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LETTER TO THE EDITOR

Comparison of Reference Genes for Transcriptional Studies in Postmortem Human Brain Tissue Under Different Conditions

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Dear Editor

Research on neurodegenerative diseases is a hot topic worldwide [1], and MRI, genetics and epigenetics, and animal models have been commonly used. Considering species differences, research on human brain samples is irreplaceable [2]. Many countries and regions have built brain banks to collect and store brain tissue from donors.

Samples provided by a brain bank can be used to study synaptic structure, protein, DNA, RNA [3, 4], and lipids to illuminate the pathological mechanisms underlying neurodegenerative diseases. When brain tissue is used for gene expression research, the quantitative real-time polymerase chain reaction (RT-qPCR) is an accurate method, which simultaneously amplifies and quantifies the expression of target genes by measuring the intensity of fluorescence in each PCR cycle; it has the advantages of high efficiency, sensitivity, accuracy, and specificity, along with low cost [5]. However, the value of the PCR cycle threshold requires accurate normalization with internal reference genes whose expression must be stable and independent of the target genes [6]. The quality of brain samples can be influenced by the time interval between death and tissue acquisition [4] and the agonal state of the donor [7]. The transcription of genes may be influenced by anamnesis of the donor, post-mortem interval (PMI), and the storage time (ST) of brain samples. Thus, choosing a suitable reference gene with a stable transcript in different brain samples to standardize the target gene transcription levels is crucial for relevant studies when using postmortem human brain samples.

Many internal reference genes have been used in transcription studies [8], but which are most suitable for human brain tissue studies needs to be explored. To identify suitable reference genes for the accurate measurement of target gene expression, transcripts of seven commonly used reference genes (*18s*, *ACTB*, *CYC1*, *GAPDH*, *HPRT1*, *UBC*, and *TBP*) were assessed in frontal pole tissue from 163 human brain samples, and subsequent data analysis was performed using the GeNorm (GMC, Ghent, Belgium) and NormFinder (MDL, Aarhus, Denmark) algorithms.

All brains were from the Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC) Human Brain Bank, which collects brains from donors through a willed whole-body donation program. All donors had given informed consent for using the donated tissues for medical research. The present study was approved by the Institutional Review Board of the Institute of Basic Medical Sciences, CAMS (Approval Number: 009-2014, 031-2017). All the brain samples were harvested according to the standardized operational protocol for human brain banking in China published in 2017 by the China Human Brain Bank Consortium [9]. The 163 brain samples were divided into four groups based on the

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anamnesis of donors, age of donors, PMI and ST (defined as the interval from brain sample storage in a −80°C refrigerator to RNA extraction). Information about the donors including age, gender, PMI, ST and the 260 nm/280 nm optical density (OD) ratio of RNA samples is summarized in Table S1.

The transcripts of seven reference genes were measured by RT-PCR in the 163 human brain samples (basic information on candidate reference genes and primer sequences in Table S2). In an RT-qPCR cycle, a positive reaction was detected by accumulation of a fluorescent signal, and the Cq value (cycle threshold) was defined as the number of cycles required for this signal to reach a defined threshold. Cq levels are inversely proportional to the logarithm of the amount of target nucleic acid in the sample, that is, a higher Cq indicates a lower transcript level of the gene. The results showed that the *18s* transcript had the highest concentration (lowest Cq value) while the *UBC* transcript had the lowest (highest Cq value) among these reference genes. The Cq values of four candidate genes, *UBC, HPRT1, TBP*, and *CYC1,* surpassed 20 cycles in all samples, and the cycles of *ACTB* and *GAPDH* were about 20 among samples. Apart from *18s*, *ACTB* and *GAPDH* had the highest content compared with other genes in brain tissue (Fig. 1A). Based on the anamnesis of donors, the samples were divided into 6 groups with: diseases of the nervous system (DNS), cancer, heart-lung disease (HLD), multiple organ failure (MOF), diabetes and hypertension (D&H), and unknown/other (Other). The transcript of each reference gene was compared among the different groups (Fig. 1B). The D&H group had higher mean Cq values of 5 candidate genes (*ACTB*, *GAPDH*, *TBP*, *HPRT1*, and *UBC*) than the other groups, indicating lower transcript levels of these genes. The transcript of *CYC1* was lower in the HLD and Other groups. With increasing donor age, the Cq value of reference genes rose, especially in *HPRT1*, *ACTB*, and *GAPDH* (Fig. 1C). The Cq values for reference genes showed an ascending tendency with increasing PMI (Fig. 1D) and an adverse weak decline with increasing ST (Fig. 1E).

The stability of the 7 reference genes was evaluated using GeNorm and NormFinder softwares. The results from GeNorm showed that *CYC1* and *TBP* had the lowest average expression stability value (most stable) among all candidate reference genes among different clinical histories (Fig. 2A) and when the cases were grouped according to

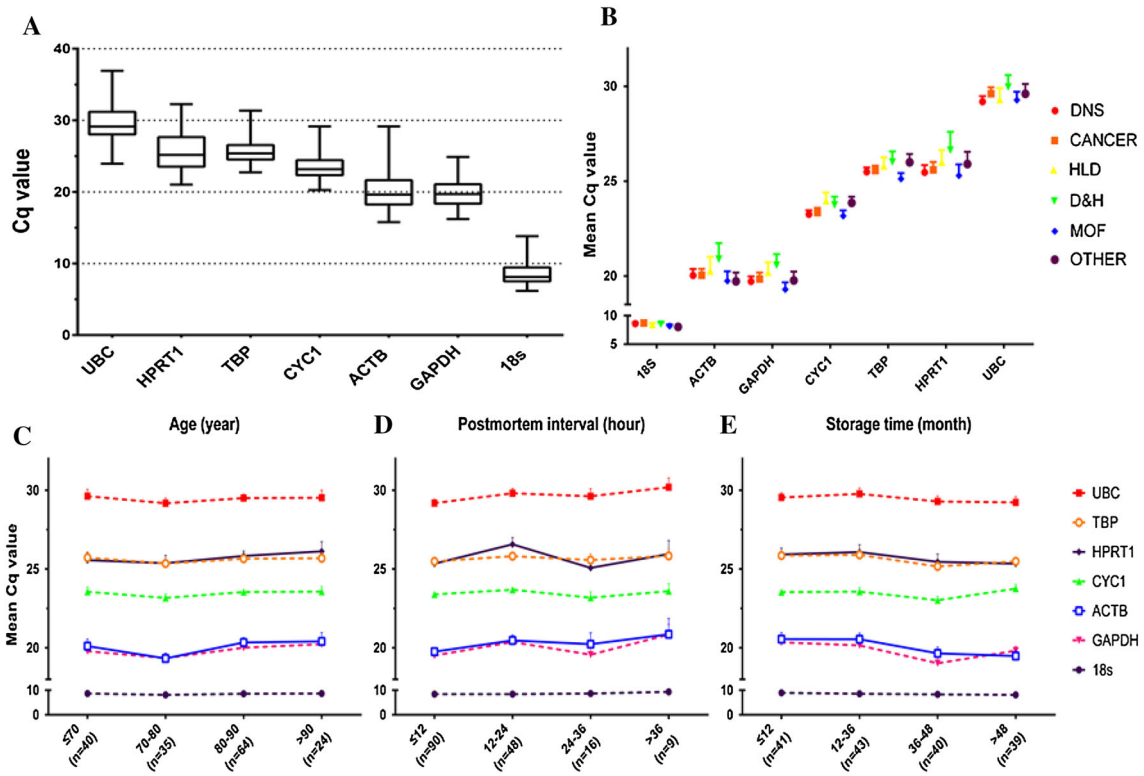

Fig. 1 Variation Cq values of reference genes. **A** Cq values of reference genes in all brain samples. Box-and-whisker plots showed the Cq value of each candidate gene in 163 brain tissues. Boxes indicated median and quartiles first and third and whiskers equivalent to the minimum and maximum of Cq value. **B** Distribution of Cq values of reference genes in different disease states. **C–E** Cq values of reference genes according to age of donors, postmortem interval and storage time. *DNS*, disease of nervous system; *HLD*, heart-lung disease; *MOF*, multiple organ failure; *D&H*, diabetes and hypertension; *Other*, anamnesis unknown or other diseases.

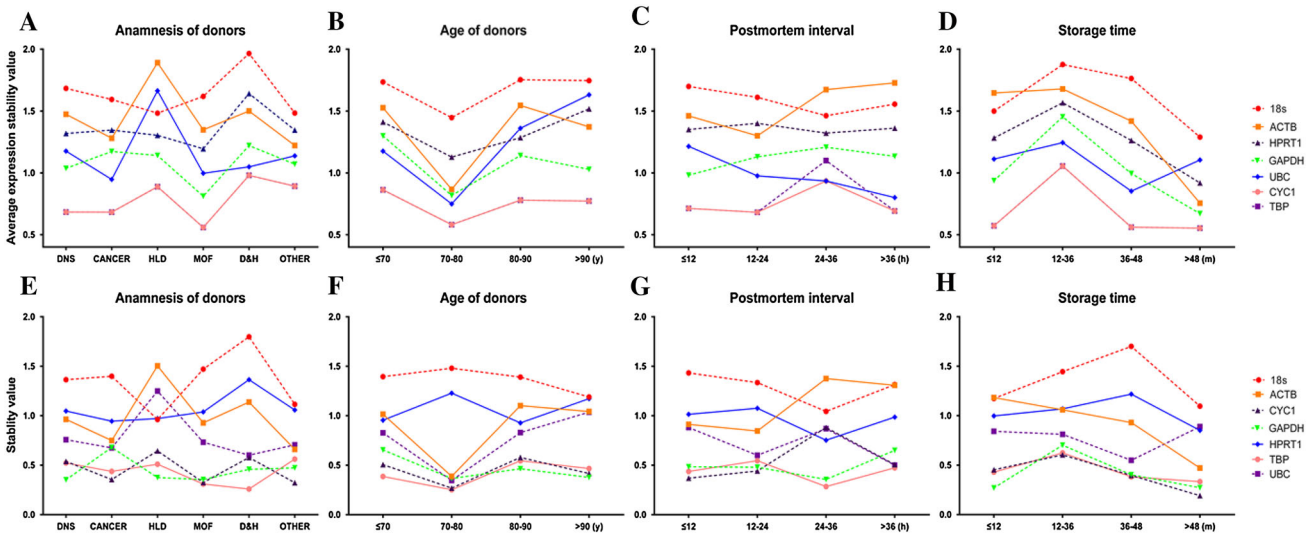

Fig. 2 Analysis of reference gene expression using GeNorm (A–D) and NormFinder (E–H) algorithms. **A**, **E** Comparison of the expression of reference genes in the brain tissue from different anamnesis of donors. **B**, **F** Comparison of the expression of reference genes in the brain tissue from different age groups of donors. **C**, **G** Comparison of the expression of reference genes in the brain tissue with different postmortem interval. **D**, **H** Comparison of the expression of reference genes in the brain tissue with different storage time.

age (Fig. 2B). Although the average expression stability value of *UBC* was less than that of *CYC1* when the PMI was 24–36 h (Fig. 2C), *CYC1* and *TBP* had the lowest value in other PMI groups and in all ST groups (Fig. 2D).

There were differences between the results of the NormFinder and GeNorm algorithms. Results from NormFinder showed that *CYC1* had a minimal stability value in the cancer, MOF, and Other groups. *TBP* had a minimal stability value in the MOF and D&H groups. *GAPDH* was the most stable reference gene in the DNS and HLD groups (Fig. 2E). *CYC1* and *TBP* were stable internal reference genes with lower stability values when the donors were <80 years old (Fig. 2F). The stability values of candidate genes by NormFinder in each group with different PMIs are shown in Fig. 2G. When PMI was <24 h, the *CYC1* was the most stable gene, but in other PMI groups the *TBP* was the most stable. Considering the influence of ST, we found that *CYC1* and *TBP* were still stable in samples with ST >12 months, but in the groups with ST <12 months *GAPDH* was the most stable (Fig. 2H).

Some transcriptional studies have verified suitable internal control genes by RT-qPCR analysis in human brain samples. *CYC1* may be a suitable reference gene in human postmortem cerebral cortex samples [10]. In human glioma [11], *GAPDH* may be recommended as the best candidate gene in transcriptional studies of astrocytoma among *GAPDH, HPRT1, POLR2A, RPLP0, ACTB,* and *H3F* [12], while *TBP* may be the most stable gene in hippocampal tissues from rats [13]. In our study, *CYC1* and *TBP* were both suitable reference genes for gene transcription studies in postmortem human brain tissue according to the results from GeNorm and NormFinder, because the two genes had a smaller stability value (more stable transcriptional expression) among all candidate reference genes, among different groupings based on clinical history, PMI, ST, and donor age. According to NormFinder, *GAPDH* can be regarded as the suitable reference gene for brain samples whose donors are older than 90 years. Other candidate genes have a large range of fluctuation in different sample groups and higher stability values, which implies that the transcript of other genes may have poor stability in our samples under different conditions.

In summary, our data showed that *CYC1* and *TBP* were the most stable among the candidate genes investigated, so they might be suitable internal genes for gene transcription studies of human brain tissue. *18s* and *HPRT1* had the worst stability in all samples, indicating that they may not be suitable as reference genes. The transcript abundance of these genes declined with prolongation of PMI, so the brain samples should be acquired as soon as possible to guarantee a shorter PMI. Our results provide several potentially appropriate reference genes for transcriptional studies in human tissue under different conditions and may facilitate the development and research of human brain banks in China and worldwide.

Limitations of this Study

When assessing RNA and brain sample quality in postmortem human brain tissue, the RNA Integrity Number (RIN) is often considered to be a critical measurement, but some studies have also shown that RIN has limited predictability for postmortem human brain tissue [14] and is not necessarily a reliable predictor of mRNA quality [15]. Therefore, we did not use RIN as an indicator of tissue RNA quality.

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Conflict of interest There are no potential conflicts in the financial and material support for this research.

References

1. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med 2013, 19: 983–997.
2. Samarasekera N, Al-Shahi Salman R, Huitinga I, Kliloueva N, McLean CA, Kretzschmar H, *et al*. Brain banking for neurological disorders. Lancet Neurol 2013, 12: 1096–1105.
3. Song CX, Clark TA, Lu XY, Kislyuk A, Dai Q, Turner SW, *et al.* Sensitive and specific single-molecule sequencing of 5-hydroxymethylcytosine. Nat Methods 2011, 9: 75–77.
4. Durrenberger PF, Fernando S, Kashefi SN, Ferrer I, Hauw JJ, Seilhean D, *et al.* Effects of antemortem and postmortem variables on human brain mRNA quality: a BrainNet Europe study. J Neuropathol Exp Neurol 2010, 69: 70–81.
5. Liu Q, Qi X, Yan H, Huang L, Nie G, Zhang X. Reference gene selection for quantitative real-time reverse-transcriptase PCR in annual ryegrass (*Lolium multiflorum*) subjected to various abiotic stresses. Molecules 2018, 23: 172.
6. Niu B, Zhang T, Hu HQ, Cao BZ. Transcriptome sequencing reveals astrocytes as a therapeutic target in heat-stroke. Neurosci Bull 2017, 33: 627–640.
7. Sherwood K, Head M, Walker R, Smith C, Ironside JW, Fazakerley JK. A new index of agonal state for neurological disease. Neuropathol Appl Neurobiol 2011, 37: 672–675.
8. Eisenberg E, Levanon EY. Human housekeeping genes, revisited. Trends Genet 2013, 29: 569–574.
9. Qiu WY, Ma C, Bao AM, Zhu KQ, Huang Y, Yan XX, *et al.* Standardized operational protocol for human brain banking in China. Acta Anat Sin 2017, 48: 282–289.
10. Penna I, Vella S, Gigoni A, Russo C, Cancedda R, Pagano A. Selection of candidate housekeeping genes for normalization in human postmortem brain samples. Int J Mol Sci 2011, 12: 5461–5470.
11. Grube S, Gottig T, Freitag D, Ewald C, Kalff R, Walter J. Selection of suitable reference genes for expression analysis in human glioma using RT-qPCR. J Neurooncol 2015, 123: 35–42.
12. Gresner SM, Golanska E, Kulczycka-Wojdala D, Jaskolski DJ, Papierz W, Liberski PP. Selection of reference genes for gene expression studies in astrocytomas. Anal Biochem 2011, 408: 163–165.
13. Swijsen A, Nelissen K, Janssen D, Rigo JM, Hoogland G. Validation of reference genes for quantitative real-time PCR studies in the dentate gyrus after experimental febrile seizures. BMC Res Notes 2012, 5: 685.
14. Sonntag KC, Tejada G, Subburaju S, Berretta S, Benes FM, Woo TU. Limited predictability of postmortem human brain tissue quality by RNA integrity numbers. J Neurochem 2016, 138: 53–59.
15. Ruzicka WB, Subburaju S, Benes FM. Circuit- and diagnosis-specific DNA methylation changes at gamma-aminobutyric acid-related genes in postmortem human hippocampus in schizophrenia and bipolar disorder. JAMA Psychiatry 2015, 72: 541–551.