Identification of a novel splice-site mutation in the CYP1A2 gene

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Aims To identify the molecular basis for a low CYP1A2 metabolic status, as determined by a caffeine phenotyping test, in a 71-year-old, nonsmoking, Caucasian woman who presented with very high clozapine concentrations despite being administered a standard dose of the drug.

Methods The nucleotide sequence of the 7 exons, exon-intron boundaries and 5′-flanking region of the *CYP1A2* gene was analysed by direct sequencing.

Results Only one heterozygous point mutation was identified in the donor splice site of intron 6 (3534G > A) of *CYP1A2*. This mutation could cause abnormal RNA splicing and therefore lead to a truncated nonfunctional enzyme. No other carrier of this mutation was identified in a population of 100 unrelated healthy Caucasians.

Conclusions This is the first report of a splice-site mutation affecting the *CYP1A2* gene. This polymorphism is a likely explanation for the low CYP1A2 activity associated with high clozapine concentrations in this patient.

Keywords: CYP1A2, cytochrome P450, pharmacogenetics, polymorphism, splicing mutation

Introduction

Cytochrome P450 1A2 (CYP1A2) is an enzyme implicated in the metabolic activation of environmental carcinogens, as well as in the metabolism of several drugs [1]. There are pronounced interindividual differences in CYP1A2 activity among humans, although the existence of a true phenotypic polymorphism in enzyme activity has not been unequivocally demonstrated to date [1–3]. Overall, it is recognized that CYP1A2-mediated slow and intermediate metabolizers represent about 50% of Caucasians [1]. Variability in CYP1A2 activity may influence individual susceptibility to cancer risk and therapeutic efficacy of some drugs. Different factors such as gender, race and environmental exposure to inducers or inhibitors are responsible for interindividual differences in the CYP1A2 phenotype [1]. Induction of CYP1A2 expression by smoking and inhibition of activity by oral contraceptives, for example, partly explain the variation in *in vivo* enzyme activity [1, 4]. Several genetic polymorphisms have been identified, in particular in the 5′-flanking region and in intron 1 of *CYP1A2* [5–7, see also http://www.imm.ki.se/CYPalleles/cyp1a2.htm]. Some of these polymorphisms may be associated with altered inducibility of gene expression in smokers [6–8]. However, no genetic polymorphism has been reported to date that results in the expression of a CYP1A2 protein lacking catalytic activity.

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Methods

A 71-year-old, nonsmoking, Caucasian woman, hospitalized with a schizoaffective disorder, mania type diagnosis (ICD 10 F25.0), was suspected of presenting with an overdose of clozapine, an antipsychotic drug. During the period of the study, the patient exhibited slight hypoalbuminaemia (35 g l^{-1}, normal range: 37–51 g l^{-1}), but with no known somatic disease or organ dysfunction, and with normal hepatic and renal function as assessed by standard clinical chemistry tests performed on several occasions (total bilirubin 17 μmol l^{-1}; aspartate aminotransferase 14 U l^{-1}; alanine aminotransferase 9 U l^{-1}; alkaline phosphatase 25 U l^{-1}; γ-glutamyltransferase 13 U l^{-1}; urea 5.6 mmol l^{-1}; creatinine 82 μmol l^{-1}; Na^+ 1.42 mmol l^{-1}; K^+ 4.0 mmol l^{-1}). Clozapine and *N*-desmethylclozapine (norclozapine) were measured by gas chromatography with the use of a nitrogen phosphorus

detector, according to the method described by Bondesson & Lindström [9] with slight modifications. Intraday and interday coefficients of variation for the determination of clozapine and norclozapine were between 5 and 10% and between 7 and 15%, respectively. The limit of quantification was 4 ng ml^{-1} for both substances.

As clozapine is metabolized to norclozapine mainly by CYP1A2 [10–12], a caffeine phenotyping test [13] was performed in order to define the CYP1A2 metabolic status of the patient. Caffeine and its metabolite (paraxanthine) were measured by gas chromatography–mass spectrometry according to a method developed in our laboratory [Eap *et al.* unpublished data, available on request]. Intraday and interday coefficients of variation for the determination of caffeine and paraxanthine were between 2 and 7% and between 6 and 15%, respectively. The limit of quantification was 0.8 ng ml^{-1} for both substances.

Using a genomic DNA sample from this woman, we analysed the nucleotide sequence of the 5′-flanking region (from nucleotide −4078 to −870), the 7 exons and the exon-intron boundaries of the *CYP1A2* gene. In order to sequence the 5′-flanking region of *CYP1A2*, a 3.2 kb fragment was first amplified by using a pair of *CYP1A2*-specific primers (forward primer: 5′-CAGGGACTTCT TGGATGCTTATGATGTCTC-3′; reverse primer: 5′-GGGTTGTAATGGCTGGTGTGGAGCTTCTGG-3′) and a TaKaRa Ex Taq™ kit (BioWhittaker, Verviers, Belgium), according to the manufacturer's instructions. Each of the 7 exons and their proximal flanking sequences were amplified according to a double step-PCR procedure, as described previously [14]. Nucleotide sequences were then determined by using an automated DNA sequencer (Model 373 A, Applied Biosystems, Foster City, USA) and the ABIPRISM Dye Terminator Cycle Sequencing Ready Reaction FS kit (Applied Biosystems) according to manufacturer's instructions. Both strands of each DNA fragment were sequenced. Approval for the study was obtained from the local Ethics Committee (Avis de la Commission d'Ethique de la Psychiatrie, University of Lausanne) and the patient gave her written informed consent for the phenotyping and genotyping tests, as well as for the possible publication of the data collected during this case study.

In order to estimate the frequency of this polymorphism, we re-examined the exon 6 of *CYP1A2* in a previously studied population of 100 unrelated healthy volunteers of French Caucasian origin, using a PCR-SSCP strategy, as described elsewhere [14].

Results

Therapeutic drug monitoring of clozapine and norclozapine was first requested due to the suspicion of a clozapine overdose, despite the patient being administered a standard dose, i.e. 300 mg day^{-1}, for 6 days. To achieve this dose, increasing amounts of the drug were given over a 7-week period.

The analysis revealed a high steady-state plasma concentration of clozapine (1296 ng ml^{-1}) and of its metabolite (760 ng ml^{-1}); (comedication: lactitol 20 ml day^{-1}). Clozapine dose was then reduced to 100 mg day^{-1} because concentrations of clozapine above 1000 ng ml^{-1} increase the risk of adverse effects on the central nervous system, causing confusion, delirium and generalized seizures [15]. Two analyses performed 2 weeks and 2 months after the dose reduction showed a corresponding decrease in the plasma concentrations of clozapine and norclozapine (first determination: clozapine: 406 ng ml^{-1}; norclozapine: 324 ng ml^{-1}; comedication: clomethiazole 300 mg day^{-1}, lactilol 20 ml day^{-1}; second determination: clozapine: 475 ng ml^{-1}; norclozapine: 205 ng ml^{-1}; comedication: flurazepam 30 mg day^{-1}, lactilol 20 ml day^{-1}).

The caffeine phenotyping test revealed a slow CYP1A2 phenotype for this patient. Her plasma caffeine and paraxanthine concentrations measured 6 h after the oral intake of 200 mg of caffeine were 7.1 μg ml^{-1} and 1.4 μg ml^{-1}, respectively, giving rise to a paraxanthine-caffeine ratio of 0.21, corresponding to a value of 0.32 ml min^{-1} kg^{-1} for systemic caffeine plasma clearance [13]. The patient was also taking clozapine 100 mg day^{-1} and flurazepam 30 mg day^{-1} at the time of the caffeine test. The paraxanthine-caffeine ratio is among the lowest found in the values (approximate range: 0.25–1.5) presented in a report that analysed retrospectively the caffeine clearance in four studies comprising a total of 78 subjects [13].

The sequencing of the 5′-flanking region, the 7 exons and their exon-intron junctions of the *CYP1A2* gene identified only one heterozygous point mutation in the patient's DNA (Figure 1b). This polymorphism is located in the 5′ splice-site consensus sequence of intron 6, substituting the invariant GT dinucleotide with AT (mutation 3534G > A according to the *CYP1A2* gene sequence published by Corchero *et al.* [16]). According to the current human CYP allele nomenclature, this novel allelic variant was termed *CYP1A*⋆7. Using the PCR-SSCP strategy previously described by Chevalier *et al.* [14], the mutation was clearly detected through an abnormal SSCP pattern (Figure 1a) and no other carrier of the 3534G > A substitution was identified in a control group comprising 100 unrelated French individuals, suggesting that the mutation is very rare in Caucasian populations.

Discussion

Our findings strongly suggest that the 3534G > A mutation in the *CYP1A2* gene identified in the patient,

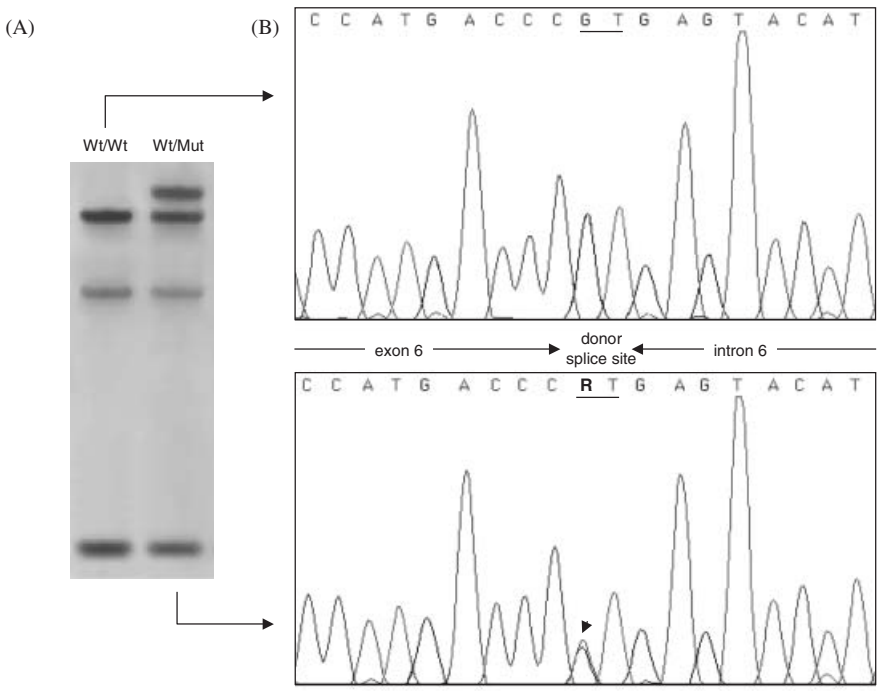

Figure 1 Detection by PCR-SSCP (a) and identification by direct sequencing (b) of the 3534G > A mutation in the *CYP1A2* gene. A PCR fragment encompassing exon 6 of *CYP1A2* and its proximal flanking sequences has been analysed by SSCP and direct sequencing as described in the **Methods** section. A DNA sample from the patient with a slow CYP1A2 activity (Wt/Mut) is compared with that from a subject with a homozygous wild-type genotype (Wt/Wt). The arrow indicates the position of the heterozygous G/A mutation in the donor splice site of intron 6. R; guanine or adenine.

although only present in a heterozygous state, is a likely explanation for the elevated plasma concentrations of clozapine and the low CYP1A2 activity as determined by the caffeine phenotyping test.

It is recognized that donor (GT) and acceptor (AG) splice-site consensus sequences are major determinants of accurate splicing [17] and therefore the observed *G* > A transition in the donor splice-site of *CYP1A2* intron 6 is likely to affect normal splicing efficiency. Considering the absence of a potential alternative donor splice site in the immediate vicinity of the mutation, exon skipping is more likely to occur than activation of a cryptic splice-site [17]. Extensive studies of the consequences of mutations in splice-sites have shown that when a mutation of the 5′ splice-site leads to exon skipping, it is always the upstream exon immediately preceding the lesion that is removed from the subsequent mRNA transcript [17]. Exon 6 skipping could alter the CYP1A2 protein sequence whereby a missense mutation (Ser388Arg) and a 29-amino acid deletion (delThr389-Pro417) would occur, leading to a truncated protein with no retained activity. *In vitro* expression analysis of an allelic minigene, consisting of exons 5–7 of the mutant gene, would allow the confirmation of our hypothesis.

Variability in CYP1A2 activity is a major determinant of the large interindividual differences in plasma concentrations of clozapine [11]. In addition, the concentrations of clozapine and norclozapine measured in this patient are compatible with a heterozygous status, as they are in the upper range of those usually measured. Thus, in one study in which 24 out of 29 patients received clozapine at a dosage of 400 mg day^{-1}, the mean ±SD (range) clozapine and norclozapine concentrations were 374 ± 233 ng ml^{-1}, 84–1088 ng ml^{-1}: 116 ± 65 ng ml^{-1}, 25–272 ng ml^{-1}, respectively [18]. In another study in which 61 patients received a fixed dose of 400 mg day^{-1} of clozapine for 6 weeks, the mean blood concentrations of clozapine were 598 ± 314 ng ml^{-1} (range: 111–1585 ng ml^{-1}) [19]. To our knowledge, a homozygous CYP1A2 poor metabolizer status has never been described. However, phenocopying can occur when a potent CYP1A2 inhibitor such as fluvoxamine, an antidepressant, is given at a medium to high dose. In one patient clozapine and norclozapine concentrations were 2166 ng ml^{-1} and 615 ng ml^{-1} after receiving 400 mg day^{-1} of clozapine and 100 mg day^{-1} of fluvoxamine [20]. After fluvoxamine, plasma concentrations of both clozapine and norclozapine were increased, which suggests that CYP1A2 was also involved in the degradation of norclozapine [21]. This is in agreement with the present case report where elevated plasma concentrations were observed both for clozapine and its metabolite. In two other patients treated with 300 mg day^{-1} of clozapine plus 75 mg day^{-1} of fluvoxamine, and with 500 mg day^{-1} of clozapine plus 150 mg day^{-1} of fluvoxamine, the concentrations of clozapine were 1678 ng ml^{-1} and a maximum of 2911 ng ml^{-1}, respectively [20].

The values of caffeine clearance measured in this patient (0.32 ml min^{-1}) are also compatible with a heterozygous status for an inactivating *CYP1A2* mutation. Thus, in other studies, systemic caffeine plasma clearance was found to vary from 0.3 to 3.3 ml min^{-1} kg^{-1} [13].

Again, when fluvoxamine is given as a comedication, a value of 0.03 ml min^{-1} kg^{-1} for systemic caffeine plasma clearance was obtained, indicative of an almost complete inhibition of CYP1A2 activity corresponding to a homozygous CYP1A2 poor metabolizer status [22].

In conclusion, this is the first report of a polymorphism affecting a splice-site consensus sequence of the *CYP1A2* gene. This mutation could cause abnormal RNA splicing and, thereby, lead to the expression of a truncated protein lacking catalytic activity.

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