EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE

Dihydrofolate reductase gene polymorphisms in *Pneumocystis carinii* f. sp. *hominis* in Japan

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This study examined polymorphisms in the dihydrofolate reductase (DHFR) gene of *Pneumocystis carinii* isolates from 27 patients with *P. carinii* pneumonia (PCP) in Japan. Four substitution sites with two synonymous and two non-synonymous changes were found. Two synonymous substitutions at nucleotide positions 540 and 312 were identified in one and 13 patients, respectively. Two amino acid substitutions (Ala67Val, Cys166Tyr) were found in two different patients. No linkage of amino acid substitutions in DHFR to those in dihydropteroate synthase was observed. The two patients whose isolates showed non-synonymous DHFR mutations were not exposed to DHFR inhibitors before they developed PCP and were treated successfully with co-trimoxazole.

Introduction

Pneumocystis carinii f. sp. *hominis* (*P. carinii*) is an important cause of morbidity and mortality in immunocompromised patients with human immunodeficiency virus type 1 (HIV-1) infection [1], haematological malignancies, organ transplantation state or connective tissue diseases, although the frequency of *P. carinii* pneumonia (PCP) has decreased since the introduction of highly active antiretroviral therapy [2].

Co-trimoxazole, which is a combination of two antifolate agents, sulphamethoxazole and trimethoprim (TMP), is the first choice for the treatment and prophylaxis of PCP [3]. Sulphamethoxazole acts on dihydropteroate synthase (DHPS), which catalyses the condensation of *p*-aminobenzoic acid and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate to produce 7,8-dihydropteroate. Lane *et al.* [4] described DHPS polymorphisms in human-derived *P. carinii* for the first time. Mutations in DHPS of *P. carinii* isolates from 24 patients with PCP in Japan were also reported recently [5].

The target enzyme of TMP is dihydrofolate reductase (DHFR), which catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate and depends on the reduced form of nicotinamide adenine dinucleotide phosphate. In *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Plasmodium falciparum*, mutations in the DHFR genes were found to confer resistance to DHFR inhibitors including TMP and pyrimethamine [6–10]. Ma *et al.* [11] have reported the cloning and sequencing of the DHFR gene in human-derived *P. carinii*. The DHFR nucleotide sequences were identical in 37 clinical isolates except one with a synonymous substitution.

This report describes the DHFR nucleotide and amino acid sequence patterns of *P. carinii* isolates from immunosuppressed patients in Japan. The linkage of amino acid substitutions in DHFR to those in DHPS for each isolate of *P. carinii* and the relationship between the substitutions in DHFR and the results of co-trimoxazole treatment in patients with PCP are also discussed.

Materials and methods

Specimens and characteristics of patients

Broncho-alveolar lavage (BAL) fluid specimens were used in the study. After diagnostic examinations including staining for *P. carinii* were completed, BAL fluid was

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centrifuged at 250 *g* for 5 min and the pellet was stored at -80°C until use. In all, 27 specimens were collected between April 1994 and Oct. 2000 in two hospitals in Tokyo (Research Hospital, Institute of Medical Science, University of Tokyo and Tokyo Metropolitan Bokutoh General Hospital). The underlying diseases for the patients included HIV-1 infection ($n = 19$), malignant lymphoma (3), renal transplantation state (2), rapidly progressive glomerulonephritis (1), polyarteritis (1) and lymphoid interstitial pneumonia (1). The numbers of CD4^{+} lymphocytes at the time of specimen collection were $<200/\text{ml}$ in 23 patients (not determined in patients 24, 25, 26 and 27). The patients were not exposed to co-trimoxazole before they developed the current episodes of PCP, except patients 5, 6 and 9 who had had prior episodes treated with co-trimoxazole. Patients 5, 6, 9, 10 and 21 were given intravenous or aerosolised pentamidine for PCP prophylaxis. No patients had taken or were taking pyrimethamine as therapy or prophylaxis for toxoplasmosis.

Amplification of DHFR by PCR

The pellet obtained after centrifugation of BAL fluid was resuspended in 100 μl of saline and the DNA was extracted with SMI TEST EX-R&D (Sumitomo Metal Ind. Ltd, Tokyo, Japan). The PCR mixture contained template DNA, PCR buffer, 0.2 μM (each) PCR primer (FR208, 5'-GCAGAAAGTAGGTACATTATTA CGAGA-3'; FR1038, 5'-AACCAGTTACCTAATCAA ACTATATTGC-3' [11]), 0.2 mM (each) deoxynucleoside triphosphate and 2.5 U of *Ex-Taq* DNA polymerase (Takara Shuzo, Shiga, Japan) in a total volume of 100 μl . After initial denaturation for 2 min at 94°C , 35 cycles of denaturation (94°C , 1 min), annealing (52°C , 1 min) and extension (72°C , 2 min) were performed. The reaction mixture was kept at 72°C for 10 min for final extension. To avoid contamination of the PCR mixtures, mixture preparation and template DNA addition were done in separate rooms. A negative control without template DNA was always included. The PCR products were electrophoresed through an agarose 1.2% gel containing ethidium bromide 0.5 $\mu\text{g}/\text{ml}$, and bands of the expected size (850 bp) were visualised with UV light, excised and purified with a gel extraction kit (Qiagen, Valencia, CA, USA).

Sequencing of PCR products

The purified PCR products were directly sequenced with an automated sequencer (ABI PRISM 377 DNA Sequencer; Perkin-Elmer, Foster City, CA, USA) with the PRISM Ready Reaction Big-Dye Terminator Cycle Sequencing kit (Perkin-Elmer). Both strands of the entire DHFR gene were sequenced with primers FR208 and FR1038. When mutations were found in the DHFR gene by direct sequencing, the mutations were confirmed by cloning of the PCR products. Briefly, the PCR products were ligated into the TA-cloning vector pGEM-T (Promega, Madison, WI, USA) and

were introduced into competent JM 109 cells (Toyobo, Osaka, Japan). Ten clones were randomly selected for each specimen and were subjected to sequencing. The DNA and amino acid sequences were aligned with Genetyx-Mac software (version 8.0, Software Development, Tokyo, Japan). ClustalW software (version 1.8, MacVector, Oxford Molecular Group, Oxford) was used to compare the alignment of the amino acid sequence of *P. carinii* DHFR with those from selected organisms.

Polymorphisms in DHFR isolated from repeated BAL specimens

Patients 4, 20, 26 and 27 had repeated fiberoptic bronchoscopies during their hospitalisation (periods between the first and the second BALs for each patient were 42, 20, 9 and 14 days, respectively). They received co-trimoxazole as therapy (patients 20, 26 and 27) or recurrence prophylaxis following treatment (patient 4) for PCP during these periods. The sequencing of the DHFR gene for isolates from the second BAL specimens was performed as described above.

Linkage of amino acid substitutions in DHFR to those in DHPS in each P. carinii isolate

Amino acid substitutions in DHPS of isolates from 27 specimens were determined as described previously [5]. Linkage of the substitutions in the DHFR to those in the DHPS was examined in each isolate.

Relationship between co-trimoxazole treatment failure and DHFR or DHPS polymorphisms

Co-trimoxazole treatment failure was defined as failure to improve clinically in terms of fever (temperature $>38^{\circ}\text{C}$), dyspnoea or respiratory failure after the administration of co-trimoxazole for >10 days. Respiratory failure included the presence of either an arterial partial pressure of oxygen of <60 Torr while the patient breathed $\geq 60\%$ O_2 or an increased arterial partial pressure of CO_2 , in addition to a need for intubation [12]. The relationship between the substitutions in DHFR or DHPS and co-trimoxazole treatment failure in patients with PCP was examined.

Results

Sequencing of DHFR gene from specimens of PCP patients

DHFR gene amplified by PCR from 27 specimens was directly sequenced with the FR208 and FR1038 primer set. Sequencing of both strands revealed that isolates from sixteen patients (patients 2–8, 10, 11, 13–15, 21, 22, 26 and 27) had nucleotide substitutions at positions 200, 312, 539 or 540 (Table 1). Nucleotide changes in the DHFR gene in these isolates were confirmed by sequencing the cloned PCR products. Ten independent

Table 1. Polymorphism of *P. carinii* DHFR in each patient

Patient no.	Sequence of DHFR		Number of clones with nucleotide substitution [†]
	Nucleotide* Amino acid*	---G ²⁰⁰ CT---GG ³¹² T---T ⁵³⁹ G ⁵⁴⁰ T--- ---67Ala---104Gly---166Cys---/	
3		---200T---312---539,540--- ---67Val---104---166--- ---200---312---539A ⁵⁴⁰ ---	10
27 (first BAL [‡])		---67---104---166Tyr--- ---200---312---539A ⁵⁴⁰ ---	6
27 (second BAL [‡])		---67---104---166Tyr--- ---200---312---539A ⁵⁴⁰ ---	10
2		---67---104---166Tyr--- ---200---312C---539,540---	8
5		---67---104---166--- ---200---312C---539,540---	10
6		---67---104---166--- ---200---312C---539,540---	4
7		---67---104---166--- ---200---312C---539,540---	5
8		---67---104---166--- ---200---312C---539,540---	4
10		---67---104---166--- ---200---312C---539,540---	10
11		---67---104---166--- ---200---312C---539,540---	10
13		---67---104---166--- ---200---312C---539,540---	10
14		---67---104---166--- ---200---312C---539,540---	5
15		---67---104---166--- ---200---312C---539,540---	5
21		---67---104---166--- ---200---312C---539,540---	10
22		---67---104---166--- ---200---312C---539,540---	2
26 (first BAL [‡])		---67---104---166--- ---200---312C---539,540---	7
26 (second BAL [‡])		---67---104---166--- ---200---312C---539,540---	10
4 (first BAL [‡])		---67---104---166--- ---200---312---539,540C---	4
4 (second BAL [‡])		---67---104---166--- ---200---312---539,540C---	7

*The sequence of the wild type.

[†]Nucleotide substitution was confirmed by cloning and sequencing (10 clones) of each PCR product.[‡]BAL, bronchoalveolar lavage.

Dots show the same nucleotides or amino acids as those in the wild type.

clones were analysed for each specimen. All 10 clones derived from patient 3 had a single nucleotide substitution at position 200 (C to T). Clones derived from patient 27 showed a mixture of wild types and mutants with a single nucleotide change at position 539 (G to A). Both nucleotide substitutions at positions 200 (C to T) and 539 (G to A) were non-synonymous and resulted in amino acid changes from Ala to Val at amino acid position 67 and from Cys to Tyr at the position 166, respectively (Table 1). All 10 clones from patients 5, 10, 11, 13 and 21 had a nucleotide substitution at position 312 (T to C). Clones from patients 2, 6, 7, 8, 14, 15, 22 and 26 were mixtures of wild types and mutants with a nucleotide change at position 312 (T to C). Clones from patient 4 showed a mixture of wild types and mutants with a nucleotide substitution at position 540 (T to C). Both nucleotide substitutions at positions 312 (T to C) and 540 (T to C) were synonymous changes (Table 1). No isolates from 27 specimens had nucleotide substitutions in an intron sequence (42 bp) from nucleotide position 267 to 308

that interrupted DHFR coding sequence (618 bp) (data not shown).

P. carinii DHFR amino acid sequence was aligned with those of five selected organisms and the positions of the polymorphisms were analysed (Fig. 1). *P. carinii* amino acid substitutions were not localised in highly conserved regions of the enzyme as the substitutions that confer resistance to TMP or pyrimethamine of the other organisms (Trp47Arg in *E. coli*, Phe98Tyr in *S. aureus*, Ile100Leu in *Str. pneumoniae* and Asn51Ile, Cys59Arg and Ser108Asn in *Pl. falciparum* [6–8, 10]).

Polymorphisms in DHFR gene of P. carinii isolated from repeated BAL fluid specimens

Among the 27 patients enrolled in this study, four patients (nos 4, 20, 26 and 27) had repeated fiberoptic bronchoscopies. The second BAL fluid specimen obtained from patient 27 had selection of mutants with Tyr at amino acid position 166 whereas the first BAL

				*		**	
<i>E. coli</i>	-----	MISLIAALAVDRVIGMENAMPWN-LPADLAWFKR					
<i>S. aureus</i>	-----	MTLSILVAHDLQRVIGFENQLPWH-LPNDLKHVKK					
<i>Str. pneumoniae</i>	-----	MTKKIVAIWAQDEEGLIGKENRLPWH-LPAELQHFKE					
<i>H. sapiens</i>	-----	MVGSINCIIVAVSQNMIGKNGDLWPPLRNEFRYFQR					
<i>P. carinii</i>	-----	MDWQKSLTLIVALTLSRGIGLKNLDPWK-LKSDMMFFSR					
<i>Pl. falciparum</i>	MMEQVCDVFDIYAICACCKVESKNEGKKNEVFNNYTFRGLGNKGVLPWKC NSLDMKYFCA						
					**		*
<i>E. coli</i>	NTLN-----	KPVIMGRHT W ESIG---RPLP					
<i>S. aureus</i>	LSTG-----	HTLMGRKTFESIG---KPLP					
<i>Str. pneumoniae</i>	TTLN-----	HAILMGCVTFDGMGR---RLLP					
<i>H. sapiens</i>	MTTSS-----	VEGKQ---NLVIMGKKTWFSIPEKNRPLK					
<i>P. carinii</i>	VTSGLL-----	VTRSTGQMNVLMLGRKT W ESL PA HSRPLK					
<i>Pl. falciparum</i>	VTTYVNESKYEKLKYKRCKYLNKETVDNVNDMPNSKKLQNVVVMGR T SWESIPKKFKPLS						
	*					**	
<i>E. coli</i>	GRKNIILSS--	QPGTDDR-VTWVKSVDIAAACGDVP-----				EIMVIGGRVYEQ	
<i>S. aureus</i>	NRRNVVLTS--	DTSFNVEGVDVIHSIEDIYQLPG-----				HVF I FGGQTLFEE	
<i>Str. pneumoniae</i>	KRETLILTR--	NPEEKIDGVATFQDVQSVLDWYQDQE-----				KNLY I IGGKQIFQA	
<i>H. sapiens</i>	GRINLVLSR--	ELKEPPQGAHFLSRSLDDALKLTEQPELAN---				KVDMVWIVGGSSVYKE	
<i>P. carinii</i>	NRINVVISR--	QEVLDLGGGAYHARSLDDALALLSQIYDSTSKIQLNRV F VIGGGELYKA					
<i>Pl. falciparum</i>	NRINVILSRTLKKEDFDEVDYIINKVEDLIVLLGKLN-----					YYKCF I IGGSVVYQE	
		* *		*		**	
<i>E. coli</i>	FL--	PKAQKLYLTHIDAEVEGDTHFPDYE-----					
<i>S. aureus</i>	MI--	DKVDDMYITVIEGKFRGDTFFPPYT-----					
<i>Str. pneumoniae</i>	FE--	PYLDEVIVTHIHARVEGDTYFPEELD-----					
<i>H. sapiens</i>	AMNHPGHLKLFVTRIMQDFESDTFFPEIDLEK-----						
<i>P. carinii</i>	AMEHSRLNRRIATVIHNEVDCDVFFPIDFRSSQSCLPWRKQDHS-----						
<i>Pl. falciparum</i>	FLEKKLIKKIYFTRINSTYECDVFFPEINENEYQIISVSDVYTSNNTTLDFIYKKTNNK						
<i>E. coli</i>	-----	PDDWESVFSEFHDADAQNSHSY					
<i>S. aureus</i>	-----	FEDWEVASSVEGKLDEKNTIPH					
<i>Str. pneumoniae</i>	-----	LSLFETVSSKFYAKDEKNPYDF					
<i>H. sapiens</i>	-----	YKLLPEYPGVLSDVQEEKGIKY					
<i>P. carinii</i>	-----	VLEAWVGSKVPQGGKINENGFIY					
<i>Pl. falciparum</i>	MLNEQNCIKGEEKNNDMPLKNDDKDTCHMKKLTEFYKNVDKYKINYENDDDEEEDDFVY						
<i>E. coli</i>	CFEILERR----						
<i>S. aureus</i>	TFLHLIRKK----						
<i>Str. pneumoniae</i>	TIQYRKRKEV---						
<i>H. sapiens</i>	KFEVYEKND----						
<i>P. carinii</i>	EFEMWIRDI----						
<i>Pl. falciparum</i>	FNFNKEKEEKNNK						

Fig. 1. Alignment of amino acid sequence of *P. carinii* f. *sp. hominis* DHFR with those from different organisms. GenBank accession numbers for these sequences are: *P. carinii*, AF090368; *E. coli*, J01609; *S. aureus*, S32014; *Str. pneumoniae*, AF055727; *Pl. falciparum*, J03772; and *Homo sapiens*, J00140. ClustalW software (version 1.8, MacVector) was used to compare the alignment of amino acid sequence of *P. carinii* DHFR with those from selected organisms. Positions of amino acid substitutions which confer resistance of trimethoprim or pyrimethamine in selected organisms, and those of the substitutions in *P. carinii* found in the present study, are shown in bold type. Identical residues are indicated by the asterisks on top of the sequences.

fluid sample from this patient had mixtures of mutant (Tyr) and wild-type (Cys) isolates (Table 1). The second BAL fluid specimens collected from patients 26 and 4 showed selection of mutants with a nucleotide change (T to C) at position 312 and mixtures of mutants with a nucleotide substitution (T to C) at position 540 and wild types, respectively (Table 1). In patient 20, the second BAL specimen did not contain

isolates with the nucleotide substitutions in DHFR gene (data not shown).

Linkage of amino acid substitutions in DHFR to those in DHPS in each P. carinii isolate

Amino acid substitutions in DHPS of isolates from 27 specimens were determined as described previously [5].

Isolates derived from patients 3 and 27 had the amino acid substitutions Ala67Val and Cys166Tyr, respectively, in DHFR, those from patients 5, 6, 10, 13, 20 and 21 showed the substitutions Thr55Ala or Pro57Ser, or both, and those from patients 18 and 19 had the substitutions His60Gln and Glu169Gly, respectively, in DHPS (Table 2). Thus, the amino acid substitutions in DHFR obtained in the study did not have linkage to those in DHPS in any isolate of *P. carinii*.

Relationship between co-trimoxazole treatment failure and DHFR or DHPS polymorphisms

Two patients (nos 3 and 27) whose isolates had the amino acid substitutions in DHFR were treated successfully with co-trimoxazole. Thus, there was no correlation between the co-trimoxazole treatment failure and the DHFR polymorphisms Ala67Val and Cys166Tyr. On the other hand, six patients (nos 10, 13, 18, 19, 20 and 21) whose isolates had the DHPS mutations Thr55Ala, Pro57Ser, His60Gln or Glu169Gly failed to respond to co-trimoxazole treatment [5]. Patients 5 and 6, whose isolates showed the DHPS mutations Thr55Ala and Pro57Ser, were treated successfully with intravenous pentamidine. The latter drug was used because these patients experienced drug-induced eruption due to co-trimoxazole during their first episodes of PCP [5].

Discussion

Edman *et al.* [13] determined the nucleotide sequences of *P. carinii* DHFR gene derived from five rat and two human specimens to evaluate the natural variability of DHFR sequences from different sources of this organism. The single base change in the sequence was shown to be an A to G transition at position 654 in the 3'-untranslated region of rat-derived *P. carinii*. No nucleotide sequence variations in the coding region were observed. The DHFR nucleotide sequence was identical in 37 isolates from 35 patients except one

with a single synonymous change, despite prior exposure to TMP in 15 patients [11]. Consistently, TMP was reported to be a poor inhibitor of *P. carinii* DHFR compared with the inhibition by other agents [14–16]. The present study found four substitution sites with two non-synonymous and two synonymous changes in the coding region of DHFR. Two synonymous substitutions were observed at nucleotide positions 312 (T to C) and 540 (T to C). The same change at position 312 has been reported previously [11]. The amino acid substitutions at positions 67 (Ala to Val) and 166 (Cys to Tyr) observed in this study are the first DHFR polymorphisms to be described in *P. carinii* isolated from immunodeficient patients. They did not match with those known to confer drug resistance in other organisms. However, analysis of successive specimens from patient 27 suggests that the Cys166Tyr substitution may have been selected by co-trimoxazole during the treatment of PCP. It is possible that this substitution influences folding of the binding site of the enzyme [17] and confers some level of resistance.

Many specimens analysed in this study contained mixtures of wild-type and mutant DHFR alleles. Most of the patients whose isolates showed DHPS mutations (five of six patients) also harboured a mixture of wild-type and mutant alleles [5]. Moreover, genotyping of *P. carinii* isolated from the same 24 patients was performed with nucleotide sequence variations in internal transcribed spacer (ITS) regions 1 and 2 of rRNA genes. Two or more ITS genotypes were present in 16 patients [18]. These mixtures of alleles could result from co-infections, which have been reported to be frequent in man [19], or mutation in a clonal population, or both.

The relationship between the results of co-trimoxazole treatment and the DHFR or DHPS polymorphisms was analysed. The two patients whose isolates showed the DHFR polymorphisms were successfully treated with co-trimoxazole, whereas six patients whose isolates had the DHPS polymorphisms failed to respond to treatment with co-trimoxazole [5]. The substitutions in DHFR in this study did not have linkage to those in DHPS of *P. carinii* isolated from each patient (Table 2). These results suggest that the polymorphisms of DHPS but not of DHFR may contribute to failures of co-trimoxazole treatment for PCP.

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Table 2. Linkage of amino acid substitutions in DHFR of *P. carinii* to those in DHPS in each patient

Patient no.	Amino acid mutations	
	DHFR*	DHPS†
3	Ala67Val	—
5	—	Thr55Ala, Pro57Ser
6	—	Thr55Ala, Pro57Ser
10	—	Thr55Ala, Pro57Ser
13	—	Pro57Ser
18	—	His60Gln
19	—	Glu169Gly
20	—	Thr55Ala, Pro57Ser
21	—	Thr55Ala, Pro57Ser
27	Cys166Tyr	—

—, no substitutions were found.

*DHFR, dihydrofolate reductase.

†DHPS, dihydropteroate synthase.

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