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Original Article

Relative exchangeable copper, a high-quality biomarker for differentiation of Traditional Chinese Medicine syndrome in Wilson's disease

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Abstract

OBJECTIVE: To investigate the value of relative exchangeable copper (REC) in diagnosing Wilson's disease (WD) and to determine its significance in the differentiation of Traditional Chinese Medicine (TCM) syndrome.

METHODS: A total of 78 patients with WD were recruited on the same day of the medical visit, and among them, 32 were suffering from non-WD (N-WD) and 37 were heterozygous ATP7B carriers (HC) enrolled as controls. Molecular genetic testing was performed for diagnosing WD and HC. Additionally, REC levels in different TCM syndromes were investigated. The correlation between REC and serum ceruloplasmin (Cp), serum copper oxidase (CO), and 24-h urinary copper was analyzed using the Global Assessment Scale and Unified Wilson's Disease Rating Scale and the significance of REC in WD diagnosis was investigated. Resting-state functional magnetic resonance imaging was used to assess the clinical symptoms of WD and analyze its severity in different TCM syndromes.

RESULTS: REC determination helped in significantly distinguishing patients with N-WD or HC from those with WD with a cut-off of 21.15%. Furthermore, the comparative analysis of REC ratios among different TCM syndromes showed markedly high REC levels in the dampness-heat internal accumulation syndrome group. Additionally, the seed-based functional connectivity value from the putamen to the cerebellum was significantly correlated with TCM syndromes.

CONCLUSION: REC level is a high-quality biomarker for diagnosing WD that effectively reflects disease severity and plays an essential role in deciding on treatment strategies and prognosis. Furthermore, REC levels are closely related to TCM syndromes in WD; thus, it is a potential objective quantitative indicator for distinguishing TCM syndromes in WD.

Keywords: Wilson's disease; relative exchangeable copper; Traditional Chinese Medicine syndrome; Global Assessment Scale; the Unified Wilson's Disease Rating Scale

1. INTRODUCTION

Wilson's disease (WD) is a rare autosomal recessive disorder related to copper metabolism, and it occurs because of mutations in the *ATP7B* gene, which results in toxic copper overload mainly in the liver and central nervous system.[1] Early and prompt diagnosis is the key to improving the therapeutic effect of a treatment strategy against the disease.[2-5] However, rapid and

accurate methods available for WD diagnosis are scarce. Furthermore, studies on the quantitative indicators of Traditional Chinese Medicine (TCM) syndrome differentiation are lacking.

Currently, no single diagnostic method is available that can exclude or confirm WD with 100% certainty and rapidity, and molecular testing is considered the gold standard;[6] however, the delay in detection time and high costs may delay the initiation of appropriate treatment.

Exchangeable serum copper (CuEXC) and relative exchangeable copper (REC) are high-quality biomarkers for WD dissemination and severity [7-9] that can be used to diagnose WD with high sensitivity and specificity.[10,11] Françoise *et al* [12] reported that REC, with a ratio > 19%, could distinguish Long-Evans rats from normal rats at every time point, and the sensitivity and specificity of the biomarker for adult rats reached 100%.

TCM plays a vital role in treating various serious diseases including WD. TCM syndrome is the core concept of TCM diagnosis, and the accurate differentiation of TCM syndromes is crucial for TCM clinics as it is considered a prerequisite for effective treatment.[13]

The diagnosis and differentiation of TCM syndromes are highly subjective, which is why classifying TCM syndromes is difficult, especially for patients with simultaneous multiple syndromes.[14-16] Therefore, identifying objective indicators is necessary to externalize TCM-syndrome classification.

One such sensitive and specific marker, REC, is a promising tool for WD diagnosis owing to its high sensitivity and specificity.[8,11,17] Thus, herein, we aimed to investigate the significance of REC in WD diagnosis and determine its potential in differentiating and classifying TCM syndromes in WD.

2. PATIENTS AND METHODS

2.1. Study population

This study was conducted at the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine, Department of Neurology, from July 2021 to August 2023. A total of 110 consecutive patients [78 patients with WD and a control population of 32 patients with non-Wilsonian liver diseases (N-WD)] and 38 heterozygous ATP7B carriers (HC) were enrolled. The 78 patients were diagnosed according to the American Association for Study of Liver Diseases, European Association for the Study of the Liver, and Indian criteria. The score of more than 4 was considered the threshold.[18-20] Participants who were pregnant or lactating or with severe mental disorders and unable to cooperate were excluded from the study. The study was conducted in accordance with the Declaration of Helsinki, approved by the First Affiliated Hospital of Anhui University of Chinese Medicine (2021AH-60), and reported according to the Standard for Reporting Diagnostic Accuracy guidelines.[21]

The control group included 32 patients with N-WD suffering from other chronic liver diseases and 38 HC who were siblings of the confirmed patients who were simultaneously identified as heterozygous by molecular testing. N-WD diagnosis was confirmed according to the usual tools. Those diagnosed with cryptogenic diseases underwent extensive testing to exclude the possibility of WD. The presence of cirrhosis was defined based on the presence of classic histological features, morphological features, and/or results of non-invasive electrometry.[22,23]

The TCM syndrome diagnosis standard refers to the TCM classification of WD, which has been established by the National Administration of Traditional Chinese Medicine Clinical Pathway for Wilson's disease (2011 trial version).[24,25]

Herein, the TCM syndrome differentiation type was diagnosed by two (deputy) chief TCM physicians. Among the patients with WD, the most frequent TCM syndrome of WD was Phlegm-stasis combination syndrome (P-SCS, 28 cases), followed by liver-kidney *Yin* deficiency syndrome (L-KYD, 26 cases) and Dampness-heat internal accumulation syndrome (D-HIA, 24 cases).

2.2. Methods

Serum ceruloplasmin (Cp), total serum copper, alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (AKP), serum copper oxidase (CO), CuEXC, and REC were detected in all patients. Additionally, molecular genetic testing was performed to detect mutations in the *ATP7B* gene to further confirm the diagnosis.

2.2.1. CuEXC and REC testing

As reported previously, blood samples were collected in Vacutainer® tubes without ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and subsequently treated within 30 min. Next, the samples were centrifuged at 3000 rpm for 10 min. Further, 1 mL of serum was diluted with 1 mL of EDTA (1∶1) in 0.9% NaCl (1∶1) and incubated for 1 h at room temperature. Then, the diluted serum was ultrafiltered through the Amicon® Ultra-4 centrifugal filter device with a 30-kD cut-off cellulose membrane (Merck Millipore Ltd., Tullagreen, Ireland). Copper levels were measured by flame atomic absorption spectrometry (5500S, Bohui, Beijing, China). REC levels were measured by determining the ratio of exchangeable copper/total serum copper.[8,10]

2.2.2. Molecular genetic testing

Molecular genetic testing was performed on 21 exons and intron-exon boundary regions of the *ATP7B* gene. Bi-directional sequencing and multiplex ligation-dependent probe amplification were performed in all patients with WD.[6] For the patients with HC, molecular genetic testing was performed based on findings in the proband.

2.2.3. Assessment of clinical symptoms

The Global Assessment Scale (GAS) and the Unified Wilson's Disease Rating Scale (UWDRS) were used to assess clinical symptoms.[26,27] This evaluation was performed by several experienced neurologists.

2.2.4. Resting-state functional magnetic resonance imaging scans analysis of brain damage

The 3.0 Tesla whole-brain magnetic resonance system (Discovery MR750, GE Healthcare, Milwaukee, WI, USA) was used to detect brain injuries. Out of 78, 58 patients with WD eventually completed the test successfully. Subregions of the lenticular nucleus were selected as regions of interest on the basis of previous studies.[28,29] The lenticular nucleus was divided into eight subregions, namely ventral anterior putamen (left and right PUT-VA), PUT-DA, dorsal anterior putamen (left and right PUT-DA), dorsal posterior putamen (left and right PUT-DP) and ventral posterior putamen (left and right PUT-VP).

A functional connectivity (FC) analysis was performed using the Analysis of Functional NeuroImages software (version 19.3.08, Medical College of Wisconsin, Milwaukee, WI, USA). The Fisher r-to-*z* transformation was performed for further conversion into a *z*-value to improve normality.

2.3. Statistical analysis

All experimental data were statistically analyzed using the SPSS software (version 25.0, IBM Corp., Armonk, NY, USA). Normally distributed data were presented as the mean ± standard deviation. Additionally, the data were analyzed by performing a one-way analysis of variance, followed by Tukey's multiple comparisons post-hoc test or Dunnett's T3 post-hoc test and χ^2 test for qualitative data. Pearson's analysis was performed to determine the correlation between the groups. *P*-values were based on two-tailed comparisons, and those less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Patient characteristics

The primary characteristics of the patients enrolled in this study are presented in Table 1. A total of 78 patients with WD [WD group, with a mean age of (24 ± 8) years] were recruited in this study on the same day of their medical visit, and their serum Cp, total serum copper, and exchangeable copper (CuEXC, with REC calculation) levels were measured. A total of 32 patients were suffering from N-WD [N-WD group, with a mean age of (22 ± 8) years], and 37 patients with HC [with a mean age of (24 ± 7) years] were enrolled as controls. Among the patients in the WD group, 54 (69.23%) patients presented with neurologic or mixed symptoms (hepatic + neurologic symptoms), whereas 22 (28.21%) patients presented with hepatological manifestations. A total of 73 patients (93.59%) showed Kayser-Fleischer rings in the cornea, as analyzed by the slit lamp examination.

The National Administration of TCM Clinical Pathway for Wilson's Disease system was used to distinguish and classify TCM syndrome types. Among the 78 patients, 28 showed the manifestation of P-SCS, and thus were categorized into the P-SCS group (28 cases), followed by 26 cases in the L-KYD group and 24 cases in the D-HIA group according to their manifestations of L-KYD or D-HIA, respectively.

3.2. REC ratios and serum Cp

Herein, immunoturbidimetry was performed to detect serum Cp levels, which showed that the serum Cp levels decreased significantly in the WD group compared with those in the N-WD and HC groups (both $P < 0.01$). No significant differences were observed in serum copper levels between the N-WD and HC groups ($P > 0.05$) (Figure 1A). A total of 77 (98.72%) patients in the WD group showed low levels of serum Cp < 200 mg/L, which was the previously reported cut-off.[19,30,31] Notably, 5 (15.63%) patients in the N-WD group and 12 patients (32.43%) in the HC group showed decreased serum Cp levels with a cut-off < 200 mg/L. Additionally, one patient in the WD group showed a normal serum Cp level. Atomic absorption spectroscopy was performed and the ratio of exchangeable copper (CuEXC)/total serum copper was calculated to detect REC levels. A total of 77 (98.72%) patients with WD showed significantly increased REC levels with a cut-off of 21.15%. The comparative analysis of the three groups showed that

Table 1 Characteristics of the study population

Item	WD group (*n* = 78)	N-WD group (*n* = 32)	HC group (*n* = 37)
Age (years)	24±8	22±8	24±7
Female [*n* (%)]	32 (41.03)	14 (43.75)	20 (54.05)
Time since first symptoms (months)	14±8	8±7	--
Neurologic symptoms at diagnosis [*n* (%)]	54 (69.23)	--	--
Kayser-Fleischer corneal rings [*n* (%)]	73 (93.59)	--	0 (0.00)
Serum ceruloplasmin (< 200 mg/L)	77 (98.72)	5 (15.63)	12 (32.43)
Dominant mutations [*n* (%)]			
c.2333G>T (R778L, exon 8)	24 (30.77)	--	14 (37.84)
c.2975C>T (P992L, exon 13)	11 (14.10)	--	6 (16.22)
c.2621C>T (A874V, exon 11)	6 (7.69)	--	3 (8.11)

Notes: WD group: Wilson's disease group; N-WD group: non-Wilsonian liver diseases; HC group: heterozygous ATP7B carriers. Data are presented as the mean ± standard deviation using one-way analysis of variance or the counting (percentage) method.

REC levels in the WD group were significantly higher than those in the N-WD and HC groups (both $P < 0.01$). Additionally, no significant difference in REC levels was observed between the N-WD and HC groups ($P > 0.05$). A total of 77 (98.72%) patients in the WD group showed REC > 21.15% (Figure 1B). These results indicated that the REC test exhibited remarkably high sensitivity and specificity for WD determination.

3.3. Distribution of REC levels in different ATP7B mutational sites in WD

Molecular genetic testing was performed in all patients with WD and HC. Among the patients with WD, the most common mutant was c.2333G > T (R778L, exon 8), accounting for 30.77% (24/78) of the total alleles, followed by c.2975C > T (P992L, exon 13, 14.10%) and c.2621C > T (A874V, exon 11, 7.69%) (Table 1). REC levels in WD increased significantly in R778L/R778L compared with that in R778L/R992L and A874V/R778L, and significant differences in REC ratios were observed between the mentioned mutants (both $P < 0.05$) (Figure 2).

3.4. Correlation between REC and laboratory indices

The Pearson analysis of the relationships of REC with serum Cp, serum CO, and 24-h urinary copper levels revealed negative correlations between REC and serum Cp, serum CO, and 24-h urinary copper levels (r-value: −0.305, −0.274, and −0.398, $P < 0.05$). Additionally, no significant correlations were observed between REC and liver function indices such as ALT, AST, and AKP ($P > 0.05$) (supplementary Figures 1A-1C).

3.5. Correlation between REC and GAS and UWDRS

The Pearson analysis of the relationships of REC with GAS and UWDRS showed a positive and significant correlation between REC ratios and GAS and UWDRS in WD (both $P < 0.01$) (supplementary Figures 2A, 2B).

3.6. REC levels in different TCM syndromes

Differences in REC ratios among the three groups (D-HIA, P-SCS, and L-KYD groups) were analyzed and investigated. The comparative analysis of these REC ratios showed that the D-HIA group showed the highest REC ratio, followed by the P-SCS group and the L-KYD group, and the differences were significant ($P < 0.01$) (Figure 3).

All patients (24/24, 100%) in the D-HIA group showed high REC ratios (REC > 50%), whereas almost all patients (24/26, 92.31%) in the L-KYD group showed relatively low REC ratios (REC < 50%). In the P-SCS group, 20 patients (20/28, 71.43%) showed high REC ratios (REC > 50%), whereas the remaining 8 patients showed relatively low REC ratios (26%-42%).

3.7. FC values in different TCM syndromes

A positive correlation between the respective FC values and REC ratios was found in the PUT-VA, PUT-DA, PUT-DP, and PUT-VP regions (r-value: 0.503, 0.551, 0.494, and 0.536, respectively; $P < 0.05$). The FC value increased in the D-HIA group (with a high level of REC) compared with that in the P-SCS and L-KYD groups, and the difference was significant ($P < 0.01$) (Table 2, Figures 4A-4C).

3.8. Serum Cp levels in different TCM syndromes

Serum Cp levels were detected to differentiate and classify TCM syndromes in WD. No significant differences were found in serum Cp levels among the three different TCM syndromes [D-HIA group, (35 ± 28) mg/L; P-SCS group, (32 ± 26) mg/L; L-KYD group, (50 ± 52) mg/L; $P > 0.05$].

4. DISCUSSION

The present study showed that REC level determination was a promising tool with high sensitivity and specificity for diagnosing WD, differentiating N-WD, and family screening. Additionally, an apparent relationship between REC and TCM syndromes in WD was observed,

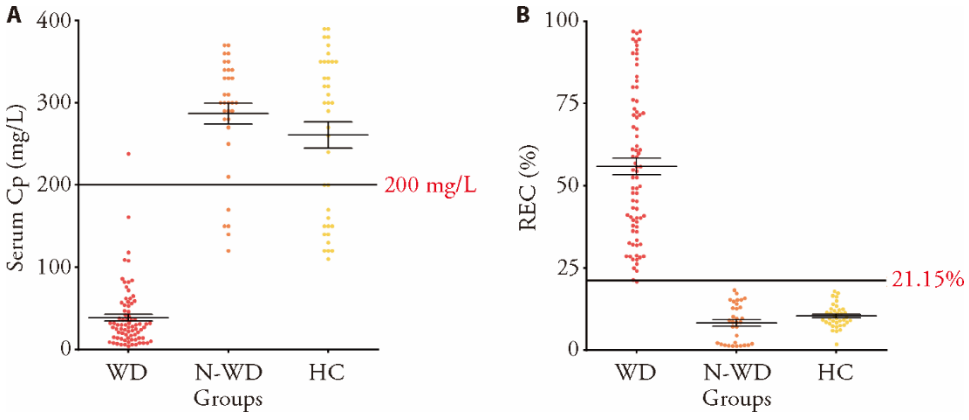

Figure 1 Comparison of the REC ratios and serum Cp levels in WD, non-Wilsonian liver diseases, and heterozygous ATP7B carriers
A: comparison of the serum copper levels in WD, non-Wilsonian liver diseases, and heterozygous ATP7B carriers. B: comparison of the REC ratios in WD, non-Wilsonian liver diseases, and heterozygous ATP7B carriers. WD group (n = 78), N-WD group (n = 32), HC group (n = 37). WD: Wilson's disease; N-WD: non-Wilsonian liver diseases; HC: heterozygous ATP7B carriers; REC: relative exchangeable copper; Cp: ceruloplasmin. Data are presented as the mean ± standard deviation using one-way analysis of variance.

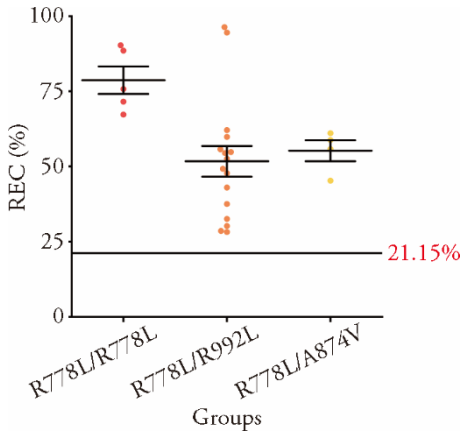

Figure 2 Comparison of the REC ratios in different ATP7B mutational sites in WD
R778L/R778L (n = 5), R778L/R992L (n = 16), and A874V/R778L (n = 4). REC: relative exchangeable copper; WD: Wilson's disease. Data are presented as the mean ± standard deviation using one-way analysis of variance.

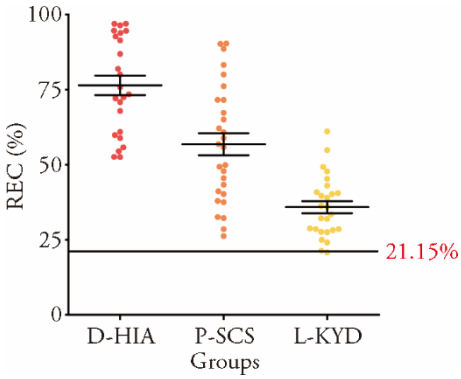

Figure 3 Comparison of the REC ratios in different TCM syndromes
D-HIA group (n = 24), P-SCS group (n = 28), L-KYD group (n = 26). REC: relative exchangeable copper; TCM: Traditional Chinese Medicine; D-HIA: Dampness-heat internal accumulation syndrome; P-SCS: Phlegm-stasis combination syndrome; L-KYD: Liver-kidney *Yin* deficiency syndrome. Data are presented as the mean ± standard deviation using one-way analysis of variance.

implying the potential value of REC in distinguishing TCM syndromes.

Serum Cp levels are commonly determined to diagnose WD, which are typically decreased in WD (lower than 100 mg/L).[32,33] However, normal serum Cp levels should not be ignored during the diagnosis because about 10% of patients with WD show normal serum Cp levels.[34,35] Herein, 5 (15.63%) patients with N-WD and 12 (32.43%) with HC showed low serum Cp levels (< 200 mg/L), and one patient with WD showed a normal serum Cp level, indicating that single serum Cp test cannot completely distinguish WD from chronic liver diseases and HC. Additionally, serum Cp levels were determined to differentiate and classify TCM syndromes in WD, and no significant differences were observed in serum Cp levels among the three TCM syndromes. This result indicated that the serum Cp level was an inaccurate and insensitive biochemical marker for differentiating and classifying TCM syndromes in WD.

REC is a specific, sensitive, and non-invasive tool for WD diagnosis with 100% sensitivity and 100% specificity.[11,31] Herein, the REC test was performed in patients who have been diagnosed with WD to confirm the diagnosis as well as to distinguish N-WD from hypoceruloplasminemia and HC. The REC ratio analysis showed that 98.72% (77/78 cases) of patients with WD, except for those with N-WD and HC, showed a REC of > 21.15% (Figure 1A). This result suggested that the REC test was extremely sensitive and specific for diagnosing WD and could effectively help distinguish WD from chronic liver diseases and HC.

The relationships between the REC ratio and the common variants R778L/R778L, R778L/R992L, and A874V/R778L were determined by performing molecular biological testing. Relatively high REC levels were observed in R778L/R778L, implying differences in REC levels between homozygous and heterozygous individuals with WD.

The analysis of the relationship between REC with serum Cp, serum CO, and 24-h urinary copper levels revealed negative correlations between the mentioned factors, further confirming that REC was crucial for diagnosing WD. The analysis of the correlation of REC with GAS and UWDRS showed positive correlations between the three factors, indicating that the REC ratio could reflect the severity of WD to some extent.

Diagnosing and differentiating TCM syndromes in some cases are highly subjective, which brings some

Table 2 FC value in all subregions of the lenticular nucleus among different TCM syndromes

Subregion	D-HIA group (n = 17)	P-SCS group (n = 23)	L-KYD group (n = 18)
Left PUT-VA	0.27±0.11	0.14±0.07	0.06±0.10
Left PUT-DA	0.26±0.09	0.15±0.04	0.05±0.08
Left PUT-DP	0.25±0.09	0.15±0.09	0.07±0.08
Left PUT-VP	0.31±0.08	0.21±0.04	0.09±0.08
Right PUT-VA	0.22±0.05	0.14±0.07	0.04±0.10
Right PUT-DA	0.25±0.07	0.15±0.06	0.03±0.14
Right PUT-DP	0.21±0.08	0.15±0.10	0.08±0.13
Right PUT-VP	0.28±0.11	0.21±0.07	0.14±0.09

Notes: D-HIA group: dampness-heat internal accumulation syndrome group; P-SCS group: Phlegm-stasis combination syndrome group; L-KYD group: Liver-kidney *Yin* deficiency syndrome group. FC: functional connectivity; TCM: Traditional Chinese Medicine; PUT-VA: ventral anterior putamen; PUT-DA: dorsal anterior putamen; PUT-DP: dorsal posterior putamen; PUT-VP: ventral posterior putamen. Data are presented as the mean ± standard deviation using one-way analysis of variance.

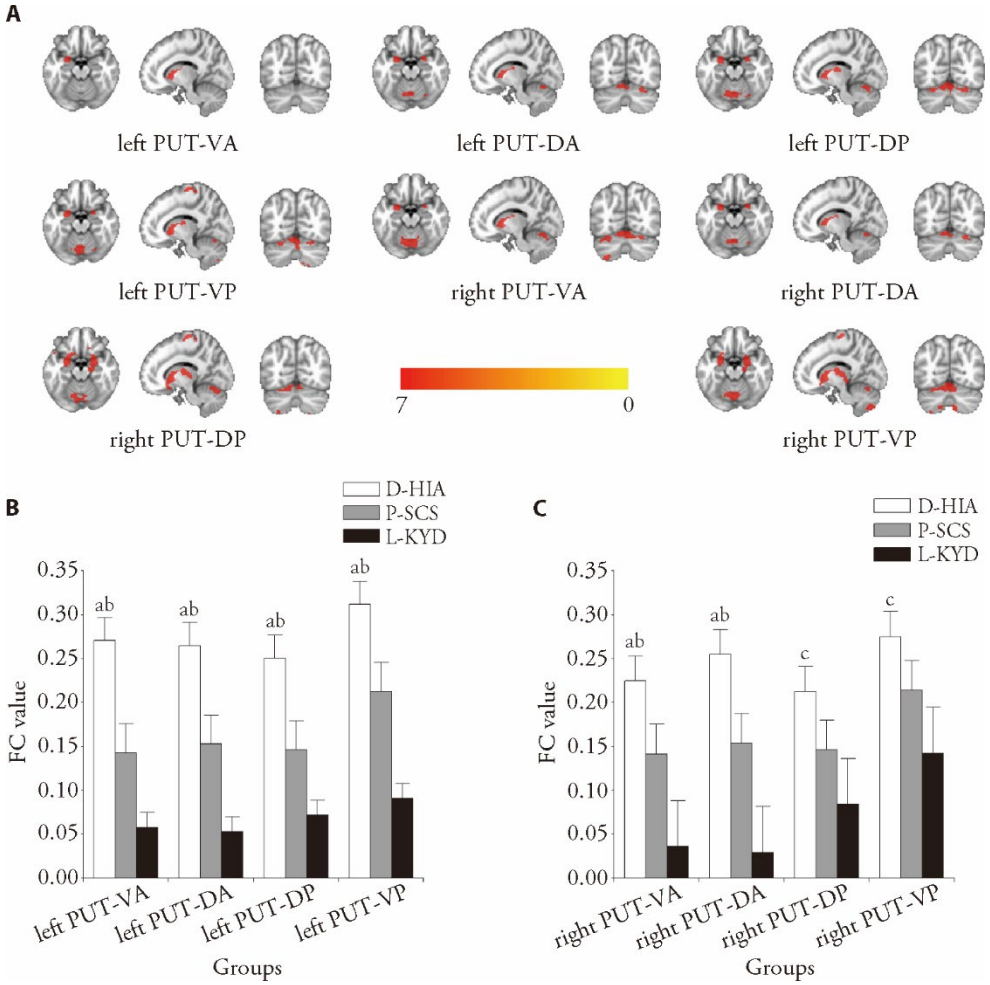

Figure 4 FC value changes in different TCM syndromes

A: the seed-based FC map in all Wilson's disease patients; B: FC value changes of left putamen in different TCM syndromes; C: FC value changes of right putamen in different TCM syndromes. D-HIA Group (n = 17), P-SCS group (n = 23), L-KYD group (n = 18). FC: functional connectivity; TCM: Traditional Chinese Medicine; D-HIA: Dampness-heat internal accumulation syndrome; P-SCS: Phlegm-stasis combination syndrome; L-KYD: Liver-kidney *Yin* deficiency syndrome; PUT-VA: ventral anterior putamen; PUT-DA: dorsal anterior putamen; PUT-DP: dorsal posterior putamen; PUT-VP: ventral posterior putamen. [a]$P < 0.05$ *vs* P-SCS group; [b]$P < 0.001$ *vs* L-KYD group, [c]$P < 0.05$ *vs* L-KYD group. Data are presented as the mean ± standard deviation using one-way analysis of variance.

challenges in classifying TCM syndromes. Thus, herein, the National Administration of Traditional Chinese Medicine Clinical Pathway for Wilson's disease (2011 trial version) system and the REC ratio were used for the syndrome differentiation and classification.

High REC ratios were observed in the D-HIA group, followed by the P-SCS and L-KYD groups in the present study. The comparative analysis of these REC ratios showed that REC was significantly higher in the D-HIA group than in the P-SCS and L-KYD groups. Additionally, REC ratios in the D-HIA group were all above 50%, whereas those in the L-KYD group (24/26, 92.31%) were below 50%, indicating that REC ratios were closely associated with TCM syndromes in WD. Notably, eight patients with WD in the P-SCS group with ratios below 50% showed a relatively high degree of dispersion. Four of these eight patients had probably taken penicillamine before their inclusion in the study, which may affected the REC-related results even though the medication was discontinued two weeks before the evaluation.

Additionally, the respective FC values were positively correlated with REC ratios in the PUT-VA, PUT-DA, PUT-DP, and PUT-VP regions. The FC value analysis revealed that the brain injury was the most serious in the D-HIA group with the highest REC ratio, followed by the P-SCS and L-KYD groups. These results reconfirmed the importance of REC in WD diagnosis, brain injury assessment, and TCM syndrome differentiation.

In conclusion, the present study indicates that REC determination (CuEXC/total serum copper ratio) is highly valuable for diagnosing WD with a high sensitivity and specificity of 100%. Furthermore, this approach can help effectively distinguish patients with WD from those with HC and N-WD. Moreover, relatively high REC levels detected in the D-HIA group and low REC ratios observed in the L-KYD group indicate that the REC level may be an objective and quantitative indicator for differentiating and classifying TCM syndromes in WD. However, the sample size of the present study was small; hence, a large cohort study should be conducted in the future to validate the present findings.

5. ACKNOWLEDGMENTS

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6. SUPPORTING INFORMATION

Supporting data to this article can be found online at http://journaltcm.cn.

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