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# Genotype—phenotype correlations in L1 syndrome: a guide for genetic counselling and mutation analysis

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► Supplementary tables A, B, C and D are published online only at <http://jmg.bmj.com/content/vol47/issue3>

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## ABSTRACT

**Objectives** To develop a comprehensive mutation analysis system with a high rate of detection, to develop a tool to predict the chance of detecting a mutation in the *L1CAM* gene, and to look for genotype—phenotype correlations in the X-linked recessive disorder, L1 syndrome.

**Methods** DNA from 367 referred patients was analysed for mutations in the coding sequences of the gene. A subgroup of 100 patients was also investigated for mutations in regulatory sequences and for large duplications. Clinical data for 106 patients were collected and used for statistical analysis.

**Results** 68 different mutations were detected in 73 patients. In patients with three or more clinical characteristics of L1 syndrome, the mutation detection rate was 66% compared with 16% in patients with fewer characteristics. The detection rate was 51% in families with more than one affected relative, and 18% in families with one affected male. A combination of these two factors resulted in an 85% detection rate (OR 10.4, 95% CI 3.6 to 30.1). The type of mutation affects the severity of L1 syndrome. Children with a truncating mutation were more likely to die before the age of 3 than those with a missense mutation (52% vs 8%;  $p=0.02$ ).

**Conclusions** We developed a comprehensive mutation detection system with a detection rate of almost 20% in unselected patients and up to 85% in a selected group. Using the patients' clinical characteristics and family history, clinicians can accurately predict the chance of finding a mutation. A genotype-phenotype correlation was confirmed. The occurrence of (maternal) germline mosaicism was proven.

## INTRODUCTION

L1 syndrome is an X-linked recessive disease caused by mutations in the *L1CAM* gene. The phenotypic spectrum includes X-linked hydrocephalus, also referred to as hydrocephalus due to stenosis of the aqueduct of Sylvius (HSAS; MIM 307000), MASA syndrome (mental retardation, aphasia, shuffling gait and adducted thumbs; MIM 303350), X-linked complicated hereditary spastic paraplegia type 1 (SPG1, MIM 303350) and X-linked complicated corpus callosum agenesis (X-linked ACC; MIM 304100).<sup>1–2</sup> The seriousness of the disease may vary from severe hydrocephalus and prenatal death (HSAS subtype) to a mild phenotype (MASA syndrome subtype). These variations may even occur in the same family.<sup>3–4</sup>

The *L1CAM* gene, coding for the neural L1 cell adhesion molecule (L1), is located on the X chromosome. The gene consists of 29 exons, the first being non-coding. The protein of 1257 amino acids is a transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily cell adhesion molecules<sup>5</sup> and contains, besides a signal peptide, 13 distinct domains—that is, six Ig and five fibronectin III-like domains at the extracellular surface, one single-pass transmembrane domain and one short cytoplasmic domain.

To our knowledge, 169 different *L1CAM* mutations have been published; 130 of these were catalogued in 2001<sup>1</sup> and an additional 39 were published more recently.<sup>6–22</sup> Most *L1CAM* mutations are unique to each family—that is, they appear to be private mutations. Only a few families harbour the same recurrent mutation.

*L1CAM* mutation analysis is offered to all patients suspected of having L1 syndrome. Once a mutation has been established, prenatal testing can be performed in subsequent pregnancies, and carriership testing can be carried out to determine the potential presence of an *L1CAM* mutation in female relatives. Optimisation of the previously reported *L1CAM* mutation detection system<sup>23</sup> has allowed us to investigate 367 L1 syndrome referrals. The analysis resulted in a mutation frequency of almost 20%. Although this is a good score, the question remains whether this relatively low frequency is due to the inclusion of large numbers of patients who do not have L1 syndrome or we have missed mutations and should extend our mutation screening procedure. To investigate this, we screened additional regulatory sequences of the *L1CAM* gene for mutations and looked for duplications and deletions. Furthermore, we evaluated the clinical data of a large set of patients to see if this could help in predicting the mutation status. Finally, we analysed genotype—phenotype correlations with the severity of the disease.

## PATIENTS, MATERIALS AND METHODS

### Patients

All patients ( $n=367$ ), from various parts of the world, analysed in our laboratory for diagnostic *L1CAM* testing were included. If no DNA from the index patient was available ( $n=47$ ), the DNA of the mother ( $n=38$ ) was analysed or that of a close family member, a sister ( $n=6$ ) or aunt ( $n=3$ ). Of these patients, 78% were referred by clinical geneticists, 12% by (paediatric) neurologists and 10% by paediatricians.

## Original article

To allow investigation of the mutation detection rate for specific subsets of patients, all referral clinicians were requested to complete a questionnaire.

### Mutation analysis using denaturing gradient gel electrophoresis (DGGE)

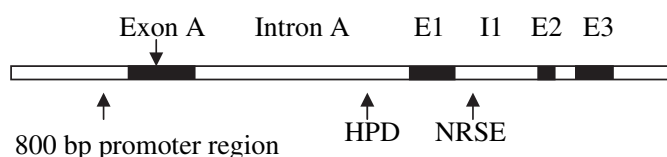
Mutation analysis of the *L1CAM* gene was performed, using DGGE analysis followed by direct sequencing of fragments showing an aberrant DGGE pattern, essentially as previously described.<sup>23</sup> The amplicons 1, 7, 15, 16B, 18, 21 and 25 are routinely analysed by direct sequencing using the same primers as developed for DGGE, but with an M13 tail instead of the GC clamp.

### Sequence analysis of regulatory regions

Analysis of *L1CAM* regulatory regions—that is, about 1000 bp of the promoter region (including the non-coding first exon of the gene,<sup>24</sup> called exon A), the neural restrictive silencer element (NRSE) and the homeodomain and paired domain binding site (HPD) (figure 1)—was performed by direct sequencing of these regions. The NRSE, within intron 1 of the gene, restricts expression of L1 to the nervous system.<sup>24</sup> The HPD is a positive regulatory element, which induces *L1CAM* gene expression.<sup>25</sup> Four primers sets were designed for the promoter region (PromA–PromD), one for the HPD region and one for the NRSE region (table 1). PCR was performed in a total reaction volume of 50 µl containing 5 µl 10×PCR buffer (Amersham Biosciences, Roosendaal, The Netherlands), 0.17 µl *rTaq* polymerase (5000 U/ml; Amersham Biosciences), 20 pmol each primer (Eurogentec, Serian, Belgium), 10 pmol dNTPs (Amersham Pharmacia Biotech, Piscataway, New York) and 100 ng DNA. In addition, 5 µl dimethyl sulphoxide was added for two PCRs (PromA and PromB). For fragments PromC and PromD, the PCRx Enhancer System (Invitrogen Life Technologies, Breda, the Netherlands) was used with a final PCRx Enhancer Solution Concentration of 3×. A standard cycling protocol of 35 cycles was used with different annealing temperatures for each fragment (table 1). The PCR products were analysed on a 2% agarose gel; the remainder were purified with ExoSAP-IT (Amersham Pharmacia Biotech) and subjected to direct sequencing using the M13 primers.

### Multiplex ligation-dependent probe amplification (MLPA) analysis

MLPA was performed using DNA from 98 individuals (75 males and 23 females randomly chosen from the cohort of 295 subjects not harbouring a mutation in the *L1CAM* gene). These were randomly chosen because we started this analysis before we had evaluated the clinical data. Probes for each exon were designed in our laboratory using the ‘Designing synthetic MLPA probes’ protocol (SOP-CUS-DE01 V5) from MRC-Holland. Twenty-four sets were judged to be suitable for use after testing and were divided over five mixes, each mix containing three control probes from the X chromosome. MLPA was carried out using the stan-



**Figure 1** 5' site of the *L1CAM* gene with regulatory regions: promoter, homeodomain and paired domain binding site (HPD) and neural restrictive silencer element (NRSE). E1, exon 1; I1, intron 1. Exon A is 125 bp. 800 bp of the promoter region were analysed.

**Table 1** Primer set regulatory regions

Primer	Sequence (5' → 3')	Length (bp)	Product size (bp)	AT (°C)
PromA_F	CGCCTGTGAGGTCATGGCCCTTG	22	357	53
PromA_R	GCCCAGTCAGGGTCCTGTTG	20		53
PromB_F	CCCAACACGCTGAGGACGAA	20	291	61.2
PromB_R	CAAGGGAGCCCGCTGTGAAA	20		61.2
PromC_F	AGGCCGAGGCTAAGGGAGCAT	21	345	49.4
PromC_R	GGAGAGGGAGGAGGAGTGAGAT	23		49.4
PromD_F	TGGCACCAGGGGCTAGGGT	19	318	57
PromD_R	GGCAGAGCGGTGTGGTGTGTC	19		57
HPD_F	GGCCCTTCCCATCTTCTT	22	237	53
HPD_R	CTGTGGGGATGAATCCCTGTAT	22		53
NRSE_F	GCGCCTACCTTCGCCGCTAT	20	331	50.9
NRSE_R	GGATGGAAGCGGAGGATCG	20		50.9

All primers are designed with an M13 tail: (f)CGACGTTGTAAACGACGCCAGT and (r)CAGGAAACAGCTATGAC.

AT, annealing temperature; HPD, homeodomain and paired domain binding site; NRSE, neural restrictive silencer element; Prom, Promoter.

ard protocol from MRC-Holland (MLPA DNA Detection/Quantification protocol V27). All probe sequences are available upon request.

### cDNA analysis of a potential RNA splicing mutation

Total RNA was extracted from peripheral blood, using the RNABee procedure (Cinna Biotech, Friendswood, Texas, USA), and cDNA was obtained using the Ready-To-Go You-Prime First-Stand Beads for reverse transcription PCR with random hexamer primers pd(N)6 (Amersham Biosciences). Primers amplifying a product from exons 5 to 8 (f)CCAAGTGGCCAAAGGAGACAG and (r)ACTGCCAGTGAGTTCTCG were used to characterise the cDNA sequence around the c.645C → T mutation in exon 6. The primers (f)CCAAGTGGCCAAAGGAGACAG and (r)TGATGGTGGGCGTGGGAAAG were used to verify the results obtained. The PCR products were loaded on to a 2% agarose gel, purified with ExoSAP-IT (Amersham Pharmacia Biotech) and subjected to direct sequencing using the primers described to confirm the presence of an aberrant transcript.

### Statistical analysis

A study was carried out to determine whether, and in what way, the mutation detection rate was influenced by (a) the number of age-independent clinical characteristics (table 2), (b) the number of affected relatives and (c) a combination of these factors.

An analysis was also performed to detect any possible genotype–phenotype correlation, notably between the severity of the disease and truncating or missense mutations. The disease was defined as severe if a child died before the age of 3.

For statistical analysis, the  $\chi^2$  test was carried out, except in cases of small numbers when the Fisher exact test was used. A logistic regression analysis was performed to calculate the odds ratio (OR).

The likely disease-causing mutations in these analyses are considered to be disease-causing.

## RESULTS

### Mutation analysis by DGGE

In total, 367 subjects—that is, 320 index patients and 47 relatives, in most cases the mother—were analysed for the presence of a mutation in the *L1CAM* gene. With this technique, a mutation was detected in 72 of the 367 subjects (online supplementary tables A and B). Sixty-seven different mutations were found: three mutations were detected in two families and one mutation

**Table 2** Characteristics of patients (n=338)

Characteristic	Selected group (n=106)		Remnant group (n=232)	
	n	%	n	%
>1 affected relatives	35	33	60	26
Died at young age	29	27	63	27
Age-independent characteristics				
Hydrocephalus	91	86	133	57
Aqueduct stenosis	46	43	15	6
Adducted thumbs	46	43	51	22
ACC/DCC	24	23	27	12
Age-dependent characteristics				
Mental retardation	56	53	46	20
Aphasia	37	35	10	4
Spastic paraplegia	36	34	49	21
No clinical data available	0	0	53	23

Characteristics of the total group of 367 patients minus the 29 patients tested for L1 syndrome exclusion.

ACC/DCC, agenesis/dysgenesis of the corpus callosum.

in three different families. Fifty-two mutations have not been published before. Five mutations have previously been reported in cooperation with us,<sup>9 10 18 19 26</sup> and 10 mutations were also found by others<sup>2 27–34</sup> (online supplementary table A). The disease-causing relevance of seven of the 67 mutations remained unclear (online supplementary table B). In addition to the 67 mutations, we also detected six variants that were probably not disease-causing (online supplementary table C). None of the variants in supplementary tables B and C match known single-nucleotide polymorphisms.

### Sequence analysis of the regulatory regions and MLPA analysis

We extended our sequence analysis to the *L1CAM* promoter and the HPD and NRSE regions and performed an MLPA analysis to determine whether additional mutations were present in the regulatory sequences of the gene, or whether deletions or insertions could partly explain why the remaining 80% of patients did not have a detectable coding sequence mutation.

No mutation was detected in the promoter region, the non-coding sequence of the first exon, the HPD region or the NRSE site in 100 of the 295 subjects (randomly chosen) of our cohort not harbouring a mutation in the *L1CAM* gene. The regulatory sequences of the other 195 subjects have not been analysed.

MLPA analysis identified one subject with a duplication from exons 2–10. So, in total, a mutation was detected in 73 of the 367 subjects.

### RNA splicing mutation and germline mosaicism

Reverse transcription PCR was carried out to determine a possible splice effect of the silent mutation c.645C→T. Figure 2 clearly shows that this mutation influences RNA splicing. Sequence data confirm that a new splice donor site is introduced in exon 6, causing a deletion of 51 bp at the 3' site of the exon (figure 2).

In this case, a maternal germline mosaicism was also demonstrated; the mother did not carry the mutation, but a second male fetus did.

### Patients

The questionnaire, covering the family anamnesis and the seven most important clinical parameters of L1 syndrome, was filled out for 135 of the 367 patients. Twenty-nine of these 135 patients were referred in order to exclude the disease. Statistical evaluation was therefore carried out on 106 patients, 31 of whom carried a mutation. The most important clinical data

have been summarised in table 2. Known data for the remaining 232 patients have also been included in this table. It is clear that this group is less well characterised. Supplementary table A contains clinical data for patients harbouring an *L1CAM* mutation. As many patients die before birth or at a very young age, a distinction is made in characteristics that can be observed at birth or post mortem and those that can be observed only in later stages of life (table 2).

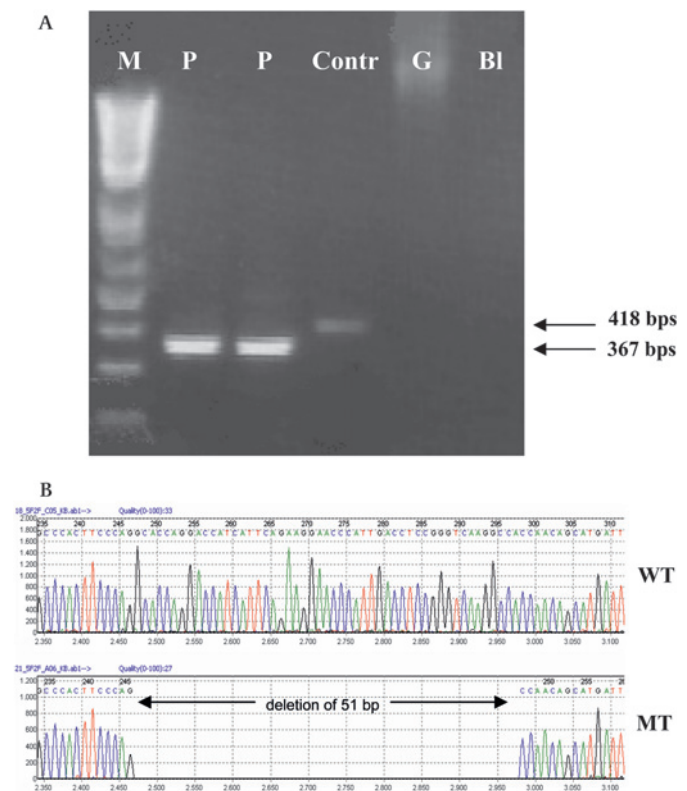
We also looked at the co-occurrence of a hydrocephalus (ventriculomegaly) and a macrocephaly (head circumference of > 2SD). In the selected group, out of 91 patients with hydrocephalus, 36 also had a macrocephaly, 25 of which needed shunting.

In the event of a positive family history, there is always compatibility with X-linked recessive inheritance. However, in a few families comprising only a limited number of affected family members, autosomal recessive inheritance cannot be excluded.

### Statistical analysis

The clinical data from 106 patients, obtained via the questionnaire, were used for statistical analysis as summarised in table 3. It can be seen that patients with three or more age-independent clinical L1 syndrome characteristics had a significantly higher percentage of mutations (66%) than patients with fewer than three characteristics (16%). This is also true for families with more than one affected family member: 51% vs 18% for families with only one affected family member. A combination of these two characteristics—that is, patients with a positive family history for L1 syndrome and having three or more clinical characteristics—resulted in a detection rate of 85%.

The OR of finding a mutation in patients with three or more clinical characteristics was 10.1 (95% CI 3.8 to 27.1). This



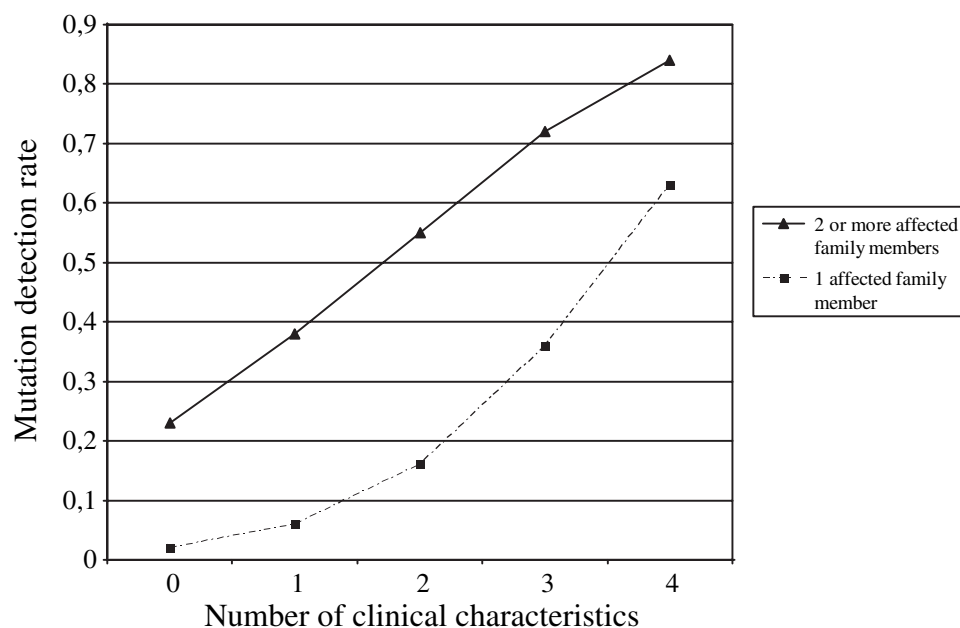
**Figure 2** (A) cDNA analysis of patient 329. Agarose gel electrophoresis of cDNA exon 6 of the patient (P) and a control sample (Contr). Wild-type exon 6 is 418 bp long. G, genomic; BI, blanco; M, marker. (B) Sequence analysis of cDNA exon 6 of patient 329. WT, wild-type; MT, mutant.

**Table 3** Mutation detection rate

	No mutation		Mutation	
	n	%	n	%
No of age-independent clinical characteristics				
<3	65	84	12	16
≥3	10	34	19	66
p<0.001				
No of affected family members				
1	58	82	13	18
≥2	17	49	18	51
p<0.001				
<3 age-independent clinical characteristics				
No of affected family members				
1	50	91	5	9
≥2	15	68	7	32
p=0.01				
≥3 age-independent clinical characteristics				
No of affected family members				
1	8	50	8	50
≥2	2	15	11	85
p=0.05				

increased slightly to 10.4 (95% CI 3.6 to 30.1) when these patients also had two or more affected family members.

Applying the same calculations to the whole group of 338 patients (367 minus the 29 who were only been analysed to exclude the presence of L1 syndrome), some lacking relevant clinical information (table 2), the mutation detection rate was 18% in patients with fewer than three characteristics. In patients with three or more characteristics but no affected relatives, this rate increased to 58%, with an OR of 6.1 (95% CI 3.0 to 12.7). In the group of patients with three or more clinical characteristics and a positive family history, the detection rate increased to 79%, with an OR of 8.1 (95% CI 3.5 to 18.6) as compared with 85% in the selected group. These results are logical, as the second group was less well defined (unselected) and therefore showed a lower detection rate, but larger and therefore showed a smaller CI.

**Figure 3** Mutation detection rate versus number of age-independent clinical characteristics and number of affected family members.

Interrelationships, as mentioned above, can be seen when the data from the whole group are used (figure 3). The chance of having a mutation increased when patients had more clinical characteristics, and even more when at least two family members were affected.

We also determined the positive predictive value of the individual clinical characteristics in relation to the presence of a mutation (table 4), showing the most predictive to be adducted thumbs (50%) and the least predictive to be mental retardation (32%).

### Genotype–phenotype correlation

A statistical analysis was performed on 33 patients who were carrying a mutation to detect any possible genotype–phenotype correlation. Children harbouring a truncating mutation were more likely to die before the age of 3 (52%) than children with a missense mutation (8%). This indicates a relationship between the seriousness of the disease and the type of mutation. These results are significant (Fisher exact  $p=0.02$ ) (table 5).

No significant difference was detected in the occurrence of one of the other clinical characteristics (including macrocephaly) comparing patients carrying a missense mutation with patients carrying a truncating mutation (online supplementary table D).

### DISCUSSION

Mutation analysis was carried out on 367 referred cases (that is 320 index patients and 47 relatives). Seventy-two patients were shown to harbour 67 different mutations. One additional mutation was found with the MLPA test, making a total of 73 patients with a disease-causing mutation. The mutations comprise 23 missense mutations, three in-frame deletions/duplications, 18 splice site mutations, 14 nonsense mutations, eight frame-shift mutations, one duplication of exons 2–10, and one deletion of the entire gene. This implies that about 60% of all mutations are truncating and therefore considered to be disease-causing. This is less straightforward for the 23 missense mutations. However, the identified missense mutations are mostly considered to be disease-causing, as they often occur in the so-called key residues<sup>35</sup> that are important for the structure of the Ig domains and the fibronectin III-like domains of L1 protein.

**Table 4** Frequency of the clinical characteristics in a group of patients carrying a mutation compared with a group without a mutation in the *L1CAM* gene and the positive predictive value

Clinical characteristic	Mutation negative				Mutation positive				p Value*	Positive predictive value (%)
	Presence of the clinical characteristic				Presence of the clinical characteristic					
	Yes		No		Yes		No			
n	%	n	%	n	%	n	%			
Hydrocephalus	61	86	10	14	30	100	0	0	0.03†	33
Aqueduct stenosis	28	68	13	32	18	78	5	22	0.56	—
ACC/DCC	14	45	17	55	10	91	1	9	0.01†	42
Mental retardation	38	73	14	27	18	100	0	0	0.01†	32
Aphasia	23	58	17	42	14	93	1	7	0.01†	38
Adducted thumbs	23	37	40	63	23	85	4	15	<0.001†	50
Spastic paraplegia	20	45	24	55	16	84	3	16	0.005†	44

Patients are taken from the selected group.

\*Fisher exact test.

†Significant.

ACC/DCC, agenesis/dysgenesis of the corpus callosum.

Basically, these conclusions relate to the findings of Holden *et al*,<sup>36</sup> who determined the three-dimensional structure of telokin, a protein with a specific domain similar to the L1 Ig domain containing 35 key residues responsible for its structure. The structure of the fibronectin III-like domain and its key residues can likewise be derived from similarities to those of neuroglian.<sup>37</sup> Other criteria are whether or not the mutation affects a highly conserved amino acid, is de novo in the index patient, or is only found in affected males and not in unaffected male family members.

Fifteen of the 23 (~65%) disease-causing missense mutations were found to be related to key residues, which is comparable to data reported by Michaelis *et al*,<sup>38</sup> who detected 52 out of 78 (~67%). In contrast with the findings of Michaelis *et al* and those of Kamiguchi *et al*,<sup>39</sup> we found no difference in the severity of hydrocephalus in patients with a missense mutation in key residues versus surface residues.<sup>35</sup> In our group, changes in a key residue led to severe hydrocephalus in 57% of the patients and surface changes in 50% of the patients, compared with 67% and 42%, respectively, found by Michaelis *et al*<sup>38</sup> and 78% and 28% determined by Kamiguchi *et al*,<sup>39</sup> indicating no correlation between the severity of hydrocephalus and the location of the missense mutation in either the key residues or the surface residues.

In five of the 73 patients carrying a mutation (~7%), the mutation was shown to be de novo or a maternal germ cell mosaicism. The latter was confirmed in one case (c.645C→T), as a second male fetus was found to have the same mutation. Germline mosaicism of *L1CAM* mutations have previously been reported.<sup>27–40</sup> Du *et al*<sup>27</sup> described an unaffected male with a somatic and germline mosaicism of an *L1CAM* mutation and his two daughters carrying the mutation.

The mutation c.645C→T proved to be a special silent mutation that influences the RNA splicing process: a splice donor site arises within exon 6 of the gene. As a consequence, 51 bp or 17 amino acids are deleted. These deleted amino acids are important for the structure of the Ig2 domain and therefore this mutation is considered to be disease-causing.

When no DNA material is available from the index patient, the diagnosis has to be carried out on a close relative, usually the mother. In this study, this was the case in 38 of the 367 patients. In 25 of these 38 mothers, no mutation was detected. Testing the mother has problems because we have shown that 7% of the mutations detected are de novo or a germline mosaicism.

Another striking finding is the very high (~10%) occurrence of de novo mutations in mothers or a germ cell mosaicism in one of the grandparents of index patients. This and the 7% de novo mutations in the index patients means that at least one-fifth of L1 syndrome families have evolved recently, which probably explains the observation that most of the mutations in the *L1CAM* gene are private mutations.

As a mutation in the *L1CAM*-encoding DNA sequence was found in 20% of referred cases, the mutation analysis was broadened by analysing 100 patients for potential mutations in the regulatory sequences of the *L1CAM* gene. This generated no extra mutations, leading to a potential detection rate in these regions of less than 1%. Screening for mutations in the 3' region was not performed because of this finding and also because too little is known about the function of specific sequences in this region. An MLPA test was used to investigate the same 100 patients for large duplications in the *L1CAM* gene. Only one duplication, exons 2–10, was detected, suggesting a very low prevalence of this type of mutation. The same seems to be true for large deletions, since, in 73 mutation-carrying patients, only one showed a deletion of the entire gene,<sup>18</sup> which is in accordance with previously published results. To date, 169 different *L1CAM* mutations have been published: 164 small point mutations and five large deletions or duplications including one deletion of the entire gene,<sup>18</sup> one deletion of the promoter region and exon 1,<sup>17</sup> one deletion of 2 kb at the distal part of the gene c.3543\_?del2kb,<sup>41</sup> one deletion of exons 2–5 and part of exon 6,<sup>16</sup> and one duplication of 1.3kb c.3543\_?dup1.3kb.<sup>42</sup> From these results, it can be concluded that broadening the DNA-screening procedure to include regulatory sequence analysis is not worthwhile. Screening for deletions and duplications may be beneficial, especially in clear clinical cases, although this does not seem to greatly influence the detection rate.

**Table 5** Genotype–phenotype correlation

	Died before the age of 3	
	No	Yes
Missense mutation		
Number	11	1
%	92	8
Truncating mutation		
Number	10	11
%	48	52*

\*p=0.02 truncating mutation compared with missense mutation.

In contrast with this, the combination of clinical data and the number of affected relatives, obtained via a questionnaire, shows a clear correlation with the mutation detection rate as shown in figure 3. Our findings are therefore of importance for clinicians and genetic counsellors, since they may be used to predict the chance of finding a mutation. Besides, the positive predictive value showing adducted thumbs to be the most predictive may also be quite useful.

Finally, it should be noted that a genotype–phenotype correlation has been identified for the *L1CAM* gene and its associated syndrome in our group of patients. As previously reported,<sup>43 44</sup> we also found that children with a truncating mutation were more likely to die before the age of 3 than children with a missense mutation. This illustrates that the type of mutation has an important effect on the severity of the L1 syndrome.

## CONCLUSIONS

Our study shows that the mutation detection system is efficient for detecting mutations. Using this system, we have clearly shown a correlation between the number of clinical characteristics, the number of affected family members and the chance of finding a mutation. Moreover, we found a genotype–phenotype correlation for L1 syndrome. This study provides a comprehensive tool for genetic counselling.

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