The site of a missense mutation in the extracellular Ig or FN domains of L1CAM influences infant mortality and the severity of X linked hydrocephalus

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Abstract

The L1 cell adhesion molecule (L1CAM) plays an important role in axon growth, fasciculation, and neural migration. Mutations in the L1CAM gene produce a phenotype characterised by X linked hydrocephalus, mental retardation, spastic paraplegia, adducted thumbs, and agenesis of the corpus callosum. We have conducted a detailed analysis of the phenotypic effects of missense mutations in the extracellular portion of L1CAM, following a study that differentiated between "key" amino acid residues critical for maintaining the conformation of the extracellular immunoglobulin type C-like (Ig) or fibronectin type III-like (FN) domains and surface residues of less certain significance. We have analysed the data from 71 published cases and seven patients whose mutations were detected in our laboratory to determine if the site of a missense mutation in the Ig or FN domains correlated with the severity of hydrocephalus, presence of adducted thumbs, or survival past infancy. Mutations affecting the key residues in either type of domain were more likely to produce a phenotype with severe hydrocephalus, adducted thumbs, and lifespan less than one year than were mutations affecting surface residues. In addition, mutations affecting the FN domains were more likely than those affecting Ig domains to produce a phenotype with severe hydrocephalus, with less certain effects on adducted thumbs and lifespan. Mutations in key residues of the FN domains were particularly deleterious to infant survival. These data provide information that may be useful in predicting some aspects of the phenotypic effects of certain L1CAM mutations.

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Keywords: L1CAM; cell adhesion molecules; missense mutations; genotype-phenotype

The L1 cell adhesion molecule (L1CAM) is a 200 kDa cell surface glycoprotein involved in cell-cell adhesion, neuronal cell migration, neurite outgrowth and fasciculation, and myelination.¹⁻⁵ Mutations in the L1CAM gene produce a phenotype in which hydrocephalus, mental retardation, spastic paraplegia, ad-

ducted thumbs, and agenesis of the corpus callosum are the most consistent features.⁶⁻¹⁰ The L1CAM phenotype is particularly noteworthy for its variability. Not only do mutations in a particular domain of the protein produce highly variable effects, but families have been reported in which different members carrying the same mutation manifested different combinations of phenotypic features.¹¹⁻¹³ As with any disorder, establishing correlations between the site of mutations and the severity or specific features of the resultant phenotype will provide useful information for families in which L1CAM mutations are found. Previous attempts to establish genotype-phenotype correlations for L1CAM mutations have separated L1CAM mutations into classes along rather broad lines.⁷⁻¹⁰ The present study represents an attempt to use previously reported information about the amino acid structure of the extracellular domains of the L1CAM protein to conduct a more detailed analysis of the phenotypic effects of missense mutations in the extracellular portion of the L1CAM gene.

The extracellular portion of the L1CAM protein contains six immunoglobulin C2-like (Ig) domains and five fibronectin type III-like (FN) domains. This is followed by a transmembrane segment and a cytoplasmic region thought to be associated with several protein kinases.^{14 15} In the first 80 families reported to carry L1CAM mutations, 75 different mutations were reported, with only three mutations appearing in multiple families.¹⁶ These mutations have been distributed throughout all portions of the L1CAM gene, and include deletions as well as frameshift, nonsense, missense, and splice site mutations.¹⁷⁻²² The original consensus that there was little correlation between mutation site and phenotype came from the general observation that different patients with mutations affecting the same domain of the L1CAM protein frequently had phenotypes that differed in their severity or the appearance of specific features.¹¹⁻¹³ More recent and systematic attempts to establish genotype-phenotype correlations have divided L1CAM mutations into groups along very broad lines. One study has reported that mutations that either truncate or grossly rearrange the structure of the L1CAM protein have more severe phenotypic effects than missense mutations that result in a single amino acid substitution.⁷ Another study has measured the severity of hydrocephalus, the presence/absence of adducted thumbs, and the incidence of death

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Table 1 Phenotypic features of the seven patients whose mutations were analysed in our laboratory

Mutation site (AA)	Hydrocephalus	Adducted thumbs	Survival past 1 y
S674C	Severe	—	Yes
A691D	Severe	—	No
	Severe	Yes	—
G698R	Severe	—	No
	Severe	—	No
	Severe	—	No
G698R	Severe	Yes	—

Patients are grouped according to family. Data not available are indicated by —.

before 1 year of age, and classified L1CAM mutations into three classes of increasing severity: mutations affecting the cytoplasmic domain, missense mutations in the extracellular domain, and mutations truncating the protein in the extracellular domain.¹⁰

Mutations truncating the protein in the extracellular domain should leave the L1CAM molecule without a means by which to attach to the cell membrane. Such a mutation would be expected to produce a complete loss of function, and the severe phenotype that results from these truncating mutations is consistent with this prediction. Similarly, the finding that mutations that grossly rearrange the structure of the L1CAM protein exert more severe phenotypic effects than mutations causing a single amino acid substitution is also consistent with our understanding of structure-function relationships. While these studies do provide information that can be useful in predicting the severity of the phenotype resulting from truncations or gross rearrangements of the L1CAM protein, these findings do not help predict the phenotypic effects of the many L1CAM mutations that involve missense mutations in the extracellular domains. Bateman *et al*²³ have recently provided a means by which missense mutations in the extracellular domains of L1CAM can be classified according to their function. These authors have differentiated between the amino acid residues necessary for maintaining conformation of the Ig and FN domains (key residues) of L1CAM and those on the surface of their respective domains. We have applied this information in an effort to increase the ability to predict the severity of the phenotype resulting from specific missense mutations in the extracellular domains of L1CAM. We have analysed the severity of hydrocephalus, the presence/absence of adducted thumbs, and survival beyond one year in 71 published patients and seven patients whose mutations were detected in our laboratory, all of whom had missense mutations in the extracellular Ig or FN domains of L1CAM.

Subjects and methods

Informed consent was obtained from all seven subjects whose mutations were analysed in our laboratory before obtaining samples for mutation analysis. This study was approved by the Institutional Review Board of Self Memorial Hospital, Greenwood, SC.

Descriptions of the mutation status and phenotypic features of the 71 patients reported

previously can be found as follows: 52 patients are described in the table describing class 2 mutations in the report by Yamasaki *et al*¹⁰; 13 patients are described in the L1 Mutation Web Page (Van Camp *et al*,¹⁶ Web Nos 21, 30, 33, 35, 41, 45, 52, 61, 63, 66, 67, 69, 70); four patients (HC25, HC5, HC29, HC11) are described by Gu *et al*¹⁹; and two patients (H18 and H12) are described by Jouet *et al*.²⁰ The phenotypic features of the seven patients whose mutations were analysed in our laboratory are listed in table 1. The mutations have been reported for five of these seven patients, two with A691D and three with G698R, by Du *et al*.¹⁷

Hydrocephalus was scored as severe if it was accompanied by aqueductal stenosis, if the patient required a ventriculoperitoneal shunt, if the hydrocephalus was marked enough to be diagnosed in utero, or if the hydrocephalus was described as progressive. Hydrocephalus involving ventricular enlargement without macrocephaly and not requiring shunting was classified as moderate. The presence versus absence of adducted thumbs was observed, as was whether the patient survived longer than one year after birth. Data regarding thumbs and survival were not available for all patients. In some cases, the patient was within a year after birth at the time of writing. Data were analysed using the chi square test. For 2 × 2 analyses, the Yates correction factor was used.²⁴

EXON NUMBERING

The L1CAM gene has historically been considered to have 28 exons, with the ATG codon signalling the beginning of translation in exon 1. It has recently been discovered, however, that there is an additional 13 kb of genomic DNA at the 5' end of the L1CAM gene.²⁵ The actual exon 1 is approximately 10 kb upstream from what has historically been considered exon 1, and the actual exon 2 is 183 bp long, composed of the 76 bp that have historically been considered exon 1 plus an additional 107 bases 5' to the original ATG. The exon numbering system used in this paper reflects the new exon numbering system. However, there are no data presented for analysis of any of the newly discovered 5' regions. Analyses for exon 2 refer not to the entire exon, but only to the 169 bp that constitute what has historically been considered exon 1 plus 43 and 50 bp of flanking sequence on the 5' and 3' sides, respectively. According to the new nomenclature, this includes the most 3' 119 bp of exon 2 plus the most 5' 50 bp of intron 2. Protocols are currently being developed to analyse the newly discovered 5' regions of L1CAM for mutations. The results of these analyses will be reported separately.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP)

Exons 2, 3, 4, 5, 20, 26, 27+28, and 29 were analysed by SSCP. The respective exon and some flanking intronic sequence were amplified by polymerase chain reaction (PCR) using standard protocols and primer sequences reported earlier.²⁰ The electrophoretic

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Table 2 Hydrocephalus, adducted thumbs, and death during the first year after birth in patients with missense mutations in the extracellular FN and Ig domains of L1CAM

		No	Hydrocephalus			Adducted thumbs	Death during first year
			Severe	Moderate	None		
Key	FN	13	13 (100)	0 (0)	0 (0)	9 (100)	10 (91)
	Ig	39	22 (56)	6 (15)	11 (28)	29 (94)	15 (39)
	FN	12	7 (58)	4 (33)	1 (8)	6 (86)	2 (17)
Surface	Ig	14	4 (29)	4 (29)	6 (43)	9 (64)	2 (14)

Entries represent the number (percentage) of patients with the specified mutation site showing the respective feature.

mobilities of the PCR products from control and patient samples were compared using either manual SSCP gels or the Automated Laser Fluorescence (ALF) system (Pharmacia Biotech). For manual analysis, PCR products were 5' end labelled using polynucleotide kinase and γ -P32 ATP, and electrophoresed through 0.5 $\times$ MDE/1 $\times$ TBE gels at room temperature, using 8 W power, for 14 hours. Fragments were visualised by autoradiography at room temperature. For automated analyses, samples were electrophoresed through a 0.5 $\times$ MDE/5% glycerol/1.2 $\times$ TBE gel, with 50 W power. To maximise detection, SSCP was performed on all samples at 14°C and 30°C, and on some others at 20°C as well. Any PCR products showing abnormal migration were regenerated and directly sequenced.

RESTRICTION ENDONUCLEASE FINGERPRINTING (REF)
Exons 6+7, 8-11, 12-16, 17-19, and 21-25 were analysed using REF. In L1CAM, these exons cluster closely enough that each exon group, along with some of its surrounding intronic sequences, can be amplified in individual PCRs. Restriction enzyme digests, end labelling, and gel electrophoresis were performed as described elsewhere.^{17 18}

PCR CYCLE SEQUENCING
Abnormally migrating PCR products (SSCP) or restriction fragments (REF) were regenerated by PCR, and purified by a spin column method (Qia-Quick, Qiagen). Purified templates were sequenced using the Promega fmol cycle sequencing kit with α -³²P-dCTP. Products were electrophoresed through 6% acrylamide/7 mol/l urea gels, and autoradiography was performed at room temperature.

Results

KEY VERSUS SURFACE RESIDUES
Table 2 illustrates the phenotypic differences resulting from mutations in the different domains. While the percentage of patients with some degree of hydrocephalus in each group was similar, mutations in key residues tended to produce more severe hydrocephalus than mutations in surface residues ($\chi^2(2)=5.44$, $p<0.10$). Adducted thumbs were more common in patients with mutations in key residues than surface residues ($\chi^2(1)=4.94$, $p<0.05$). In addition, patients with mutations in key residues were more likely to die within the first year of life than patients with mutations in surface residues ($\chi^2(1)=7.6$, $p<0.01$).

FN VERSUS IG DOMAINS
Mutations in the FN domains were more likely than mutations in the Ig domains to produce hydrocephalus, especially severe hydrocephalus ($\chi^2(2)=8.7$, $p<0.02$). In contrast, there was no significant difference between FN and Ig mutations with respect to the presence of adducted thumbs or the percentage of patients who died within the first year of life.

KEY FN RESIDUE MUTATIONS ARE ESPECIALLY DELETERIOUS TO SURVIVAL
There was a marked difference in the effect that key versus surface mutations exerted on survival depending on whether the mutations were in FN or Ig domains ($\chi^2(3)=18.5$, $p<0.01$). For the Ig domains, survival rates were relatively high for patients with mutations in either key or surface residues ($\chi^2(1)=1.9$, NS). However, for the FN domains, mutations in key residues had a significantly more deleterious effect on survival than did mutations in surface residues ($\chi^2(1)=9.8$, $p<0.01$). No such interaction was seen with respect to the severity of hydrocephalus or the presence of adducted thumbs.

Discussion
The fact that mutations in key residues of L1CAM carry more severe phenotypic consequences than mutations in surface residues is predictable given our understanding of the importance of protein conformation to protein function. There is too little known regarding the relative importance of the extracellular Ig and FN domains of L1CAM to brain development, however, to make a similar judgement regarding the greater incidence of hydrocephalus seen in patients with mutations in the FN versus Ig domains. The specific roles played by the Ig and FN domains may be cell specific. It has been suggested that the second Ig domain is involved in the homophilic binding that promotes neurite outgrowth from retinal cells.^{26 27} In contrast, all six Ig domains and the first two FN domains have been shown to promote neurite outgrowth from small cerebellar neurones.²⁸
The fact that FN domain mutations are more likely to produce hydrocephalus than Ig domain mutations may reflect either an important ligand binding function of the FN domain or the relative positions of these domains on the L1CAM molecule. Mutations in key residues will no doubt disrupt ligand binding in the specific domain where the mutation occurs. However, mutations in the key residues of the FN domains could also exert indirect effects on Ig domain function, by either inducing a conformational change in the L1CAM protein that leaves the Ig domains less than maximally accessible to their ligands, or by interfering with signal transduction normally initiated by ligand binding at the Ig domains.
Consistent with previous reports, most patients with L1CAM mutations, regardless of the mutation site, had adducted thumbs,⁶ while more variability was seen with respect to the severity of hydrocephalus and survival past infancy. Our findings suggest that mutations in

key residues can be expected to produce more severe hydrocephalus and poorer odds for survival than mutations in surface residues. In addition, mutations in the extracellular FN domains can be expected to produce more severe hydrocephalus, and to a lesser extent poorer chances for survival past infancy, than mutations in the Ig domains. Finally, mutations in key residues of the extracellular FN domains are particularly deleterious to survival. There is still a lot that is unclear regarding the genotype-phenotype relationships for L1CAM mutations. However, separating amino acids from the extracellular Ig and FN domains on the basis of their role as key versus surface amino acids may help predict some aspects of the phenotype that results from a missense mutation in one of the L1CAM extracellular domains.

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