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A novel mutation of the calcium sensing receptor gene is associated with chronic pancreatitis in a family with heterozygous SPINK1 mutations

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Abstract

Background: The role of mutations in the serine protease inhibitor Kazal type 1 (*SPINK1*) gene in chronic pancreatitis is still a matter of debate. Active SPINK1 is thought to antagonize activated trypsin. Cases of *SPINK1* mutations, especially N34S, have been reported in a subset of patients with idiopathic chronic pancreatitis. However, the inheritance pattern is still unknown. Some cases with N34S heterozygosity have been reported with and without evidence for CP indicating neither an autosomal recessive nor dominant trait. Therefore *SPINK1* mutations have been postulated to act as a disease modifier requiring additional mutations in a more complex genetic model. Familial hypocalciuric hypercalcemia (FHH) caused by heterozygous inactivating mutations in the calcium sensing receptor (*CASR*) gene is considered a benign disorder with elevated plasma calcium levels. Although hypercalcemia represents a risk factor for pancreatitis, increased rates of pancreatitis in patients with FHH have not been reported thus far.

Methods: We studied a family with a FHH-related hypercalcemia and chronic pancreatitis. DNA samples were analysed for mutations within the cationic trypsinogen (N29I, R122H) and *SPINK1* (N34S) gene using melting curve analysis. Mutations within *CASR* gene were identified by DNA sequencing.

Results: A N34S *SPINK1* mutation was found in all screened family members. However, only two family members developed chronic pancreatitis. These patients also had FHH caused by a novel, sporadic mutation in the *CASR* gene (518T>C) leading to an amino acid exchange (leucine->proline) in the extracellular domain of the CASR protein.

Conclusion: Mutations in the calcium sensing receptor gene might represent a novel as yet unidentified predisposing factor which may lead to an increased susceptibility for chronic pancreatitis. Moreover, this family analysis supports the hypothesis that *SPINK1* mutations act as

disease modifier and suggests an even more complex genetic model in *SPINK1* related chronic pancreatitis.

Background

Chronic pancreatitis (CP) remains one of the big challenges among the GI-tract disorders. Since 1996 the understanding of genetic risk factors for CP extended with the discovery of different gene defects causing hereditary pancreatitis (HP). A French and two American groups were the first to identify chromosome 7q35 as a putative locus for HP [1-3]. The disease gene was mapped and a heterozygous point mutation A>T in exon 3 (R122H) of the protease serine 1 (*PRSS1*) was detected in a majority (~70%) of cases with cationic trypsinogen mutations [4]. Subsequently the *PRSS1* mutations N29I (25%), A16V (<3%), K23R, D22G, R122C and N29T (several kindreds) were discovered [4-9]. The second gene that was characterized to be clearly associated with idiopathic chronic pancreatitis (ICP) was the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [10,11].

The third gene that was postulated to be associated with ICP is the serine protease inhibitor Kazal type 1 (*SPINK1*). Initially an autosomal recessive inheritance pattern was suspected for the N34S *SPINK1* mutation resulting in CP in homozygous patients while another heterozygous subgroup seems to suffer from disease due to a combination of genetic defects of *SPINK1* and yet unidentified genes [12]. Pfützer et al. showed that neither an autosomal dominant disorder with a low penetrance pattern (observed frequency of N34S mutation in the population [0.77%] versus ICP prevalence [0.0066%]) nor an autosomal recessive trait can be used as a model for disease inheritance [13]. The prevalence for the N34S mutation in tested healthy controls stemming from these countries ranges in between 1.6 % (USA) [13], 1.58% (France) [14] and 0.36% (Germany) [12]. Thus the role of *SPINK1* mutations in ICP which have a much lower disease prevalence (<0.01%) remains to be further defined and the hypothesis of a modifying but not causative factor for *SPINK1* mutations was proposed [13,15].

One of the toxic metabolic risk factors for CP is hypercalcemia. The correlation was first observed in patients with hyperparathyroidism while trypsin activation, secretion stimulus and stabilization are suspected mechanisms [16].

Therefore, mutations in genes involved in calcium homeostasis might be associated with an increased risk for CP. We describe a family with members suffering from both CP and familial hypocalciuric hypercalcemia (FHH) to investigate the association of calcium sensing receptor gene (*CASR*) and *SPINK1* mutations with CP.

Case Presentation

We report on a 35-year-old caucasian male (II:4, Fig. 3) who was admitted to the hospital with an acute exacerbation of chronic pancreatitis in 2002. In 1993, he had been hospitalized for a first episode of acute pancreatitis that was followed by 40 recurrent exacerbations necessitating 27 periods of prolonged hospitalization. While the aetiology of pancreatitis remained undefined between 1993 and 1996, prolonged hypercalcemia in combination with an elevated level of parathormone (PTH) were suspected as the dominating causes in 1996.

A familial hypocalciuric hypercalcemia (FHH) was diagnosed biochemically by measuring a persisting high serum calcium level and a low urinary calcium excretion. The ratio of the creatinine and calcium clearance was below 0.01. The fact that his older brother (II:2, Fig. 3) had the same complex of symptoms (recurrent pancreatitis, elevated serum calcium level and low urinary calcium excretion) supported the hypothesis of a genetic cause and the diagnosis of FHH. In the younger brother, the disease onset occurred at the age of 27 years. In the patient, treatment with bisphosphonates and octreotide (200 μg TID) was initiated in 1995 and continued through 2001 with little to none clinical improvement.

In 1996, the two lower parathyroid glands were removed surgically because of persistent elevated PTH serum levels and calcitonin substitution was started (100 μg BID). This therapy also failed to suppress the recurrent episodes of pancreatitis.

In December 1999, the patient was the first time admitted to our clinic with another exacerbation of his recurrent pancreatitis. After endoscopic investigation (ERCP and endoscopic ultrasound), indicating neither signs of chronic pancreatitis nor any evidence for the pathology of his disease, we suspected that additive genetic alterations might cause the disease. An analysis for *PRSS1* mutations was performed which revealed neither an R122H or N29I mutation.

In 2001, during another episode of acute pancreatitis, clinical evaluation revealed marked signs of chronic pancreatitis according to the Marseilles-Rome criteria. The endoscopic ultrasound was consistent with chronic calcifying pancreatitis with intraductal stones within the head

of the pancreas. Persistent pain and impaired exocrine function with an abnormal fecal elastase accompanied the disease progress. A heterozygous N34S mutation was detected by DNA sequence analysis of the *SPINK1* gene.

Blood samples and DNA studies

We collected blood samples for clinical chemistry and 24 h urine of all family members who accepted testing. Blood samples for mutation analysis were taken after counselling according to the guidelines of the Consensus Committees of the European Registry of Hereditary Pancreatic Diseases [17].

Informed consent was given by the patient's mother (62), the elderly brother (43), while a half-sister of the index patient (24) denied genetic testing. The daughter (8) of the index patient was tested giving her and her parents informed consent. We extracted genomic DNA from whole blood according to established protocols.

Coded DNA samples of the patients were analyzed for *PRSS1* mutations (N29I, R122H) and the *SPINK1* variant N34S by melting point curve analysis. DNA sequencing of the *CASR* gene were performed using PCR products of the exons.

Polymerase Chain Reaction and melting point curve analysis

Primers flanking the designated coding regions of the *CASR*, *PRSS1* and *SPINK1* genes were designed according to the nucleotide sequences published (*CASR*: GenBank #U20759 [18], *PRSS1*: GenBank #U66061 [19] and *SPINK1*: GenBank #AF286028 [12]).

Melting curve analyses for the N29I, R122H *PRSS1* mutations were performed using specific pairs of fluorescence resonance energy transfer (FRET) probes (N29I: LC-Red attached to 5'-CTGTCCCCTACCAGGTGTCC and FL attached to 5'-GGGCTACAACTGTGAG GAGAA-FL ; R122H: LC-Red 5'-TCTCTGCCCACCGCCCCTCCAGCC and 5'-CAACGCCCACGTGTCCACCA-FL) synthesized by TIB MOLBIOL (Berlin, Germany) and the LightCycler (Roche Diagnostics, Germany). Primers for the SPINK1 melting curve analysis have been described previously [12].

DNA sequence analysis

Genomic DNA was prepared from whole blood using standardized protocols. The patient was screened for published mutations in the 6 translated exons (exon 2–7) of the *CASR* gene. PCR products of all 6 exons were gel-purified and sequenced on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster, CA) using the listed primers (Table 1). DNA from relatives of the patient was screened for mutations in exon 4 identified in the index patient's DNA c.518T>C.

Results

Family history

The family history revealed no other family members with pancreatitis, with the exception of the two previously mentioned brothers (36 and 39 years), who both experienced a first episode at the age of 27. The father had died of cardiac arrest in 1971 and never had any documented illness or symptoms indicating pancreatitis. The daughter of the index patient is asymptomatic but still at risk to develop pancreatitis. The brother who had a documented history of recurrent pancreatitis and FHH could not be located. Looking at the family tree and his medical history of recurrent episodes of pancreatitis, a N34S mutation can only be suspected.

Clinical chemistry

Serum plasma levels of Ca^{2+}, creatinine and urinary calcium excretion were normal in the mother and asymptomatic brother. The ratio of creatinine clearance and calcium clearance was within normal limits and thus not indicating FHH. The index patient (12/2001) had a normal PTH serum concentration of 19.0 ng/l (normal 12–72 ng/l) and an elevated plasma calcium between 2.75 and 3.25 mmol/l. Urinary calcium excretion was 180 mg/24 h, creatinine clearance was 164 ml/min and the ratio of creatinine and calcium clearance was 0.014.

Polymerase Chain Reaction, melting point curve analysis and DNA sequencing

The data of the melting point curve analysis revealed a heterozygous N34S mutations for the index patient, the unaffected brother, mother and daughter (Figure 1).

The sequence similarity search of the *CASR* gene showed a point mutation in exon 4 at position c.518T>C leading to a heterozygous missense mutation L173P in the extracellular domain of the calcium sensing receptor (CaR) protein in the index patient and his daughter (Figure 1 and 2).

The other family members who had no clinical signs for FHH (normal plasma and urinary Ca^{2+} excretion; Tab.2) did not have a *CASR* gene mutation in exon 4. The results of the mutational analysis are summarized in the family tree (Fig. 3).

Discussion

Hypercalcemia is considered a risk factor of acute pancreatitis. Elevated serum calcium levels can lead to premature activation of trypsinogen inducing autodigestion of the pancreatic parenchyma [20]. Clinical examples for this association are patients with endocrine disorders such as

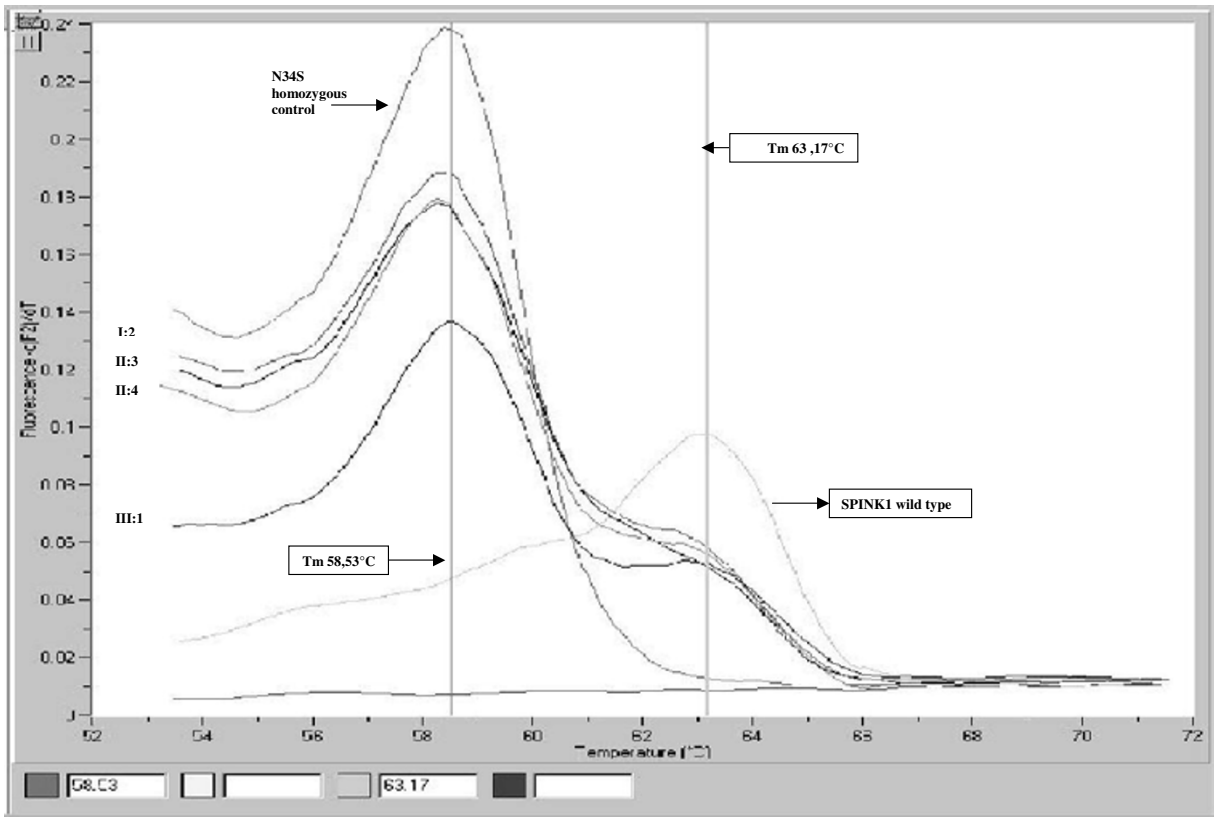


Figure 1

Melting curve analysis (*SPINK1* N34S) of the index patient and family members To assess the *SPINK1* genotype, representative gene fragments of the *SPINK1* gene were amplified by PCR. The melting point (Tm) of each amplified double-stranded DNA fragment was determined using the LightCycler thermocycling device and designated probes. Curves with a melting point at 58,53°C represent the *SPINK1* (N34S) control, index patient (II:4), daughter of the index patient (III:1), healthy brother (II:3) and healthy mother of the index patient (I:2). The melting point curve with the higher melting point at 63,17°C, due to a more stable complex (donor probe was complimentary to the wild type) represents the *SPINK1* wild type.

primary hyperparathyroidism (pHPT) characterized by hypercalcemia with an elevated risk for pancreatitis. However, this interrelation is questioned by some investigators and for the specific case of pancreatitis in patients with primary hyperparathyroidism the prevalence rates range from 1 to 19 % [21]. In our case, FHH-associated hypercalcemia was thought to be required for recurrent episodes of acute pancreatitis but even parathyroidectomy and treatment with bisphosphonates did not bring the expected result. FHH is a benign disorder with a slight elevation of plasma Ca^{2+}. It is inherited in an autosomal dominant order and shows almost 100% penetrance. In 1992, the disease gene (*CASR*) was mapped to the long arm of chromosome 3 [22] and today more than 70 mutations have been described [23]. The CaR plays a key role in calcium homeostasis and is widely expressed in tissues including kidney, parathyroid glands and bone cells which are involved in calcium metabolism [24]. Recently the CaR was characterized in human pancreatic acinar and duct cells [25]. However, the functional significance of CaR expression in pancreatic acinar and duct cells in the pathogenesis of pancreatitis remains to be defined.

While a heterozygous inactivating *CASR* mutation leads to FHH, homozygous mutations result in severe neonatal pHPT. In contrast to pHPT, pancreatitis is an unusual complication of FHH.

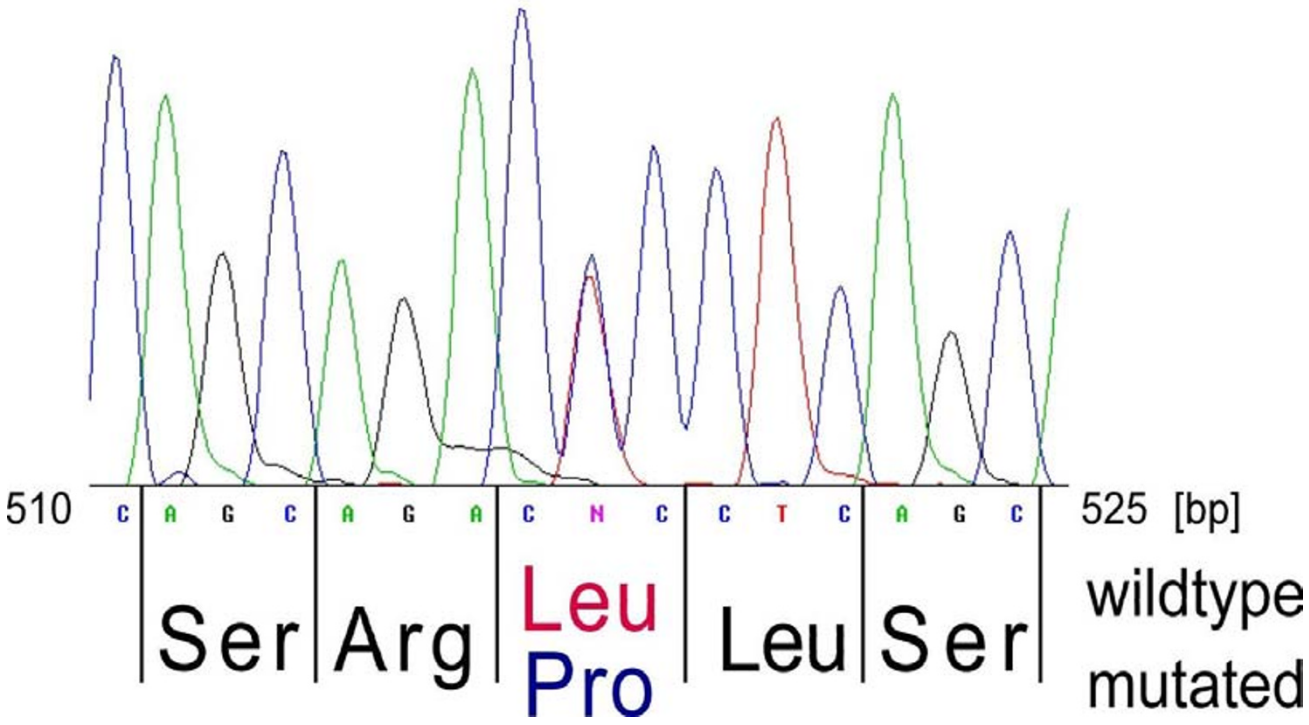
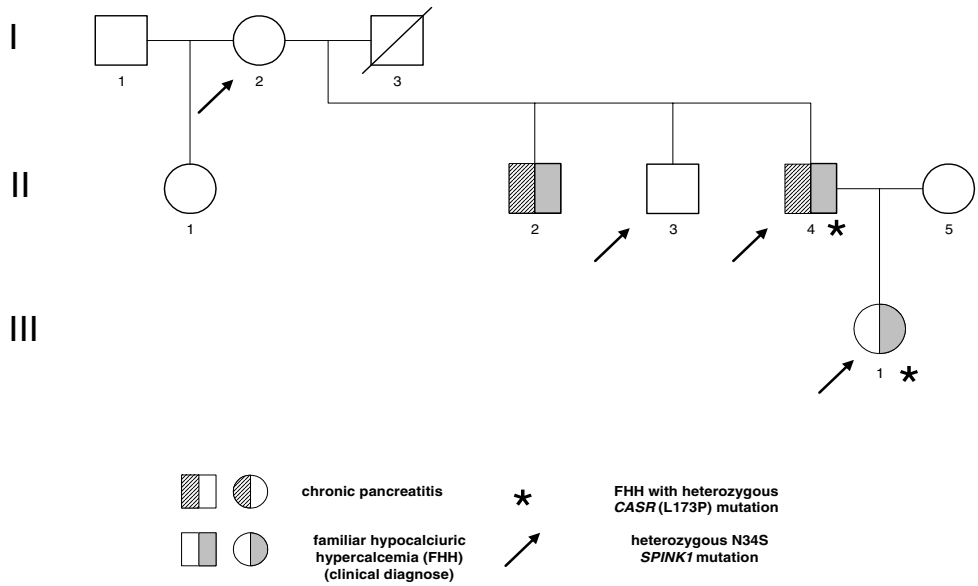


Figure 2
Calcium-sensing receptor gene mutation L173P The DNA sequence electropherogram of the index patient revealed a heterozygous mutation of nucleotide 518T>C. At the bottom, the amino acid sequence corresponding to this DNA region is shown. The identified mutation at base 518 results in a T to C transition (518T>C) which leads to a substitution of leucine 173 by proline in exon 4 (L173P). This corresponds to the extracellular domain of the CaR protein.

Interestingly, there are a few reports of as yet unexplained cases with FHH and recurrent episodes of pancreatitis [26,27]. Pearce et al. (1996) suspected in their collective of three FHH kindreds [26] that patients with FHH and CP may represent a distinct subgroup. Subsequently they identified three different *CASR* mutations, suggesting that they may represent distinct FHH variants with pancreatitis. From our point of view, it cannot be ultimately ruled out, that other genetic factors may have contributed to the development of pancreatitis in the presented cases, as the researchers did not exclude additional mutations in other genes including the *SPINK1*, *PRSS1* and *CFTR* genes. However, our observation of an individual with a combination of heterozygous *SPINK1* (N34S) and *CASR* gene (L173P) mutations, who developed chronic pancreatitis, may indicate the specific pathogenetic background of CP within this family.

There is still an ongoing controversy about the relevance of *SPINK1* mutations in CP although different groups postulated an association of N34S mutations in patients with ICP [12,13]. Pfützer et al. reported that approximately 40 % of their collective of patients with ICP (n = 57) had a N34S mutation (12 % were homozygous) [13] while Witt and coworkers detected N34S mutations in 23 % of their cases (n = 96) in a similar study [12].

Even though these observations support the importance of N34S mutations in ICP, it still remains to be



Subject	I:2	II:2	II:3	II:4	III:1
chronic pancreatitis	Ø	X	Ø	X	Ø
age (years)	62	39	43	36	8
N34S heterozygous	X	unknown	X	X	X
FHH	Ø	X	Ø	X	X
CASR mutation (c.518T>C)	wild type	unknown	wild type	X	X
Plasma Ca^{2+} [mg/dl] normal range [8.0-10.4] (year)	9.2 (2002)	11.7 (1996)	9.5 (2002)	11.4 (1996) 12.6 (2002)	11.2 (2002)

Figure 3

Family tree and clinical parameters of the family with *SPINK1* (N34S) and *CASR* (518T>C) gene mutations Two family members were diagnosed with chronic pancreatitis and FHH (II:2 and II:4). Mutational analysis of the index patient (36 years) and three other individuals, respectively, mother (62), unaffected brother (43) and daughter (8) (I:2, II:3, II:4 and III:1) showed a heterozygous SPINK1 mutation (N34S). In addition, the index patient and his daughter (II:4, III:1) had a point mutation in exon 4 at position 518 (518T>C) of the *CASR* gene. As for the individual II:3 in generation I no family member complained about abdominal pain (I:1 deceased in 1971). DNA from the symptomatic brother (39 years) was unavailable for genetic testing, but CP and FHH was diagnosed clinically.

determined whether N34S mutations are causative. Threadgold et al. postulated that SPINK1 mutations "rather act as a disease modifying factor but not causing the disease" [15]. Today their role is further substantiated as independent groups showed a strong association of *SPINK1* mutations (N34S) in patients with tropical pancreatitis [28-30].

Conclusion

Family and clinical history may indicate that the combination of the N34S *SPINK1* and *CASR* gene mutation induces CP in this kindred. Our observation supports the hypothesis that *SPINK1* mutations rather modify the disease phenotype than actually cause CP.

This is therefore the first report to show an association between *CASR* mutations and *SPINK1* related CP. Larger association studies or animal models are required to elucidate whether the *CASR* gene plays a more important role in *SPINK1* related CP. The current investigation may thus represent another part of the mosaique in the complex model of genetic causes for CP.

Abbreviations

CaR, calcium sensing receptor; *CASR*, calcium receptor gene; CP, chronic pancreatitis; FHH, familial hypocalciuric hypercalcemia; HP, hereditary pancreatitis; ICP, idiopathic chronic pancreatitis; *PRSS1*; cationic trypsinogen gene; *SPINK1*, serine protease inhibitor Kazal type 1 gene.

Competing interests

None declared.

Authors' contributions

All authors contributed to treatment and characterization of this kindred. PF and HE carried out the *CASR* gene analysis, while FS gave his broad support concerning all aspects. All authors read and approved the final manuscript

Note

Table 1: Sequences of oligonucleotide primers used for the PCR of the *CASR* gene The patient was screened for published mutations in exons 2–6 (exon 1 is untranslated) of the *CASR* gene. PCR products were gel-purified and sequenced on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster, CA) using the listed primers for PCR and sequencing.

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