Bioinformatics method combined with logistic regression analysis reveal potentially important miRNAs in ischemic stroke

Running title: miRNAs to ischemic stroke.

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Abstract

Purpose: This study aimed to investigate the comprehensive differential expression profile of miRNAs by screening for miRNA expression in ischemic stroke and normal samples.

Methods: Differentially expressed miRNA analysis was conducted using limma R Bioconductor package. Target genes of differential expression miRNAs (DEMs) were identified from TargetScanHuman and miRTarBase databases. Functional enrichment analysis of the target genes was performed using clusterProfiler R Bioconductor package. The miRNA-based ischemic stroke diagnostic signature was constructed via logistic regression analysis.
**Results:** Compared with the normal cohort, a total of 14 DEMs, including 5 upregulated miRNAs and 9 downregulated miRNAs, were identified in ischemic stroke patients. These DEMs have 1600 regulatory targets. Using a logistic regression model, the top 5 miRNAs were screened constructing a miRNA-based ischemic stroke diagnostic signature. Using the miRNA–mRNA interaction pairs, two target genes (SP1 and AGO1) were speculated to be the primary genes of ischemic stroke.

**Discussion and Conclusion:** Here, several potential miRNAs biomarkers were identified and a miRNA-based diagnostic signature for ischemic stroke was established, which can be a valuable reference for future clinical researches.

**Key words:** miRNAs; ischemic stroke; machine learning algorithms
1 Introduction

Stroke is an acute predominant cerebrovascular disease, which causes brain tissue damage owing to a sudden rupture of blood vessels in the brain or the inability of blood to flow into the brain because of vascular occlusion [1]. In 2010, the incidence of stroke was approximately 16.9 million, which added to a pool of 33 million stroke survivors worldwide. Currently, stroke is considered to be the second leading cause of death after ischemic heart disease [2]. Typical symptoms of stroke include sudden unilateral weakness, numbness, visual loss, diplopia, altered speech, ataxia, and non-orthostatic vertigo [3-4]. Stroke can be categorized into ischemic and hemorrhagic stroke. The incidence of ischemic stroke is higher than that of hemorrhagic stroke, accounting for 60%–70% of the total number of strokes [5-6]. Cardioembolic stroke accounts for approximately 25%–30% of ischemic stroke cases, and 25% of such cases pass all diagnostic tests and have no known causes. Because of the acute onset, treatment difficulty, and the lack of detection methods, such cases pose a great challenge to clinicians.

MicroRNAs (miRNAs) are a class of small endogenous RNAs that regulate gene expression post-transcriptionally and play a role in gene silencing and translation inhibition by binding to target genes. The miRNAs are a highly conserved class of tissue-specific genes that have been found in all eukaryotic cells preserved across species since their discovery in 1993 [7-8]. In general, they are short RNA molecules measuring 19–25 nucleotides in size. A single miRNA can target hundreds of mRNAs and influence the expression of many genes often involved in a functional interacting pathway [9]. Appropriate maintenance of miRNA expression is required for a balanced physiological environment because these small molecules influence almost every genetic pathway from cell cycle checkpoint and cell proliferation to apoptosis, with a wide range of target genes [10]. In recent years, miRNA regulation has been extensively studied for their role in biological processes (BPs) as well as in the development and progression of various human diseases including ischemic stroke [11-12].

In mammals, the brain exhibits a high level and activity of several miRNAs that
show region-specific expression [13-14]. Studies conducted using conditional knock-out Dicer (RNA enzymes are critical for miRNAs biogenesis) have demonstrated the indispensable functional significance of miRNAs in controlling processes that include cellular differentiation, proliferation, synaptic morphogenesis, and vascular formation [15-17], indicating an overlap between ischemic stroke and miRNAs. It has been suggested that stroke alters the expression levels of many miRNAs in human blood and brain [18-19].

Despite decades of research, treatment for ischemic stroke is limited to thrombolytic therapy and symptom management. Moreover, a considerable number of patients remain asymptomatic and cannot be detected at onset. To this end, a more comprehensive approach to predict potential ischemic stroke patients based on the differential expression of specific miRNAs is required. To address these issues, we performed bioinformatic analysis combined with machine learning algorithms to identify potential candidate diagnostic miRNAs. This study will help us screen for ischemic stroke-associated miRNA biomarkers and can be tremendously useful for ischemic stroke patients.

2 Material and Methods

2.1 Study materials

The materials used in this study were obtained from the Gene Expression Omnibus (GEO, https://ncbi.nlm.nih.gov/geo) with the accession number of GSE55937, which included 24 blood samples from healthy individuals and 24 blood samples from ischemic stroke. The miRNA expression profiles of the abovementioned samples were detected based on Affymetrix Multispecies miRNA-3 Array chip platform.

2.2 Differential expression analysis

The miRNA expression profiles were normalized using robust multi-array (RMA) method via the affy R Bioconductor package and standardized by logarithmic transformation. Differentially expressed miRNAs (DEMs) were screened using the limma R Bioconductor package by employing the criteria of absolute log-transformed fold change (|log2FC|) > 0.5 and p value ≤ 0.05.
2.3 Construction of miRNA–mRNA regulatory network

The target genes of DEMs were searched from TargetScan (Release 7.2: March 2018 www.targetscan.org) and miRTarBase (Release 7.0: Sept. 15, 2017 mirtarbase.mbc.nctu.edu.tw). Target genes that were common between the two databases were used for constructing the miRNA–mRNA regulatory network. Cytoscape software was applied for visualizing the regulatory network.

2.4 Functional enrichment analysis

Functional enrichment analysis of the target genes of DEMs was conducted using clusterProfiler Bioconductor package. Ultimately, Gene Ontology (GO, including Biological Process, Molecular Function, and Cellular Component) and KEGG pathways that satisfied Benjamini–Hochberg (BH)-adjusted p value of <0.05 were retained.

2.5 Construction of logistic regression model

Here, we proposed to test if DEMs could help distinguish stroke samples from normal ones. For this purpose, logistic regression analysis was performed by considering DEMs and sample groups as continuous predictor and categorical responsory, respectively, based on the glm basic R function. Each DEM with a p value of <0.05 was retained for constructing the prediction model.

2.6 Statistical analysis

Statistical analyses were performed using R software v3.5.2. Affy R Bioconductor package for the normalization of raw expression profiles. Limma R Bioconductor package was used for conducting differential expression analysis. A p value of <0.05 was considered to be statistically significant in all of the abovementioned analyses.

3 Results

3.1 DEMs

Expression of all miRNAs contained in the miRNA chip after normalization is shown in Figure S1A, which indicates that the normalization process successfully eliminated the batch effects. After calculation, we obtained a total of 14 DEMs in ischemic stroke samples in comparison with the normal samples, including 5 upregulated miRNAs and 9 downregulated miRNAs, as shown in Figure S1B.
Expression of those 14 DEMs in normal and ischemic stroke groups were illustrated as a heatmap in Figure 1.

3.2 Target genes of DEMs

A total of 1,600 target genes (Supplemental Table 1) were simultaneously predicted by TargetScanHuman and miRTarBase databases for 14 DEMs; functional enrichment analysis of these 1,600 target genes led to the identification of a total of 499 and 87 significantly enriched GO terms, respectively. Figure 2A and Figure 2B illustrates the top 30 most significant GO terms and KEGG pathways.

3.3 DEMs effectively characterizes ischemic stroke

Correlation of expression of the 14 DEMs in ischemic stroke and normal samples is shown in Figure 3A, indicating that there was no particularly strong collinear relationship among them. Thus, all these 14 DEMs were used for the construction of logistic regression model. Receiver operating characteristic (ROC) analysis was used for evaluating the performance of the model. Consequently, the area under curve (AUC) value of the model was 0.8645, as shown in Figure 3B, which proved that the logistic model could robustly determine the sample type. More importantly, we found that the p values of the five miRNAs, hsa_mir_122, hsa_mir_99b, hsa_mir_339, hsa_mir_145, and hsa_mir_3130_1, were less than 0.05, indicating that those five miRNAs had a greater contribution to the model than the remaining nine miRNAs. Hence, we reconstructed the logistic model using those five miRNAs, and it was found that the AUC value of the logistic model could reach 0.8589 (Figure 3C). Additionally, we conducted 5-fold cross validation basing on the dataset, and result illustrated high AUC value (Figure 3D). The abovementioned results proved that the model constructed based on these five miRNAs could effectively predict the sample type and should be more cost-effective for the diagnosis of ischemic stroke.

3.4 SP1 and AGO1 are highly connected in miRNA–mRNA regulatory network

We constructed the miRNA–mRNA network for all 14 DEMs and the 5 DEMs that had a p value of <0.05 in logistic regression analysis, as shown in Figures S2A and S2B, respectively. Nodes in the network were colored according to their connectivity, i.e., number of their direct neighbors; furthermore, of the 1,600 target genes, 499 and 87 significantly enriched GO terms were found for the ischemic stroke and normal samples, respectively.
genes, SP1 and AGO1 were the 2 genes that were regulated by at least two miRNAs in both regulatory networks. Therefore, they might be pivotal biomarkers in the development of ischemic stroke.

4 Discussion

Ischemic stroke is a leading cause of death and disability, resulting in over six million deaths per year worldwide [20]. In addition to the high mortality rate, the timely monitoring of undiagnosed stroke patients is also a very critical issue. All these issues highlight the dire need for effective forecasting targets or biomarkers. Because miRNAs are implicated in a wide variety of diseases and have been shown to be essential for diverse proper physiological functions in the human brain, it is beneficial to develop a comprehensive specific expression profile of miRNAs in ischemic stroke patients for identifying potential miRNAs candidates as well as for targeting mRNA. Here, compared with the normal samples, a total of 14 DEMs in ischemic stroke patients were screened, including 5 upregulated and 9 downregulated miRNAs. Moreover, 1,600 target genes have been further identified. We also studied the BPs related to these genes via GO and KEGG enrichment analysis. We found that these 1,600 target genes were significantly enriched in ischemic stroke-related BPs, such as response to oxygen levels and response to decreased oxygen levels. The results of this biomarker selection study demonstrate not only the rationality of our method (many related BPs can be selected) but also the importance of two processes (response to oxygen levels and response to decreased oxygen levels) in ischemic stroke. The responses to oxygen levels and to decreased oxygen levels have been researched with regard to transport, oxygen homeostasis, translation, nitrogen fixation, and angiogenesis, which are involved in hypoxia, retinal neoplasms, tumor angiogenesis, retinoblastoma, and neoplasms [21-22]. A laboratory study conducted by Perez-Alvarez using an in vivo model revealed that mTORC1 (mammalian Target of Rapamycin Complex-1), a protein complex downstream of PI3K-Akt pathway, was dysregulated after ischemic stroke and oxygen–glucose deprivation [23]. This evidence highlights the importance of understanding the relation between response to oxygen levels and ischemic stroke. Few other topics concerning response to oxygen levels and response...
to degraded oxygen levels in ischemic stroke may provide scope for more novel studies in the future.

We further reconstructed the logistic model and identified top five miRNAs (hsa_mir_122, hsa_mir_99b, hsa_mir_339, hsa_mir_145, and hsa_mir_3130_1). Previously, based on the screening of miRNA functional synergistic network, miR-145, miR-122, and miR-99b have been implicated to be associated with ischemic stroke by participating in the processes of post-ischemic neuronal damage and thrombosis, respectively. Based on our search, we found that the hsa_mir_339 and hsa_mir_3130_1 have not been well studied in ischemic stroke, which provide a very valuable starting point for future biomarker selection studies. The miR-339 and miR-3130 were mainly reported to be tumor suppressors in previous studies owing to their biological roles in the suppression of cell proliferation. Additionally, Martinez et al. also illustrated that miR-339 in the cerebellum and plasma of rats could be perturbed by in vitro stimulation with agents ethanol and caffeine; this indicates the potential of miR-339 as a novel biomarker for ischemic stroke.

From the miRNA–mRNA regulatory network, two target genes (SP1 and AGO1) are speculated to be the primary genes of ischemic stroke. Argonaute 1 (AGO1) plays critical roles in RNA interference among the many regulators participating in microRNA formation. According to the findings reported by Shi et al., the expression of miR-103 is modulated by hypoxia-inducible factor 1α, which can target argonaute 1 (AGO1) to promote tumor vessel formation. Meanwhile, miR-103 can substantially affect angiogenesis and vascular density after ischemic stroke by targeting vascular endothelial growth factor (VEGF). SP1 (specificity protein 1) is a member of a family of transcription factors that include SP2, SP3, and SP4; these factors are implicated in various essential BPs and have been established to play important roles in cell growth, differentiation, apoptosis, and carcinogenesis. SP1 reportedly interacts with zinc finger protein 179 (Znf179), which is a neuroprotective factor for the accumulation of reactive oxygen species (ROS). Znf179 autoregulation through Sp1-dependent mechanism plays an important role in neuroprotection, and NGF-induced Sp1 signaling may help attenuate more extensive (ROS-induced)
damage following brain injury [30]. Both genes are related to ischemic stroke on some levels, and both those genes deserve to be investigationed further in detail.

In conclusion, in light of the fact that no gold standard treatment is currently available and that disease-specific prediction for ischemic stroke remains unreliable despite the presence of several standard criteria, we summarized the differential expression profile of miRNAs in ischemic stroke. The manner of expression of five miRNAs as well as of the two specific target genes (SP1 and AGO1) may provide new insights into the discovery of therapeutic biomarkers. Because many pathological states are known to alter miRNA profiles and functions, understanding those changes and developing new target genes to rectify them might lead to the formulation of novel therapeutic strategies.

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**Conflict of interest:** The authors declare no conflict of interests.

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**Figure legends**

**Figure 1. The overview of DEMs’ expression profile between ischemic stroke and normal samples.** Heat map display of the 14 differentially expressed miRNAs in all samples. The horizontal axis is the sample and the vertical axis is miRNA. Red represents high expression and blue represents low expression.

**Figure 2. The GO and KEGG enrichment analysis of the 1,600 target genes.** GO and KEGG enrichment results are shown in (A) and (B), respectively. The horizontal
axis in the figure represents the ratio of genes enriched, and the vertical axis represents the name of each biological process or pathway.

Figure 3. The collinear analysis and ROC curve of the differentially expressed miRNAs. (A) The collinear analysis of the 14 differentially expressed miRNAs. The darker the blue or red color, and the larger the area of blue or red color in the Pie, the greater the collinearity between them. (B) The ROC curve of the model. The AUC value of the Logistic model was found to be 0.8645. (C) The top five miRNAs were used to reconstruct the logistic model with the AUC value of the logistic model reached 0.8589. (D) The ROC curve of the model basing 5-fold cross validation.

Figure S1. Normalization of the raw miRNA expression profiles and differential expression miRNA analysis. (A) The distribution of miRNAs’ expression values in each sample after the data normalization; the horizontal axis is the sample and the vertical axis is the miRNAs expression level. (B) Volcano plot of the differentially expressed miRNAs. The horizontal axis is the log2-based fold change (Log2FC) and the vertical axis is -log10 (P-value). The blue dots in the figure represent up-regulated miRNAs and the red dots represent down-regulated miRNAs. The green dots are non-differentially expressed miRNAs.

Figure S2. The prediction of miRNAs target genes. (A) The regulatory network between 14 miRNAs and target genes. (B) The regulatory network between 5 identified miRNAs and target genes. Each rectangle in the figure represents a node (miRNA or mRNA). SP1 and AGO1 as well as their regulatory miRNAs were highlighted in yellow.
Figure 3

A. Corrgram of miRNA

B. 14miRNA AUC=0.8645

D. 5miRNA AUC=0.8589

Legend:

- AUC=0.84
- AUC=0.76
- AUC=0.65
- AUC=0.96
- AUC=0.65
Table S1 Target genes of DEMs.

ORA12
ZFYVE27
POU2F3
APOBEC3A
ANKRD36
ADM2
PHLD3A
CLCN6
THY1
SLC35E3
MAVS
RUNDC1
FBXO27
YIPF4
ORC6
HEYL
RRP36
ST3GAL1
VIM
DUSP18
ZNF790
POLR2D
CUBN
ABL2
FHDC1
LAMTOR3
DNAJB13
MYO1C
SHISA9
CDC37
CCDC142
SMAGP
BSCL2
HOXA7
TNFAIP8L1
UBE2G2
SPON2
NFIC
RAB11FIP1
COL13A1
MAFF
PRR23A
ARIH2OS
IYD
TMEM40
DSN1
ARSK
PIK3R2
PLXNA1
YME1L1
APOM
PHLDA2
CYP20A1
MYCN
FAM9B
SLC16A5
PRPF38A
MKLN1
SIK2
IBA57
ISY1-RAB43
MYH11
ZNF713
MFAP2
KCNA7
MAGT1
LONP2
SLC2A8
REL
FADS6
MLXIP
GPRIN3
SMYD4
SNTB2
CD3E
RBM43
HEATR5A
MED18
AKR7L
RPS15A
ACOT9
HM13
SEC14L4
GALNT6
LRRC3C
DCAF16
WDR17
KDELRC2
NFX1
HS3ST1
PLEKHS1
ZNF573
NOM1
SLC4A1
ZNF154
NUDT3
GPR161
OLA1
MRPS25
NKD1
ZYG11B
CCDC170
RAB43
PIGO
ZNF34
PRSS16
FLG2
GDF7
XIAP
FHL2
NKAP
BIRC5
TAS2RS5
ZNF841
PNMA2
TIMM50
PARVB
ADCY2
GMEB1
LYN
DCAF7
NOL10
TTLL12
ROBO1
OSBPL10
MED7
VWC2
ERC1
KRBA2
GNB1
QSOX1
PDP2
PLEKH3M
PGBD5
| Gene    |
|---------|
| ZNF347  |
| SPIB    |
| TEP1    |
| JPH2    |
| PLXDC2  |
| FAM217B |
| RAB10   |
| SBO1    |
| RACGAP1 |
| ARHGEF5 |
| PNPT1   |
| ZFP14   |
| HEBP2   |
| HSPA4L  |
| MMP17   |
| NFE2L1  |
| XPC     |
| SLC35E2B|
| SCAMP4  |
| SLC26A2 |
| HMOX1   |
| INTS3   |
| KDM6B   |
| ATP5F1  |
| TSHZ2   |
| HLA-E   |
| CENPM   |
| ECE1    |
| ANKRD9  |
| METTL2B |
| GPSM2   |
| TIMM8A  |
| THAP2   |
| C3orf62 |
| PIGX    |
| COPA    |
| TERF2   |
| ARSA    |
| SMG1    |
| NKPD1   |
| GK5     |
| ESYT2   |
| LRCH3   |
| GFPT1   |
| CINP    |
SLC16A10
MTAP
GTF2F1
DGKE
APOA1
RNF170
PSMB5
KAT7
MASTL
ALG1
LIMS1
IDS
DPY19L4
PRMT3
RFX7
SAR1A
NAGK
PTPN2
TFDP2
EEF2K
CDK4
ORC1
LMNB2
MEAF6
ANG
MGAT1
MYCBP
ABCF1
RGS9BP
FMN1
DNPEP
RAB32
BAMBI
SLC25A33
SLC1A5
CALCOCO2
AP1S1
NAA50
MTO1
FLYWCH2
YWHAB
RBM23
MSRB1
MED16
RPS6KA5
UROS
TIGD6
ISPD
OPTN
ZNF74
ITPA
RNF19B
BMS1
PIGG
CEP89
QPRT
SLC38A9
KDEL1
ESF1
TIAL1
MAPKAPK5
MAP4K2
DNAJC10
LYRM4
ZC3H15
ROM01
EMC3
KLHL7
ACP6
PTCD3
ALG14
SOD2
XPO5
LUC7L
C3
KCMF1
BROX
SF3A1
FFAR1
POTED
CCS
FIG4
GRSF1
ZNF786
MRPS23
BTN3A2
ZNF281
CYB5D1
LILRA2
ALOX5AP
| Gene Symbol | Gene Symbol |
|-------------|-------------|
| NEGR1       | STAT2       |
| CCDC80      | GTF3C6      |
| SETD7       | TNRC6B      |
| DDX19B      | PRIM1       |
| ODF2L       | PNRC1       |
| ZNF670      | ZFPM1       |
| RBM27       | ORC4        |
| NNT         | FUS         |
| IER3        | SHISA6      |
| ZNF444      | SLC38A7     |
| ABHD12      | HIF3A       |
| TMEM164     | MPDU1       |
| BAX         | NOVA2       |
| CACNG7      | CS          |
| SMIM7       | EN2         |
| RAD51B      | WIPF2       |
| CD209       | CCL11       |
| PEX11B      | CLPSL1      |
| DNAJC8      | WNT8B       |
| SNAP25      | FAM168A     |
| KIAA1671    | SIX3        |
| DNAH10OS    | GBA2        |
| NPLOC4      |             |
RPL13A
RAB34
ZBTB4
HBP1
LCE1A
KDM2A
HPCAL1
C16orf58
ANKRD42
DFFB
EDA2R
FCRL2
DBNDD2
ZNF317
FAM131B
HNF4A
LRRC59
PFN1
KSR2
RAB15
CBX7
NIPAL3
RAPGEF1
PHC2
CLMP
BTF3L4
DAD1
KCN5
C4orf26
ADAMTS11
PIP4K2A
HYOU1
CDCA8
DPCR1
GABRB2
ALKBH5
PIP4K2C
LIF
CRCP
C6orf106
KIAA1755
PHB2
NCEH1
MYLK3
SLC2A4
KLK10
EZH1
SET
WDR13
YWHAZ
COX6B1
TPD52L2
SOWAHA
FN3K
ZNFS29
VAV3
SAP18
PPP1R18
TGF1
ZMAT5
MEX3A
BMP3
MIDN
REG3A
MCF2L2
PNRC2
STMN3
AOC3
ZNFS90
AFF3
SC5D
TMEM170A
TFPI
PPP2R2A
SMAD7
AEN
GMEB2
ZNFS460
CRTC2
ENTPD1
LINGO1
RNF125
GAN
RPS9
GLG1
ATG13
CBL
ZDHHC9
TAF8
VPS4A
SLC25A37
CYP4F3
TIRAP
ARL2
CNNM4
NUAK2
CDK2
CBS
TET3
GATAD2B
EPB41
PEG10
PAX2
PLA2G2F
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GATAD2A
CLPB
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SORCS2
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CENPL
SSH3
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POFUT2
NUFIP2
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| BTD    |
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| ERG    |
| DUSP6  |
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| KLF5   |
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| ARF6   |
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| CFTR   |
| PSAT1  |
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| Gene       |
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| MAP3K3     |
| MTDH       |
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| DDI2       |
| VGLL4      |
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| DDX6       |
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| PRKRA      |
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| FUNDC2     |
| G6PC3      |
| FOXK2      |
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| PDK4       |
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| MIPOL1     |
| DUSP2      |
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| SUCLA2     |
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| CALM3      |
| OCLN       |
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| KIF5B      |
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| HECTD3     |
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| NDRG3      |
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| EGLN3      |
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| LONRF1       |
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| KLF7         |
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| SHCBP1       |
| NFIB         |
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| TRAK2        |
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| TMBIM6       |
| RHOB         |
| HOMER1       |
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| KCNJ2        |
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| OTUD1 |
| RGL1  |
| SNX17 |
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| KPNA6 |
| BTBD7 |
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| LDLR  |
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| PFN2  |
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| REEP3 |
| MIER1 |
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| MMGT1 |
| FBXO28 |
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| RAPGEF2 |
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| TNPO2 |
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| CREBL2 |
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| GIYF1 |
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ARID2  
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SUZ12  
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BLCAP  
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MECP2  
DICER1  
NF1  
UBE2A  
ZFYVE26  
ZBTB7B
