Cloning, functional characterisation and population analysis of a variant form of the human glycine type 2 transporter

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Abstract **Two forms of glycine transporter have been described to date, GlyT-1 and GlyT-2. The GlyT-2 form is expressed mainly in the spinal cord, brainstem and cerebellum. Here we describe the identification of a variant form of the human GlyT-2 (SC6), showing three amino acid changes to the previously reported protein. Population analysis identified the allele causing one of the polymorphisms, D463N, at 10% within the population with 3% being homozygous for the change. We also transfected our new variant into mammalian cells and compared it to the published cDNA, showing that the three amino acid changes present have no major effect on the biochemical properties of the transporter.**

Key words: Glycine T2 transporter; Polymorphism; Population analysis

1. Introduction

The amino acid glycine is an important neurotransmitter in the mammalian CNS. Two distinct glycinergic systems have been described. Firstly, glycine acts as a major inhibitory neurotransmitter in the spinal cord and brainstem. The inhibitory effects of glycine are mediated by strychnine-sensitive glycine receptors, via gating of a Cl^- channel [1]. Secondly, glycine modulates excitatory neurotransmission by acting via strychnine-insensitive regulatory sites on NMDA receptors to potentiate the action of glutamate [2].

The actions of glycine are thought to be terminated by rapid uptake via glycine transporters. These transporters are members of the Na^+/Cl^--dependent family, which also includes transporters for GABA and monoamines [3]. The first class of glycine transporter to be described (GlyT-1) was reported to occur in at least three separate isoforms (GlyT-1a, -1b, -1c) encoded by a single gene [4]. GlyT-1 is widely expressed in brain and spinal cord, and is present predominantly on glial cells [5]. Tissue localisation studies suggest that GlyT-1 may be co-localised with the glycine regulatory sites present on NMDA receptors [6].

The second class of glycine transporters, GlyT-2, is encoded by a unique gene and shares 48% amino acid sequence identity with GlyT-1 [7,8]. GlyT-2 shows substantially different properties to those of GlyT-1. It is expressed mainly in spinal cord, brainstem and cerebellum, and is present mostly on neuronal cells [5]. Localisation studies indicate that the distribution of GlyT-2 correlates with that of strychnine-sensitive glycine receptors [9]. GlyT-2 is also pharmacologically distinguishable from GlyT-1 by its insensitivity to inhibition by sarcosine, which is a substrate of GlyT-1 [8,10,11].

Many polymorphisms in the human GlyT-2 gene have been identified [9,12,13]. However, it is not clear which of these exist in the human population, or what their relative frequencies are. In addition, the influence of the variations on the functional activity of the resultant protein in terms of glycine transport is not known. Here we describe the cloning, expression, functional analysis and frequency population assessment of a novel GlyT-2 variant not reported previously.

2. Materials and methods

2.1. Cloning of human GlyT-2 SC6 variant

Human spinal cord Poly(A)$^+$ RNA (Clontech) was used as a template for the production of first strand cDNA template. This was carried out using the Reverse Transcription System (Promega). The reaction mixture contained final concentrations of 1×RT buffer, 1 mM dNTPs, 5 mM $MgCl_2$, 10 U RNasin ribonuclease inhibitor, 7.5 U AMV reverse transcriptase, 0.25 μg Oligo $(dT)_{15}$ primer, and 500 ng spinal cord Poly(A)$^+$ RNA template. Reverse Transcription was carried out at 42°C for 60 min and followed by 5 min at 99°C. The first strand cDNA was then diluted 1 in 10 for subsequent PCR reactions.

For full length PCR, human gene specific primers were used as follows: 5′ primer (5′-GCC ACC ATG GAT TGC AGT GCT CCC AAG GA-3′), and 3′ primer (5′-GGA CTA GCA CTG AGT GCC CAG TTC C-3′). Final concentrations of reagents in PCR were 1×Klentaq cDNA Polymerase mix (Clontech), 1×Klentaq PCR buffer, 0.2 mM dNTPs, 0.5 μM each primer, and 5 μl spinal cord first strand cDNA template. Thermal cycling was then carried out at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 62°C for 30 s and 68°C for 4 min and ended with 10 min at 68°C.

PCR products were separated by agarose gel electrophoresis and purified by a silica membrane based method (QIAquick Gel Extraction Kit, Qiagen). The products were then cloned into topoisomerase activated pcDNA3.1 vector (Invitrogen), and transformed into TOP10 *Escherichia coli* cells (Invitrogen). Clones containing the plasmid with the insert in the correct orientation were identified by PCR colony screening. Plasmid DNA was then extracted from suspension cultures

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of the selected clones by alkaline lysis followed by a silica membrane based method (QIAprep Spin Miniprep Kit, Qiagen).

2.2. Sequence analysis

All sequencing was carried out with vector or gene specific primers using an ABI automated sequencer. Data obtained were analysed using DNASTAR software.

2.3. Site directed mutagenesis

The full length variant hGlyT-2 clone in pcDNA3.1 (Invitrogen) designated SC6, was used as template for site directed mutagenesis by PCR (QuikChange Site-Directed Mutagenesis Kit, Stratagene). The PCR was carried out with three gene specific primers covering the three sites of variation, and corresponding antisense primers. Thermal cycling was carried out at 95°C for 30 s followed by 14 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 16 min. The PCR reaction mix was then digested with the enzyme *Dpn*1 for 2 h and transformed into *E. coli* XL1-Blue supercompetent cells. A number of colonies were picked and grown in suspension cultures. DNA was extracted by alkaline lysis followed by a silica membrane based method (QIAprep Spin Miniprep Kit, Qiagen). Appropriate clones were chosen for expression.

2.4. PCR reactions and sequence analysis from pituitary gland cDNA

PCR was carried out from a pool of 59 pituitary gland cDNAs (Clontech) to determine sites of variation. Gene specific primers designed to amplify the full length sequence in six overlapping sections were used. Final PCR reactions included 1×PCR buffer, 0.2 mM dNTPs, 0.5 μM each primer, 1.25 U Platinum Taq DNA polymerase (Life Technologies), 1.5 mM $MgCl_2$ and 2.5 μl pituitary gland cDNA template. Thermal cycling was carried out at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, 68°C for 1 min, and finished with 7 min at 68°C. PCR products were purified by a silica membrane based method (QIAquick PCR Purification Kit, Qiagen) and sent for direct sequencing with gene specific primers.

2.5. Transient transfection of HEK293 cells

HEK293 cells were cultured in MEM supplemented with 10% foetal bovine serum and 1×non-essential amino acids (all supplied by GIBCO-BRL). The expression construct pcDNA3.1-GlyT-2.SC6 and the equivalent published sequence construct were transfected into HEK293 cells by the Lipofectamine-Plus procedure following the manufacturers protocol (GIBCO-BRL). After transfection, cells were seeded onto poly-lysine coated 96-well plates (Becton Dickinson) at 50 000 cells/well, and assayed for glycine transport.

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GT2sc6.pro : MDCSAPKEMNKLPANSPEAAAAQGHPDGPCAPRTSPEQ :  38
GT2wt.pro  : MDCSAPKEMNKLPANSPEAAAAQGHPDGPCAPRTSPEQ :  38
GT1c.pro   : M----------------------AAAHGPVAPSSPEQV :  16

GT2sc6.pro : ELPAAAAPPPPRVPRSASTGAQTFQSADARACEAERPG :  76
GT2wt.pro  : ELPAAAAPPPPRVPRSASTGAQTFQSADARACEAERPG :  76
GT1c.pro   : TLLP-------VQRS------FFL-------------- :  27

GT2sc6.pro : VGSCKLSSPRAQAASAALRDLREAQGAQASPPPGSSGP : 114
GT2wt.pro  : VGSCKLSSPRAQAASAALRDLREAQGAQASPPPGSSGP : 114
GT1c.pro   : -------------------------------PPFSGATP :  35

GT2sc6.pro : GNALHCKIPSLRGPEGDANVSVGKGTLERNNTPVVGWV : 152
GT2wt.pro  : GNALHCKIPSLRGPEGDANVSVGKGTLERNNTPVVGWV : 152
GT1c.pro   : STSL-----------AESVLKVWHGAYNSGLLPQL--- :  59

GT2sc6.pro : NMSQSTVVLGTDGITSVLPGSVATVATQEDERGDENKA : 190
GT2wt.pro  : NMSQSTVVLGTDGITSVLPGSVATVATQEDEQGDENKA : 190
GT1c.pro   : -MAQHSLAMAQNG----------AVPSEATKRDQNLK- :  85

GT2sc6.pro : RGNWSSKLDFILSMVGYAVGLGNVWRFPYLAFQNGGGA : 228
GT2wt.pro  : RGNWSSKLDFILSMVGYAVGLGNVWRFPYLAFQNGGGA : 228
GT1c.pro   : RGNWGNQIEFVLTSVGYAVGLGNVWRFPYLCYRNGGGA : 123

GT2sc6.pro : FLIPYLMMLALAGLPIFFLEVSLGQFASQGPVSVWKAI : 266
GT2wt.pro  : FLIPYLMMLALAGLPIFFLEVSLGQFASQGPVSVWKAI : 266
GT1c.pro   : FMFPYFIMLIFCGIPLFFMELSFGQFASQGCLGVWRIS : 161

GT2sc6.pro : PALQGCGIAMLIISVLIAIYYNVIICYTLFYLFASFVS : 304
GT2wt.pro  : PALQGCGIAMLIISVLIAIYYNVIICYTLFYLFASFVS : 304
GT1c.pro   : PMFKGVGYGMMVVSTYIGIYYNVVICIAFYYFFSSMTH : 199
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Fig. 1. Multiple alignment of the amino acid sequence of the SC6 clone (GT2sc6), the wild-type GlyT-2 (GT2wt) and the GlyT-1 gene (GT1c). The amino acid positions where variations were found are highlighted.

2.6. Glycine uptake assays

Transiently transfected cells were washed with Krebs/HEPES buffer containing (mM): NaCl 140, KCl 5.5, $MgSO_4$ 0.8, $CaCl_2$ 1.8, HEPES 10, pH 7.4 (NaOH). Cells were then incubated for 30 min at 37°C in the same buffer containing 0.5 μM [^{3}H]-glycine (15.7 Ci/mmol; Amersham) and 0–1000 μM unlabelled glycine. The incubation was terminated by washing with ice-cold buffer. Cells were then dissolved in 0.5 M NaOH, and the amount of radioactivity taken up by the cells was measured by liquid scintillation counting. The protein content of selected wells was also assessed using the Biorad protein assay. The rate of uptake at each glycine concentration was calculated in terms of pmol glycine/mg protein/min. Graphs of rate of uptake against glycine concentration were plotted, and K_m and V_{max} values were calculated (GraFit; Erithacus Software). Results are presented as means ± S.E.M. of three separate assays.

2.7. Genotyping

Allele specific amplification primers for the D463N polymorphism were used in conjunction with a common reverse oligo. Two PCRs were conducted for each DNA sample (one for each allele specific oligonucleotide) using the following conditions: 94°C for 40 s, 60°C for 30 s, 35 cycles in 20 μl reactions containing 10 pmol of each primer, 1 U TaqExpress (GenPak Ltd.), 50 mM Tris-HCl pH 9.1, 16 mM ammonium sulphate, 3.5 mM $MgCl_2$, 150 μg/ml bovine serum albumin, plus 25–100 ng of DNA. For each DNA, the two ASA reactions [14] were run independently on 3% agarose gels and each lane scored for presence and absence of each allele. Allele specific oligos used were: GlyT-2-2FG (D) 5′-ccaagtgggagaaactgacgg, GlyT-2-2FA (N) 5′-ccaagtgggagaaactgacga. Common reverse oligo: GlyT-2-2R 5′-gtaccaagatcatcaggctc.

2.8. DNA samples

Blood was collected from USA residents and anonymised for name and ethnic origin. DNA was extracted from leukocytes using the Scotlab (Glasgow, UK) Nucleon kit.

3. Results and discussion

The full length human GlyT-2 [8,9,12] was obtained by PCR from spinal cord cDNA template. This cDNA template had been prepared from a pool of Poly(A)$^+$ RNAs (11 individuals). The PCR reaction produced a band of the expected 2.4 kb size for the full length GlyT-2 gene, this band was subcloned directly into the expression vector pcDNA3.1. Se-

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GT2sc6.pro : VLPWGSCNNPWNTPECKDKTKLLLDSCVISDHPKIQIK : 342
GT2wt.pro  : VLPWGSCNNPWNTPECKDKTKLLLDSCVISDHPKIQIK : 342
GT1c.pro   : VLPWAYCNNPWNTHDCAG----VLDASNLTNGSRP--- : 230

GT2sc6.pro : NSTFCMTAYPNVTMVNFTSQANKTFVSGSEEYFKYFVL : 380
GT2wt.pro  : NSTFCMTAYPNVTMVNFTSQANKTFVSGSEEYFKYFVL : 380
GT1c.pro   : ------AALPS-NLSHLLNHSLQR-TSPSEEYWRLYVL : 260

GT2sc6.pro : KISAGIEYPGEIRWPLALCLFLAWVIVYASLAKGIKTS : 418
GT2wt.pro  : KISAGIEYPGEIRWPLALCLFLAWVIVYASLAKGIKTS : 418
GT1c.pro   : KLSDDIGNFGEVRLPLLGCLGVSWLVVFLCLIRGVKSS : 298

GT2sc6.pro : GKVVYFTATFPYVVLVILLIRGVTLPGAGAGIWYFITP : 456
GT2wt.pro  : GKVVYFTATFPYVVLVILLIRGVTLPGAGAGIWYFITP : 456
GT1c.pro   : GKVVYFTATFPYVVLTILFVRGVTLEGAFDGIMYYLTP : 336

GT2sc6.pro : KWEKLTNATVWKDAATQIFFSLSAAWGGLITLSSYNKF : 494
GT2wt.pro  : KWEKLTDATVWKDAATQIFFSLSAAWGGLITLSSYNKF : 494
GT1c.pro   : QWDKILEAKVWGDAASQIFYSLGCAWGGLITMASYNKF : 374

GT2sc6.pro : HNNCYRDTLIVTCTNSATSIFAGFVIFSVIGFMANERK : 532
GT2wt.pro  : HNNCYRDTLIVTCTNSATSIFAGFVIFSVIGFMANERK : 532
GT1c.pro   : HNNCYRDSVIISITNCATSVYAGFVIFSILGFMANHLG : 412

GT2sc6.pro : VNIENVADQGPGIAFVVYPEALTRLPLSPFWAIIFFLM : 570
GT2wt.pro  : VNIENVADQGPGIAFVVYPEALTRLPLSPFWAIIFFLM : 570
GT1c.pro   : VDVSRVADHGPGLAFVAYPEALTLLPISPLWSLLFFFM : 450

GT2sc6.pro : LLTLGLDTMFATIETIVTSISDEFPKYLRTHKPVF-TL : 607
GT2wt.pro  : LLTLGLDTMFATIETIVTSISDEFPKYLRTHKPVF-TL : 607
GT1c.pro   : LILLGLGTQFCLLETLVTAIVDEVGNEWILQKKTYVTL : 488
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Fig. 1 (*continued*).

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GT2sc6.pro : GCCICFFIMGFPMITQGGIYMFQLVDTYAASYALVIIA : 645
GT2wt.pro  : GCCICFFIMGFPMITQGGIYMFQLVDTYAASYALVIIA : 645
GT1c.pro   : GVAVAGFLLGIPLTSQAGIYWLLLMDNYAASFSLVVIS : 526

GT2sc6.pro : IFELVGISYVYGLQRFCEDIEMMIGFQPNIFWKVCWAF : 683
GT2wt.pro  : IFELVGISYVYGLQRFCEDIEMMIGFQPNIFWKVCWAF : 683
GT1c.pro   : CIMCVAIMYIYGHRNYFQDIQMMLGFPPPLFFQICWRF : 564

GT2sc6.pro : VTPTILTFILCFSFYQWEPMTYGSYRYPNWSMVLGWLM : 721
GT2wt.pro  : VTPTILTFILCFSFYQWEPMTYGSYRYPNWSMVLGWLM : 721
GT1c.pro   : VSPAIIFFILVFTVIQYQPITYNHYQYPGWAVAIGFLM : 602

GT2sc6.pro : LACSVIWIPIMFVIKM-HLAPGRFIERLKLACSPQPDW : 758
GT2wt.pro  : LACSVIWIPIMFVIKM-HLAPGRFIERLKLVCSPQPDW : 758
GT1c.pro   : ALSSVLCIPLYAMFRLCRTDGDTLLQRLKNATKPSRDW : 640

GT2sc6.pro : GPFLAQHRGERYKNMIDPLGTSSLGLK-LPVKDLEL-- : 793
GT2wt.pro  : GPFLAQHRGERYKNMIDPLGTSSLGLK-LPVKDLEL-- : 793
GT1c.pro   : GPALLEHRTGRYAPTIAPSPEDGFEVQPLHPDKAQIPI : 678

GT2sc6.pro : ----------GTQC : 797
GT2wt.pro  : ----------GTQC : 797
GT1c.pro   : VGSNGSSRLQDSRI : 692
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Fig. 1 (*continued*).

quencing analysis carried out on a number of full length clones revealed several single nucleotide differences compared to the 'consensus' sequence reported by Albert et al. [12]. One clone, described here as SC6, showed four nucleotide differences which resulted in three amino acid differences at positions 184, 463 and 751 of the translated protein (see Fig. 1 and Table 1).

We proposed at this stage that the changes identified derived from differences between individuals that represented the pooled samples in the starting RNA template. Interestingly, the amino acid difference at position 463 from an aspartic acid residue (D) to an asparagine (N) (see Fig. 1 and Table 1) was reported among a number of variations in this gene [12]. In light of this, we proceeded to validate the presence and frequency of the three amino acid variations in our SC6 clone in a larger number of individuals, along with evaluating functional and pharmacological characteristics in vitro.

Table 1
Description of nucleotide and amino acid changes identified in hGlyT-2 variant SC6

Difference	Nucleotide change in SC6		Amino acid change	
1	129	gcT→gcC	43 (none)	A→A
2	551	cAa→cGa	184	Q→R
3	1387	Gat→Aat	463	D→N
4	2252	gTg→gCg	751	V→A

This clone was obtained from a pool of 11 spinal cord Poly(A)$^+$ RNAs. The A of the ATG is taken as position number 1 in the nucleotide sequence.

Initially, PCR was carried out from a pool of 59 human pituitary gland cDNAs. Sequencing results from PCR products obtained confirmed one of the differences initially identified in the SC6 clone. This was the change at nucleotide position 1387 in the coding region, D463N. Other potential polymorphisms were also identified (results not shown).

A detailed genotyping analysis was performed, to assess the population frequency of this confirmed variation. This analysis was carried out on a random selection of 312 US citizens. Results obtained from this analysis confirmed the existence of this variation in the population. The gel in Fig. 2 illustrates the identification of individuals containing the GAT→AAT (D463N) variation. We discovered that the allele containing the AAT codon is present at a frequency of approximately 10% within the population, with 3% of this sample being homozygous for this change. Genotyping results are summarised in Table 2.

Another of the SC6 variations, the change at position 751 from a valine residue (V) to an alanine (A), was confirmed in PCR reactions from a number of different genomic templates. This again confirmed a real polymorphism of the gene. A

Table 2
Genotype totals and calculated allele frequencies for the GlyT-2 D463N polymorphism in 312 US citizens

Genotype	DD	DN	NN	
N	260	42	10	total = 312

allele frequencies D = 90%, N = 10%

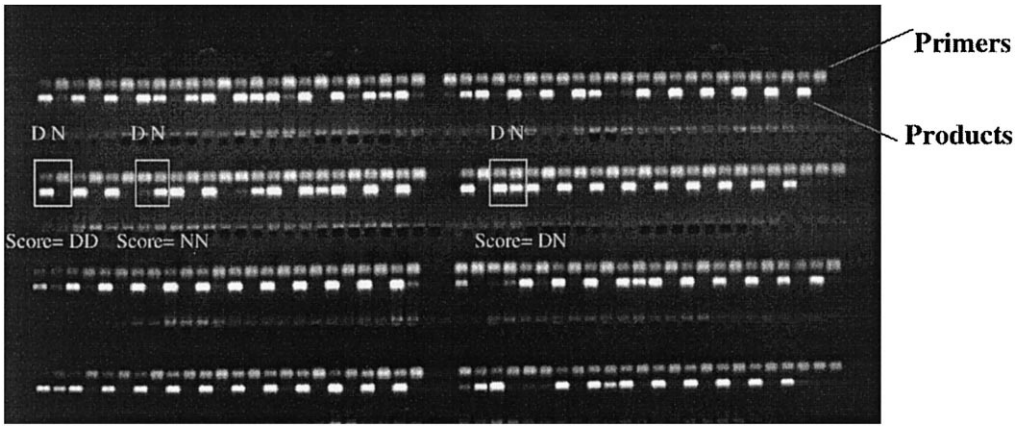

Fig. 2. An ASA genotyping agarose gel for the GlyT-2 D463N polymorphism. PCRs are in pairs for each DNA sample for the D and N alleles from left to right respectively. Thus the first five genotypes from the top left are: DD, DD, DD, DN, NN. Three examples of the possible genotypes are also boxed.

detailed population analysis was not performed in this case. We were unable to confirm in the large number of templates the third difference found in our SC6 clone (at position 184 of the translated protein).

In order to test the effects of the three variations found in the SC6 on the functionality of the protein we transiently transfected HEK293 cells and assessed glycine uptake. Fig. 3 shows the results obtained from HEK293 cells transiently transfected with either SC6 or the previously reported clone, evaluated in a [^{3}H]-glycine uptake assay. Results showed that the two transfects had similar K_m values for glycine uptake (162 ± 33 μM for SC6; 160 ± 45 μM for wild-type), though V_{max} values were somewhat higher for the wild-type sequence (3734 ± 533 pmol/mg protein/min) than for SC6 (1648 ± 414 pmol/mg protein/min). This could be due to differences in protein expression levels between the two transfects. Overall, the functional characteristics of the variant were not different from the published form of the gene, suggesting that the three amino acid changes reported have no major effect on functional activity of hGlyT-2.

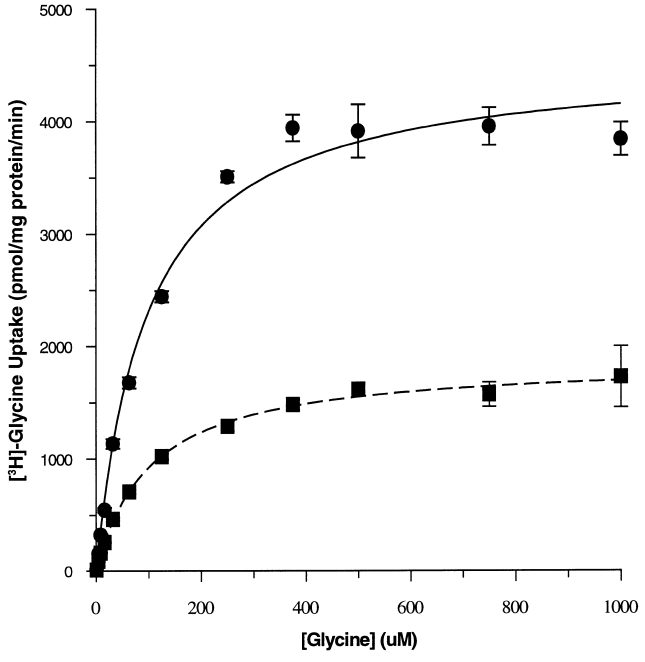

Fig. 3. Rate of glycine uptake as a function of substrate concentration in HEK293 cells transiently transfected with published GlyT-2 sequence (●) or SC6 sequence (■). Results are means ± S.E.M. of eight replicates from a single representative experiment.

Many individual variations have been reported in the human GlyT-2 gene [12] although no work has described whether there are significant pharmacological differences associated with any of them. In this report we have identified the presence of a variant form (SC6) of this gene and showed that two of the amino acid changes reported are present in our test population. We reported the presence of a variation in amino acid 463 D→N of the GlyT-2 gene. We also confirmed the presence in the population of a change from V→A at position 751 of the protein and the presence in our population of further variations.

We have seen no major differences in glycine transport between the published GlyT-2 sequence and our variant. Further genotyping work would be required to look in detail at the polymorphisms within the population for this gene. It would be relevant to assess the frequencies of combinations of all the different polymorphisms reported for the GlyT-2 gene, and to test their functionalities.

The array of polymorphisms found within the GlyT-2 gene provides a useful tool for future pharmacogenetic and disease association studies, which may help target relevant diseases for GlyT-2 based agonists and antagonists.

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