Involvement of claudin 3 and claudin 4 in idiopathic infantile hypercalcaemia: a novel hypothesis?

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Abstract

Background. Idiopathic infantile hypercalcaemia (IIH) is a rare disease that generally resolves spontaneously between the age of 1 and 3 years. Similar symptoms may occur in patients suffering from Williams–Beuren syndrome (WBS), which is caused by a microdeletion on chromosome 7. Two of the genes, named CLDN3 and CLDN4, located within this region are members of the claudin family that has been shown to be involved in paracellular calcium (Ca\(^{2+}\)) absorption. Based on the hemizygous loss of CLDN3, CLDN4 and TRPV6, we conclude that IIH is neither caused by mutations in these candidate genes nor by deletions or duplications in the genome of these patients.

Methods. Biochemical characteristics, including calciotropic hormone levels, were obtained from three typical IIH patients. CLDN3 and CLDN4 sequences were also analysed for the presence of deletions or duplications in the genome of these patients. The major intestinal Ca\(^{2+}\) transporter TRPV6 was also screened for the presence of mutations, since hypercalcaemia in IIH and WBS has been shown to result from intestinal hyperabsorption. All three patients were also analysed for the presence of deletions or duplications using a single-nucleotide polymorphism (SNP) array for genomic DNA.

Results. The serum Ca\(^{2+}\) levels of patients were 2.9, 3.3 and 3.8 mmol/L (normal $<2.7$ mmol/L). Levels of 25-hydroxyvitamin D\(_3\) and 1,25-dihydroxyvitamin D\(_3\) were normal, parathyroid hormone (PTH) and PTH-related peptide (PTHrP) levels were appropriately low. Sequencing of coding regions and intron–exon boundaries did not reveal mutations in CLDN3, CLDN4 and TRPV6. Identified SNPs were not correlated with the disease phenotype. A SNP array did not reveal genomic deletions or duplications.

Conclusions. Biochemical analysis did not reveal inappropriate levels of calciotropic hormones in IIH patients in this study. Furthermore, based on the lack of mutations in CLDN3, CLDN4 and TRPV6, we conclude that IIH is neither caused by mutations in these candidate genes nor by deletions or duplications in the genome of these patients.

Keywords: claudin 3; claudin 4; hypercalciuria; idiopathic infantile hypercalcaemia; Williams–Beuren syndrome

Introduction

Idiopathic infantile hypercalcaemia (IIH) is a rare disorder of thus far unknown aetiology with a reported prevalence of 1 in 50 000 live births [patients with Williams–Beuren syndrome (WBS) included] [1]. Children suffering from IIH usually present during their first year of life with clinical...
Claudin 3 and 4 in idiopathic infantile hypercalcaemia a novel hypothesis?

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</table>

Table 1. Human primer sequences for CLDN3, CLDN4 and TRPV6

features of hypercalcaemia, such as irritability, constipation, vomiting, increased thirst and a failure to thrive. Hypercalcaemia in IIH patients is the result of intestinal hyperabsorption, which was shown by Barr et al. using oral calcium (Ca2+) loading tests, and is emphasized by a cellulosic phosphate treatment which normalizes the serum Ca2+ levels [2,3]. Hypercalcaemia generally resolves between the ages of 1 and 3 years; however, hypercalcemia may persist up to the age of 12 [4,5]. If diagnosed and treated in an early stage, the prognosis of IIH patients can be improved, and the extent of nephrocalcinosis can be limited [5,6].

IIH was first described in the UK in the 1950s by Lightwood and Fanconi [7,8]. Originally, a subdivision of ‘mild’ and ‘severe’ IIH was made in order to distinguish between patients suffering solely from hypercalcaemia and patients that also display additional abnormalities such as distinctive facial features [9]. The ‘severe’ form has subsequently been shown to be part of a larger group of symptoms common to patients suffering from WBS [10]. WBS is nowadays known to result from a hemizygous microdeletion at 7q11.23 which arises from a recombination between misaligned repeat sequences flanking the region [11,12]. The WBS deletion region encompasses 24 genes [13]. Among the genes located in the deleted region are CLDN3 and CLDN4 which encode tight junction proteins claudin 3 and 4, respectively [14,15]. Members of the claudin protein family have been shown to be involved in paracellular Ca2+ absorption [16]. Also, they have been described to play an important role in the regulation of ion transport in both the intestine and the kidney [16]. The deletion of claudin 3 and 4 increases the permeability of the intestinal epithelium [17–20]. Changes in expression of various Claudins in murine intestine throughout time indicate that the absence of a particular Claudin can, in time, be compensated for by the maturation of other Claudins [21].

Similarities in the course of development of hypercalcaemia in IIH and WBS patients could be explained by a shared underlying mechanism such as a gene defect. The hemizygous loss of CLDN3 and CLDN4 in WBS, combined with their function in ion transport, makes them good candidate genes underlying the similarities in disease phenotype in patients suffering from IIH and WBS. In order to validate this hypothesis, three typical IIH patients were analysed for the presence of mutations in CLDN3 and CLDN4. Since the IIH phenotype is the result of intestinal hyperabsorption, the major intestinal Ca2+ transporter TRPV6 was also analysed in order to exclude changes in Ca2+ uptake due to mutational changes in this transporter. Finally, the presence of deletions and duplications was analysed in all three patients using a single-nucleotide polymorphism (SNP) array.

Materials and methods

Patient selection and phenotype assignment

Patient 1 and 2 were selected and treated in Australia. They have been described in two previous Australian studies as Patient II2 and III3 and as Patient 1 and 2 [4,22]. Observation and treatment of Patient 3 took place in the Netherlands, and this patient has not been previously described.

Serum and urine biochemistry

Biochemical analysis of blood and urine was routinely performed using standard laboratory techniques at the Royal Children’s Hospital and Mater Children’s Hospitals in Brisbane, Australia (Patient 1 and 2) or the Radboud University Nijmegen Medical Centre in the Netherlands (Patient 3). Parathyroid hormone (PTH) measurements in both hospitals were performed using an immunometric assay measuring only intact PTH.

DNA extraction

DNA sample collection and storage were carried out according to standard methods. In short, 10 mL of peripheral blood was collected into ethylene-diamine-tetraacetic acid (EDTA) containing tubes and centrifuged for 10 min at 3000 g to separate buffy coats and plasma. Total genomic DNA was isolated from the buffy coat using a standard blood
and body fluid protocol. Extracted DNA was quantified by spectrophotometrical absorbance measurements and stored in aliquots.

Genotyping

Oligonucleotide primers (Biolegio, Malden, The Netherlands) for the amplification of genomic DNA by polymerase chain reaction (PCR) were developed based on genomic sequences. Since CLDN3 and CLDN4 both consist of one large exon, primers were designed to amplify overlapping parts of the exon and included intron–exon boundaries. For TRPV6, a similar approach was used. Because of short intron sequences between exons 2 and 3, and 7 and 8, as well as 9 and 10, PCR amplification of these exons was combined, and PCR products were sequenced as single fragments. Primer sequences used for the amplification of CLDN3, CLDN4 and TRPV6 are listed in Table 1. PCR products were purified using a GenElute PCR Clean-up Kit (Sigma, St Louis, MO, USA). DNA sequencing was carried out by standard procedures at the sequence facility of the Radboud University Nijmegen Medical Centre. Subsequently, patient sequences were aligned and compared with human CLDN3 (NM_001306), CLDN4 (NM_001305) or TRPV6 (NM_018646) mRNA sequences, obtained from the NCBI nucleotide database.

Affymetrix NspI SNP array hybridization and analysis

Microdeletion analyses were carried out on the Affymetrix GeneChip 250k (NspI) SNP array platform (Affymetrix, Inc., Santa Clara, CA, USA), which contains 25-mer oligonucleotides representing a total of 262 264 SNPs. Hybridizations were performed according to the manufacturer’s protocols. Copy numbers were determined using the 2.0 version of the CNAG (Copy Number Analyzer for Affymetrix GeneChip mapping) software package, by comparing SNP intensities from patient DNA with those of a sex-matched pooled reference DNA sample (DNA from either 10 healthy male or 10 healthy female individuals) [23]. The average resolution of this array platform is 150–200 kb.

Results

Patient phenotype and biochemistry

An overview of selected clinical and biochemical characteristics of all patients and the corresponding reference ranges for infants <12 months of age are provided in Table 2. Family background and clinical and biochemical characteristics of Patient 1 and Patient 2 have been described in previous publications [4,22,24]. In short, Patient 1 and 2 both showed elevated serum Ca^{2+} levels and a urinary Ca/Cr ratio above the normal reference range. PTH levels were below the reference range in Patient 1 and normal in Patient 2. PTH-related peptide

![Image](https://example.com/image.png)

**Fig. 1.** Ultrasound of the left kidney of Patient 3 at the age of 2 months. Nephrocalcinosis in the medullar regions is indicated by white arrows.
(PTHrP) levels were normal in both patients, as were 25-hydroxyvitamin D$_3$ levels. 1,25-dihydroxyvitamin D$_3$ levels were not changed in Patient 1 and slightly elevated in Patient 3.

Patient 3 was born at 40 weeks gestational age with normal birth weight of 3150 g. Both parents were healthy without consanguinity. Ultrasonography during pregnancy showed increased echogenicity of both kidneys of the fetus. She was first seen at our outpatient clinic at the age of 2 months because of failure to thrive and to evaluate the increase of fluid intake, no other therapy was applied, and her growth resumed at a normal rate. Since the age of 2 years, she suffered from recurrent urinary tract infections. A cystogram showed no reflux. Figure 2 depicts the development of Ca$^{2+}$, phosphate and creatinine levels over time. After an initial period of extremely hypercalcaemia, serum Ca$^{2+}$ levels normalized at a level within the normal range, but remained in the upper percentiles (2.6–2.7 mmol/L). At the age of 8 years, her serum creatinine was 92 μmol/L with an estimated creatinine clearance of 76 mL/min/1.73 m$^2$. This slightly decreased clearance rate can be explained by the combination of recurrent urinary infections and nephrocalcinosis.

**Sequence analysis**

Analysis of coding regions and intron–exon boundaries of CLDN3, CLDN4 and TRPV6 genomic sequences revealed a number of SNPs in our three patients. Two SNPs were identified in CLDN3 of Patient 1 and located in the 5’ untranslated region (UTR) of the exon. CLDN4 showed five identical SNPs in both Patient 2 and 3. One was located outside the exon near its 5’ end, one in the 5’ UTR and three in the 3’ UTR. Sequence analysis of TRPV6 revealed one synonymous SNP in the coding region of exon 11 in Patient 3. Table 4 provides an overview of all SNPs identified in our patients in CLDN3, CLDN4 and TRPV6. All SNPs found in this study were previously identified and

### Table 3. Biochemical characteristics Patient 3

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<th>Source</th>
<th>Analysed compound</th>
<th>Patient 3</th>
<th>Normal reference range</th>
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</thead>
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<td>Blood</td>
<td>Ca$^{2+}$</td>
<td>3.41–3.76 mmol/L</td>
<td>&lt;2.7 mmol/L</td>
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<tr>
<td></td>
<td>Ionized Ca$^{2+}$</td>
<td>1.26–1.34 mmol/L</td>
<td>1.0–1.3 mmol/L</td>
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<tr>
<td></td>
<td>Mg$^2$</td>
<td>0.8 mmol/L</td>
<td>0.78–1.04 mmol/L</td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>1.05–1.14 mmol/L</td>
<td>1.20–2.2 mmol/L</td>
</tr>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
<td>278–397 U/L</td>
<td>150–350 U/L</td>
</tr>
<tr>
<td></td>
<td>Creatinine</td>
<td>46–62 μmol/L</td>
<td>20–80 μmol/L</td>
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<tr>
<td></td>
<td>Intact PTH</td>
<td>0.3 pmol/L</td>
<td>1.0–6.5 pmol/L</td>
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<tr>
<td></td>
<td>PTHrP</td>
<td>&lt;0.3 pmol/L</td>
<td>&lt;0.3 pmol/L</td>
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<tr>
<td></td>
<td>25-hydroxy-vitamin D$_3$</td>
<td>51 μmol/L</td>
<td>35–100 μmol/L</td>
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<tr>
<td></td>
<td>1,25-dihydroxy-vitamin D$_3$</td>
<td>132 pmol/L</td>
<td>50–150 pmol/L</td>
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<td>Calcitonin</td>
<td>5 pmol/L</td>
<td>&lt;3.5 pmol/L</td>
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<tr>
<td>Urine</td>
<td>Ca/Cr ratio</td>
<td>8.2–8.7 mmol/mmol</td>
<td>0.09–2.2 mmol/mmol [34]</td>
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<tr>
<td></td>
<td>Phosphate reabsorption</td>
<td>78–82%</td>
<td>78–96% [35]</td>
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Ca$^{2+}$; calcium; Mg$^2$, magnesium; PTH, parathyroid hormone; PTHrP, PTH-related peptide; Ca/Cr, calcium/creatinine.

As the serum Ca$^{2+}$ levels decreased spontaneously upon the increase of fluid intake, no other therapy was applied, and her growth resumed at a normal rate. Since the age of 2 years, she suffered from recurrent urinary tract infections. A cystogram showed no reflux. Figure 2 depicts the development of Ca$^{2+}$, phosphate and creatinine levels in Patient 3 throughout time. After an initial period of severe hypercalcaemia, serum Ca$^{2+}$ levels (squares) normalize in the upper percentiles of the normal reference range (<2.7 mmol/L). Serum phosphate levels (triangles) are generally within the reference range (1.2–2.2 mmol/L), but remain in the lower percentiles. Serum creatinine levels (circles, right y-axis) are within the reference range (20–80 μmol/L) and seem to increase throughout time.

**Fig. 2.** Development of serum Ca$^{2+}$, phosphate and creatinine levels in Patient 3 throughout time. After an initial period of severe hypercalcaemia, serum Ca$^{2+}$ levels (squares) normalize in the upper percentiles of the normal reference range (<2.7 mmol/L). Serum phosphate levels (triangles) are generally within the reference range (1.2–2.2 mmol/L), but remain in the lower percentiles. Serum creatinine levels (circles, right y-axis) are within the reference range (20–80 μmol/L) and seem to increase throughout time.

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<th>dbSNP cluster ID</th>
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CLDN3, claudin 3; CLDN4, claudin 4; TRPV6, transient receptor potential vanilloid 6; UTR, untranslated region.
are listed in SNP databases (http://ncbi.nlm.nih.gov/SNP/). SNP array analysis of genomic DNA from the patients did not reveal significant deletions or duplications.

Discussion

To date, the diagnosis IIH is still based on exclusion. Only if disorders such as Williams–Beuren syndrome, benign familial hypocalciuric hypercalcaemia, primary hyperparathyroidism, thyroid disease, malignancy, excessive vitamin D and A intake, diuretic administration, sarcoidosis, and other granulomatous diseases are excluded the diagnosis IIH can be made [25]. No genetic defects causing IIH are known thus far.

Certain polymorphisms in TRPV6 have been associated with hypercalcaemia due to intestinal hyperabsorption [26]. In IIH and WBS, correlations between the absorptive hypercalcaemic phenotype and mutations or polymorphisms in specific genes have not been identified thus far. Most studies on \( \text{Ca}^{2+} \) homeostasis in IIH and WBS patients have focused on the abnormalities in \( \text{Ca}^{2+} \) and \( \text{Ca}^{2+} \)-regulating hormones including PTH and vitamin D. Studies consistently show that infants with IIH and WBS suffer from hyperabsorption of \( \text{Ca}^{2+} \) in the intestine, and evidence for excessive vitamin D intake is generally lacking [1,2,27]. Although some reports showed elevated levels of 25-hydroxyvitamin \( D_3 \) and 1,25-dihydroxyvitamin \( D_3 \), other studies were not able to confirm these findings [5]. We found no increased levels of 25-hydroxyvitamin \( D_3 \) in our patients. 1,25-dihydroxyvitamin \( D_3 \) levels were normal in one patient; however, in the two patients with the highest \( \text{Ca}^{2+} \) levels, 1,25-dihydroxyvitamin \( D_3 \) levels were normal. Alternative mechanisms to explain the hypercalcaemia are an increased sensitivity for vitamin D, deficient suppression of PTH by hypercalcaemia or an increased release of PTHrP which is highly similar to PTH in structure and function [5,28–30]. PTH levels of our patients were below the normal reference range indicating an appropriate suppression of PTH in response to hypercalcaemia. None of the patients described in this study had elevated PTHrP levels. PTHrP is, therefore, not likely to be involved in the increased intestinal \( \text{Ca}^{2+} \) absorption seen in IIH patients. Patient 3 showed slightly decreased serum phosphate concentrations and low renal phosphate reabsorption (Table 3). Decreased tubular phosphate reabsorption as a result of chronic hypercalcaemia is a known phenomenon of unspecified aetiology [31]. The correlation between serum phosphate and \( \text{Ca}^{2+} \) levels in Patient 3 is also shown in Figure 2.

The leading hypothesis on IIH and WBS pathogenesis is a genetic defect in \( \text{Ca}^{2+} \) homeostasis resulting in intestinal hyperabsorption of \( \text{Ca}^{2+} \), which is compensated later in life [32,33]. As described before, CLDN3 and CLDN4, which are hemizygously deleted in WBS, are members of a large family of claudin proteins that are part of the tight junction complex regulating the paracellular permeability. Holmes et al. studied the expression of intestinal claudins in the mice of different ages and showed that some claudins are significantly up- or downregulated throughout life [21]. The transient character of hypercalcaemia in IIH and WBS patients could therefore potentially be explained by the altered expression of claudins that regulate the permeability of the intestinal epithelium. The partial loss of claudin 3 or claudin 4 expression would, in this case, be compensated for after a certain period of time, explaining the disappearance of hypercalcaemia. This way our hypothesis would explain the clinical phenotype as observed in our patients on both a genetic and molecular level.

Patient DNA was analysed for the presence of mutations in CLDN3 and CLDN4. Alternatively, the major intestinal \( \text{Ca}^{2+} \)-channel TRPV6 was also analysed to exclude the presence of gain of function mutations and activating polymorphisms that could be involved in the development of hypercalcaemia. All SNPs found in this study were previously identified and are listed in SNP databases (http://ncbi.nlm.nih.gov/SNP/). None of the polymorphisms known to be related to disease, nor did our study indicate such a correlation. An SNP array did not reveal significant deletions or duplications in any of the patients. Together, these results show that mutations in CLDN3 and CLDN4 are not involved in IIH. Also, the results indicate that the IIH phenotype does not result from deletions or duplications in the genome of these patients.

In summary, this study showed no aberrant levels of vitamin D, PTH and PTHrP in IIH patients, thereby invalidating a number of previous proposed hypotheses. Furthermore, no mutations in CLDN3, CLDN4 and TRPV6 were found in these patients, who also did not have genomic deletions or duplications. The sporadic occurrence and general lack of a clear inheritance pattern in IIH, together with the limited number of patients known to suffer from IIH, complicate identification of genes involved in the development of transient hypercalcaemia.

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Conflict of interest statement. None declared.

References

Pre-existing renal failure worsens the outcome after I/R in rats

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Abstract

Background. Chronic kidney disease (CKD) serves as a risk factor in the development of acute kidney injury (AKI) requiring renal replacement therapy. Furthermore, superimposed AKI on CKD is associated with an increased mortality and risk of progression to end-stage renal disease. We aim to examine whether CKD increases the

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Pre-existing renal failure worsens the outcome after intestinal ischaemia and reperfusion in rats