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Research paper

Polymorphisms involving the *Pneumocystis jirovecii*-related genes in AIDS patients in eastern China

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ABSTRACT

Objective: To investigate the genetic polymorphisms of mitochondrial large ribosomal subunit (*mtLSU*)-rRNA, dihydrofolate reductase (*DHFR*), dihydropteroate synthase (*DHPS*), cytochrome *b* (*CYB*), and superoxide dismutase (*SOD*) genes and its correlation with clinical outcomes of *Pneumocystis jirovecii* pneumonia in acquired immune deficiency(AIDS) patients.

Methods: Eighty AIDS patients with *P. jirovecii* pneumonia that were admitted to our hospital from 2016 to 2018 were included in this study. Their demographic information and clinical data were collected, as well as corresponding saliva specimens for PCR and sequencing of *mtLSU*-rRNA, *DHFR*, *DHPS*, *CYB*, and *SOD* genes to analyze genetic polymorphisms, different polymorphic combinations, and clinical outcomes.

Results: Of the 80 saliva specimens, *mtLSU*-rRNA was successfully amplified and sequenced in 30 cases; *CYB* was successfully amplified and sequenced in 26 cases; and *SOD*, *DHFR*, and *DHPS* were successfully amplified and sequenced in 18 cases. These results indicate that The *mtLSU*-rRNA, *CYB*, and *SOD* genes were highly polymorphic. *mt85T* and *CYB1* were the variants dominantly detected at the *mtLSU*-rRNA and *CYB* loci, respectively. The *SOD1* and *SOD2* variants (each in 50% of the cases) were detected at the *SOD* locus. Among the 18 cases that were successfully amplified and sequenced for *DHFR* and *DHPS*, three *DHFR* nonsense mutations and no *DHPS* mutation were observed. The *mt85C*, *CYB1*, *SOD1*, and *DHFR312T* genes harbored common polymorphisms ($n = 4$; 22.22%) and the mt85T, CYB1, SOD1, DHFR312T genes were associated with poor clinical outcomes.

Conclusion: The types of genetic polymorphisms and polymorphic combinations of *mtLSU*-rRNA, *DHFR*, *DHPS*, *CYB*, and *SOD* in *P. jirovecii* were related to the clinical outcomes of patients with *P. jirovecii* pneumonia in Zhejiang Province, China.

1. Introduction

Pneumocystis jirovecii pneumonia is a common opportunistic infection in human immunodeficiency virus (HIV)-infected individuals with < 200 $CD4^+$ T cells/mm^3 in the blood (Thomas Jr. and Limper, 2004). These patients often have trouble breathing, and exhibit coughing, a low-grade fever, chest pain, acute dyspnea, and tachycardia. Although the emergence of highly active antiretroviral therapy (HAART) has greatly reduced the incidence of AIDS, *Pneumocystis* pneumonia continues to endanger public health because of an increase in the size of the immunocompromised population due to other causes, drug-resistant strains, and HIV-infected individuals in developing countries (Kelly and Shellito, 2010). In addition, infected individuals are prone to pulmonary opportunistic infections within six months after solid organ transplantation and have a higher mortality rate than those with opportunistic infections involving other organs; the most common microorganisms isolated from these patients include *Aspergillus* spp. and *P. jirovecii* (Hoyo et al., 2012).

P. jirovecii, as a fungal pathogen, can cause severe pneumonia(PCP) in patients with immunodeficiency. Because of difficulty in culturing this pathogen *in vitro*, molecular typing is often used to distinguish differences in the microorganisms that are isolated from different patients (Miller et al., 2003). The common molecular typing methods include PCR-based DNA sequencing, single-strand conformation polymorphism (SSCP), and multilocus sequence typing (MLST) (Curran et al., 2013; Miller et al., 2003). A MLST study has shown that genotype

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polymorphisms and the recombination of some multilocus genotypes in *P. jirovecii* are prevalent, and that some combinations are associated with the severity of *PCP* (Matos and Esteves, 2010). MLST has recently become the gold standard for epidemiological observation of *PCP* in hospitals (Maitte et al., 2013; Matos and Esteves, 2010; Phipps et al., 2011). Selection of MLST loci is particularly important. Among the MLST loci, mitochondrial large ribosomal subunit (*mtLSU*)-rRNA, dihydrofolate reductase (*DHFR*), dihydropteroate synthase (*DHPS*), cytochrome *b* (*CYB*), and superoxide dismutase (*SOD*) are suitable for the identification and classification of *P. jirovecii,* as well as the correlation analysis between drug resistance and clinical outcomes of *PCP* (Matos and Esteves, 2010).

This study analyzed the polymorphisms of *mtLSU*-rRNA, *DHFR*, *DHPS*, *CYB*, and *SOD* loci in *P. jirovecii* in AIDS-PCP patients from Zhejiang Province, China, to identify the most common multilocus genotypes and to establish the relationship between different combinations and clinical outcomes.

2. Materials and methods

2.1. Patients

Eighty patients admitted to the First Affiliated Hospital of College of Medicine, Zhejiang University, China, from June 2016 to June 2018 due to AIDS-PCP were included in this study. The inclusion criteria of patients were as follows: (1) clearly diagnosed with AIDS; (2) ≥18 years old; (3) with initial treatment for *P. jirovecii* pneumonia; and (4)comply with PCP diagnosis. And diagnosis of PCP was based on the combination of symptoms, signs, laboratory data, arterial oxygenation at rest, and computed tomography. The exclusion criteria for patients were as follows: (1) undergoing HAART at the disease onset; (2) accompanied by other immunocompromised diseases, such as tumors or congenital diseases, and receiving chemotherapy; (3) immune reconstitution inflammatory syndrome (IRIS); (4) severe allergies and/or allergies to sulfa drugs; and (5) severe heart, brain, liver, kidney, and other important organ diseases, including severe circulatory or endocrine system disease, and medical history.

Bemographic information, treatment regimens after admission, clinical outcomes, and laboratory indicators, including blood routine, biochemical, D-dimer, ferritin, C-reactive protein (CRP), lactate dehydrogenase (LDH), alpha-hydroxybutyrate dehydrogenase (HBDH), CD4 cell count, and arterial blood gas analysis (ABG), were collected within 12 h of admission.

All patients provided written informed consent and this study was approved by Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. (reference number 2014320). All procedures and methods were performed in accordance with the relevant international guidelines and regulations in order to reduce physical discomfort of the subjects.

2.2. Sample collection and gene extraction

Saliva specimens were collected from each patient after no food consumption for at least 30 min. After rinsing the mouth with normal saline for one minute and spitting out the normal saline, 2 mL of saliva were collected from each patient using a saliva collection and storage device and were temporarily stored at 4 °C. DNA was extracted from each saliva specimen using a swab genomic DNA kit(CWBIO , Hangzhou , China).

2.3. Amplifications and primers

Locus-specific nest PCR amplification for five loci in the *P. jirovecii* genome was performed using the primers listed in Table 1. For the external round PCR, 1 μL of DNA(< 500 ng/μL) were added to the reaction mixture which contained 25 μL of 2 × Es Taq MasterMix and 1 μL of the PCP forward and reverse primers and 22 μL of ddH2O. The nest PCR reaction was carried out using 1 μL of undiluted external round PCR product. PCR conditions for both external and nested round involved initial denaturation for 10 min at 95 °C, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 1 min), with a final extension at 72 °C for 5 min.

Table 1
Primer sequences of different genes.

Locus	Primer	Size of PCR products
mtLSU rRNA (Wakefield, 1996)	pAZ102-E GATGGCTGTTTCCAAGCCCA pAZ102-H GTGTACGTTGCAAAGTACTC PAZ102-X GTGAAATACAAATCGGACTAGG PAZ102-Y TCACTTAATATTAATTGGGGACC	252 bp
CYB (Esteves et al., 2010a)	CytbFw CCCAGAATTCTCGTTTGGTCTATT CytbRw AAGAGGTCTAAAAGCAGAACCTCAA CytbF3 TCTCGTTTGGTCTATTGGTG CytbR4 AAGCAGAACCTCAAATTCAAGATA	590 bp
SOD (Esteves et al., 2010b)	MnSOD-Fw GGGTTTAATTAGTCTTTTTAGGCAC MnSOD-Rw CATGTTCCCACGCATCCTAT SODF3 AGTCTTTTTAGGCACTTGAACCT SODR4 TCCAAGAATAACTTTGCCTTGAGT	560 bp
DHPS (Ma et al., 1999)	FR208 GCAGAAAGTAGGTACATTATTACGAGA FR1018 AAGCTTGCTTCAAACCTTGTGTAACGCG FR242 GTTTGGAATAGATTATGTTCATGGTGTACG FR1038 GCTTCAAACCTTGT GTAACGCG	798 bp
DHFR (Lane et al., 1997)	DHPS1 CAAATTAGCGTATCGAATGACC DHPS2 GCAAAATTACAATCAACCAAAGTA DHPS3 AGCGCCTACACATATTATGG DHPS4 GTTCTGCAACCTCAGAACG	278 bp

2.4. Sequence analysis and genotyping

Based on the selective incorporation of chain-terminating dideoxynucleotides, Sanger sequencing of the PCR products(> 50 ng/μL) was performed. Each sample was sequenced twice to confirm the result. The BioEdit 7.1.3 sequence alignment editor was used to analyze the DNA sequence chromatogram. ClustalW software was used to align paired forward and reverse sequences and the sequences were manually refined to obtain better consensus sequences. Reference numbers of the loci sequence of *mtLSU*–rRNA, *CYB*, *SOD*, *DHFR*, and *DHPS* are M58605, AF320344, AF146753, AF090368, and AY628435, respectively. The genotype was named according to the previously published nomenclature (Maitte et al., 2013).

2.5. Statistical analysis

The measurement data were tested for differences between consecutive data using Mann-Whitney *U* test (non-parametric) or Student's *t*-test (normal distribution and variance). All statistical analyses were performed using SPSS version 19 (SPSS, Armonk, New York, USA) using a statistical significance threshold of $P < 0.05$.

3. Results

Of the 80 patients included in this study, there were 72 males and eight females, and six deaths occurred within two months. None of the patients received trimethoprim-sulfamethoxazole (TMP-SMZ) for prophylaxis before hospital admission. Specific clinical data and baseline analyses are listed in Table 2.

The *mtLSU*-rRNA was used as the target gene amplification, and the positive saliva specimens were detected in 30 patients. Genotyping was performed in the 30 *mtLSU*-rRNA-positive cases and showed that

Table 2
Characteristics of AIDS patients with PCP.

Characteristics	
Gender (M/F)	80 (72/8)
Age, years	42.04 ± 13.31
The most recent CD4+ cell count	61.89 ± 95.72
Treatment	
SMZ-TMP prophylaxis	0 (0%)
HAART before PCP	5 (6.25%)
Anti-PCP therapy	80 (100%)
Clinical outcome	
Survivor	74 (92.5%)
Non-survivor	6 (7.5%)

genotype 3 (*mt85T*; *mt248C*, *n* = 15) was the most common in our study cohort, followed by genotype 1 (*mt85C*; *mt248C*, *n* = 10) and genotype 2 (*mt85A*; *mt248C*, *n* = 5).

The P. jirovecii CYB primers yielded an amplification product in 26 of the 30 saliva specimens that were positive for the mtLSU-rRNA locus. The results showed that *CYB1* (279C; 348A; 516C; 547C; 566C; 838C, n = 15) was the most common variant, followed by *CYB2* (279C; 348A; 516C; 547C; 566C; 838 T, *n* = 8), *CYB6* (279C; 348A; 516 T; 547C; 566C; 838C, *n* = 2), and *CYB4* (279C; 348A; 516C; 547 T; 566C; 838C, *n* = 1) (Table 3).

The *P. jirovecii* SOD primers yielded an amplification product in 18 of the 30 saliva specimens that were positive for the mtLSU-rRNA locus. The result indicated that *SOD1* (110C; 215 T, *n* = 9) and *SOD2* (110 T; 215C, n = 9) were common variants. In addition, only three out of 18 cases of amplified *DHFR* showed T-C same-sense mutations at the 312 nucleotide position, and all 18 cases of amplified *P. jirovecii DHPS* had the wild-type sequence (no substitution observed at codons 55 and 57).

This study obtained 10 different combinations of genes by observing different types of genes as previously described. 1) mt85T, CYB2, SOD2, DHFR312C (*n* = 1; 5.56%); 2) mt85A, CYB1, SOD1, DHFR312T (*n* = 2; 11.11%); 3) mt85C, CYB1, SOD1, DHFR312T (*n* = 4; 22.22%); 4) mt85C, CYB2, SOD1, DHFR312C (n = 1; 5.56%); 5) mt85C, CYB1, SOD2, DHFR312T (n = 2; 11.11%); 6) mt85T, CYB2, SOD2, DHFR312T (n = 2; 11.11%); 7) mt85T, CYB1, SOD1, DHFR312T (n = 2; 11.11%); 8)mt85T, CYB1, SOD2, DHFR312T (n = 2; 11.11%); 9) mt85C, CYB2, SOD2, DHFR312C (n = 1; 5.56%); 10) mt85T, CYB4, SOD2, DHFR312T (n = 1; 5.56%). By analyzing the genotypes of the 18 saliva specimens, we found that the probability of each gene combination was between 5.56% and 22.22% (Table 4), with the gene combination of *mt85C*, *CYB1*, *SOD1*, and *DHFR312T* showing the highest frequency. Moreover, one out of two patients with the gene combination of *mt85T*, *CYB1*, *SOD1*, and *DHFR312T* had died, and another patient had recurrent *P. jirovecii* pneumonia, suggesting that the gene combination of *mt85T*, *CYB1*, *SOD1*, and *DHFR312T* may be associated with poor clinical outcome.

4. Discussion

This study amplified, sequenced and classified the *P. jirovecii* DNA (*mtLSU*–rRNA, *CYB*, *SOD*, *DHFR*, and *DHPS* loci) to assess multilocus genotype combinations of *P. jirovecii*. Furthermore, this study determined its correlation with clinical outcomes or the disease severity because some studies demonstrated that certain genotypes were associated with the severity or clinical outcomes of PCP (Crothers et al., 2005; Esteves et al., 2010a, 2010b; Helweg-Larsen et al., 1999; Matos and Esteves, 2010).

In this study, we analyzed the *mtLSU*-rRNA genotype and found that the allele frequency distribution at the 85th nucleotide consisted of Genotype 3 (15/30, the most common), Genotype 1 (10/30, common), and Genotype 2 (5/30, the least common). A previous study in Spain showed that *P. jirovecii* DNAs were detected in healthy adults(without underlying lung disease or immunosuppression), with Genotype 2 commonly observed and Genotype 3 the least common (Medrano et al., 2005). However, Genotype 2 had the least frequency and Genotype 3 commonly observed in AIDS-PCP patients in this study, suggesting that Genotype 3 may have a strong virulence to immune-impaired population and invade the lungs. Another study conducted in India showed that Genotype 3 was the most common in patients with malignant diseases, whereas Genotype 2 was more common in HIV-positive patients. In addition, Genotype 2 was most common in patients with autoimmune diseases and chronic respiratory diseases (Singh et al., 2017). In contrast, several studies involving some cities or countries showed that Genotype 1 was the most common (Esteves et al., 2008; Montes-Cano et al., 2004; Respaldiza et al., 2005; van Hal et al., 2009). These results indicate that the prevalence of the *mtLSU*-rRNA gene varied among countries or cities, suggesting that the geographical and climatic changes may result in different distribution patterns of *P. jirovecii*.

A retrospective cohort study has demonstrated that *CYB* mutations (Qo region , coenzyme Q binding site)are strongly associated with atovaquone(a ubiquinone analog that inhibits electron transport at the cytochrome *bc*1 complex)exposure in AIDS patients with PCP (Kazanjian et al., 2001). In the present study, atovaquone was not used as a treatment or preventive regimen in our patients. Therefore, CYB mutations were not caused by atovaquone in this study, suggesting that mutations have occurred in *P. jirovecii*. Among the 30 samples *mtLSU*-rRNA-positive, 26 were successfully amplified for the CYB locus and CYB1 and CYB2 genotypes were relatively common, which is consistent with the results of a study done the Portugal (Esteves et al., 2010b), suggesting that the distribution of allele frequencies in the study regions in Portugal and China is stable. Previous studies have shown that *SOD* mutations occur at two nucleotide positions: 110 and 215 (Denis et al., 1998; Denis et al., 2000; Wakefield et al., 2003). The most common *SOD* genotypes detected in previous studies were *SOD1* and *SOD2* (Denis et al., 2000; Miller et al., 2003, 2005a, 2005b), followed by *SOD3* and *SOD4* (Esteves et al., 2010b). The *SOD* genotypes detected in this study were *SOD1* (9/18) and *SOD2* (9/18), and the other *SOD* genotypes were not detected. Many studies have shown that the *SOD1* genotype is commonly observed in PCP patients with poor prognosis (Esteves et al., 2012, 2010a; Singh et al., 2017), suggesting that the *SOD1* genotype may be associated with the toxicity of *P. jirovecii*.

Several studies have shown that *DHPS* and *DHFR* gene have high prevalence of mutation rates and these mutations are associated with TMP-SMZ treatment failure (Ponce et al., 2017; Queener et al., 2013), whereas other studies have demonstrated that the mutation rates of

Table 3
Genotyping of *Pneumocystis jirovecii* MtLSUrRNA and CYB genes.

Locus (number)	Genotype	Number	Nucleotide position and identity
MtLSUrRNA (30)	Genotype 1	10	mt85C; mt248C
	Genotype 2	5	mt85A; mt248C
	Genotype 3	15	mt85T; mt248C
CYB (26)	CYB1	15	279C; 348A; 516C; 547C; 566C; 838C
	CYB2	8	279C; 348A; 516C; 547C; 566C; 838 T
	CYB4	1	279C; 348A; 516C; 547 T; 566C; 838C
	CYB6	2	279C; 348A; 516 T; 547C; 566C; 838C

Table 4
Results of genotyping of five loci of *Pneumocystis jirovecii*.

Patient No.	Sample type	Genotype					Outcome
		mtLSUrRNA	CYB	SOD	DHPS	DHFR	
10	Saliva	mt85T	CYB2	SOD2	WT	312C	Survived
15	Saliva	mt85A	CYB1	SOD1	WT	WT	Survived
14	Saliva	mt85C	CYB1	SOD1	WT	WT	Survived
23	Saliva	mt85C	CYB1	SOD1	WT	WT	Survived
25	Saliva	mt85C	CYB2	SOD1	WT	312C	Survived
33	Saliva	mt85C	CYB1	SOD2	WT	WT	Survived
34	Saliva	mt85T	CYB2	SOD2	WT	WT	Survived
36	Saliva	mt85T	CYB1	SOD1	WT	WT	Survived
37	Saliva	mt85T	CYB1	SOD2	WT	WT	Survived
40	Saliva	mt85T	CYB1	SOD2	WT	WT	Survived
45	Saliva	mt85C	CYB1	SOD1	WT	WT	Survived
46	Saliva	mt85A	CYB1	SOD1	WT	WT	Survived
47	Saliva	mt85C	CYB1	SOD1	WT	WT	Survived
73	Saliva	mt85T	CYB1	SOD1	WT	WT	Not survived
74	Saliva	mt85C	CYB2	SOD2	WT	312C	Survived
77	Saliva	mt85C	CYB1	SOD2	WT	WT	Survived
78	Saliva	mt85T	CYB4	SOD2	WT	WT	Survived
79	Saliva	mt85T	CYB2	SOD2	WT	WT	Survived

DHPS and *DHFR* are very low (Mane et al., 2015; Singh et al., 2015). In the present study, no missense mutations in *DHPS* or *DHFR* were detected, which may be related to the lack of prevention of TMP-SMZ in AIDS patients in Zhejiang Province. Because the patients included in this study had not received TMP-SMZ for prophylaxis.

The genotype combinations of *mtLSU*-rRNA, *CYB*, and *SOD* loci in *P. jirovecii* were used to further observe the diversity of multilocus genotype combinations. Multilocus genotypes could be used to integrate polymorphic information at multiple loci to achieve the analysis of strain diversity. This study obtained 10 genotypes, of which two genotype combinations are relatively important; the combination of mt85C, CYB1, SOD1, DHFR312T genotypes (22.2%) is the most common, and the combination of mt85T, CYB1, SOD1, DHFR312T genotypes is associated with the poorest prognosis.

5. Conclusions

In this study, we explored the multilocus polymorphisms in *P. jirovecii* and combined multiple genotypes. This work facilitated our epidemiological observation on genotypic and strain variations in *P. jirovecii* in Zhejiang Province, China, and our understanding of the toxicities in different strains and their relationship to clinical outcomes.

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Declaration of Competing Interest

There is no conflict of interest.

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