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## Functional Effects of Genetic Polymorphisms in the N-acetyltransferase 1 Coding and 3' Untranslated Regions

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### Abstract

**BACKGROUND**—The functional effects of N-acetyltransferase 1 (NAT1) polymorphisms and haplotypes are poorly understood, compromising the validity of associations reported with diseases including birth defects and numerous cancers.

**METHODS**—We investigated the effects of genetic polymorphisms within the NAT1 coding region and the 3'-untranslated region (3'-UTR) and their associated haplotypes on N- and O-acetyltransferase catalytic activities, and NAT1 mRNA and protein levels following recombinant expression in COS-1 cells.

**RESULTS**—1088T>A (rs1057126; 3'-UTR) and 1095C>A (rs15561; 3'-UTR) each slightly reduced NAT1 catalytic activity and NAT1 mRNA and protein levels. A 9-base pair (TAATAATAA) deletion between nucleotides 1065-1090 (3'-UTR) reduced NAT1 catalytic activity and NAT1 mRNA and protein levels. In contrast, a 445G>A (rs4987076; V149I), 459G>A (rs4986990; T153T), 640T>G (rs4986783; S214A) coding region haplotype present in *NATI\*11* increased NAT1 catalytic activity and NAT1 protein, but not NAT1 mRNA levels. A combination of the 9-base pair (TAATAATAA) deletion and the 445G>A, 459G>A, 640T>G coding region haplotypes, both present in *NATI\*11*, appeared to neutralize the opposing effects on NAT1 protein and catalytic activity, resulting in levels of NAT1 protein and catalytic activity that did not differ significantly from the *NATI\*4* reference.

**CONCLUSIONS**—Since 1095C>A (3'-UTR) is the sole polymorphism present in *NATI\*3*, our data suggests that *NATI\*3* is not functionally equivalent to the *NATI\*4* reference. Furthermore, our findings provide biological support for reported associations of 1088T>A and 1095C>A polymorphisms with birth defects.

### Keywords

N-acetyltransferase 1 polymorphisms; haplotypes; acetylator genotype; acetylator phenotype; arylamines

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## INTRODUCTION

The human genome contains two functional arylamine N-acetyltransferase genes, which code for N-acetyltransferase 1 (NAT1) and 2 (NAT2) enzymes (EC 2.3.1.5). *NAT1* and *NAT2* both possess a single intronless exon with an open reading frame of 870 bp, encoding a 290 amino acid protein. Whereas *NAT2* expression is limited primarily to the liver and gut, *NAT1* is expressed widely across human tissues (Husain et al., 2007) including placenta (Sim et al., 2000; Smelt et al., 2000).

Human N-acetyltransferase catalyzes conjugation of an acetyl motif, usually from acetyl coenzyme A (AcCoA), to the exocyclic amine (N-acetylation) or hydroxyl (O-acetylation) of substrates. N-acetylation of the exocyclic amine usually results in detoxification (Hein, 2009). However, following N-oxidation, the N-hydroxyl metabolite undergoes O-acetylation (usually activation). The resulting N-acetoxy arylamines are highly unstable, spontaneously forming electrophilic intermediates that readily bind to protein and DNA (Hein, 1988).

Genetic polymorphisms have been observed in both *NAT1* and *NAT2* (Hein, 2009). *NAT2* polymorphisms are well established as the basis of rapid, intermediate and slow acetylation phenotypes. Excellent *NAT2* genotype/phenotype correlations have been reported (Chen et al., 2006; Ma et al., 2009; Doll et al., 2010). Although studies have investigated the functional effects of *NAT1* single nucleotide polymorphisms (SNPs) and alleles (Walraven et al., 2008), there are remaining gaps regarding effects of *NAT1* genetic variation, particularly polymorphisms in the *NAT1* 3'-untranslated region (3'-UTR).

The functional effects of individual SNPs in the *NAT1* coding region recently were reviewed (Hein, 2009). *NAT1\*11* alleles have SNPs in both the *NAT1* coding region and the 3'-UTR. Individuals possessing *NAT1\*11* alleles have been reported to have significantly lower N-acetyltransferase activities in human red blood cells (Bruhn et al., 1999) but not human leukocytes (Zhangwei et al., 2006). The latter finding is consistent with previous studies following recombinant expression of *NAT1\*11* in bacteria (Hughes et al., 1998) and COS-1 cells (de Leon et al., 2000). However, recombinant expression in COS-1 cells that focused entirely on the coding region SNPs in *NAT1\*11* showed increased protein and catalytic activity (Zhu and Hein, 2008).

*NAT1* plays an important role in the bioactivation of arylamine carcinogens such as 4-aminobiphenyl in cigarette smoke. *NAT1* also has been shown to play an important role in folate metabolism (Wakefield et al., 2007). *NAT1* polymorphisms have been investigated for associations with birth defects related to maternal smoking and with periconceptional supplementation of multivitamins that include folic acid. Two of the most common *NAT1* polymorphisms are present in the *NAT1* putative polyadenylation signal sequence: 1088T>A (rs1057126; 3'-UTR) and 1095C>A (rs15561; 3'-UTR). Oral clefts (lip with or without the palate) were strongly associated with maternal cigarette smoking in infants homozygous for 1088A or 1095A in the *NAT1* 3'-UTR (Lammer et al., 2004b). Some evidence for an interaction between maternal smoking and the homozygous 1095A genotype also has been shown towards risk of spina bifida (Jensen et al., 2005). Infants homozygous for 1088A or 1095A had a slight increase in oral clefts (lip with or without the palate) that was more robust in those mothers who did not take multivitamins (including folate) during pregnancy (Lammer et al., 2004a). Similarly, infants homozygous for 1088A or 1095A also are associated with increased limb deficiency defects, particularly in mothers with both active and passive smoke exposures, who did not take multivitamin supplements containing folate (Carmichael et al., 2006). As recently reviewed (Shi et al., 2008), although interactions between genes, environment and behavior on birth defects have been reported, further work towards understanding these interactions is needed.

Molecular epidemiological studies have reported associations between *NAT1* genetic polymorphisms and disease risk (Hein, 2009) including urinary bladder (Taylor et al., 1998; Katoh et al., 1999; Gago-Dominguez et al., 2003; Sanderson et al., 2007), colorectal (Bell et al., 1995; Chen et al., 1998; Ishibe et al., 2002; Lilla et al., 2006; Shin et al., 2008), breast (Millikan et al., 1998; Zheng et al., 1999; Lee et al., 2003; Ambrosone et al., 2007), lung (Wikman et al., 2001; Gemignani et al., 2007), prostate (Hein, 2002; Rovito, Jr. et al., 2005), and pancreatic cancers (Li et al., 2006; Jiao et al., 2007; Suzuki et al., 2008), and non-Hodgkin lymphoma (Morton et al., 2006; 2007; Kilfoy et al., 2010).

Although human epidemiological studies suggested a role for *NAT1* genetic polymorphism in susceptibility to birth defects and numerous cancers, the present poor understanding of the relationship between *NAT1* genotype and phenotype compromises the accuracy and the validity of the association observed. Understanding the molecular mechanisms of *NAT1* polymorphisms is needed to improve understanding of human epidemiological studies. Therefore, the purpose of this study was to investigate functional effects of some of the more common genetic polymorphisms in the *NAT1* open reading frame and the 3'-UTR.

## MATERIALS AND METHODS

### Materials

Cloning vector pCDNA3.1, transfection control plasmid pCMV SPORT-βgal, O-nitrophenyl beta-D-galactopyranoside and lipofectamine plus reagent were obtained from Invitrogen Life Technologies (Carlsbad, CA USA). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA USA). The protein assay kit was purchased from Bio-Rad Laboratories (Hercules CA, USA). P-Amino-benzoic acid (PABA), 4-aminobiphenyl (ABP), acetyl coenzyme A (AcCoA) and polyclonal rabbit anti-β-galactosidase antibody were purchased from Sigma Chemical Company (St. Louis, MO USA). COS-1 cells (SV40-transformed African green monkey kidney cells) were obtained from American Type Culture Collection (Manassas, VA USA). N-hydroxy-2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (N-OH-PhIP) was obtained from Toronto Research Chemicals (North York, ON, Canada). Polyclonal antibody against human NAT1 was kindly provided by Dr. Edith Sim, University of Oxford, Oxford UK.

### Vector Construction

Human lung surgical samples (de-identified) were obtained from the National Cancer Institute Cooperative Human Tissue Network. To generate mammalian expression constructs that possess genetic variations in *NAT1* 3'-UTR (Supplemental Figure), genomic DNA from individuals possessing *NAT1*\*4, *NAT1*\*3, *NAT1*\*10 or *NAT1*\*11 alleles was used as template for polymerase chain reaction (PCR). The PCR amplification was performed with 10 μg genomic DNA, 200 nM each primer (forward primer: 5'-AGTATGGACATTGAAGCATAT-3', underlined letters indicate the first Metcodon; reverse primer: 5'-GAATTCAACAATAAACCAACATTA-3', the 5'-end of reverse primer localized at nucleotide 1175 of *NAT1*), 200 μM each dNTP in 1× reaction buffer (10 mM Tris-HCl (pH 7.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>) with final volume of 50 μl. The PCR cycling parameters were: 94 °C for 5 min, 30 cycles of 94 °C for 30 sec, 55°C for 30 sec, 72°C for 1.5 min, followed by additional extension for 8 min at 72 °C. By this application, an 1175 bp *NAT1* fragment containing the 873 bp open reading frame plus 302 bp of the 3'-UTR region was obtained for *NAT1*\*4 reference allele as well as *NAT1* variant alleles possessing 1088T>A, 1095C>A or the 9 bp deletion between nucleotides 1065-1090 in the 3'-UTR (Fig. 1). Following PCR amplification of each allele, 120 ng PCR product and 60 ng pCDNA3.1 (Invitrogen Life Technologies, CA USA) were ligated overnight at 15°C with T4 DNA Ligase. Transformants were grown and positive colonies were confirmed by

automated DNA sequencing. For mock transfections, 800 ng pCMV sport-βgal plasmid (without NAT1) was delivered into COS-1 cells with identical conditions to the transfection of NAT1 alleles described above.

Since the cloned *NAT1* fragment contained a putative polyadenylation signal, the original bovine growth hormone (BGH) polyA signal (nucleotides 1110-1324) was deleted with *Ava* I digestion. Because two *Ava* I sites have different recognition sequences (c↓tcgac at 1030, and c↓ccggg at 2186, arrow indicates cut position), the linear vector would not be re-ligated directly. It was treated with T4 DNA polymerase to produce blunt ends, followed by re-ligation with T4 DNA Ligase. The deletion of BGH polyA signal for each construct was verified by automated DNA sequencing.

Two additional vectors were constructed in order to investigate coding and non-coding genetic polymorphisms separately. One contained 445G>A (rs4987076; V149I), 459G>A (rs4986990; T153T), and 640T>G (rs4986783; S214A) in the open reading frame; the other contained the 9-bp deletion between nucleotides 1065-1090 in the 3'-UTR. The *NAT1\*4* vector was modified to eliminate one *Ssp* I site in the vector portion of the plasmid to facilitate the usage of *Ssp* I site in *NAT1* open reading frame. The plasmid was digested with *Ssp* I, which produced blunt ends. The small fragment that contained part of *NAT1* coding region was treated with *Bsp*HI, followed by treatment with T4 DNA polymerase to produce a blunt end. This blunt end-produced small fragment and the larger fragment generated in digestion of *Ssp* I were ligated with T4 DNA Ligase. This modified plasmid (identified as *NAT1\*11*) was confirmed with automated DNA sequencing. The modified *NAT1\*11* and *NAT1\*4* vectors were digested with *Bam*HI and *Ssp* I (New England Biolabs), which cut at a restriction site in the polylinker of the vector and at nucleotide 814 in the *NAT1* open reading frame, respectively. *NAT1*-vector possessing 445G>A (rs4987076; V149I), 459G>A (rs4986990; T153T), and 640T>G (rs4986783; S214A) in the open reading frame was generated by ligation of the small fragment from the *NAT1\*11* vector and the linear modified *NAT1\*4* vector. The *NAT1*-vector possessing the 9-bp deletion between nucleotides 1065-1090 (3'-UTR) was obtained by digestion of the same vectors with *Ssp* I and *Eco*R I (in polylinker of vector), and re-ligation of the small fragment from the *NAT1\*11* vector and the linear modified *NAT1\*4* vector. The positive colonies were confirmed by automated DNA sequencing.

### Cell Culture, Transfection and Cell Lysate Preparation

The cells were cultured in Dulbecco's modified eagle's medium (DMEM), supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 4mM L-glutamine. The cells were maintained at 37 °C with 10% CO<sub>2</sub>, and sub-cultured every two or three days.

Transfections were repeated three times in duplicate. The three transfections started with cell recovery from cell stock of the same cell passage. After two passages, COS-1 cells were seeded 2.0×10<sup>6</sup> cells/10 cm dish in DMEM medium with 4 mM L-glutamine, incubated at 37 °C to allow 90-95 % confluence, and transfected using Lipofectamine plus reagent (Invitrogen Life Technology, Carlsbad, CA USA). To facilitate the binding of Lipofectamine to DNA, 8.8 μg plasmid containing *NAT1* fragment and 800 ng pCMV SPORT-βgal plasmid were incubated with 40 μl Plus reagent at room temperature for 15 min. After formation of DNA-Plus complex, 60 μl Lipofectamine was added and incubated at room temperature for a second 15 min, allowing the plasmid to be wrapped with Lipofectamine. The cells were pre-washed with basic medium (DMEM with 4 mM L-glutamine) and incubated with 5 ml basic medium for 1 hr. Transfection was initiated by the addition of DNA-Lipofectamine complex into the cells. Three hours after transfection, the

medium was brought back to normal (10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM L-glutamine) with another normal medium change after 24 hr.

Forty-eight hours after transfection, cells were washed with ice cold PBS and scraped in 10 ml PBS. A portion (1.5 ml of 10 ml cell suspension) was collected and pelleted by centrifugation (15,000 rpm 3 min) then stored at -70 °C for future mRNA quantification. The remaining 8.5 ml of cell suspension were pelleted by centrifugation then disrupted by sonication (three pulses for 15 sec at 10 sec intervals) in 1.2 ml lysis buffer (20 mM sodium phosphate pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10 µM phenylmethanesulfonyl fluoride, 10 µM leupeptin, 1 µM pepstatin, 4 µM aprotinin, 2 mg/ml bovine serum albumin). Precipitation of cell debris was done by centrifugation at 15,000 rpm for 15 min at 4°C. The cell lysate in 50% glycerol was stored at -20 °C for enzyme assays. Total protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

### RNA Isolation and Quantitative RT-PCR

RNA was isolated from cell pellets stored at -70 °C using RNeasy® Mini kit and QIAshredder (RNeasy® Mini kit, Qiagen) according to manufacturer's instructions. Crude RNA products were treated with Turbo-DNase (Ambion) at 37 °C for 30 min to remove any contaminating plasmid DNA, followed by incubation at 70 °C in presence of 5 mM EDTA to inactivate Turbo-DNase. The inactivated DNase and EDTA were removed using RNeasy Mini cleanup protocol. The procedure was repeated once. The third time RNA samples were treated with Turbo-DNase followed by heat-inactivation (70°C for 10 min) of the Turbo-DNase.

First strand of *NAT1* cDNA was synthesized and used as template in real-time PCR quantitation of *NAT1* mRNA using SuperScript™ First-Strand Synthesis system for RT-PCR (Invitrogen Life Technology, Carlsbad, CA USA). With 50 ng random hexamer and 10 nmoles dNTPs, 500-800 ng template (total RNA) was denatured at 65°C for 5 min in 10 µl volume, and then chilled on ice. The volume of the system was adjusted to 19 µl with final concentrations of 100 nM MgCl<sub>2</sub>, 200 nM DTT, 40 U RNaseOUT™ (recombinant Ribonuclease inhibitor) in 1 ×RT buffer (120 mM Tris-HCl (pH 8.4) 50 mM KCl). Following pre-warming at 25 °C for 2 min, the reaction was initiated with the addition of 50 units SuperScript™ II RNase H-Reverse transcriptase. The amplification was initiated with a hold at 25 °C for 10 min, followed by incubation at 42° C for 50 min, and then the transcriptase was inactivated at 70°C for 10 min.

The quantitative real time PCR was performed as described previously (Zhu and Hein, 2008). The cycling parameters were 50 °C for 2 min, 95°C for 10 min, followed by 40 cycles of two-step amplification (95 °C for 15 sec, 60 °C for 1 min). Reactions were carried out in 20 µl volume with 1×master mix, 2 µl total RNA from transfected COS-1 cells as templates (200-800 ng), 300 nM forward and reverse primers (forward primer: 5'-AGACATCTCCATCATCTGTGTGTTTACTAGT-3'); (reverse primer: 5'-TTCCTCACTCAGAGTCTTGAAGTCTATT-3'); and 100 nM probe (5'-TTCCTGTTTGGTGGGCTTCACCC-3'). The endogenous control COS-1 β-actin was amplified with the same conditions (reaction components, cycling parameters) in separate tubes as previously described (Zhu and Hein, 2008). The primers and probe were (forward primer: 5'-CGCCCAGCACGATGAAA-3'; reverse primer: 5'-CCGCCGATCCACACAGA-3'; probe: 5'-AGATCATTGCTCCTCCTGAGCGCAAGT-3').

## Western Blot Analysis

NAT1 protein (1.25 µg total protein) was loaded onto 12% PAGER® Gold Tris-Glycine SDS-PAGE gel (Carmbex Bio Science Rockland, INC., Rockland, ME USA), and subjected to electrophoresis at 120V for 60 min. Protein was transferred to Immuno-blot PVDF membrane at 100V for one hour using Mini Trans-Blot®cell (Bio-Rad) apparatus. The blockage of nonspecific sites was performed at 4°C overnight with 5% nonfat milk in 0.05 % T-TBS (20 mM Tris, 500 mM sodium chloride pH7.5, 0.05% Tween-20). The NAT1 specific bands were detected by hybridization with anti-NAT1 rabbit derived polyclonal antiserum (1:4000) in 2.5% milk in 0.01 % T-TBS for 2 hours. NAT1 bands were detected by peroxidase labeled anti-rabbit secondary antibody (1: 20,000) for one hour, visualized with ECL plus western blotting detection reagents. The membrane then was stripped, and re-probed to visualize β-galactosidase bands as loading control. The membrane was incubated at 50 °C for 30 min with occasional agitation in strip buffer (100 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol) and washed twice with 0.01 % T-TBS for 10 min. With the exception of the primary antibody (rabbit anti-β-galactosidase in 1:2000 dilution), the conditions for blotting β-galactosidase were the same as that for NAT1. The intensity of the individual protein (NAT1 or β-galactosidase) band was quantified by densitometry analysis and expressed as density units.

## Enzyme Assays

Cell lysates were assayed for N-acetyltransferase and O-acetyltransferase catalytic activities as described previously (Zhu and Hein, 2008). Briefly, arylamine substrate PABA (750 µM), ABP (300 µM) or N-OH-PhIP (100 µM) were incubated with AcCoA (1 mM) and diluted cell lysate at 37 °C. The cell lysate dilution and incubation time was varied to ensure less than 5 percent substrate converted to product. Reactants and products were separated and quantitated by high performance liquid chromatography. The activities were normalized to co-transfected β-galactosidase activity as previously described (Zang et al., 2004).

## Statistical Analysis

One-way analysis of variance followed by Newman-Keuls Multiple Comparison test was employed to test for differences among NAT1 haplotypes in catalytic activities, and NAT1-specific mRNA and protein. Statistical significance was set at  $p < 0.05$ .

## RESULTS

### Vector Construction

Vectors constructed for this study all possessed 1175 bp *NAT1* fragments including the 873 bp coding sequence and 302 bp of the 3'-UTR (Fig. 2) and the native *NAT1* polyA signal altered by the 1088T>A SNP (supplemental figure). The original BGH polyA signal was deleted for each vector to eliminate the influence of vector pcDNA3.1V5-His-TOPO® BGH polyadenylation signal on *NAT1* mRNA processing.

### Effects of 1088T>A and 1095C>A in NAT1 3'-UTR

As shown in Figure 1, both 1088T>A and 1095C>A resulted in slight reduction in N- and O-acetylation activities compared to the *NAT1*\*4 reference allele. The reduction caused by 1088T>A was significant ( $p < 0.01$ ) for the N-acetylation of PABA and ABP, but not towards the O-acetylation of N-hydroxy-PhIP. The reduction caused by 1095C>A was significant ( $p < 0.05$ ) only for the N-acetylation of ABP. Both 1088T>A and 1095C>A caused significant reduction ( $p < 0.01$ ) in NAT1 mRNA (Fig. 1). NAT1 protein levels also were reduced significantly ( $p < 0.001$ ) compared to the *NAT1*\*4 reference allele (Fig. 3).

### Effects of 9-bp (TAATAATAA) Deletion Between Nucleotides 1065-1090 in NAT1 3'-UTR

As shown in Figure 4, the 9-bp (TAATAATAA) deletion between nucleotides 1065-1090 in the 3'-UTR resulted in significant reductions in N-acetylation of PABA ( $p < 0.001$ ), ABP ( $p < 0.01$ ) and the O-acetylation of N-OH-PhIP ( $p < 0.05$ ). The 9-bp deletion also caused significant reduction in NAT1 mRNA (Fig. 4). NAT1 protein levels also were reduced significantly ( $p < 0.05$ ) compared to the 445G>A, 459G>A, and 640T>G and 9-bp deletion haplotype (Fig. 3).

### Effects of 445G>A, 459G>A, and 640T>G Haplotype

The 445G>A, 459G>A, and 640T>G haplotype resulted in significantly higher PABA ( $p < 0.05$ ) and ABP ( $p < 0.01$ ) NAT1 catalytic activities but not in NAT1 mRNA ( $p > 0.05$ ) compared to the *NAT1*\*4 reference (Fig. 4). This haplotype resulted in significantly ( $p < 0.01$ ) higher levels of NAT1 protein than the *NAT1*\*4 reference allele (Fig. 3).

### Effects of Combination 445G>A, 459G>A, and 640T>G and 9-bp (TAATAATAA) Deletion Between Nucleotides 1065-1090 in NAT1 3'-UTR

Although the 445G>A, 459G>A, and 640T>G haplotype enhanced catalytic activities and the 9-bp (TAATAATAA) deletion between nucleotides 1065-1090 in the 3'-UTR reduced catalytic activities, the combination 445G>A, 459G>A, 640T>G, and 9-bp deletion haplotype resulted in N- and O-acetylation catalytic activities that did not differ significantly ( $p > 0.05$ ) from the *NAT1*\*4 reference allele (Fig. 4). The NAT1 protein level for the combination 445G>A, 459G>A, 640T>G, 9-bp deletion haplotype was significantly higher ( $p < 0.05$ ) than the 9-bp deletion haplotype and significantly lower ( $p < 0.05$ ) than the 445G>A, 459G>A, and 640T>G haplotype (Fig. 3).

## DISCUSSION

The functional effects of *NAT1* polymorphisms and haplotypes are poorly understood, compromising the validity of associations reported with diseases including birth defects and numerous cancers. The *NAT1* reference allele is *NAT1*\*4. We investigated the effects of polymorphisms within the *NAT1* coding region and the 3'-UTR and their associated haplotypes on N- and O-acetyltransferase catalytic activities, and mRNA and protein levels via recombinant expression of cDNAs possessing the polymorphisms in COS-1 cells.

1088T>A (3'-UTR) and 1095C>A (3'-UTR) reduced NAT1 catalytic activity slightly secondary to a reduction in NAT1 mRNA and protein levels. As noted in the introduction, these polymorphisms have been associated with oral clefts (Lammer et al., 2004b) and evidence for an interaction between maternal smoking and the homozygous 1095A genotype has been shown towards risk of spina bifida (Jensen et al., 2005). Infants homozygous for these polymorphisms show increased oral clefts that was more robust in those mothers who did not take multivitamins such as folate during pregnancy (Lammer et al., 2004a). Similarly, infants homozygous for 1088A or 1095A also are associated with increased limb deficiency defects, particularly in mothers with both active and passive smoke exposures, who did not take multivitamin supplements containing folate (Carmichael et al., 2006). Transgenic A/J mice overexpressing human NAT1 are protected from both teratogen-induced and sporadic cleft palate suggesting a protective role of human NAT1 (Erickson et al., 2008). Whether or not the slight reductions in NAT1 caused by 1088A and 1095A in our study are sufficient to explain the increased risk for oral clefts and limb deficiencies observed previously requires further study.

Since 1095C>A (3'-UTR) is the sole polymorphism present in *NAT1*\*3, our data suggest that *NAT1*\*3 is not functionally equivalent to the *NAT1*\*4 reference. This result has major

impact for investigations of associations between NAT1 alleles and disease risk (see introduction), since the *NAT1*\*3 allele is commonly grouped with the *NAT1*\*4 as reference alleles.

Previous studies have investigated the role of individual NAT1 coding region polymorphisms following recombinant expression in bacteria (Grant et al., 1997), yeast (Fretland et al., 2001, 2002) and COS-1 cells (Zhu and Hein, 2008). Our current study focused on functional effects of the 445G>A, 459G>A, and 640T>G coding region haplotype present in *NAT1*\*11. We observed that this haplotype caused an increase in NAT1 catalytic activity in association with an increase in NAT1 protein, but not mRNA levels, a result different from that observed following recombinant expression in yeast (Fretland et al., 2001) but in agreement with a previous study following recombinant expression in COS-1 cells (Zhu and Hein, 2008).

Individuals possessing *NAT1*\*11 alleles have been reported to have significantly lower N-acetyltransferase activities in human red blood cells (Bruhn et al., 1999) but not human leukocytes (Zhangwei et al., 2006). The latter finding is consistent with previous studies following recombinant expression of *NAT1*\*11 in bacterial (Hughes et al., 1998) and COS-1 cells (de Leon et al., 2000). Recombinant expression studies in COS-1 cells that focused entirely on the coding region SNPs in *NAT1*\*11 showed increased protein and catalytic activity (Zhu and Hein, 2008). The present results clarify some of these conflicting findings. Although the 445G>A, 459G>A, and 640T>G coding region haplotype present in *NAT1*\*11 increased NAT1 catalytic activity in association with an increase in NAT1 protein, but not mRNA levels (as noted before in Zhu and Hein, 2008), the 9-bp (TAATAATAA) deletion between 1065-1090 also present in *NAT1*\*11, reduced NAT1 catalytic activity associated with a reduction in NAT1 mRNA and protein levels. Consistent with these results, the combination of the 9-bp (TAATAATAA) deletion between 1065-1090 in the 3'-UTR and the 445G>A, 459G>A, 640T>G coding region haplotypes, both present in *NAT1*\*11, resulted in levels of NAT1-specific protein and catalytic activity that did not differ significantly from the *NAT1*\*4 reference. Thus, our data suggest that *NAT1*\*11 does not encode NAT1 protein with a higher activity than that encoded by the *NAT1*\*4 reference as had been suggested from observations of the functional effects of the *NAT1*\*11 coding region polymorphisms alone.

In summary, our data suggest that *NAT1*\*3 should not be included in the reference group and *NAT1*\*11 should not be included in a "rapid" acetylator group in gene-environment association studies. Since transgenic A/J mice overexpressing human NAT1 are protected from both teratogen-induced and sporadic cleft palate (Erickson et al., 2008) the findings of the present study provide biological support for reported associations of NAT1 1088T>A and 1095C>A polymorphisms with birth defects.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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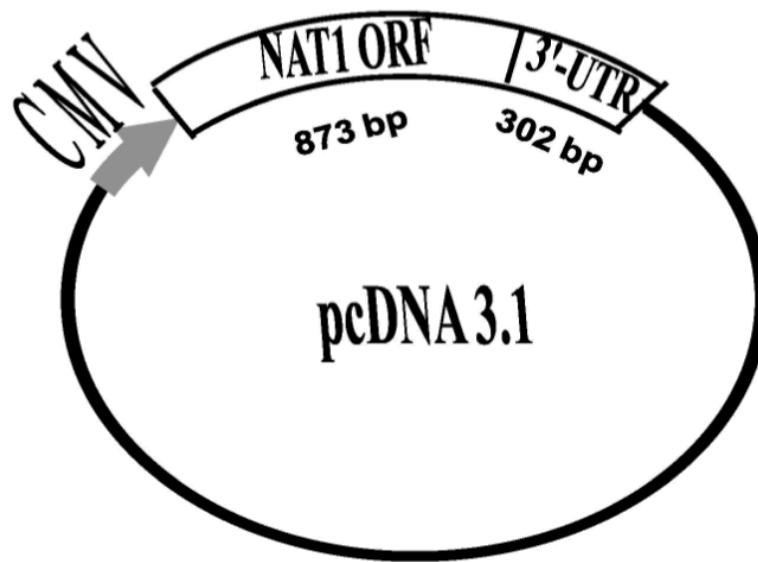


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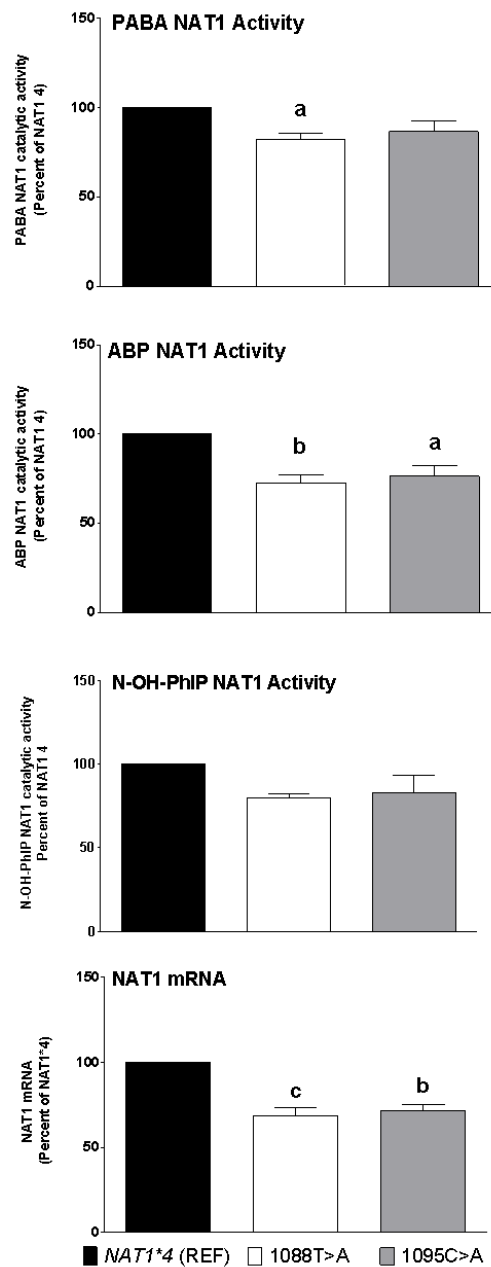
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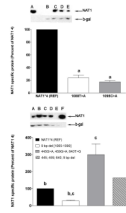


**Figure 1.**

Vector structure of the cloned NAT1 fragments containing the 873-bp *NAT1* open reading frame (ORF) and 302 bp of the *NAT1* 3'-UTR. *NAT1* mRNA transcription was driven by cytomegalovirus (CMV) promoter, whereas RNA processing at the 3-end was facilitated by the *NAT1* putative polyA signals since the BGH polyA signal in the vector was deleted.

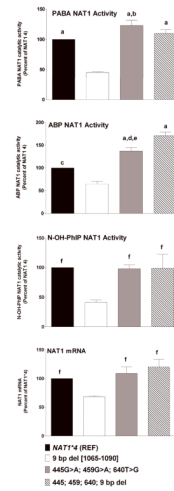


**Figure 2.** Effects of 1088T>A and 1095C>A on NAT1 activities towards PABA and ABP N-acetyltransferase, and N-OH-PhIP O-acetyltransferase activities and NAT1 mRNA. Each bar represents Mean  $\pm$  SEM for three transfections. a)  $p < 0.05$ ; b)  $p < 0.01$ ; c)  $p < 0.001$  significantly different from the NAT1\*4 reference.



**Figure 3.**

Effects of NAT1 polymorphisms on protein expression. Ratios of NAT1 protein to beta-galactosidase protein are shown following densitometric scanning of the representative Western blots shown above the graph. Top Western blot: **A**: Positive control (recombinant NAT1 from yeast); **B**: Negative control (mock transfection); **C**: *NAT1*\*4 reference; **D**: 1095C>A; **E**: 1088T>A. Bottom Western blot: **A**: 9-bp deletion haplotype; **B**: 445G>A, 459G>A, 640T>G haplotype; **C**: *NAT1*\*4 reference; **D**: Combination 445G>A, 459G>A, 640T>G, 9-bp deletion haplotype; **E**: Negative control (mock transfection); **F**: Positive control (recombinant NAT1 from yeast). Each bar represents Mean  $\pm$  SEM for three transfections. a)  $p < 0.001$  from *NAT1*\*4 reference; b)  $p < 0.01$  from 445G>A, 459G>A, 640T>G haplotype; c)  $p < 0.05$  from combination 445G>A, 459G>A, 640T>G, 9-bp deletion haplotype.



**Figure 4.**

Effects of combination 445G>A, 459G>A, and 640T>G and 9-bp (TAATAATAA) deletion between 1065-1090 in the 3'-UTR on PABA and ABP N-acetyltransferase and N-OH-PhIP O-acetyltransferase activities and NAT1 mRNA. Each bar represents Mean  $\pm$  SEM for three transfections. a)  $p<0.001$  from 9-bp deletion; b)  $p<0.05$  from *NAT1\*4* (REF); c)  $p<0.01$  from 9-bp deletion; d)  $p<0.01$  from *NAT1\*4* (REF); e)  $p<0.01$  from combination 445G>A, 459G>A, and 640T>G and 9-bp deletion haplotype; f)  $p<0.05$  from 9-bp deletion.