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LETTER TO THE EDITOR

Simultaneous genotyping of four functional loci of human *SLC6A4*, with a reappraisal of *5-HTTLPR* and rs25531

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The human serotonin transporter gene (*SLC6A4*, *5-HTT*) possesses several polymorphic loci that affect its expression or function. Rare gain-of-function coding mutations such as Ile425Val and Gly56Ala have recently been discovered,[1,2] while common noncoding polymorphisms that impact transcription include an intron 2 VNTR and the *5-HTT*-linked polymorphic region (*5-HTTLPR*).[3,4] The latter polymorphism is commonly subdivided into *S* (short, lesser expressing) and *L* (long, greater expressing) alleles based on the presence of a 43 bp indel (initially described as 44 bp)[3,5,6] and has been extensively analyzed in over 300 behavioral, psychiatric, pharmacogenetic and other medical genetics papers over the past 10 years.[7,8]

Recent reports have raised the possibility that the established subdivision of *5-HTTLPR* into *S* and *L* alleles requires reconsideration. Several variants of *S* and *L* were originally described by Nakamura, Sakai and colleagues, although they were found to not be functionally significant in reporter gene assays; these studies also did not replicate the original differential activities of *S* vs *L*.[3,5,9] Hu *et al.*,[10] however, recently noted an A/G SNP *within* the *5-HTTLPR* insertion that renders only the A variant of the *L* allele (designated L_A, corresponding to allele 16-A,[5] GenBank accession number AB031251) as yielding high *5-HTT* mRNA levels, while the G variant (L_G, 16-D, AB031254) did not. Thus, L_G apparently behaves equivalent to the low-expressing *S* allele. Another report, in turn, presented data on a potentially functional SNP (rs25531) located immediately *upstream* of the indel in an AP-2 binding region.[6]

While at first sight these two SNPs might appear to be two physically distinct loci, a detailed alignment presented in Figure 1a shows that, in fact, both refer to the same nucleotide located at the 10th position after the ε element denoted by Nakamura *et al.*[5] This

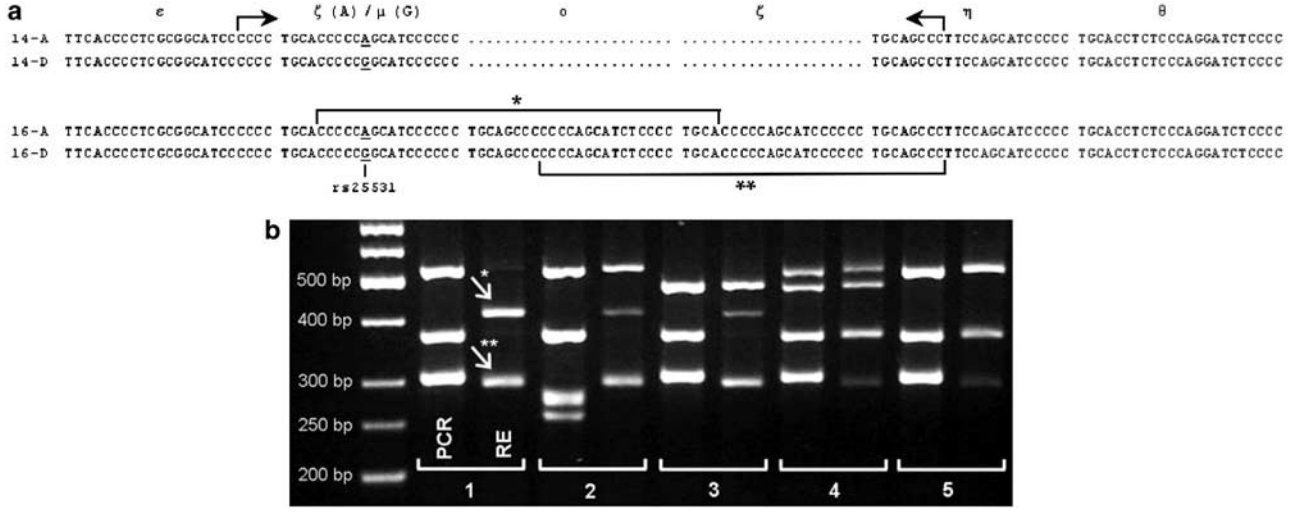

Figure 1 (**a**) Multiple sequence alignment of *5-HTTLPR* variants in the context of SNP rs25531. Labeling of alleles (14-A—16-D, see text for GenBank accession numbers) and of repetitive elements by Greek letters follows the nomenclature introduced by Nakamura *et al.*[5] Note that the 43 bp indel (element o and the second ζ) can be aligned over a range spanning the fourth-last cytosine of element ε to the fourth cytosine of η, as indicated by arrows. Brackets depict alternative indel boundaries as reported by *Heils *et al.*[3] and **Kraft *et al.*[6] (**b**) Agarose gel electrophoresis photograph of amplified genomic DNA from five individuals before (left, 'PCR') and after (right, 'RE') restriction endonuclease digestion. Arrows depict origin of digested bands in the RE lane, that is, from **5-HTTLPR* and **Ile425 amplicons. Intron 2 amplicons are all digested to multiple small fragments < 100 bp not visible here. Samples 1 and 5 served as controls, samples 2–4 are derived from Coriell samples NA17233, NA18062 and NA18024, respectively. Genotypes thus are: (1) L_G/L_G, STin2.12/STin2.12, Ile/Ile; (2) L_A/L_G, STin2.9/STin2.10, Ile/Ile; (3) S_A/S_G, STin2.12/STin2.12, Ile/Ile; (4) S_A/L_A, STin2.12/STin2.12, Ile/mutated; (5) L_A/L_A, STin 2.12/STin2.12, Ile/mutated. Mutated codon 425 alleles from samples 4 and 5 were sequenced and found to be Val425.

alignment also demonstrates that the boundaries of the 43 bp indel can be set in different ways; Hu *et al.* report the SNP as being located at the sixth cytosine of the insertion and use the boundaries as originally described,[3,10] while Kraft *et al.*[6] locate the insertion further downstream and thus the same SNP then lies upstream of the indel (Figure 1a).

The same SNP can also be used to subdivide *S* alleles, which we designate S_A (14-A, AB031257) and S_G (14-B, AB031248 and 14-D, AB031259). The fact that the SNP can be found in the context of both *S* and *L* alleles and the finding that rs25531, but not *5-HTTLPR*, was associated with selective serotonin reuptake inhibitor response[6] demand that both *5-HTTLPR* and rs25531 be viewed as two independent loci for genotyping and analysis purposes. These new developments also justify a re-evaluation of previous association studies and more detailed genotyping in future analyses. This, in turn, entails the need for more comprehensive genotyping procedures.

We present here a triplex PCR protocol followed by double restriction endonuclease digestion, which enables the determination of phase-certain *5-HTTLPR* and rs25531 (S_A, S_G, L_A and L_G) as well as triallelic intron 2 VNTR genotypes (9, 10 and 12 repeats). At the same time, it identifies Ile425Val (A→G SNP) mutations at nucleotide position 1568 (RefSeq NM_001045.2). In a total volume of 20 μl, 25 ng of genomic DNA were amplified in the presence of 1× multiplex master mix (Qiagen, Valencia, CA) and oligonucleotide primers (Operon, Huntsville, AL; Table 1) at final concentrations of 200, 350 and 100 nM each for *5-HTTLPR*, intron 2 and Ile425, respectively. Thermal cycling consisted of 15 min of initial denaturation at 95°C followed by 35 cycles of 94°C (30 s), 65.5°C (90 s) and 72°C (60 s) each with a final extension step of 10 min at 72°C. Subsequently, 7 μl of PCR product were double-digested by *Hpa*II (an isoschizomer of *Msp*I)[5] and *Bcc*I (5 U each; New England Biolabs, Ipswich, MA) in a 20 μl reaction assay containing 1× NEBuffer 1 and 1× BSA at 37°C for 3 h. Finally, 4 μl of remaining PCR product and 18 μl of restriction enzyme assay solution were loaded onto a 3.5% UltraPure agarose gel (Invitrogen, Carlsbad, CA), run for 1 h at 160 V in TBE and visualized by ethidium bromide (Sigma-Aldrich, St Louis, MO). All genotyping was performed in duplicate, with an overall completion rate of 99%. Sequenced controls and no template controls consistently yielded expected results. All samples were also genotyped by established singleplex PCR protocols for each locus, revealing no discrepancies. As Table 1 and Figure 1b show, accurate and comprehensive genotyping can easily be performed by analysis of amplicon and restriction fragment sizes between 250 and 512 bp. The rare mutations of Ile425 require further analysis, such as sequencing since a different variant (Ile425Leu, A→C SNP) with unknown functionality has recently been discovered at the same nucleotide position.[2]

Table 1 Allelic frequencies of functional *SLC6A4* polymorphic loci in 200 individuals and amplicon/restriction fragment lengths of presented genotyping assay

SLC6A4 polymorphic loci and oligonucleotide primer sequences	*Amplicon and restriction fragment lengths of alleles*			*Allelic frequencies*
	PCR product	*HpaII*	*BccI*	
5-HTTLPR and rs25531 (phase-certain)				
Forw.: 5′-TCCTCCGCTTTGGCGCCTCTTCC-3′	S–469 bp	S_A–469 bp (uncut) S_G–402 + 67 bp	Unaffected	S_A–43.2% S_G–0.25%
Rev.: 5′-TGGGGGTTGCAGGGGAGATCCTG-3′	L–512 bp	L_A–512 bp (uncut) L_G–402 + 110 bp		L_A–50.0% L_G–6.5%
Intron 2 VNTR				
Forw.: 5′-GGGCAATGTCTGGCGCTTCCCCTACATA-3′	STin2.9–250 bp	All alleles digested to multiple fragments		STin2.9–1.25%
Rev.: 5′-TTCTGGCCTCTCAAGAGGACCTACAGC-3′	STin2.10–267 bp STin2.12–300 bp	< 100 bp		STin2.10–33.2% STin2.12–65.6%
Ile 425				
Forw.: 5′-TGGAAGCCCCACCCTTCCTG-3′	All alleles–365 bp	Unaffected	Mutated-365 bp (uncut)*	Ile425–99.75%
Rev.: 5′-CATCCTCCCACAGCCCATTTCC-3′			Ile425–288 + 77 bp	Val425–0.25%

The restriction endonuclease *Hpa*II specifically detects the 16-D (L_G) variant among all six known *L* alleles and the 14-B and 14-D variants (both of which we designate S_G) among all four known S alleles.[5,10] The assay can also be performed as duplex PCR amplifying the *5-HTTLPR*/rs25531 and intron 2 loci followed by single *Hpa*II digestion to genotype common polymorphisms only. *The exact genotype determination of undigested Ile425 amplicons requires sequencing as both the Val425 and Leu425 variants alter the *Bcc*I restriction site.

We genotyped the human variation panel of 200 (101 males, 99 females) self-declared, apparently healthy Caucasian Americans obtained from the Coriell cell repository (Camden, NJ; catalog ID HD200CAU). Allelic frequencies are shown in Table 1 and are in agreement with earlier reports.[5] We observed one S_G allele (sample NA18062) and one Ile425Val heterozygote (NA18024, Figure 1b). The presented method allows rapid, cost-effective and detailed determination of common functional polymorphisms at the *5-HTTLPR,* rs25531 and intron 2 loci and simultaneously screens for rare mutations at the Ile425 codon. It is our hope that it will facilitate research on *SLC6A4* in the future.

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