ORIGINAL ARTICLE

Genome-wide linkage analysis in families with infantile hypertrophic pyloric stenosis indicates novel susceptibility loci

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Infantile hypertrophic pyloric stenosis (IHPS) is a common cause of upper gastrointestinal obstruction during infancy. A multifactorial background of the disease is well established. Multiple susceptibility loci including the neuronal nitric oxide synthase (*NOS1*) gene have previously been linked to IHPS, but contradictory results of linkage studies in different materials indicate genetic heterogeneity. To identify IHPS susceptibility loci, we conducted a genome-wide linkage analysis in 37 Swedish families. In regions where the Swedish material showed most evidence in favor of linkage, 31 additional British IHPS families were analyzed. Evidence in favor of significant linkage was observed in the Swedish material to two loci on chromosome 2q24 (non-parametric linkage (NPL) = 3.77) and 7p21 (NPL = 4.55). In addition, evidence of suggestive linkage was found to two loci on chromosome 6p21 (NPL = 2.97) and 12q24 (NPL = 2.63). Extending the material with British samples did not enhance the level of significance. Regions with linkage harbor interesting candidate genes, such as glucagon-like peptide-2 (GLP-2 encoded by the glucagon gene *GCG*), *NOS1*, motilin (*MLN*) and neuropeptide Y (*NPY*). The coding exons for GLP-2, and *NPY* were screened for mutations with negative results. In conclusion, we could confirm suggestive linkage to the region harboring the *NOS1* gene and detected additional novel susceptibility loci for IHPS.

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INTRODUCTION

Infantile hypertrophic pyloric stenosis (IHPS; OMIM 179010) is one of the most common causes of upper gastrointestinal obstruction in infants and a frequent cause of surgery during the first months of life.¹ The pyloric smooth muscle hypertrophy causes gastric outlet obstruction resulting in projectile vomiting, which if not treated can cause lethal dehydration and electrolyte imbalance. Incidence is generally reported to 1.5–3 per 1000 live births in white populations.^{2,3} IHPS is a well-defined clinical entity but the underlying disease causing mechanism remains unclear. A genetic contribution to the disease is well established by Carter and Evans description of families with IHPS (1961 and 1969) and IHPS is regarded as a complex disease where both genetic and environmental factors are of importance for development of the disease.^{2,4–6} Elucidation of the molecular background of the disease could give a better understanding of the pathophysiology, and possibly lead to identification of environmental risk factors that may be target for preventive measures.

The disorder is more common in boys with a sex ratio of 5:1⁵ and estimated heritability is 87%.³ Concordance rate of 46% is reported in monozygotic twins compared with 8% in dizygotic twins.³ In accordance with the postulated complex nature of the disease, there are also a few reports of families with inheritance pattern compatible with an autosomal dominant disease.⁷ Reanalysis of data from several published family studies suggested the existence of two or three loci, where each loci is associated with a relative risk up to 5.² Multiple susceptibility loci have been reported associated with IHPS. Linkage to chromosome 16p12–p13 and 16q24 was found in two different large Caucasian families with autosomal dominant inheritance. However, this could not be replicated in any other families of same ancestry investigated, indicating locus heterogeneity of the disease.^{8,9} Previously, a SNP-based genome-wide scan identified linkage to chromosome 11q14–q22 and chromosome Xq23 in a material of 81 Caucasian pedigrees.¹⁰ IHPS is also described in association with duplication of chromosome 9q11–q33, but linkage analysis of this region did not

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provide evidence for an IHPS locus among 20 families with several affected IHPS cases.¹¹ Neuronal nitric oxide synthase (*NOS1*) is to date the only gene reported with evidence as an IHPS susceptibility locus. However, the gene has not been mutation analyzed in IHPS patients, but linkage to an intragenic marker¹² and association to a promoter polymorphism¹³ are reported. Neither of these findings could be confirmed when studied in different materials^{14–16} supporting the presence of locus heterogeneity.

Aim of this study was to identify IHPS loci in a Swedish material of 37 families with 184 individuals, 92 affected and 58 affected relative pairs (ARPs), by performing genome-wide linkage analysis using a non-parametric ARP method. Thirty-one British families with 256 individuals, 101 affected and 103 ARPs were included in order to either confirm or exclude findings of non-parametric linkage (NPL) in the Swedish material.

MATERIALS AND METHODS

Material

Thirty-seven Swedish families with 2–5 affected individuals identified from hospital records of the Pediatric Surgery clinics in Stockholm, Uppsala, Lund and Gothenburg were recruited to the study. The families included a total of 184 individuals, 92 affected and 58 ARPs. Ratio of affected males to females was 1.7:1. One family had five affected, 2 had four affected, 11 had three affected and 23 had two affected. Table 1 describes the details of the patient material. Pedigrees of the three largest Swedish families are shown in Figure 1, the remaining pedigrees are available on request.

In collaboration with University College London, 31 British IHPS families with 256 individuals, 101 affected and 103 ARPs were included to be analyzed in chromosome regions where the Swedish material showed most evidence in favor of linkage. Ratio of affected males to females in the British material was 2.1:1. One family had six affected, 2 had five affected, 7 had four affected, 14 had three affected and 7 had two affected individuals. Informed consent was obtained from all the Swedish and British participants. The study was approved by the Ethics Committee of Karolinska Institutet and the Ethics Committee of University College London Hospital.

Genotyping

Genomic DNA was extracted from peripheral blood using standard protocols. For the initial genome scan, a total of 353 fluorescently labeled microsatellite markers evenly located on the 22 autosomes and the X chromosome were analyzed in the Swedish material. The basis for the microsatellite markers was the Weber 6 screening set.¹⁷ In sparsely covered regions, new markers were added from the Genome Database and Marshfield Medical Research foundation. The average marker density for the initial genome scan was 10.24 cM. For fine mapping of the loci on chromosome 2p, 2q, 6p, 7p, 8q, 12q and 13q, an additional set of 6, 4, 11, 10, 2, 2 and 6 markers were genotyped, respectively. Also, two markers on chromosome 16p were added because of a recently published report of linkage in this region.⁸ Average marker density of the fine-mapped regions was 2.9 cM. Mean heterozygosity for autosomal markers was 0.76, and the mean success rate for included markers was 0.80.

Each marker was amplified separately according to standard PCR protocols (conditions available upon request). The PCR products were pooled and size fractionated on an ABI 377 DNA Sequencer and ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The resulting electrophoretic data were analyzed with Genescan 3.1.2 and Genotyper2.0 software (Applied Biosystems). The British material was genotyped with fine mapping markers on chromosome 2p, 2q, 7p, 8q, 12q and 13q, and analyzed as described.

All genotyping data were analyzed for Mendelian inheritance and relationship patterns of the families using the zGenStat 1.126 software (Zazzi, unpublished). Any marker genotypes violating the Mendelian rules of transmission were rechecked and ambiguous marker genotypes were excluded from the analysis. Allele frequencies were calculated from all genotyped individuals using the zGenStat 1.126 software. To identify markers with allele dropout, a homozygosity test was performed using the Pearson χ^2 -test implemented in zGenStat 1.126 software. Any marker with significant deviation from expected

Table 1 Characteristics of the Swedish and British families

	Swedish families	British families
<i>Number of affected</i>		
Affected total	92	101
Ratio boys:girls	1.7:1	2.1:1
<i>Relationship between affected</i>		
Sib pair	18	43
Parent–child	19	23
Grandparent–grandchild	3	3
Cousins	14	13
Uncle, aunt	23	27
Other		17

Figure 1 Pedigrees of the three largest Swedish families included in the linkage analysis. Affected individuals are indicated by filled squares (male) or circles (female).

homozygosity frequency ($P < 0.001$) was reanalyzed. Marker order and genetic distances were based on the deCODE linkage map.

Statistical analysis

NPL method was used as the mode of inheritance is unknown. Analysis was performed in Allegro software version 1.2¹⁸ using NPL ARP method with option arguments for linear statistics and S_{all} scoring function. Majority of the pedigrees in the Swedish material were relatively small and homogenous in structure with 2–3 affected, which motivates the use of a statistical model with equal weight assigned to all pedigrees. However, the material also included a few extended pedigrees with up to five affected individuals. If an equal weight model is used, there is a risk that these families exert excessive influence on the total result because of their higher information content. This motivates instead the use of a statistical model where a weighting scheme is applied for the different pedigrees. Consequently, we chose to analyze data with both an equal-weighted model and a model with weighting factor power 0.5, and evaluate possible differences between these two analyses.

We used Lander and Kruglyak's¹⁹ criteria to define the threshold value for genome-wide linkage. According to this definition, the threshold value for significant linkage equals the NPL score randomly occurring 0.05 times in a dense genome-wide scan. The threshold value for suggestive linkage equals the score randomly occurring once per genome scan. Allegro software¹⁸ was used to simulate genotype data for 10 000 genome-wide scans. The simulation marker set included the fine mapping markers and the simulated genotypes had identical pedigree structures, affection status, success rate and allele frequencies

Figure 2 Results of multipoint NPL analysis for the IHPS genome-wide scan using a statistical model with equal weight for each family. The NPL score is given on the y axis and the position expressed in centimorgans (cM) from p-ter to q-ter on the x axis. Blue curve represents the initial genome-wide scan and the red curve the fine-mapping results of the Swedish material. The green curve represents the results after addition of the British material in fine-mapped regions.

as in the original analysis. Only individuals having genotype data in the original analysis were assigned genotype data in the simulations. The 10 000 simulated genome-wide scans were analyzed in Allegro software¹⁸ using the same settings as in the original analysis, resulting in separate threshold values for equal and weighted analysis models. The NPL scores that occurred in average 0.05 times respectively once per simulated genome wide scan, were defined as the threshold NPL score for significant and suggestive linkage. Double peaks of a NPL score were not considered independent if they occurred within 20 cM of each other.

Candidate gene analysis

The function and expression pattern of genes located in the chromosome regions with evidence of significant or suggestive linkage were investigated by mining the Ensembl and NCBI databases. In addition, the gene prioritizing

software Endeavour^{20,21} was used to identify possible candidate genes in these regions. The coding exons in the neuropeptide Y gene (*NPY*) and exon 5 in the glucagon gene (*GCG*) coding for glucagon-like peptide-2 (*GLP-2*) were examined with sequencing in 18 and 52 affected patients, respectively. Primer sequences and cycling conditions are available on request. Purified PCR products were sequenced using ABI PRISM BigDye Terminator v1.1 kit and ABI 3730 DNA sequencer (Applied Biosystems).

RESULTS

Nonparametric linkage

The multipoint NPL results of the Swedish material with equal and weighted analysis are plotted as blue curves in Figures 2 and 3, respectively. Seven regions on chromosomes 2p, 2q, 6p, 7p, 8q, 12q

Figure 3 Results of multipoint NPL analysis for the IHPS genome-wide scan using a statistical model with weighting factor power 0.5. The NPL score is given on the y axis and the position expressed in centimorgans (cM) from p-ter to q-ter on the x axis. Blue curve represents the initial genome-wide scan and the red curve represents the fine-mapping results of the Swedish material. The green curve represents the results after addition of the British material in fine-mapped regions.

and 13q demonstrating NPL scores between 1.63 and 3.83 in either the equal or weighted analysis were further investigated with additional fine mapping markers. Fine mapping regions and their flanking markers are listed in Table 2. Two additional markers were also added to an earlier identified IHPS locus on chromosome 16p.⁸ The fine mapping results of the Swedish material are plotted as red curves in Figures 2 and 3. Inclusion of the fine mapping markers enhanced the NPL score in the fine-mapped regions on chromosome 2q, 6p, 7p, 12q and 13q demonstrating NPL scores between 1.75 and 4.55 (Tables 3 and 4). Threshold scores for significant and suggestive genome-wide linkage obtained from simulations were: equal weight model NPL 3.04 (significant linkage), NPL 2.18 (suggestive linkage), weighted model

NPL 3.46 (significant linkage) and NPL 2.60 (suggestive linkage). Four fine-mapped regions showed evidence in favor of linkage (Figure 4). Significant linkage was found at the locus 2q24 in both equal (NPL=3.10) and weighted model (NPL=3.77). The locus 7p21-p22 showed evidence in favor of significant linkage in the weighted model (NPL=4.55) and suggestive linkage in the equal weight model (NPL=2.98). Suggested linkage was also found at locus 12q24 in the equal model (NPL=2.63) and 6p21 in the weighted model (NPL=2.97). The fine-mapped regions at 2p, 8q and 13q did not show evidence of linkage after fine mapping, even though the fine mapping increased the NPL score in the 13q region. The British material was analyzed with fine mapping markers only. When adding

Table 2 Fine mapping regions and their flanking markers

Chromosome	Position (cM) ^a			Flanking markers	
2p23-2p21	49.6 ^b	—	68.84	D2S2144	D2S2298
2q23-2q31	159.43 ^b	—	177.98	D2S2299	D2S335
6p25-6p21	11.3	—	68.9 ^b	D6S1617	D6S1650
7p22-7p14	3.5 ^b	—	47.84	D7S1532	D7S2496
8q24	132.43 ^b	—	135.57 ^b	D8S1774	D8S1801
12q24	136.23 ^b	—	139.04 ^b	D12S2082	D12S385
13q12-13q14	17.41	—	50.11 ^b	D13S1254	D13S1312

^adeCODE map.^bPosition missing in deCODE map, position estimated based on Marshfield map.**Table 3** Fine mapping results for equal weight analysis. Highest NPL score for each fine-mapped region listed in descending order

Chromosome	Marker	Position (cM) ^a	NPL-score	Level of significance ^b
2q24	D2S111	171	3.10	Significant linkage
7p22	D7S1532	3.5 ^c	2.98	Suggestive linkage
12q24	D12S366	137.36	2.63	Suggestive linkage
13q12	D13S1244	18.72	1.75	NS
6p24	D6S470	23.75	1.65	NS
8q24	D8S1774	132.43 ^c	0.85	NS
2p23	D2S405	51.48	0.58	NS

Abbreviation: NPL, non-parametric linkage.

^adeCODE map.^bDefined by 10 000 simulations; NS, not significant.^cPosition missing in deCODE map, position estimated based on Marshfield map.**Table 4** Fine mapping results for weighted analysis. Highest NPL score for each fine-mapped region listed in descending order

Chr	Marker	Position (cM) ^a	NPLscore	Level of significance ^b
7p21	D7S2514	14.94	4.55	Significant linkage
2q24	D2S111	171	3.77	Significant linkage
6p21	D6S1568	53.85	2.97	Suggestive linkage
13q12	D13S217	22.17	2.55	NS
2p22	D2S1788	58.96 ^c	2.16	NS
12q24	D12S366	137.36	1.14	NS
8q24	D8S1774	132.43 ^c	0.17	NS

Abbreviation: NPL, non-parametric linkage.

^adeCODE map.^bDefined by 10 000 simulations; NS, not significant.^cPosition missing in deCODE map, position estimated based on Marshfield map.

the result to the Swedish material, NPL scores decreased in all regions except at the locus on 8q (green curves Figures 2 and 3).

Candidate genes

Candidate genes located in vicinity of loci showing evidence in favor of linkage were identified. The boundaries of the candidate regions where to primarily search for candidate genes, were defined as the position where the NPL score fell 1 unit below the region's maximum value. The obtained candidate regions according to this definition were 2q24-2q31 and 7p21-7p22 with evidence in favor of significant linkage, 6p21-6p22 and 12q24 with evidence in favor of suggested linkage. We regard the genes for glucagon (*GCN*, coding for GLP-2, 2q24.2), motilin (*MLN*, 6p21.31) and neuronal *NOS1* (12q24.22) genes as the most interesting candidate genes based on location and function. Also, the *NPY* (7p15.3) located in close proximity to the

candidate region on chromosome 7 was considered as a candidate gene of interest for further study. Exon 5 in the *GCG* gene coding for GLP-2 was sequenced in 52 patients. We found one patient heterozygote for a missense variant (A>G) exchanging an isoleucine to valine (I13V) that was inherited from the non-affected father. Sequencing of the *NPY* gene in 18 patients did not show any mutations in the coding exons. Previously, we have published results of mutation screening of the *MLN* gene²² and an association study of one single *NOS1* promoter polymorphism.¹⁵

DISCUSSION

We report a genome-wide linkage analysis with 353 microsatellite markers in 37 Swedish IHPS families forming 58 ARPs. We performed fine mapping analysis in seven chromosomal regions adding 41 markers and included a British sample set. In the Swedish material, we identified two candidate regions with significant linkage on chromosome 2q24 and 7p22, and additional suggestive linkage on chromosome 6p21 and 12q24.

Earlier studies have identified five IHPS regions located on chromosomes 12q24 (*NOS1*), 16p12-p13, 11q14-q22, Xq23 and 16q24.¹ We could confirm suggestive linkage to the *NOS1* locus in the Swedish material, when the statistical model where all pedigrees were assigned equal weight was used. Previous speculation on the pathogenesis of IHPS has focused on the idea that an overactivity or prolonged spasm of the pyloric muscle is mandatory for the development of the pyloric muscle hypertrophy.²³ Mediators that affect gastric contractility are thus likely to be key factors in the development of the disease. Nitric oxide is a major inhibitory neurotransmitter in the gut causing smooth muscle relaxation. The synthesis of nitric oxide is catalyzed by the enzyme neuronal nitric oxide synthase coded by the *NOS1* gene. A mouse model with targeted disruption of the *nos1* gene shows a phenotype consistent with IHPS with a hypertrophic pyloric muscle and distended stomach.²⁴ Also, in hypertrophic pyloric muscle tissue, the *NOS1* gene expression has been found to be significantly reduced,²⁵ and the *NOS1* gene has therefore been subject to extensive investigation in IHPS patients. Evidence of linkage to the *NOS1* gene using intragenic markers was reported from a study comprising 27 families,¹² but could not be confirmed in three extended pedigrees from another population.¹⁴ Furthermore, a functional polymorphism in the *NOS1* gene promoter that affects the transcription was found to be associated with IHPS in a small material of 16 patients.¹³ This finding could neither be confirmed in our material consisting of 83 IHPS patients,¹⁵ nor by another recent study of 56 cases.¹⁶ The diverging results of the *NOS1* gene studies could be explained by genetic heterogeneity of IHPS. Our finding of suggestive linkage to the *NOS1* locus, supports its involvement in the IHPS etiology. Apart from the *NOS1* locus, the other loci with evidence in favor of linkage identified in this study are novel candidate regions not described in previously performed genome-wide linkage studies. The candidate region 2q24 harbor the Glucagon gene (*GCG*) coding for GLP-2, which is a pleiotrophic hormone affecting multiple aspects of gastrointestinal physiology. It is formed from proglucagon produced and secreted from the intestinal enteroendocrine L-cells in a nutrient-dependent manner. GLP-2 has an intestinotrophic effect stimulating epithelial cell proliferation and inhibiting apoptosis in addition to a role in regulation of gastrointestinal motility.^{26,27} Both these effects could possibly promote pyloric hypertrophy, and we considered *GCG* as an IHPS candidate gene. Sequencing of the gene in 52 patients revealed a missense variation in one patient inherited from the non-affected father, which neither supports nor excludes the significance of the sequence variation. *NPY* is a neurotransmitter present in enteric

Figure 4 Chromosomal regions exhibiting significant or suggestive linkage after fine mapping using equal weight or weighted analysis.

neurons suggested to exert an inhibitory influence on smooth muscle cells.²⁸ Furthermore, NPY is often colocalized in neurons with nNOS, supporting its function of mediating relaxation.²⁹ The NPY gene (*NPY*) is located adjacent to the candidate locus on chromosome 7, which makes it an interesting IHPS candidate gene. The gene consists of four exons, which were screened for mutations in 18 IHPS patients, however, without any positive finding. It has been reported that infants with phenylketonuria has an increased risk for IHPS.³⁰ Phenylketonuria is an autosomal recessive genetic disorder caused by the deficiency of the enzyme phenylalanine hydroxylase. The phenylalanine hydroxylase gene is located adjacent to the suggestive candidate region on chromosome 12, but was not further investigated in this study. Motilin is a hormone inducing contractions in the gastrointestinal tract. The *MLN* gene is considered as an IHPS candidate gene as treatment with the motilin agonist erythromycin in newborn children gives an increased risk for developing IHPS.^{31,32} In a previous study, we have investigated the *MLN* gene as an IHPS candidate gene without finding evidence of association to the disease in that material.²² However, the *MLN* gene is located in the candidate region with suggestive linkage on chromosome 6p21, which motivates further studies of this candidate gene. Interestingly the motilin receptor gene (*MLNR*) is located adjacent to the fine-mapped region on chromosome 13q, but mutation analysis has not revealed any mutations (E Chung, personal communication).

Extending the fine mapping sample size by including the British pedigrees did not enhance the NPL scores. A reason for this could be the still relatively small material with total 68 families. Even though the European populations in general have a common genetic background, there can exist a founder effect resulting in different susceptibility genes in the Swedish and British families. The two populations may also have been exposed to different environmental risk factors, affecting which functional polymorphisms that are being involved in disease susceptibility. The fact that no mutations were found in the

coding regions of the suggested candidate genes does not exclude them being associated with the disease. For example, alterations in regulatory elements of the genes have not been studied. Also, a weak association between a sequence variant and the disease may not be detected if the sample size is not big enough. An interesting feature of the Swedish material is the high occurrence of girls among cases with a ratio 1.7:1 of boys to girls. If this is just a coincidence, or a true indication of hereditary cases having a higher share of girls compared with sporadic cases remains to be studied.

In conclusion, this genome-wide linkage study shows evidence for significant or suggestive linkage on chromosomal regions 2q24, 7p21-22, 6p21 and 12q24 to IHPS. We are aware of the limitations of linkage studies in small materials, and that negative results do not exclude existence of a true candidate region. In case of finding a region with suggestive linkage, a large material is of course preferable. However, if simulations are performed to set the threshold for linkage specific for the material studied, the significance of a positive finding cannot be ignored even if the material is small.

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