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## Genes in Glucose Metabolism and Association With Spina Bifida

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### Abstract

The authors tested single nucleotide polymorphisms (SNPs) in coding sequences of candidate genes involved in glucose metabolism and obesity for associations with spina bifida (SB). Coding SNPs on 12 candidate genes was investigated. Genotyping was performed on 507 children with SB and their parents plus anonymous control DNAs from Hispanic and Caucasian individuals. The transmission disequilibrium test was performed to test for genetic associations between transmission of alleles and SB in the offspring ( $P < .05$ ). A statistically significant association between Lys481 of *HK1* (G allele), Arg109Lys of *LEPR* (G allele), and Pro196 of *GLUT1* (A allele) was found ( $P = .019, .039$  and  $.040$ , respectively). Three SNPs on 3 genes involved with glucose metabolism and obesity may be associated with increased susceptibility to SB.

### Keywords

Spina bifida; glucose metabolism; obesity; single nucleotide polymorphism

Neural tube defects (NTDs) constitute a group of malformations that result from failure of normal neural tube closure between the third and fourth week of embryologic development. Spina bifida (SB) and anencephaly are the most common types of NTDs in humans, with spina bifida meningocele (SBMM) being the most common form among individuals who survive.<sup>1</sup> They are multifactorial in origin with an incidence of approximately 1 to 2 per 1000 in the United States.<sup>1,2</sup> The multifactorial inheritance pattern seen with NTDs implicates both environmental and genetic factors. The methylenetetrahydrofolate reductase (*MTHFR*) gene involved in folate metabolism has been previously associated with NTDs.<sup>1</sup> Other environmental factors with genetic controls that have been implicated are derangements in glucose metabolism and obesity.<sup>3-5</sup> Maternal obesity before pregnancy has been found to be associated with an increased risk of approximately 2-fold or more for NTDs.<sup>5</sup> Mexican American women are of particular interest since they have the highest risk in the United States for having a child with an NTD as well as the highest rates of obesity and type 2 diabetes mellitus.<sup>2,6</sup>

At the cellular level, recent animal studies have demonstrated that increased glucose levels disturb expression of genes that regulate embryonic development and cell cycle progression, causing premature cell death of emerging organ structures.<sup>7-11</sup> This results in defective organogenesis. In preimplantation embryogenesis, hyperglycemia triggers exaggerated

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apoptosis at the blastocyst stage. Apoptosis at this early stage of development has been shown to result in either spontaneous miscarriage or congenital malformations.<sup>9</sup> In 1 postimplantation diabetic model, alterations in apoptotic pathway-related gene expression directly resulted in NTDs.<sup>12</sup> Another postimplantation model of diabetes-associated anomalies suggested a role for apoptosis triggered by the increased generation of oxygen free radicals.<sup>13</sup>

Single nucleotide polymorphisms (SNPs) can alter human response to disease. SNPs within the coding region of genes are more likely to alter biological functions because only about 3% to 5% of a person's DNA sequence codes for the production of proteins. We hypothesized that coding SNPs (cSNPs) within genes involved in glucose metabolism and obesity are associated with risk for development of SBMM. Twelve candidate genes known to regulate glucose homeostasis and obesity were selected: solute carrier family 2, members 1 and 4 (*GLUT1*, *GLUT4*), hexokinases 1 and 2 (*HK1*, *HK2*), glyceraldehyde 3 phosphate dehydrogenase (*GAPD*), insulin (*INS*), insulin receptor (*INSR*), leptin (*LEP*), leptin receptor (*LEPR*), transforming protein 53 (*TP53*), superoxide dismutase 2 (*SOD2*), and catalase (*CAT*).

The *GLUT* genes, also known as solute carrier family (*SLC2A*) genes, are facilitated glucose transporters.<sup>14</sup> Hexokinases and *GAPD* assist in blood glucose regulation. Hexokinases phosphorylate glucose, committing glucose to the glycolytic pathway. *GAPD* catalyzes an important energy-yielding step in carbohydrate metabolism. *HK1* has been found to be a Gli2-responsive gene, which has an important role in neurogenesis,<sup>15</sup> and *HK2* facilitates glucose uptake in response to insulin. Leptin is an antiobesity hormone that exerts insulin- and glucose-lowering effects by enhancing peripheral insulin sensitivity and glucose uptake. The leptin receptor is a single transmembrane protein that also plays a role in glucose metabolism.<sup>16</sup>,<sup>17</sup> Binding of insulin to the insulin receptor stimulates glucose uptake.

The process of neural tube formation relies on the balancing of cell proliferation and cell death events among cells on the neural ridge. One hypothesis for the cause of diabetes-associated malformations, including NTDs, is hyperglycemia-induced apoptosis.<sup>12</sup> A *TP53* gene-dependent apoptosis pathway has been identified in murine embryos.<sup>18</sup> An alternative effect of this is generation of reactive oxygen species (ROS). Diabetes may induce teratogenesis by disturbing the embryonic metabolism of arachidonic acid, prostaglandin, or myoinositol by increased DNA mutation rates possibly due to excess production of ROS. The ROS pathway is of particular importance because antioxidants may protect from disturbances of both the inositol and the arachidonic pathway. The intracellular antioxidant defense mechanism is attained mainly by the scavenging enzymes superoxide dismutase and catalase.<sup>19</sup> Accentuation of oxidative injury may occur when catalase and superoxide dismutase functions are impaired.

In summary, we investigated coding polymorphisms in 12 candidate genes involved in glucose metabolism and obesity for their association with SB by using the trio-based transmission disequilibrium test (TDT).

## MATERIALS AND METHODS

Patients with SBMM and their parents were enrolled into the study from 1996 to 2006. Informed consent was obtained, with the Institutional Review Board (IRB) of University of Texas Health Science Center at Houston being the primary IRB. Blood samples were obtained from the patients and both parents when possible, and genomic DNA was extracted from blood lymphocytes using the Puregene DNA extraction kit (Gentra Systems Inc, Minneapolis, MN). When a parent was not available for blood sampling, a saliva DNA collection kit (Oragene) was sent to the family to complete the family trio. Working DNA stocks of 10 ng/ $\mu$ L were prepared for polymerase chain reaction (PCR). Our affected case population consisted of 207

males, 227 females, and 73 of unknown gender, for a total of 507 families. Most of the patients were North American Caucasians of European descent and Mexican Americans from northern Mexico and were recruited in Houston, Texas; Los Angeles, California; and Toronto, Canada. The location and size of spinal defect were available for most patients (Table 1). In addition, anonymous control DNA from 92 Hispanic individuals 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. Sociodemographic as well as pregnancy history, maternal health history, and maternal exposures were obtained from the mothers of the affected patients. Information regarding vitamin supplementation during the pregnancy was not collected for the initial study; however, a survey to collect this information is currently ongoing. There was no excess reporting of maternal diabetes. Genotyping quality control was accomplished using DNA samples from 30 families collected by the Centre d'Etude du Polymorphisme Humain (CEPH) that were also used in the HapMap (haplotype map of the human genome) project.

### SNP Selection

We searched the public database (<http://www.ncbi.nlm.nih.gov/SNP/> and <http://genome.cse.ucsc.edu/>) for cSNPs in each of our candidate genes. For this study, we selected SNPs that met several criteria: (1) potentially functional SNPs such as coding SNPs and SNPs in the promoter region, (2) SNPs that are validated, (3) SNPs with a reported population frequency of minor allele greater than 5% to allow power of statistical analyses for our patients' population size, and (4) SNPs that are reported to have an impact on the function of our selected genes. Therefore, some genes have only 1 SNP, while others have many (eg, *LEPR*). Both synonymous and nonsynonymous SNPs were chosen, with nonsynonymous SNPs being those that result in an amino acid change. A total of 38 cSNPs, most of them with heterozygosity present in at least 5% of the CEU population (30 trios of Utah residents of northern and western European ancestry from the CEPH collections), were chosen (Table 2). Information on the heterozygosities of the selected SNPs for Mexican Americans was not available at the time of SNP selection and is still unavailable. We successfully examined 7 cSNPs in the *INSR* and *LEPR* genes, 4 in *SOD2*, 2 in *INS* and *HK1*, and 1 in *LEP*, *HK2*, *GLUT1*, *TP53*, and *GAPD*. Once we identify SNPs that are significantly associated with SBMM, we will resequence SNPs spanning the whole gene with high density (eg, *GLUT1*, *HK1*, and *LEPR*).

### DNA Genotyping

SNP genotyping was carried out using a recently developed high-throughput SNPlex Genotyping System protocol (ABI) based on an oligonucleotide ligation/PCR/probe hybridization assay that can interrogate 48 SNPs simultaneously in 1 reaction. Working DNA stocks of 50 ng were used for each SNPlex reaction. PCR amplification was performed in a 10- $\mu$ L reaction volume. Raw genotyping files were generated by running the samples through a 3730 xl DNA analyzer, and GeneMapper version 4.0 software was used to analyze the raw data and assign SNP genotypes. The genotypes initially assigned by the software were examined by at least 2 investigators before exporting and compiling for statistical analyses. Eleven cSNPs were excluded from statistical analysis because they did not meet our criteria for having at least an 85% genotype call rate from GeneMapper.

### Statistical Analysis

All analyses were conducted on the entire sample set (Caucasians and Hispanics) and stratified by parent-identified ethnicity of the child. We evaluated genotypes in both a codominant and dominant fashion among all tested samples. The overtransmitted alleles are the tested at-risk alleles. The significance level was set at  $P < .05$ .

For family-based analyses, we used the reconstruction-combined transmission disequilibrium test (RC-TDT). The TDT is a family-based association test that detects a linkage between a marker and a disease-susceptibility locus in the presence of linkage disequilibrium between the 2 loci. It is not subject to population stratification. The RC-TDT permutes information on missing parents and is considered to be a robust method for assessing transmission in data containing both complete and incomplete trios.<sup>20,21</sup>

## RESULTS

To verify the SNPlex genotyping platform accuracy, we included 45 CEPH CEU individuals used in the HapMap project for comparison. For all the SNPs successfully genotyped, 100% concordance was observed. The 27 SNPs genotyped our patient samples and showed Hardy-Weinberg equilibrium. Duplicates of positive controls used in separate SNPlex experiments produced reproducible genotypes.

In our RC-TDT analysis, our experiments showed higher transmission from parents to offspring with SBMM of the G allele at Arg109Lys of *LEPR*, the A allele at Pro196 of *GLUT1*, and the G allele at Lys481 of *HK1*, suggesting possible risk association to SBMM. Although not statistically significant, a trend of significance was observed in the transmission of the G allele at Arg223Gln of *LEPR*. No statistical significances were found in the remaining SNPs (Table 3).

## DISCUSSION

We analyzed 27 genetic variants thought to have functional consequences in 10 genes involved in glucose homeostasis and obesity to explore their relationship with SBMM. Three SNPs in 3 different genes showed statistically significant transmission disequilibrium from parents to affected offspring. Here, we report the association for the G allele at Arg109Lys of *LEPR*, the A allele at Pro196 of *GLUT1*, and the G allele at Lys481 of *HK1* with SBMM. Both Pro196 and Lys481 are synonymous SNPs, while Arg109Lys results in an amino acid change.

### LEPR

Kawamura and colleagues<sup>22</sup> demonstrated that leptin promotes the development of mouse preimplantation embryos in vitro together with the expressed LEPR, suggesting that the leptin signaling pathway plays a critical role in embryonic development before fat development and feeding. Park and colleagues<sup>23</sup> sequenced the *LEPR* gene in patients with type 2 diabetes mellitus (DM) and in nondiabetic controls in an effort to identify genetic polymorphisms in a potential candidate gene for obesity and type 2 DM. They found that Arg109Lys was weakly associated with body mass index among nondiabetic controls. We genotyped this same SNP in our patients and found a significant association with Arg109Lys (amino acid change from arginine to lysine at codon 109). Our patients had significantly higher 109Lys (G allele) transmitted from parents to affected offspring. Lakka and colleagues<sup>24</sup> showed that the 109Arg variant of *LEPR* exhibits higher activities than the 109Lys variant in terms of improved insulin sensitivity and glucose tolerance. Therefore, inheriting this SNP with its resultant amino acid change may alter the embryologic response to glucose homeostasis with subsequent development of SBMM.

A previous association study of SB with the *LEP* and *LEPR* genes did not find association using microsatellites proximal to these genes.<sup>25</sup> However, this study was based on a very small sample size (approximately 50 cases and 125 controls), and the power for significant statistical analyses was minimal. In addition, the microsatellite markers used in the study were more than 40 Kbp away from the *LEPR* gene, which also limits the power of the linkage disequilibrium study. The *LEPR* gene spans more than 200 Kbp on human chromosome 1; therefore, testing

SNP markers inside the gene is better than using remote microsatellite markers to study disease association.

## GLUT1

Animal models have demonstrated that *GLUT1* is present in both preimplantation and postimplantation embryos.<sup>26-28</sup> Its expression has been identified in the neural tube of rat embryos during the early organogenesis period.<sup>28</sup> Chi and colleagues<sup>29</sup> showed that decreased expression of GLUT1 in the mouse blastocyst results in decreased glucose transport and an increase in apoptosis. They were also able to demonstrate that these preimplantation apoptotic events were linked to malformations, including NTDs. Heilig and colleagues<sup>30</sup> produced *Glut1*-deficient transgenic mice in the range reported with maternal diabetes and demonstrated that reduced embryonic glucose transporter 1 was associated with the appearance of apoptosis and neural tube defects. They showed that isolated *Glut1* suppression in mice impaired embryonic development from the earliest stages on, leading to developmental malformations including NTDs, and they concluded that hyperglycemic suppression of embryonic *Glut1* may play an important role in the malformations that develop in response to maternal diabetes. We showed an overtransmission of the A allele of Pro196 from parents to offspring with SBMM. It involved no amino acid change, so the function of this is not quite known. It could be in linkage disequilibrium (LD) with an unknown risk variant of the *GLUT1* gene that has yet to be identified by resequencing the gene of the patients having the risk allele. This change could also affect transcription or splicing. Recent studies have revealed these heretofore unknown effects sometimes caused by synonymous changes in genes. Analyses of the 2 rs2229682 (Pro196) polymorphic variants using the Exonic Splicing Enhancer Finder program (ESEFinder; <http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>)<sup>31</sup> indicate that the A allele variant has higher SRp40 binding (from 0.5317 to 3.0862) and Splicing Factor 2/Alternate Splicing Factor (SF2/ASF) binding (from 1.1734 to 2.4703) and is predicted to enhance splicing of this exon. The G allele variant has values less than the thresholds (1.956 and 2.67, respectively) and is not considered an exonic splicing enhancer. Use of other software programs (PESX, RESCUE-ESE, and FAS-ESS)<sup>32-35</sup> to examine exonic splice enhancers/silencers resulted in similar conclusions regarding the potential functional significance of this change. As recently reported, studies performed with other genes have demonstrated that silent mutations can affect transcription and splicing.<sup>36-38</sup>

## HK1

*HK1* expression is induced by Gli2 and is detected in distinct groups of cells in the neural tube of frog embryos.<sup>15</sup> Since the primary energy source of the brain is derived from glucose metabolism, and hexokinase catalyses the first and rate-limiting step in this process, there is potentially a link between the regulation of neuronal induction and differentiation. We showed an overtransmission of the G allele of Lys481 from parents to affected offspring. It involved no amino acid change, so the functional consequence of this change is not known. It is possible that the Lys481 SNP is in LD with an unknown SBMM variant of the *HK1* gene that has yet to be identified by resequencing the gene of the patients having the risk allele. This variant could also affect transcription or splicing. Analyses of the 2 rs748235 (Lys481) polymorphic variants using ESEFinder indicate that the A allele variant does not constitute any ESE binding motif with a predicted value of 1.0514. However, the G allele variant is predicted to bind the exonic splicing enhancer protein SF2/ASF (value of 2.7479).<sup>31</sup>

Derangements in glucose metabolism and obesity have been associated with birth defects such as SB.<sup>3-5</sup> Twofold or more SB cases were attributed to maternal obesity in a large-scale study involving more than 10 000 cases from the National Birth Defects Prevention Study (1997-2002).<sup>39</sup> Among the highest risk are Mexican American women having a child with an NTD, and the same group also has the highest rates of obesity and type 2 DM.<sup>2,6</sup> The cellular

mechanism of the disturbed glucose homeostasis leading to NTDs began to emerge from recent animal studies demonstrating that increased glucose levels disturb the expression of genes that regulate embryonic development and cell cycle progression, causing premature cell death of emerging organ structures.<sup>7-11</sup> Disrupted apoptotic pathway-related gene expression was shown in a postimplantation diabetic mouse model to result directly in NTDs.<sup>12</sup> Increased generation of free radical oxygen species, as shown in a postimplantation model associated with diabetic pregnancies, can trigger cellular apoptosis and organ anomalies.<sup>13</sup> More evidence has begun to link cell survival and apoptotic pathway kinases, such as serine/threonine kinase Akt, Bcl2 associated-X protein, and c-Jun N-terminal kinase, with the activities of leptin, glucose transporters, and hexokinases in disrupted glucose homeostasis.<sup>40-45</sup> Akt increases mitochondria-associated hexokinase activity. The antiapoptotic activity of Akt requires the first committed step of glucose metabolism catalyzed by hexokinase.<sup>42</sup> Akt can prevent cell death following growth factor withdrawal and was found to regulate multiple steps in glycolysis via posttranscriptional mechanisms, including localization of GLUT1 to the cell surface and maintenance of hexokinase function.<sup>40</sup> Thus, our findings may support the theory that excess apoptosis is a key mechanism in the induction of NTDs.

## CONCLUSION

In summary, the RC-TDT analyses were able to show a statistically significant association with SBMM for 3 SNPs on 3 genes. Our study is limited by the fact that it is not able to differentiate the specific contribution to the development of SBMM from the overtransmitted alleles on the SNPs versus maternal or fetal contributions during formation of the neural tube. This study was undertaken to identify risk polymorphisms associated with SBMM and not to attempt to establish a cause and effect relationship. Future testing may include genotyping denser SNPs to locate SNPs that are closer to the risk variant. The design of functional assays to test if the variants affect splicing and resequencing of exons of these genes to find risk variants are other areas of interest for future research. Case-control analyses are currently ongoing in our laboratory.

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**Table 1**

## Characteristics of the Study Population

	<b>Trios</b>	<b>Duos</b>	<b>Total</b>
Race			
Caucasian	114	76	190
Mexican American	138	146	284
African American	3	12	15
Asian American	2	1	3
Other	3	2	5
Unknown	2	8	10
Gender			
Male	110	97	207
Female	119	108	227
Unknown	33	40	73
SB Lesion			
≥L1	73	72	144
≤L2	169	147	316
Mixed <sup>a</sup>	3	4	7
Unknown	18	22	40

<sup>a</sup>Mixed SB lesion = lesion spanning L1 and L2.

**Table 2**

## Genes and Coding SNPs Investigated

Gene	dbSNP ID	SNP Type	Amino Acid Polymorphism	Population Heterozygosity
<i>INS</i>	rs11557616	s	Leu105	NA
<i>INS</i>	rs11557608	s	Tyr103	NA
<i>INS</i>	rs11557614	ns	Val38Ala	NA
<i>INS</i>	rs13306444	ns	Thr23Ala	NA
<i>INS</i>	rs11557610	s	Pro9	NA
<i>INSR</i>	rs1799817	s	His1085	0.392
<i>INSR</i>	rs1799815	s	Tyr1011	0.077
<i>INSR</i>	rs2229433	s	Ala906	0.092
<i>INSR</i>	rs16994192	s	Ile881	0.106
<i>INSR</i>	rs13306457	s	Asn865	0.178
<i>INSR</i>	rs2229434	s	Thr790	0.068
<i>INSR</i>	rs2963	s	Leu640	0.200
<i>INSR</i>	rs2229435	s	Ser366	0.032
<i>INSR</i>	rs2229428	s	Ser296	0.013
<i>INSR</i>	rs891087	s	Asp261	0.216
<i>LEP</i>	rs17151919	ns	Met94Val	0.055
<i>LEPR</i>	rs1137100	ns	Arg109Lys	0.498
<i>LEPR</i>	rs1137101	ns	Arg223Gln	0.450
<i>LEPR</i>	rs3790419	s	Ser343	0.308
<i>LEPR</i>	rs8179183	ns	Asn656Lys	0.825
<i>LEPR</i>	rs1805095	s	Tyr986	0.015
<i>LEPR</i>	rs6413506	s	Ser1008	0.067
<i>LEPR</i>	rs1805096	s	Pro1019	0.483
<i>GLUT1</i>	rs2229682	s	Pro196	0.335
<i>GLUT1</i>	rs1385129	s	Ala15	0.379
<i>GLUT4</i>	rs8192702	ns	Val358Ala	0.020
<i>HK1</i>	rs748235	s	Lys481	0.325
<i>HK1</i>	rs1054203	ns	Met776Leu	0.020
<i>HK2</i>	rs11887192	s	Asp251	0.478
<i>HK2</i>	rs2229629	ns	Lys844Arg	0.375
<i>GAPD</i>	rs1803621	s	Phe295	0.391
<i>CAT</i>	rs11032709	s	Leu419	0.119
<i>P53</i>	rs1800371	ns	Ser47Pro	0.014
<i>SOD2</i>	rs5746129	ns	Trp156Arg	0.022
<i>SOD2</i>	rs4987023	ns	Arg76Gly	0.010
<i>SOD2</i>	rs5746097	ns	Val66Glu	0.012
<i>SOD2</i>	rs1799725	ns	Ala16Val	0.484
<i>SOD2</i>	rs5746096	ns	Ile10Ser	0.012

Abbreviations: NA, information not available; ns, nonsynonymous; s, synonymous; SNP, single nucleotide polymorphism.

**Table 3**

Statistically Significant Transmission Disequilibrium Test (TDT) Genes and Their Single Nucleotide Polymorphisms (SNPs)

Gene	dbSNP ID	Amino Acid	Reconstruction-Combined TDT (P Value)	Overtransmitted Allele
LEPR	rs1137100	Arg109Lys	0.0393	A
	rs1137101	Arg223Gln	0.0585	G
	rs3790419	Ser343	0.9150	G
	rs8179183	Asn656Lys	0.7830	C
	rs1805095	Tyr986	—	—
	rs6413506	Ser1008	—	—
	rs1805096	Pro1019	0.3960	C
	rs2229682	Pro196	0.0396	A
GLUT1	rs1385129	Ala15	0.7540	A
	rs748235	Lys481	0.0187	G
HK1	rs748235	Lys481	0.0187	G
	rs1054203	Met776Leu	—	—