NAT1 genotypes do not predict response to mesalamine in patients with ulcerative colitis

Abstract

BACKGROUND: 5- Aminosalicylic acid (5-ASA) is metabolised in colonic mucosa by N-acetyltransferase 1 (NAT1). Common genetic polymorphisms in this enzyme result in rapid or slow acetylation. 5-ASA treatment causes side effects in up to 10 % of patients with ulcerative colitis (UC). We therefore determined genetic variations of NAT1 in patients with UC and looked for a possible association with the clinical response to 5-ASA. **METHODS:** DNA was obtained from 78 patients with UC. 77 % of the patients were in remission during 5-ASA treatment, whereas 23 % suffered from active disease. NAT1 genotyping was performed for 23 known alleles using RFLP and sequence analysis. Clinical response to 5-ASA was determined by medical record review and associated with NAT1 genotypes. **RESULTS:** Utilising PCR we amplified a 570-bp coding region of the human NAT1 gene in addition to 240 bp in the 3'-untranslated region (UTR). 4 NAT1 alleles previously known as NAT1*3, *4, *10 and *11 were recovered. 31 % of the patients were heterozygous and 4 % homozygous for the NAT1*10 allele. 6 % were heterozygous for the NAT1*3 allele. 6 % were heterozygous for the NAT1*11 allele. No association was found between NAT1 genotype and clinical response as well as side effects to 5-ASA in patients with UC. **CONCLUSIONS:** NAT1 genotypes do not predict response or side effects to mesalamine in patients with UC. Variations caused by non-genomic effects may be associated with the clinical response to 5-ASA.

NAT1 genotypes do not predict response to mesalamine in patients with ulcerative colitis

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Running Title: NAT1 and ulcerative colitis therapy with 5-ASA

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Abbreviations: 5-ASA = 5-Aminosalicylic acid, bp = base pairs, CD = Crohn's disease, EDTA = ethylenediaminetetraacetic acid, IBD = inflammatory bowel disease, min = minutes, NAT1 = N-acetyltransferase 1, NI = non-inflamed, RFLP = Restriction fragment length polymorphism, UC = ulcerative colitis, UTR = untranslated region

Summary

Background: 5- Aminosalicylic acid (5-ASA) is metabolized in colonic mucosa by N-acetyltransferase 1 (NAT1). Common genetic polymorphisms in this enzyme result in rapid and slow acetylation. 5-ASA treatment causes side effects in up to 10% of patients with ulcerative colitis (UC). We therefore determined genetic variations of NAT1 in patients with UC and a possible association with the clinical response to 5-ASA.

Methods: DNA was obtained from 78 patients with UC. 77% of the patients were in remission during 5-ASA treatment, whereas 23% suffered from active disease. NAT1 genotyping was performed for 23 known alleles using RFLP and sequence analysis. Clinical response to 5-ASA was determined by medical record review and associated with NAT1 genotypes.

Results: Utilizing PCR we amplified a 570-bp coding region of the human NAT1 gene in addition to 240 bp in the 3'- untranslated region (UTR). 4 NAT1 alleles prior known as NAT1*3, *4, *10 and *11 were recovered. 31% of the patients were heterozygous and 4% homozygous for the NAT1*10 allele. 6% were heterozygous for the NAT1*3 allele. 6% were heterozygous for the NAT1*11 allele. No association was found between NAT1 genotype and clinical response as well as side effects to 5-ASA in patients with UC.

Conclusions: NAT1 genotypes do not predict response or side effects to mesalamine in patients with UC. Variances caused by non-genomic effects may be associated with the clinical response to 5-ASA.

Introduction

5-Aminosalicylate (5-ASA, mesalazine) is a therapeutically active agent during acute phase and remission maintenance treatment of UC [1]. Intestinal metabolite tissue levels of 5-ASA correlate with the therapeutic effect [2]. Efficacy of 5-ASA correlates with tissue delivery and, therefore, tissue delivery, intestinal metabolism and elimination may be important in determining drug efficacy [3].

5-ASA is metabolised by N-acetylation catalysed by N-acetyltransferases (NAT). Two isozymes NAT1 and NAT2 have been described in intestine [4][5] but NAT1 has been shown to be significantly more important for 5-ASA metabolism than NAT2 [6]. Expression of NAT1 and NAT2 mRNAs in humans is found in the whole gastrointestinal tract (esophagus, stomach, small intestine and colon) [5][7][8]. The specific NAT1 and NAT2 mRNAs were also present in small intestinal epithelial cells [9]. NAT1 activity is detected along the whole length of the [10].

It has been shown that intestinal metabolism of 5-ASA contributes to a greater extent to total body clearance than systemic N-acetylation of 5-ASA (as measured by urinary excretion) when the drug is administered as a delayed release dosage form [11][12][13][14].

There is up to a 20-fold difference in acetylation rates of NAT1 substrates amongst different individuals [10][15][16][17][18][19]. NAT1 and NAT2 have been shown to differ significantly with respect to their intrinsic stability and their kinetic selectivity for amine-containing acceptor substrates [5][20]. N-acetylation of 5-ASA by intestinal bacteria is minimal [21].

NAT1 polymorphic enzymes [22][23][24], and thus individuals, segregate into rapid and slow acetylator phenotypes. The variation in the acetylation of NAT1-selective substrates has been shown to have a genetic basis [25]. For patients with ulcerative colitis (UC) it was suggested that NAT1 polymorphisms may have a role in the therapeutic response to 5-ASA and for side effects [26]. It was speculated that intestinal NAT-1 activity may correlate with efficacy of 5-ASA treatment and could be important in determining drug efficacy before therapy. In a previous phenotype study by Ricart *et. al.* [27] no significant association between the clinical response to mesalamine and the NAT1 acetylator status univariately or adjusted for dose or duration of therapy was found. However, the NAT1 acetylator status has not been associated with adverse effects related to the therapy. This led us to investigate known allelic variants of NAT1 in 78 UC patients and to correlate it with 5-ASA induced side effects and the therapeutic response to 5-ASA treatment.

To date 26 allelic variants of NAT1 have been reported (<http://www.louisville.edu/medschool/pharmacology/NAT.html>). Allelic variants of NAT1 found in this study are listed in table 1. A map showing all described nucleotide substitutions proved is given in figure 1. NAT1*4 is the most common allele and was classified as wild-type. The NAT1*10 allele has been associated with rapid acetylator phenotype both in vitro [7][25] and in vivo [25][28]. NAT1*10 was mentioned to exhibit altered functional capacity [7][25]. The NAT1*11 allele has been associated with slow acetylator phenotype with low levels of activity [29].

Material and methods

Patients and DNA-isolation

Blood samples were obtained from 78 patients with UC from the German IBD competence network serum bank (Core facility Regensburg, table 2). The study was approved by the University of Regensburg Ethics Committee. Genomic DNA was purified from human whole blood samples with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, DNA was isolated following cell lysis and proteinase K digestion (56°C, 10 min). DNA was loaded on a QIAamp spin column, washed twice and eluted in 200 µl distilled water.

Medication of 78 patients UC: all patients (100%) received 5-ASA *p.o.* (median dose 3.0 g/day, range 1.0 – 8.0 g/day, median duration of treatment 365 days, range 1380 - 30 days). Glucocorticosteroids were given to 56% of the UC patients and 26% of the patients received a 6-mercaptopurine therapy.

Polymerase chain reaction (PCR)

Amplifications of NAT1 DNA fragments by PCR were performed according to a modification of a method previously described [30]. Briefly, for the initial NAT1 PCR 150 ng of genomic DNA, 150 ng of upstream primer (described in [30]), 90 ng of downstream primer were mixed in a 30 µl reaction with 15 µl master mix (Taq PCR Master Mix Kit, Qiagen, Hilden, Germany). After pretreatment at 94°C for 5 min the PCR for NAT1 comprised 30 cycles with denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and concluded with a extension step at 72°C for 7 min in a TRIO-Thermoblock (Biometra, Goettingen, Germany).

For the detection of C⁵⁵⁹T, G⁵⁶⁰A and T¹⁰⁸⁸A nucleotide changes nested PCRs with 0.5 µl NAT1 PCR product were performed in a 20 µl reaction with 200 ng of appropriate primers [30] as described above. C¹⁰⁹⁵A was detected in a 35 µl reaction.

Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis was realized according to a method previously described [30]. In brief, to confirm NAT1 amplification or to analyse the nucleotide substitution G^{350,351}C 10 µl of the NAT1 PCR product were mixed in a 20 µl reaction including 10 units *Aat*II or *Ban*I (New England BioLabs, Frankfurt, Germany), respectively in the recommended buffer at 37°C for 14 hours.

To analyse the nucleotide substitution C¹⁰⁹⁵A 20 µl of the according nested PCR product were mixed in a 35 µl reaction including 5 units *A*/w26I (MBI Fermentas, St. Leon-Rot, Germany) in the recommended buffer at 37°C for 14 hours.

To distinguish nucleotide substitutions C⁵⁵⁹T, G⁵⁶⁰A and T¹⁰⁸⁸A 17 µl of the according nested PCR product were mixed in a 20 µl reaction including 5 units of *Sc*aI, *Hin*fI or *As*eI (each New England BioLabs), respectively in the recommended buffer at 37°C for 14 hours.

Digested samples were size fractionated on a 1,2% E-gel[®] gel (Invitrogen, Leek, Netherlands) and visualized and photographed under ultraviolet light.

To detect an insertion or deletion between nucleotide 1089 and 1091 or 1065 and 1090, respectively, 30 µl of the C¹⁰⁹⁵ nested PCR product were mixed in a 50 µl reaction including 2 units of *M*seI (New England BioLabs) in the recommended buffer at 37°C for 14 hours. Digested samples were size fractionated on a 5% Metaphor gel (FMC

BioProducts, Rockland ME) containing ethidium bromide and then visualized and photographed under ultraviolet light.

DNA Sequence analysis

Results from RFLP analysis were confirmed by sequence analysis. 150 ng of genomic DNA, 140 ng of upstream primer 5' ATC TCC TAG AAG ACA GCA AAT AC 3', 140 ng of downstream primer downstream primer 5' GCT TTC TAG CAT AAA TCA CCA A 3' were mixed in a 50 µl reaction including 5 µl dNTP (10 µM each, BD Biosciences Clontech, Heidelberg, Germany) and 1 µl AdvantageTM-HF2 Polymerase Mix (BD Biosciences Clontech, Heidelberg, Germany) in the recommended buffer. After pretreatment at 94°C for 2 min the PCR comprised 30 cycles with denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 30 seconds and concluded with a extension step at 68°C for 7 min. The reactions were performed in a TRIO-Thermoblock (Biometra, Goettingen, Germany). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), sequenced and proved for heterozygous and homozygous mutations.

Statistical analysis

Expected gene frequencies were calculated from four respective single allele frequencies determined according to the Hardy-Weinberg equation for four alleles $[(p + q + r + s)^2 = 1; \text{ where } p, q, r \text{ and } s = \text{allele frequency}]$.

Results

NAT1 genotypes

We amplified a 1158-bp NAT1 PCR product containing 278 bp in the 3'-UTR. *AatII* digestion confirmed NAT1 amplification (figure 2A). NAT1 genotyping was performed for 23 alleles (NAT1*3, 4, 5, 10, 11^{A/B/C}, 14^{A/B}, 15, 16, 18^{A/B}, 21, 22, 23, 24, 25, 26^{A/B}, 27, 28 and 29) using RFLP analysis and DNA sequencing.

The nucleotide substitution G^{350,351}C (according to a mutation occurring in the NAT1*5 allele) was determined by *BanI* digests of the 1158-bp NAT1 PCR product. Gel electrophoresis revealed a 713 and 445 bp band in all analyzed samples (figure 2B) indicating that G^{350,351}C substitutions were not present in any of the patients.

The substitution C⁵⁵⁹T (allele NAT1*15) was determined by *Scal* digests of the appropriate NAT1 nested PCR product. A single 138 bp band in all analyzed samples (figure 2C) indicated that no C⁵⁵⁹T substitutions were present.

The substitution G⁵⁶⁰A (NAT1*14) was determined by *HinfI* digests of the appropriate NAT1 nested PCR product. 146, 90 and 25 bp bands in all analyzed samples (figure 2D) indicated that no G⁵⁶⁰A substitutions were present.

An insertion of a trinucleotide (AAA) (NAT1*16) and a deletion of 9 nucleotides (Δ 9, NAT1*11) was determined by *MseI* digests of the appropriate NAT1 nested PCR product. Size fractionation on a 5% Metaphor gel revealed 62, 33, 25 and 12 bp bands in 71 samples indicating no mutation. In 5 samples evidence for a heterozygous 9-bp deletion was found indicated by bands with 62, 53, 33, 25 and 12 bp. Absence of AAA-insertion and heterozygous 9-bp deletions were confirmed by DNA sequencing of all samples (figure 3). With the chosen test arrangement differentiation between NAT1*11^A and NAT1*11^C was not possible. NAT1*11^B could be excluded.

The substitution T¹⁰⁸⁸A (NAT1*10 and 14 allele) was determined by *Asel* digests of the appropriate NAT1 nested PCR product. 64 and 32 bp bands in 54 analyzed samples indicated no mutation. In 3 samples homozygous loss of the *Asel* site yielded in a single 90 bp product. In 24 samples evidence for a heterozygous T¹⁰⁸⁸A substitution was found indicated by bands with 90, 64 and 32 bp. Substitutions were confirmed by DNA sequencing of all samples (figure 3B and C).

The substitution C¹⁰⁹⁵A (according to a mutation occurring in the NAT1*3, 10, 11, 14 and 16 allele) was determined by *A/w26I* digests of the appropriate NAT1 nested PCR product. 107 and 25 bp bands in 42 analyzed samples indicated no mutation. In 3 samples homozygous loss of the *A/w26I* site yielded in a single 132 bp product. In 33 samples evidence for a heterozygous C¹⁰⁹⁵A substitution was found indicated by bands with 132, 107 and 25 bp. Substitutions were confirmed by DNA sequencing of all samples (figure 3B and C).

NAT1 genotyping for the remaining alleles was performed using DNA sequencing only. No NAT1*18^{A/B}, 21, 22, 23, 24, 25, 26^{A/B}, 27, 28 or 29 alleles were present (table 3 and 4).

Clinical response

Overall, 16 of 78 patients (21%) treated with mesalazine experienced toxicity related to the therapy with symptoms severe enough to discontinue mesalazine (table 5 and 6). The observed adverse events were as follows: nausea (3 patients), elevated liver enzymes (3 patients), stomach pain (3 patients), headache (2 patients), heartpain (2 patients), hemolysis (2 patients) and arthralgia (one patient). In all, 16 of 73 (22%) NAT1 rapid acetylator patients experienced toxicity to mesalazine (table 7). None of 5 slow acetylator patients experienced toxicity to mesalazine.

Patients with rapid and slow acetylator phenotype did not differ significantly with respect to dose ($p = 0.18$, table 7), duration of therapy ($p = 0.83$), remission time ($p = 0.80$), age at initial diagnosis ($p = 0.29$), time after initial diagnosis ($p = 0.73$) and bowel movements ($p = 0.56$).

Patients experiencing toxicity to mesalazine and patients continuing mesalazine treatment did also not differ significantly with respect to duration of therapy ($p = 0.89$), remission period ($p = 0.13$), age at initial diagnosis ($p = 0.80$), time after initial diagnosis ($p = 0.13$) and bowel movements ($p = 0.20$). Multifactorial analysis with respect to 5-ASA medication was not intended due to major dose range between 1.0 – 8.0 g/day and undersized representing patient numbers.

Discussion

In the present study we investigated allelic variants of NAT1 in UC patients treated with mesalamine. Confirming earlier data we found no relationship between the NAT1 genotype and the clinical response to mesalamine [27][31]. Interestingly, none of the UC patients with a slow acetylator phenotype experienced toxicity related to the therapy with symptoms severe enough to discontinue mesalazine.

The classification of patients with NAT1 polymorphisms into rapid and slow acetylator phenotypes is discussed controversially. The NAT1*10 allele has been associated with rapid acetylator phenotype both in vitro [7][25] and in vivo [25][28]. But recombinant expression studies have failed to confirm this conclusion [32]. Others confirmed the absence of functional consequences of enzyme activity in carriers of NAT1*10 allele [17]. The NAT1*11 allele has been associated with slow acetylator phenotype [33] and with low level of activity [29]. However, NAT1*11 was also classified as potential high activity allele [33]. In our study acetylator phenotypes were classified according to Bruhn et. al. [17]. Here the N-acetylation velocity was stratified for different genotypes of NAT1 by an assay quantifying the formation of N-acetyl-para-acetamidobenzoic acid by a HPLC method.

In a previous study by Ricart et. al. [27] NAT1 genotypes from 77 white patients of both sexes were determined. In both studies the observed frequencies of NAT1 were similar for *3/*4 (3 vs 5%), *3/*11 (0 vs 1%), *4/*4 (56 vs 54%), *4/*10 (25 vs 31%), *4/*11 (5 vs 5%), *10/*10 (5 vs 4%). However, we did not find the NAT1*3/*14A and *3/*18 allele in our patients. No association of the clinical response to mesalamine with NAT1 rapid versus slow acetylator status was found confirming the data of Ricart and coworkers [27].

Further, we found that UC patients with a slow acetylator phenotype (NAT1*3/*11 and NAT1*4/*11) experienced no toxicity related to the therapy with symptoms severe enough to discontinue mesalazine. In contrast 20% of the UC patients with normal or rapid acetylator phenotype discontinued mesalazine due to 5-ASA intolerance. The side effects documented during treatment of the UC patients included in this study were as expected from the unwanted effects described in the literature: mainly stomach pain (mild or severe); headache (mild or severe) and nausea. No case of mesalazine-induced acute renal dysfunction or failure was documented.

Consistent with previous data from other groups our data indicate that NAT1 genotypes do not predict response to mesalamine in patients with UC. However, UC patients with a slow acetylator phenotype may be less affected from toxicity to mesalamine. Variation in the acetylation of NAT1-selective substrates may have a non-genetic or environmental basis not provable with the provided study design.

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Legends

Table 1: Prior known allelic variants of NAT1 recovered in this study. NAT1*11 includes additional nucleotide substitutions not shown.

Table 2: Characteristics of patients.

Table 3: NAT1 mutations determined in 78 patients and designation to NAT1 haplotypes. The chosen test did not allow differentiation between NAT1*11^A and NAT1*11^C arrangement.

Table 4: Observed frequency distribution of NAT1 genotypes among 78 patients. Expected frequencies are calculated from the allelic frequencies (table 3) by the Hardy-Weinberg law.

Table 5: Association between NAT1 alleles and side effects, remission status or bowel movements.

Table 6: Association between NAT1 genotypes and side effects, remission status or bowel movements.

Table 7: Association between different acetylator phenotypes. None of the UC patients with slow acetylator phenotype showed 5-ASA intolerance.

Figure 1: Nucleotide substitutions proven in this study. Δ : deletion, ins: insertion, black line: 3' and 5' untranslated region, box: coding sequence. According to Cascorbi et al. [34].

Figure 2: Gel electrophoresis following restriction enzyme digests on the 1158-bp NAT1 PCR and the appropriate nested PCR products. (A) Molecular weight marker 100 bp DNA ladder (M) and *AatII* digestion (lane 1-5). 1158-bp confirmed correct NAT1 amplification. (B) Molecular weight marker ϕ X174/*HaeIII* ϕ (Promega) and *BanI* digestion (lane 1-5). 713 and 445 bp indicated no G^{350,351}C substitution. (C) 1007-bp undigested positive control (lane 1). *ScaI* digestion. 762 and 245-bp digested positive control demonstrated enzyme viability (lane 2 and 3). 138 bp indicated no C⁵⁵⁹T substitution (lane 4-6). (D) *HinI* digestion. 146, 90 and 25 bp indicated no G⁵⁶⁰A substitution (lane 1-5).

Figure 3: Position 1085–1099 of the sequenced 1158-bp NAT1 PCR product. (A) wt, (B) homozygous substitutions T¹⁰⁸⁸A (*) and C¹⁰⁹⁵A (**), (C) heterozygous substitutions T¹⁰⁸⁸A (*) and C¹⁰⁹⁵A (**), (D) heterozygous substitution C¹⁰⁹⁵A (**) and heterozygous 9 bp deletion (#).

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