Supplementary File

Leukocyte transcriptome of Cushing's disease are associated with nerve impairment and psychiatric disorder

1 Supplementary methods

1.1 Detailed diagnostic criteria of CD patients

Functional diagnosis was first made to confirm the presence of endogenous hypercortisolism: elevated cortisol secretion rates (reference range at 08:00: 198.7–797.5 nmol/L), 24-hour urinary free cortisol (24 h-UFC, reference range 98.0–500.1 nmol/24 h), late-night salivary cortisol (>4 nmol/L), clinical features associated with cortisol overexposure (e.g. moon face, central obesity, purpura, diabetes, dorsocervical fat pad, hypertension, psychiatric deficits and neurocognitive deficits, immunosuppression), absence of low-dose dexamethasone suppression (1 mg overnight or 2 mg/d for 48 h) and abnormal cortisol circadian. The aetiological diagnosis was also made to identify ACTH as dependent or independent: normal or high serum ACTH (reference range at 08:00: <10.12 pmol/L). We also performed localization diagnosis to determine eutopic or ectopic: existence of high-dose dexamethasone suppression (>50% suppressed, 8 mg/d for 48 h) and positive dynamic gadolinium-enhanced pituitary MRI. Postoperative pathology verified the diagnosis and excluded the possibility of malignancy.

1.2 Detailed methods for RNA extraction, quality control, sample preparation, transcriptome sequencing, and upstream analysis

RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit®2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext®UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo attached magnetic beads.

Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLV reverse transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 250~300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and 150 bp paired end. Use fastp v 0.19.3 to filter the original data, mainly to remove reads with adapters; when the N content in any sequencing reads exceeded 10% of the base number of the reads, the paired reads were removed; when any sequencing reads When the number of low-quality (Q<=20) bases contained in reads exceeds 50% of the bases of the reads, this paired reads were be removed. All subsequent analyses were based on clean reads. The reference genome and its annotation files can be downloaded from http://ftp.ensembl.org/pub/release-105/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz and http://ftp.ensembl.org/pub/release-105/gtf/homo_sapiens/Homo_sapiens.GRCh38.105.chr.gtf.gz. HISAT v2.1.0 and featureCounts 1.6.1 wre used to construct the index, and to compare clean reads to the reference genome to get the raw count data. Quality control data can be found in Table S13. Overall distribution of gene expression can be seen in Figure S1, which showed no batch effect.

1.3 Detailed methods for WGCNA

The raw count data was first normalized using the variance stabilizing transformation (VST) method by the DESeq2 R package (v1.34.0). Correlation matrices estimated by the robust correlation measure of biweight midcorrelation were calculated and converted into an adjacency matrix holding correlation symbols. Next, these adjacent matrices were raised to 26 soft power (Figure 1A; Tab. S10). This soft power was selected by the function pickSoftThreshold() in the the WGCNA R package. Next, the soft power threshold adjacency matrix was converted into a topological superposition matrix (TOM) and then a 1-TOM matrix. Then the average linkage method was used to put the 1-TOM matrix into the agglomerative hierarchical clustering. Gene modules were defined from the clustering tree, and the branches were cut using the hybrid dynamic tree cutting algorithm (deepSplit parameter = 4) (Fig. 1B). The module was merged with 0.25 cutting height and the minimum module size was set to 100. Only genes with a module membership of $r > 0.2$ were retained within modules. For each gene module, module eigengene was calculated as the first PC of the scaled (standardized) module expression profiles (Tab. S11). Module membership for each gene and module was also calculated. The module membership represents the correlation between each gene and the module eigengene (Tab. S12). Genes that could not be clustered into a particular module remained in the M0 module and will not be considered in future analysis.

1.4 PCR

We recruited additional subjects with the same diagnostic and enrolment criteria as before, including 5 patients with CD as well as 4 healthy subjects. Venous blood and leukocytes werde obtained under the same conditions. Total RNA was extracted using the RNeasy MinElute Cleanup Kit (QIANGEN, Germany). Reverse transcription of mRNA was carried out using FastKing gDNA Dispelling RT SuperMix (Tiangen, China) according to the manufacturer's instructions. The relative quantity of mRNAs was measured by SYBR Green PCR assays by Talent qPCR PreMix(SYBR Green) (Tiangen, China). ACTB was used as internal control. Primers were synthesized by Biomed (Beijing, China). Primer sequences were obtained from the PrimerBank [1]: ACTB: Forward 5'- CATGTACGTTGCTATCCAGGC-3' Reverse 5'-CTCCTTAATGTCACGCACGAT-3'; CAMP: Forward 5'- AGGTCCTCAGCTACAAGGAAG-3' Reverse 5'- TCTTGAAGTCACAATCCTCTGGT-3'; MMP9: Forward 5'- TGTACCGCTATGGTTACACTCG-3' Reverse 5'- GGCAGGGACAGTTGCTTCT-3'; TIGIT:

Forward 5′- TGGTCGCGTTGACTAGAAAGA-3′ Reverse 5′- GGGCTCCATTCCTCCTGTC-3′; ABCB1: Forward 5′-GGGATGGTCAGTGTTGATGGA-3′ Reverse 5′- GCTATCGTGGTGGCAAACAATA-3′; AKR1C3: Forward 5′-GAGACAAACGATGGGTGGACC-3′ Reverse 5′- TGGAACTCAAAAACCTGCACG-3′; GPER1: Forward 5′-CACCAGCAGTACGTGATCGG-3′ Reverse 5′- CATCTTCTCGCGGAAGCTGAT-3′. Data were represented as means ± standard deviation (SD). One-sample t-test was used to check the significance of the differences in mean values using Graphpad Prism 8.0 (CA, USA).

1.5 References of the questionnaire and R packages used in this research

Self-Rating Depression Scale (SDS) [2]

Self-Rating Anxiety Scale (SAS) [3]

DESeq2 R package(v1.34.0) [4]

WGCNA R package(v1.71) [5]

References

1. Spandidos A, Wang X, Wang H, et al. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res. 2010; 38(Database issue): D792–D799, doi: 10.1093/nar/gkp1005, indexed in Pubmed: 19906719.
2. Zung W. The depression status inventory: An adjunct to the self-rating depression scale. Journal of Clinical Psychology. 1972; 28(4): 539–543, doi: 10.1002/1097-4679(197210)28:4<539::aid-jclp2270280427>3.0.co;2-s.
3. Zung WW. A rating instrument for anxiety disorders. Psychosomatics. 1971; 12(6): 371–379, doi: 10.1016/S0033-3182(71)71479-0, indexed in Pubmed: 5172928.
4. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014; 15(12): 550, doi: 10.1186/s13059-014-0550-8, indexed in Pubmed: 25516281.
5. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 2008; 9: 559, doi: 10.1186/1471-2105-9-559, indexed in Pubmed: 19114008.

Figure S1. Box plot of the overall gene expression of every sample. The y-axis is the log10 (FPKM), while the x-axis represents every sample. No batch effect was found.

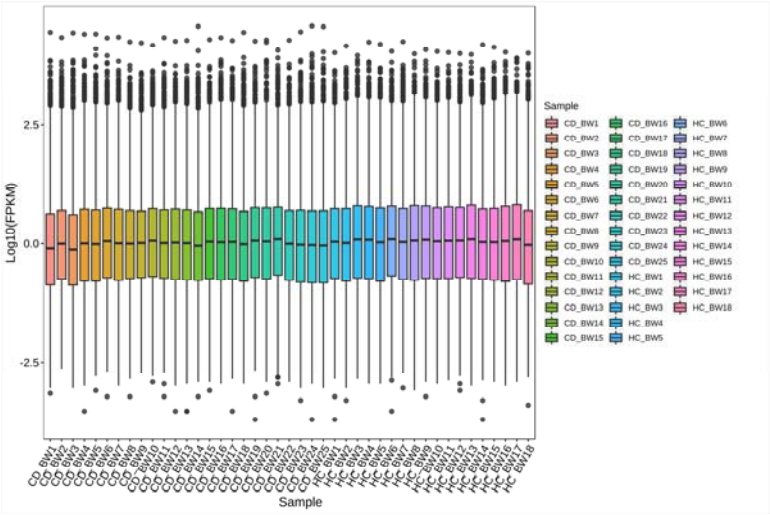

Figure S2. Soft power of group CD (top), group HC (down). The meaning is the same as Figure 1A.

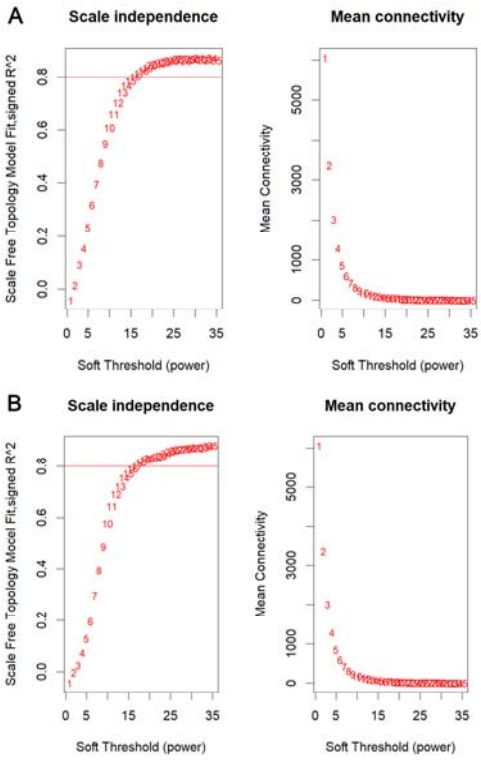

Figure S3. Polymerase chain reaction (PCR) results. Left: up-regulated genes; Right: down-regulated genes. All results passed one-sample t-test $p < 0.05$.