


# A 10 bp Deletion Polymorphism and 2 New Variations in the *GLUT1* Gene Associated With Meningomyelocele

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## Abstract

We sought to examine the diversity and extent of sequence variations in *GLUT1* in patients with myelomeningocele (MM) and to identify variations conferring risk of MM. Sequences of the 10 exons and exon-intron boundaries of *GLUT1* for 96 patients with MM (48 Caucasians and 48 Mexican Americans) were determined by direct sequencing of DNA. Two new variants were identified. One is located within intron 7 (c.972+17t>a), 17 bases from exon 7. The other is within exon 8 (c.1016T>C) and results in an amino acid change at isoleucine 339 (p.Ile339Thr). A 10 base pair (bp) deletion within intron 9 was genotyped for 457 patients with MM and showed it to be more common in Caucasian MM patients than in Caucasian controls ( $P = .02$ ). The physiologic role of the 2 newly identified variants in the *GLUT1* gene and the 10 bp deletion associated with risk of MM in Caucasian patients is under investigation.

## Keywords

glucose metabolism, glucose transporters, single nucleotide polymorphisms, spina bifida meningomyelocele

## Introduction

Neural tube defects (NTDs) are a common category of central nervous system anomalies affecting 0.5 to 2 per 1000 pregnancies worldwide.<sup>1</sup> Failure of the neural tube to close during neurulation gives rise to this heterogeneous group of birth defects. Defects can occur cranially (anencephaly) or in the lower spine (myelomeningocele [MM] or spina bifida). Individuals who survive with NTDs typically have increased morbidity and physical handicap.

Neural tube defects exhibit a multifactorial inheritance pattern, which implicates both environmental and genetic factors. Environmental factors with possible genetic controls include alterations in glucose metabolism and obesity.<sup>2-9</sup> In the United States, Mexican American women are especially interesting as they have the highest risk for having a child with an NTD as well as the highest rates of obesity and type 2 diabetes mellitus.<sup>10,11</sup>

Animal studies have revealed that increased glucose levels impair expression of genes that regulate embryonic development and cell cycle progression, which results in premature cell death within growing organ structures.<sup>12-16</sup> This subsequently results in disruption of organogenesis. Moley et al demonstrated that hyperglycemia during pregnancy in mammals increased apoptosis in preimplantation blastocyst.<sup>15</sup> In a previously studied postimplantation diabetic model, alterations in apoptotic pathway-related gene expression appeared to have a causal effect on NTD formation.<sup>17</sup> Another postimplantation model of diabetes-associated anomalies suggested a possible role for apoptosis triggered by the increased oxygen free

radicals generation.<sup>18</sup> Yazdy et al, recently demonstrated in humans that diets with high dietary glycemic load may put the developing fetus at risk of an NTD.<sup>9</sup>

*GLUT1* is a transmembrane glycoprotein that transports glucose across blood-tissue barriers.<sup>19,20</sup> The gene that encodes for this transporter, *GLUT1* (SLC2A1), locates on chromosome 1 (1p35-31.3).<sup>21</sup> It contains 10 exons, 9 introns, a promoter region, and several putative enhancers.<sup>22</sup> In the nervous system, *GLUT1* exclusively facilitates the ingress of D-glucose across the blood-brain barrier and works together with other glucose transporters mediating glucose transport into astrocytes and neurons.<sup>23,24</sup>

*GLUT1* expression has been demonstrated in preimplantation and postimplantation animal embryos.<sup>25-27</sup> Expression of *GLUT1* has been localized to the neural tube of rat embryos in early organogenesis.<sup>25</sup> Preimplantation apoptotic events, previously shown to be related to decreased expression of

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Group 1-24 Caucasian American patients with lesion levels at or above vertebrae L1 ( $\geq$ L1).

Group 2-24 Caucasian American patients with lesion levels below vertebrae L1 ( $<$ L1).

Group 3-24 Mexican American patients with lesion levels at or above vertebrae L1 ( $\geq$ L1).

Group 4-24 Mexican American patients with lesion levels below vertebrae L1 ( $<$ L1).

**Figure 1.** Demographic breakdown of the 4 groups chosen from the previously acquired patient DNA database.

*GLUT1*, have been linked to NTDs.<sup>28</sup> Also, 1 previous study examining mice lacking the *GLUT1* functional allele show increased fetal demise and congenital deformities similar to diabetic embryopathies.<sup>29</sup>

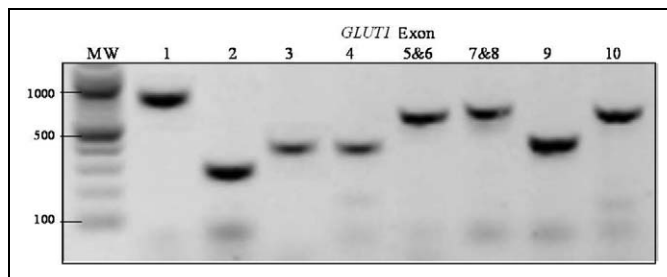
In a previous study, we examined the association of coding single nucleotide polymorphisms (cSNP) within 12 candidate genes known to regulate glucose homeostasis with MM.<sup>30</sup> One synonymous SNP (rs2229682; Pro196Pro) within the *GLUT1* gene showed significant association with SBM risk. In our current study, we sought to further define the relationship between *GLUT1* and MM. Our objective was to examine the diversity and extent of sequence variations in *GLUT1* in patients with MM, report any novel variants discovered in the *GLUT1* gene, and to identify any variations conferring increased risk of MM. We accomplished our objective by systematically identifying genetic variations in *GLUT1* through sequencing of the coding exons and portions of adjacent introns in a set of patients with MM.

## Materials and Methods

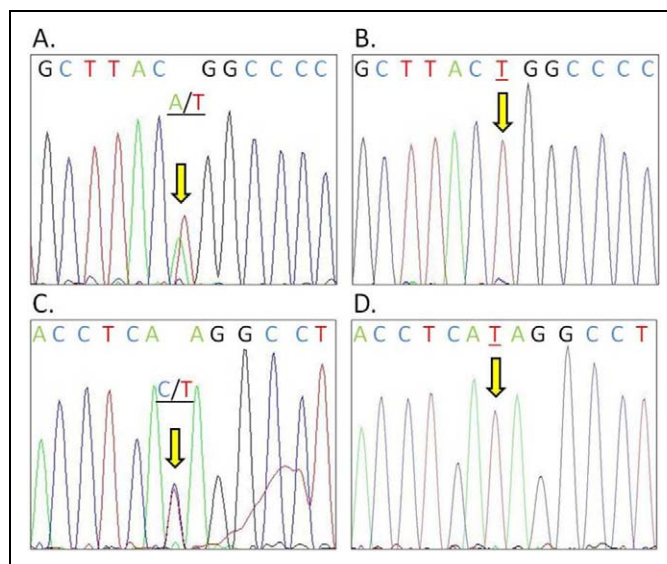
Patients diagnosed with MM and their parents were enrolled into the study from 1996 to 2006 at 3 sites (Houston, Texas, Los Angeles, California, and Toronto, Canada). Informed consent was obtained through the Institutional Review Board (IRB) of University of Texas Health Science Center at Houston. Patient blood samples were obtained and genomic DNA was extracted from blood lymphocytes using the Puregene DNA extraction kit (Gentra Systems Inc, Minneapolis, Minnesota). Stock DNA was then stored at  $-80^{\circ}\text{C}$ . Working DNA aliquots of 10 ng/ $\mu\text{L}$  were prepared for polymerase chain reaction (PCR) at the beginning of this study.

From our patient database, DNA from 96 patients with MM was initially selected at random from our patient cohort. These were chosen in 4 blocks of 24 based on ethnicity (Caucasian vs Mexican American) and location of the neural tube defect lesion (at or above L1 vertebrae or below L1 vertebrae; see Figure 1).

Polymerase chain reaction amplifications were performed using each of the 96 DNA samples as template, with primers designed to amplify each of the 10 exons and 50 to 100 bases of flanking introns comprising *GLUT1*. Success of amplification was verified via agarose gel electrophoresis (see Figure 2). Amplified exon DNA was then treated with EXOI/SAP enzyme to remove excess PCR primers and nucleotides. The

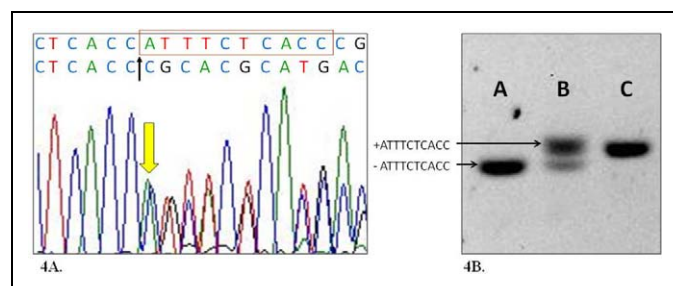


**Figure 2.** Gel electrophoresis of each amplified exon of *GLUT1*. Notes: MW – 100 bp DNA ladder with 1000 bp, 500 bp, and 100 bp marked. *GLUT1* exons are labeled from 1 to 10. Exons 5 and 6, and exons 7 and 8 are amplified together in 1 polymerase chain reaction (PCR) product.



**Figure 3.** Two novel DNA sequence variants of *GLUT1* in patient with myelomeningocele (MM). A, Novel variant identified within intron 7 (c.972+17t>a), 17 bases from exon 7. B, Sequence from same area of intron 7 as (A), showing sequence without variant. C, Novel variant within exon 8 (c.1016T>C) which results in an amino acid change at isoleucine 339 (p.Ile339Thr). D, Same locus as C, without variant seen.

purified exon DNA was then sequenced using the BigDye Terminator method (Applied Biosystems, Inc, Foster City, California; ABI). All sequencing products were separated according to length and recorded using the ABI 3100 genetic analyzer. Sequences (see Figure 3) were then analyzed with



**Figure 4.** Characteristics of a 10 bp deletion polymorphism in intron 9 of *GLUT1*. A, DNA sequence electropherogram showing heterozygous 10bp deletion (in red box on top sequences) with the deletion allele sequences shown below the wild type allele. B, Agarose gel electrophoresis of intron 9 fragments containing homozygous 10bp deletion (A), heterozygous deletion (B), and homozygous wild type (C).

Sequencing Analysis v5.1 software (ABI) and heterozygote variants were identified manually. Sequences were also analyzed with BioEdit 7.04 gene alignment software (Carlsbad, California) to identify homozygote variants for known single nucleotide polymorphisms (SNPs) and novel mutations with reference to the sequences of *GLUT1* at the GenBank (NM\_006516). Potentially novel variants were verified through sequencing from opposite end, and by sequencing of parental DNA (when available). Frequency of SNPs were compared based on patient ethnicity and MM lesion level within each group and to known population frequencies published in the reference SNP database (dbSNP).

A deletion of ATTCTCACC (10 bp del) 30 bases from end of exon 9 within intron 9 (Figure 4A) was examined using a fluorescence labeled primer (6-FAM-5'-GCTTCTCCAACG-GACCTC-3') and reverse primer (5'-GGGCCAGCACTTTG-CACAG-3') flanking the deletion. The size of the allele with a deletion is 126 bp and the allele without deletion is 136 bp. For the initial 96 patient cohort, verification of the deletion was

performed by agarose gel electrophoresis (Figure 4B). Further investigation of our entire patient cohort of MM patients (457 subjects) was performed via ABI3730 Genetic Analyzer (ABI), and the results were analyzed using Genemapper v4.0 software (ABI). Anonymous control DNA from 92 unaffected Mexican American volunteers from the Houston area and 93 Caucasian individuals from the Human Variation Panel-Caucasian Panel of 100 (HD100CAU) without a personal or family history of NTDs was used as controls.

Statistical analysis included use of 2-sided Fisher exact test to compare frequencies of SNPs between groups of patients and to the known control population frequencies. STATA 10.0 (College Station, Texas) was used for all statistical calculations.

## Results

In sequencing all 10 exons of 96 patients with MM, we identified 2 new variants that were not seen in the dbSNP. The first is located within intron 7 (c.972+17t>a), 17 bases downstream of exon 7 with reference to the human *GLUT1* RefSeq NM\_006516 at the GenBank. The second is in exon 8 (c.1016T>C) and resulted in an amino acid change at isoleucine 339 (p.Ile339Thr). These were confirmed through reverse sequencing as well as by sequencing of parental DNA when available.

The sequencing results also recorded allele frequencies for 10 known SNPs in the exon areas we sequenced (Table 1). These allele frequencies were compared between ethnic groups as well as between differing lesion levels. Overall allele frequencies observed were then compared to expected frequencies as noted by dbSNP.<sup>31</sup> Among all patients with MM, the rs1385129 variant G allele is more frequent in Caucasians than Mexican American (82% vs. 67%,  $P = .02$ ). This is similar to previously genotyped controls (both Caucasian and Mexican American) in our laboratory of 78% and 67% (unpublished data). Comparison of overall MM SNP frequencies to known

**Table 1.** Allele Frequencies for Known SNPs in Patients With MM, Categorized by Race and Lesion Level<sup>a</sup>

SNP	Location	Major Allele	Common Allele Frequency					Expected Frequency (dbSNP) <sup>b</sup>	P Value <sup>b</sup>
			All MM Patients; N = 96	Cauc Amer (≥LI); N = 24	Mex Amer (≥LI); N = 24	Cauc Amer (<LI); N = 24	Mex Amer (<LI); N = 24		
rs11537639	Exon 1	A	1.0	1.0	1.0	1.0	1.0	NA	–
rs11537640	Exon 1	A	0.841	0.875	0.738	0.833	0.870	0.810	NS
rs28365848	Exon 1	A	0.833	0.875	0.738	0.833	0.875	0.780	NS
rs1385129	Exon 2	G	0.745	0.833	0.625	0.792	0.729	0.750	NS
rs11537641	Exon 4	C	0.849	0.854	0.833	0.854	0.854	0.770	NS
rs2229682	Exon 5	G	0.839	0.896	0.813	0.792	0.854	0.825	NS
rs2229681	Exon 8	C	1.0	1.0	1.0	1.0	1.0	1.0	NS
rs2306662	Exon 8	A	1.0	1.0	1.0	1.0	1.0	1.0	NS
rs2236574	Exon 9	C	1.0	1.0	1.0	1.0	1.0	1.0	NS
rs13306758	Exon 10	C	1.0	1.0	1.0	1.0	1.0	1.0	NS

Abbreviations: NA, not available; NS, not significant; MM, myelomeningocele; SNPs, single nucleotide polymorphisms; Cauc Amer, Caucasian American; Mex Amer, Mexican American.

<sup>a</sup> Expected frequency from dbSNP in CEU samples.

<sup>b</sup> P value indicates 2-sided Fisher exact test comparing patients with MM to the expected frequency.

**Table 2.** Comparison of a 10 bp Deletion (rs35565219) in *GLUT1* Intron 9 in Patients with MM and Controls<sup>a</sup>

MM Subpopulation	n	Minor Allele	Patient Frequency	Control Frequency	Odds Ratio (95%CI)	P Value 2-Sided
All MM patients	457	10 bp del	0.454	0.392	1.24 (0.98-1.56)	.07 (NS)
Cauc Amer	172	10 bp del	0.515	0.401	<b>1.23 (1.05-1.45)</b>	<b>.0148</b>
Mex Amer	285	10 bp del	0.401	0.374	1.046 (0.92-1.19)	.5485(NS)
Lesion level $\geq$ L1	137	10 bp del	0.427	0.387	1.07 (0.94-1.22)	.3332(NS)
Lesion level <L1	320	10 bp del	0.451	0.387	<b>1.12 (1.00-1.24)</b>	<b>.0498</b>
Cauc Amer ( $\geq$ L1)	53	10 bp del	0.500	0.401	1.20 (0.96-1.50)	.1131 (NS)
Cauc Amer (<L1)	119	10 bp del	0.521	0.401	<b>1.25 (1.05-1.49)</b>	<b>.0151</b>
Mex Amer ( $\geq$ L1)	84	10 bp del	0.381	0.374	1.01 (0.86-1.19)	.9133 (NS)
Mex Amer (<L1)	201	10 bp del	0.410	0.374	1.06 (0.93-1.22)	.4190 (NS)

Abbreviations: NS, not significant; MM, myelomeningocele; Cauc Amer, Caucasian American; Mex Amer, Mexican American.

<sup>a</sup> Mex Amer controls N = 92, Cauc Amer controls N = 93, Combine controls N = 185. The minor allele (10 bp del, ATTCTCACC) shows increased risk for some MM subpopulations (**Bold**).

<sup>b</sup> Cauc Amer = Caucasian American; Mex Amer = Mexican American.

dbSNP expected frequencies showed no difference. Similar comparisons among all other known SNPs are not significant (Table 1).

We identified and characterized a 10 bp deletion (ATTCTCACC) 30 bp downstream from exon 9 within intron 9 of *GLUT1* (Figure 4A). After examining all 96 patients with MM, we determined that this 10 bp del is the minor allele. This 10 bp del within intron 9 has been previously reported and is known as rs35565219; however, no population frequency information is available.

We genotyped 457 patients with MM for rs35565219 and the results are shown in Table 2. When considered together, MM-affected patients trended toward increased frequency of the deletion when compared to all controls, without reaching statistical significance ( $P = .07$ ). However, when Caucasian and Mexican American patients with MM were considered separately, Caucasian patients were more likely to carry the deletion than Caucasian controls ( $P = .01$ ). Mexican American patients did not show significant difference to ethnic-matched controls when considered separately. To evaluate whether our ethnic matched controls were different, their deletion frequencies were compared based on race (Caucasian vs Mexican American) and there was no significant difference ( $P = .6$ ). Further analysis by subpopulation (race and lesion level) shows the presence of the minor allele (10 bp deletion) appears to increase MM risk for some subpopulations (Table 2). The largest increase in risk is seen in Caucasian patients with MM having lesion level below L1 ( $P = .05$ ).

## Discussion

In the present study, we used DNA sequencing of MM-affected individuals to identify 2 new variants and quantify common and rare variants within the *GLUT1* gene, each at an estimated frequency of  $\sim 0.5\%$  (1/192). Completion of the 1000 genomes project will help verify whether the variants we identified in patients with MM are present in the general population. Future population-based studies should aid in revealing population frequency information.

A novel variant (c.972+17t>a) is identified within intron 7 of the *GLUT1* gene of a Caucasian MM patient with  $\leq$ L1 lesion. The variant is predicted to alter splice factors binding activity using the Human Splicing Finder online tool.<sup>32</sup> However, actual demonstration of the loss or gain of splicing activity in relation to the c.972+17a allele is necessary.

Another novel variant (c.1016T>C; p.Ile339Thr) is identified in exon 8 of the *GLUT1* gene of a Mexican American patient with MM having  $\geq$ L1 lesion. The 339Ile is located at the cytoplasmic side of the transmembrane domain #9 of the GLUT1 protein. A change to 339Thr is predicted to possibly damage the transmembrane domain using PolyPhen online tool (<http://genetics.bwh.harvard.edu/pph/>).<sup>33</sup> However, using the SIFT online tool ([http://sift.jcvi.org/www/SIFT\\_seq\\_submit2.html](http://sift.jcvi.org/www/SIFT_seq_submit2.html)), the 339Thr change is predicted to be tolerated with a score of 0.12 (a score of <0.05 is deleterious). Several distinct missense mutations of *GLUT1* cause GLUT1 deficiency syndrome (MIM #606777) or paroxysmal exertion-induced dyskinesia (PED, DYT18, MIM #612126). The SIFT predicted 339Thr tolerated change is consistent with the clinical presentation of a patient with MM in which no DYT18 or GLUT1 deficiency symptoms were observed. Further functional tests are necessary to verify if GLUT1 with the 339Thr variant has a deficit in function.

Animal studies have shown that *GLUT1* is expressed in the neural tube of rat embryos during early embryogenesis.<sup>25</sup> Also, GLUT1-deficient mice have been shown to have increased apoptosis and neural tube defects.<sup>29</sup> We have demonstrated significant association of a synonymous SNP rs2229682 in exon 5 of *GLUT1* with MM in a previous report<sup>30</sup> and here we demonstrated significant MM association between another *GLUT1* variant rs35565219, 2.4 kbp downstream of rs2229682.

This variant, rs35565219, is a 10 bp deletion within intron 9, 30 bases downstream from exon 9, and has lower allele frequency in our patient population. We showed the minor allele (presence of the deletion) to be significantly associated with risk of MM in Caucasians, individuals with lesion level below L1, and in Caucasians with lesion level below L1. The strongest effect was in the last group, where there was a 1.25 times risk for MM. It is important to note that the relationship

between the deletion and MM was similar among patients with low level lesions and high level lesions. Also, there was a trend toward difference when examining the entire group of patients with MM compared to all controls, but it fell short of statistical significance (OR 1.24 [0.98-1.56]). Our separate examination of racial groups is justified by the significantly different MM incidence rates seen in Caucasians vs. Mexican Americans,<sup>34</sup> which suggests potentially different genetic causes. We were careful to examine our ethnic-matched control groups, since they were obtained from different sources. When compared to one another, the frequencies of the deletion were not significantly different (40% vs. 37%).

Due to its location within the intron, this variant does not directly affect amino acid translation. However, analysis using the Exonic Splicing Enhancer Finder program (ESEFinder; <http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>)<sup>35</sup> indicates the 10 bp deletion (ATTTCTCACC) consists of sequences that match mRNA splicing factor target motifs. Previous studies have shown with other genes that intronic mutations can affect transcription and splicing.<sup>36-38</sup> Further, assays to demonstrate splicing alteration will be needed to verify functional significance of the deletion variant.

We applied information regarding a 10 bp deletion to a case-control analysis of MM according to race and lesion level. To our knowledge, this is the first large-scale sequencing study of the *GLUT1* gene with respect to MM (PubMed search, May 2010). While our findings are interesting, it is important to note that we have only studied one MM-affected population; therefore, to validate the importance of our findings, it is important for the study to be replicated in one or more independently ascertained MM populations. Our study is limited because we have not defined the functional effect of the 10 bp deletion variant that showed association. One of our future areas of research interest is to design functional assays to test whether the variants we identified in this report affect the physiology of *GLUT1* and contribute to the development of MM.

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The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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