Slow acetylator phenotype and genotype in HIV-positive patients with sulphamethoxazole hypersensitivity

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Aims To test the role of acetylator status, and to investigate the reported discrepancy between acetylator phenotype and genotype in HIV-positive patients with sulphamethoxazole (SMX) hypersensitivity.

Methods Forty HIV-positive patients (32 of whom were SMX-hypersensitive), and 26 healthy volunteers, were genotyped by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis, and phenotyped using dapsone (50 mg) as a probe, for acetylator status. Sequencing of the NAT2 exon was performed where discrepancy between phenotyping and genotyping was detected. Our results were also pooled with published studies addressing slow acetylator status in HIV-positive SMX-hypersensitive patients.

Results Slow acetylator genotype and phenotype frequencies did not differ between HIV-positive SMX-hypersensitive and nonhypersensitive patients, and healthy controls, which was further confirmed in a meta-analysis of published studies (pooled odds ratio 2.25, 95% confidence interval 0.45, 11.17). Discordance between phenotype and genotype was resolved in four of the subjects by sequencing of the whole NAT2 exon, which revealed rare mutations, leaving three (9%) HIV-positive SMX-hypersensitive patients and one (4%) healthy volunteer who continued to demonstrate the discordance.

Conclusions Slow acetylator phenotype or genotype is unlikely to predispose to SMX hypersensitivity in HIV-positive patients, although a minor role cannot be excluded. Phenotype–genotype discrepancies are partly due to nondetection of all rare alleles by PCR methodology, and can be circumvented by sequencing of the gene in patients showing a discrepancy.

Keywords: adverse drug reactions, HIV, hypersensitivity, NAT2, polymorphism, sulphamethoxazole

Introduction

In the UK, the use of sulphamethoxazole–trimethoprim (cotrimoxazole) has decreased greatly in the general population since 1995. Nonetheless, it remains the drug of choice in the prophylaxis and treatment of *Pneumocystis carinii* pneumonia (PCP) in patients infected with HIV [1, 2]. However, its use is associated with hypersensitivity reactions, which has been attributed to the sulphamethoxazole (SMX) component, rather than trimethoprim [3]. In HIV-positive patients, the frequency of

hypersensitivity reactions is higher (SMX) (30–50%) when compared with patients not infected with HIV (3%) [4, 5]. The pathogenesis of SMX hypersensitivity is unclear, but is thought to involve the formation of toxic metabolites from SMX. In particular, the oxidative hydroxylamine and nitroso metabolites have been implicated [6, 7]

SMX is extensively metabolized by both forms of N-acetyltransferase, NAT1 and NAT2. It has been postulated that in the individuals who are slow acetylators, a larger proportion of the parent drug would be available for oxidative metabolism by the cytochrome P450 isoform CYP2C9 [8], making them more susceptible to the hypersensitivity reactions. Indeed, several groups have shown that in HIV-negative patients, approximately 90% of SMX-hypersensitive patients were slow acetylators phenotypically [9] and genotypically [10, 11].

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Interestingly, more than 90% of acutely ill HIV-infected patients were found to be slow acetylators using caffeine as a phenotyping probe [12]. A higher frequency of the slow acetylator phenotype has also been shown in SMX-hypersensitive HIV-positive patients [8, 13]. However, Delomenie *et al.* [14] and Pirmohamed *et al.* [15] failed to show an association with slow acetylator genotype and SMX hypersensitivity, suggesting that in HIV-positive patients there may be a discrepancy between phenotype and genotype. Indeed, a high degree of acetylator phenotype/genotype discordance has been reported [16, 17] in the HIV-positive population. In contrast, in HIV-negative individuals, there is a high correlation between NAT2 phenotype and genotype, the prediction rate being between 88% and 100% [17, 18].

In this study we have therefore undertaken a further investigation of whether there is a discrepancy between the NAT2 genotype and phenotype in another HIV-positive patient cohort, and pooled our results with those of previously published studies, to answer the question as to whether either NAT2 genotype or phenotype predisposes to SMX hypersensitivity.

Patients and methods

Patients and controls

The total population ($n = 800$) in the HIV clinic at North Manchester General Hospital were screened to identify those on dapsone for primary or secondary prophylaxis against PCP. Forty patients who were on a standard dose of dapsone (50 mg day^{-1}) were identified and took part in the study. HIV-negative controls were recruited from Liverpool. Ethical approval was obtained from the Ethics Committees in Liverpool and Manchester, and subjects gave informed consent. Of the 40 patients with HIV infection who took part in the study, 35 were Caucasians, two patients were of African origin, two were of mixed race and one was Asian. Slow acetylator phenotype and genotype were determined in all patients and controls using a blinded protocol.

Following completion of the analysis, the patients were unblinded. Thirty-two of the patients were found to be SMX hypersensitive. Hypersensitivity reaction was defined as fever and widespread erythematous maculopapular rash that disappeared on drug withdrawal. Another patient who developed Stevens-Johnson syndrome 23 days after starting therapy with cotrimoxazole (960 mg day^{-1}) was also included in the hypersensitive group. Eighteen patients were taking dapsone as secondary prophylaxis and 22 as primary prophylaxis. Patients were staged according to the Centers for Disease Control and Prevention classification (16 each were in stage B and stage C).

Eight patients (five stage B and three stage C) were found to be intolerant of cotrimoxazole because of nausea and vomiting (seven of them on 960 mg day^{-1} ; one of them 1920 mg twice daily). They were assigned into a nonhypersensitive control group. Further nonhypersensitive patients were not recruited, as administration of a single dose of dapsone to these patients was felt to be unethical because (i) there was the theoretical risk of inducing resistance, and (ii) there might be a possibility of changing the phenocopy due to concomitant intake of cotrimoxazole. In addition, 26 healthy Caucasian volunteers were genotyped and phenotyped 3 h after a single oral dose of dapsone (50 mg).

Phenotyping

Phenotyping was performed using dapsone as the acetylation probe by determination of the ratio of monoacetyldapsone (MADDS) to dapsone (DDS) in human plasma [19]. Plasma was prepared by centrifugation (3000 g , 10 min) and an aliquot ($300 \mu\text{l}$) was spiked with internal standard (pyrimethamine, $0.5 \mu\text{g}$) before extraction with ethyl acetate ($2 \times 1 \text{ ml}$). The organic extracts were combined and evaporated to dryness at 30°C under a steady stream of nitrogen. The residue was reconstituted in mobile phase ($150 \mu\text{l}$) and vortexed. An aliquot ($50 \mu\text{l}$) of this solution was then injected onto a Hypersil Elite column ($5 \mu\text{m}$ HyPURITY Elite C_{18} , $150 \times 4.6 \text{ mm}$ internal diameter), and the compounds eluted with a mobile phase consisting of acetonitrile/ammonium acetate 25 mM , pH 3.5 (20:80 v/v) flowing at 1.0 ml min^{-1} . The eluate was monitored at 254 nm . Quantification of DDS and MADDS was by comparison of the metabolite peak in relation to the area of the internal standard peak. The retention times for dapsone, monoacetyldapsone and pyrimethamine were 6, 8 and 13 min, respectively. The limit of quantification was 100 ng ml^{-1} for both dapsone and monoacetyldapsone. The determined values of accuracy, interday and intraday precision of the method at the lowest working concentrations were within an acceptable range of $\pm 10\%$. Probit analysis showed the antimode to be 0.35, and thus subjects with a MADDS to DDS molar ratio >0.35 were classified as rapid acetylators, while the remaining subjects were classified as slow acetylators.

Genotyping

NAT2 genotyping was performed using the method described by Hickman and Sim [20] and Hickman *et al.* [21]. Amplification of genomic DNA (approximately 100 ng) was carried out by the polymerase chain reaction (PCR) in total volume of $100 \mu\text{l}$ using a Perkin Elmer GeneAmp 9700 thermal cycler. Two sets of oligonucle-

otide primers were used. Nat-Hu7 (sense) and Nat-Hu8 (antisense) generated a 840-bp fragment which was separately digested with *KpnI* and *TaqI* restriction enzymes. The PCR product (1000 bp) from the Nat-Hu14 (sense) and Nat-Hu16 (antisense) amplification was separately digested with *BamHI*, *DdeI* and *HindII* enzymes. The combination of restriction fragments generated allows the determination of the wild-type *NAT2**4, and the allelic variants *NAT2**5A, *NAT2**5B, *NAT2**5C, *NAT2**5F, *NAT2**6A to *NAT2**6E and *NAT2**7A, *NAT2**7B alleles. The *HindII* digest was carried out to confirm that *NAT2* had been amplified and not *NAT1*, as *NAT1* coding region does not contain a *HindII* restriction site. The digested PCR product was separated on a 3% Nusieve :Agarose (3 : 1) gel along with DNA molecular weight markers and visualized by ethidium bromide and ultraviolet transillumination. All genotype assignments were blind to case–control status.

Sequencing of discordant genotype/phenotype results

All *NAT2* genotype/phenotype discordant results were analysed by direct sequencing of the purified PCR product (Nucleon QC for PCR/oligo cleanup; Tepnel Life Sciences PLC, Manchester, UK). Automated DNA sequencing was performed on an ABI 377 sequencer by using BigDye Terminator Version 2 reactions (Perkin Elmer/Applied Biosystems, Warrington UK) (Figure 1). The coding region of the gene was compared with the *NAT2**4 sequence (wild type) to identify mutations that were not detected by the PCR–restriction fragment length polymorphism (RFLP) genotyping methods described above.

Systematic literature review

Relevant published studies were identified from MEDLINE. The search was restricted to papers with abstracts in English. The following keywords were used: *NAT2*, N-acetyltransferase, phenotype, genotype, sulphamethoxazole, hypersensitivity. All studies that reported *NAT2* phenotype in HIV-positive SMX-hypersensitive patients compared with nonhypersensitive patients regardless of the acetylator probe were included. We did not include unpublished observations or personal communications.

Statistical analysis

Arcus Quickstat Biomedical software (Research Solutions, Cambridge, UK) was used for statistical analysis. Frequencies of the slow acetylator phenotype, genotype and allelic frequencies were compared using χ^2 test and Fisher's exact test. Meta-analysis of published studies was performed by the Mantel–Haenzsel test and data pre-

Figure 1 Sequencing details of the *NAT2* gene in two patients in whom there was a discordance between genotype and phenotype. One of the patients was heterozygous for a mutation on position 341 (T→C) (shown in top panel). Genotype *NAT2**5B/*12A was determined from the additional mutations 481 (C→T) and 803 (A→G) found in the same patient by sequencing and restriction enzyme patterns. The other patient was homozygous for mutation on position 191 (G→A), the inactivating mutation for *NAT2**14 (lower panel). The same patient was also homozygous for mutation 282 (C→T).

sented graphically as Cochrane plots. Tests for heterogeneity were performed using the Review Manager 4.1 software (Update Software, Oxford, 2000).

Results

Acetylator status was determined in 32 HIV-positive SMX-hypersensitive patients (median age 39 years; range 26–55 years), eight HIV-positive SMX-nonhypersensitive patients (median age 37 years, range 24–54 years) and 26 healthy volunteers (median age 33 years; range 22–48 years).

The frequencies of the slow acetylator genotype are shown in Table 1 and Figure 2. All the polymorphism frequencies in the patients and controls met the Hardy–Weinberg equilibrium. The slow acetylator *NAT2* genotype (and thus the deduced phenotype) in SMX-hypersensitive patients was not different from SMX-

Table 1 Frequencies of the slow acetylator genotypes and phenotypes in HIV-positive patients with and without hypersensitivity to SMX, and in healthy volunteers.

NAT2 genotype/ phenotype	HIV-positive patients		Healthy volunteers (n = 26)
	SMX- hypersensitive (n = 32)	SMX- nonhypersensitive (n = 8)	
Slow genotype	20 (63%)	3 (38%)	18 (69%)
Slow phenotype	17 (53%)	3 (38%)	17 (65%)

NAT2, N-acetyl transferase type 2; SMX, sulphamethoxazole.

nonhypersensitive patients [odds ratio (OR) 4.4; 95% confidence interval (CI) 0.98, 20.1] or healthy volunteers (OR 0.7; 95% CI 0.3, 2.2).

The slow acetylator phenotype in the patients and controls was determined using dapsone as a probe. The frequencies of the slow acetylator phenotype determined by this method are shown in Table 1. There was no difference in the frequency of the slow acetylator phenotype between SMX-hypersensitive and nonhypersensitive patients (OR 1.9; 95% CI 0.38, 9.3). There was also no difference between SMX-hypersensitive patients and healthy volunteers (OR 0.6; 95% CI 0.2, 1.74).

An initial analysis of the discrepancies between phenotype and genotype based on the above results revealed discordance in eight patients. Six of the patients had a slow acetylator genotype, but had a measured fast phenotype, while two who were genotypically fast had a slow phenotype. In order to ensure that these patients did not have any rare alleles that were missed by the PCR-RFLP genotyping, the whole exonic region was amplified and sequenced (Figure 1). Using this procedure, we were able to resolve the discrepancies in four of the patients. In one hypersensitive and one nonhypersensitive patient, both of whom were African in origin, an inactivating mutation for NAT2*14 at position 191 (G→A) was found which changed the assignment of the genotype from fast to slow [22, 23]. In the other two patients with discrepancies, both of whom were Caucasians, the genotype assignment was changed from slow to fast after identification of the NAT2*12 cluster, which is associated with a fast acetylator phenotype [23, 24].

The final relationship between NAT2 genotype and MADDs/DDS ratio in SMX-hypersensitive patients is shown in Figure 2. In our cohort, discordance between genotype and phenotype was therefore found in three patients with SMX hypersensitivity (Figure 2a) and in one healthy control (Figure 2c). All the discrepancies were due to assignment of a slow genotype but a fast (borderline) phenotype. In SMX-nonhypersensitive patients, there were no discrepant findings (Figure 2b).

Figure 2 A graphical representation of the relationship between the NAT2 genotypes and the acetylator phenotype (as assessed by the MADDs/DDS ratio) for (a) SMX-hypersensitive subjects, (b) SMX-nonhypersensitive subjects, and (c) healthy volunteers. The horizontal line represents antimode (MADDs/DDS ratio of 0.35).

We also undertook a systematic review to identify studies that addressed the topic of NAT2 acetylator status in HIV-positive patients with SMX hypersensitivity; three such studies satisfied our predefined criteria [8, 13, 25]. The results from these studies were pooled together with our findings using the Mantel-Haenszel test (Figure 3). Of the different studies, only the study by Carr *et al.* [8] showed an association between slow acetylator phenotype and SMX hypersensitivity, while the other two published studies and our findings did not support such an association. Pooling of the data revealed that there was no difference in the frequency of the slow

Figure 3 Cochrane plots illustrating the DerSimonian Laird (DL) pooled odds ratio for the random effects model (derived from the Mantel-Haenszel test) utilizing data from different studies reporting NAT2 phenotype in HIV-positive SMX-hypersensitive patients. The size of each diamond represents the weighting of each study in providing the DL odds ratio and its 95% confidence intervals (CI).

acetylator phenotype between hypersensitive and nonhypersensitive patients (pooled OR 2.25; 95% CI 0.45, 11.17; Figure 3).

Discussion

Cotrimoxazole is the drug of choice for the treatment and prophylaxis of PCP, even in patients infected with mutant forms of *P. carinii* [26]. SMX, a component of cotrimoxazole, is metabolized by the N-acetyltransferases and cytochrome P450 enzymes. Although a relatively small proportion of SMX undergoes oxidation at the N4-position (approximately 2%) [27], it is thought that the toxic hydroxylamine and nitroso metabolites formed in this way are responsible for the idiosyncratic adverse reactions [28]. Both NAT isozymes, NAT1 and NAT2, are involved in SMX metabolism [29], with about 40% of the SMX undergoing N-acetylation. In subjects with a decreased capacity for N-acetylation, the balance between the different routes of metabolism is disturbed, allowing more of the parent drug to become available for oxidative metabolism [9, 30]. Both NAT1 and NAT2 are also capable of converting SMX-hydroxylamine into N-acetoxy-SMX, which could also be involved in sulphonamide hypersensitivity [31].

In HIV-negative patients, slow acetylator status, assigned by both phenotype [9] and genotype [10, 11], has been shown to predispose to SMX hypersensitivity. However, the number of patients studied was small, and a nonhypersensitive control group was included in only one study [11]. In HIV-positive patients studied at the time of an acute illness, a high prevalence of the slow

acetylator phenotype was found in two of the studies [12, 25], but not in other studies [16, 32]. The latter studies also reported a high degree of discordance between NAT2 phenotype and genotype in HIV patients using caffeine as a probe for acetylator status [16, 32]. A more recent study has reported an even higher discordance rate of 35% [25]. By contrast, a high degree (>90%) of genotype/phenotype concordance using caffeine as a probe has also been reported in HIV-positive patients [13], which accords with the healthy population, where genotype and phenotype are generally interchangeable measurements [33].

In view of these contradictory findings, we have undertaken a study in another HIV patient cohort to (i) determine whether slow acetylator phenotype predisposes to SMX hypersensitivity, and (ii) investigate the causes of discrepancy between phenotype and genotype. Our results show that there was no difference in frequency of slow acetylator phenotype between hypersensitive and nonhypersensitive patients, and healthy volunteers. This is in accordance with some [13, 25] but not all [8] of the previously published studies. A limitation of our study, and indeed of all the other previously published studies, was that only small numbers of patients were investigated. Given that the frequency of the slow acetylator phenotype is approximately 50% in the local population [34], the numbers studied here only provide adequate statistical power (α 0.05) to detect an OR of approximately 5 between hypersensitive patients and controls. For ethical reasons, we studied only patients who were already on dapsone, rather than administer another phenotyping probe, which limited the numbers recruited. Furthermore, the advent of highly active anti-retroviral therapy has led to a decrease in the use of cotrimoxazole prophylaxis in the HIV population.

In view of the difficulty in recruiting adequate numbers, we performed a meta-analysis of all previously published studies (Figure 3). This has increased the statistical power to detect an OR of 2.5 between hypersensitive and nonhypersensitive patients. Thus, it can be concluded from the primary and secondary data presented in this study that slow acetylator phenotype is unlikely to be a risk factor in predisposing to SMX hypersensitivity, although its role as a minor predisposing factor cannot be excluded completely.

In this study, there was also no association between acetylator genotype and SMX hypersensitivity. This confirms findings from our previous larger study [15], which also included a meta-analysis of previously published genotyping studies, and has also been reiterated by a study published subsequently [25].

The other aim of our study was to determine the degree of discordance between acetylator genotype and

phenotype in our patients and controls. We found discordance in three out of 32 SMX-hypersensitive patients (9%), and one out of 26 healthy volunteers (4%), which is lower than that reported previously [25]. Initially, however, a higher rate of discordance was found, due to the NAT2 genotyping method used in the study. We did not include assays that detect the T341C polymorphism directly or screen for NAT2*14 allele. In four patients we were able to resolve the conflict between phenotype and genotype by direct sequencing of the exon of the NAT2 gene. This revealed other mutations that were not detected by PCR-RFLP analysis used to genotype patients. Our PCR-RFLP analysis detects approximately 95% of nucleotide changes in the NAT2 gene [35, 36]. Thus, the higher rates of discordance reported in the previous studies may have been due to nondetection of the less common nucleotide changes in the NAT2 gene [13, 16, 32].

The reasons for the discrepancy between phenotype and genotype in the four other individuals reported are unclear. All the discrepant results were due to a genotypically slow acetylator status but measured fast acetylator phenotype. This seems illogical given that the polymorphisms identified in our subjects within the NAT2 gene have previously been shown to reduce or abolish NAT2 activity [23], and thus would suggest interference with the phenotyping methodology used in HIV-positive patients. It is interesting to note that the MADDS/DDS ratio for all of the patients displaying discordance between phenotype and genotype was close to the anti-mode of 0.35 identified by probit analysis; this has also been used previously [16]. Our anti-mode has wide 95% confidence limits (0.2, 0.6), and thus discordance could be due to the ratio used for detecting phenotypically slow acetylators. It is also possible that there may be another minor pathway involved in the N-acetylation of dapsone; whether NAT-1 can also acetylate dapsone is unknown. Another probe that has been used to determine acetylator status is caffeine. However, we chose to use dapsone instead of caffeine for several reasons. Firstly, our HIV-positive patients who experienced SMX hypersensitivity reaction were given dapsone as prophylaxis for opportunistic infection, which eliminated the need to increase further their very high pill burden. Secondly, the genotype/phenotype discrepancy rate is higher when caffeine rather than dapsone is used as a probe in HIV-positive patients [16]. Thirdly, determination of acetylator phenotype with dapsone is dependent upon determination of a primary metabolite [37], while caffeine relies upon determination of a secondary metabolite without knowing the intermediate [38]. Fourthly, acetylator status determined using dapsone as a probe correlates well with other acetylator probes, is stable 2 h after a single oral

dose and remains unchanged during long-term therapy [37, 39]. Another factor that might have contributed to the discrepancy is that metabolic phenotype is a continuous measure of activity and should be treated as a continuous rather than a dichotomous variable. Meisel and coworkers have found a gene-dose effect of the mutation pattern in the NAT2 gene on enzymatic activity, and that interindividual variability in phenotype is only partially related to genotype [40].

In conclusion, our data suggest that there is unlikely to be an association between slow acetylator phenotype and genotype and hypersensitivity to SMX in HIV-positive patients, although a minor role cannot be excluded. The previously reported discrepancy between phenotype and genotype may be due partly to nondetection of rare alleles using conventional PCR-RFLP genotyping methodology. Ideally, all studies investigating acetylator status should include assessment of both phenotype and genotype, although the former may be difficult and more expensive to perform in large-scale population-based studies.

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