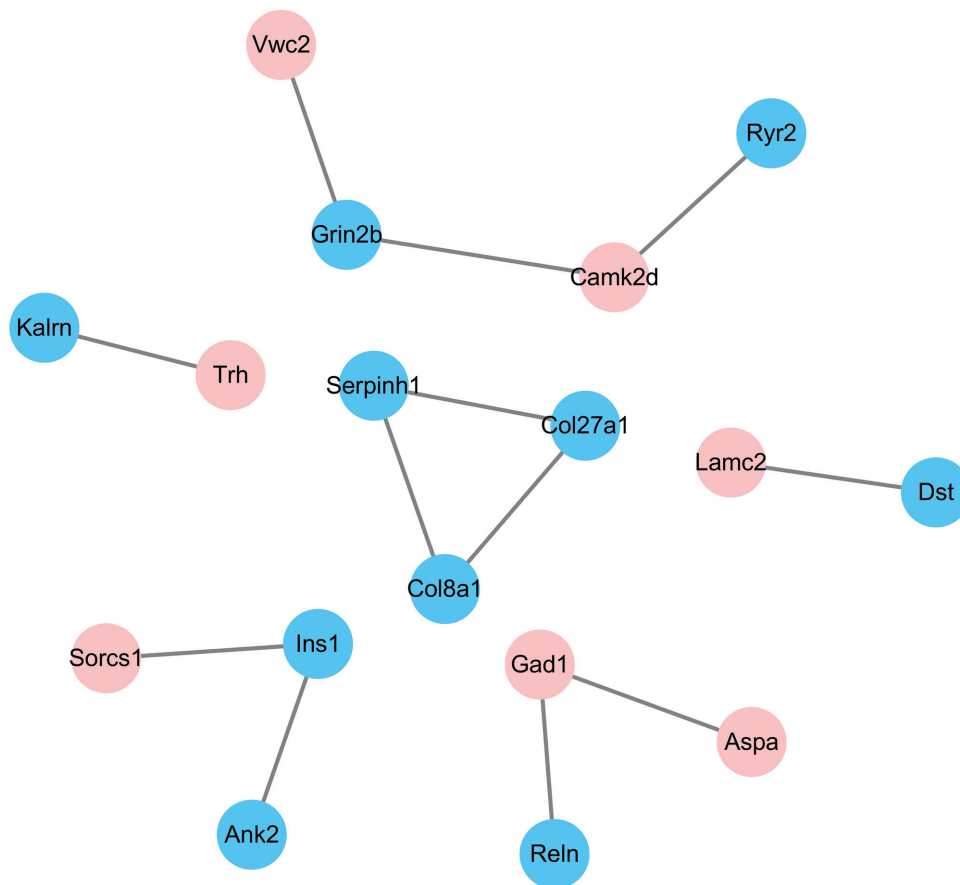


Figure S1 PPI network diagram of differentially expressed genes in miRNA-mRNA regulatory network. PPI, protein-protein interaction network.