Two Novel Mutations in a Zinc Transporter Gene Slc39a4 in Patients with Acrodermatitis Enteropathica

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ABSTRACT

Background: Acrodermatitis Enteropathica (AE) is a rare autosomal recessive disorder, characterized by severe zinc deficiency. The human AE gene, *SLC39A4* encodes the zinc transporter hZIP4. A large number of different mutations of the SLC39A4 gene have been identified in AE.

Objective: We received blood samples from four families of AE for the determination of the molecular basis of this disorder.

Design: We utilized genomic DNA analysis by PUREGENE Kit for DNA isolation and PCR analysis by Advantage GC Genomic PCR kit. The PCR products were purified by QIA quick PCR Purification kits and then sequenced.

Results: We describe 2 novel mutations in the *SLC39A4* gene in patients from the Middle East. The genetic analysis of SLC39A in one patient revealed a mutation in exon 7, p.Gly 409→ Arg and in the other, the genetic mutation was in exon7, p. Leu 415→ Pro. We identified a mutation in *SLC39A4* in one family from the USA in exon 6 which consisted of a single nucleotide substitution G→A (c.1120 G>A), p Gly 374 Arg. In another USA family the mutation consisted of a single nucleotide substitution of G→A in exon 5 (c.900 G>A), p.-GluL284ys). These latter mutations have been reported before in other countries, but not in the USA. In the fourth case, the patient and his father were heterozygous for the mutation in exon 5. Although the patient had AE, the father was normal.

Conclusion: We identified 2 novel mutations in the *SLC39A4* genes in Middle Eastern patients. We also report *SLC39A* mutations in two USA families, although previously reported outside the USA. In one USA family, the heterozygote subject showed symptoms of AE. Previously only three other cases of symptomatic AE were hetero zygotes. The pathogenesis of this phenomenon needs further investigation.

ABBREVIATIONS

AE: Acrodermatitis Enteropathica; hZIP4: SLC39 a solute carrier; DNA: Deoxyribonucleic Acid; PCRs: Polymerase Change Reaction; RE: Restriction Endonuclease-Based Assays; ZNT: SLC30 a solute carrier; SLC: Solute Carrier; MREs: Metal Regulatory Elements

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INTRODUCTION

AE is a rare autosomal recessive disease characterized by clinical manifestations of severe zinc deficiency, due to the inability of the affected individual to absorb intestinal zinc. It was first described by Barnes and Moynahan (1973) in a two-year old girl with AE whose symptoms and skin lesions dramatically improved with oral zinc sulfate therapy [1]. It was clear at that time that zinc might be fundamental to the pathogenesis of AE. The essentiality of zinc for humans was first recognized in 1963 and its deficiency is known to affect adversely growth and development and cell mediated immunity [2,3].

Clinical manifestations of AE are diarrhea, peri-orificial and acral skin lesions of erythematous vesicular-bullous type, growth retardation, inter current infections (due to T cell mediated immune dysfunction), alopecia, and death if left untreated. The human AE gene identified as *SLC39A4* is located on chromosomal region 8q24.3 and encodes a zinc transporter protein belonging to the zinc/iron-regulated transporter-like protein (hZIP) family, and is expressed in the kidney, colon, duodenum and jejunum [4,5]. Thirty-one different mutations or variants of the *SLC39A4* gene have been identified in AE patients throughout the world, including France, Tunisia, Japan, Austria, Turkey, Germany, Morocco and Sweden [6].

In this paper, we describe 2 novel mutations in the *SLC39A4* gene (one from a family originating in the United Arab Emirates (UAE) and the other from Turkey. We also include two mutations reported earlier from outside the USA, but observed in these families in the USA for the first time.

METHODS

Venous blood was collected from the individuals diagnosed with AE, and their unaffected parents (as available). Genomic DNA was isolated using the PUREGENE kit (Gentra Systems, Inc.). Polymerase chain reactions (PCRs) using genomic DNA were performed using the Advantage GC Genomic PCR kit (Clontech). PCR primer sequences and conditions to amplify the 12 exons of iso form 1 (NM _017767) of SLC39A4 were obtained from Kury and coworkers [4] and primers designed in our laboratory. PCR products were purified using QIA quick PCR Purification Kits (Quiagen), and sequenced. To verify the presence of the mutations, Restriction Endonuclease-based (RE) assays detailed in were designed. Digests were run on 4%

Synergel/agarose mix or 1.5% agarose gel (depending on the fragment size), and visualized using ethidium bromide.

Zinc concentration in plasma, hair and erythrocytes were determined by Atomic Absorption Spectrophotometery (Perkin-Elmer) [7]. The reference values used are based on the International Zinc Nutrition Consultative Group serum zinc reference levels [7].

All research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained, and the author's institutional review board at Wayne State University approved the study.

Case 1

A 19 month old Caucasian male from Abu-Dhabi UAE came to Children's Hospital of Michigan (CHM) diagnosed with of zinc deficiency since age 7 months. The plasma/serum zinc assay was done in an American hospital in Bangkok, Thailand. He was referred to the USA for determining the genetic basis of zinc deficiency.

The patient was born at full term. His mother was a 21-yr old female, gravida one, para one with uncomplicated prenatal, postnatal and perinatal courses. The birth weight of the baby was 3 kg. He was growing well until 6 months of age (8 kg body weight) when he first developed rashes in the diaper area followed by a rash on the face. After 4-5 days of developing the rash, he started having non-bloody watery diarrhea 5-6 times/day. The rash progressed from simple erythema to excoriated weeping skin lesions and it spread to the extremities and trunk within one month. The mother also noticed loss of scalp hairs in the baby. His weight declined to ~6 kg. There was no fever, vomiting, or other symptom. The rash was treated by a local physician with various creams and vitamin preparations but without any benefit. The parents took him to Bangkok where he was started on oral zinc supplementation (75 mg chelated zinc twice a day). Rashes healed and diarrhea deceased in 4 days following the start of supplementation. He was discharged with instructions to continue zinc therapy. He remained asymptomatic since then except some redness of cheeks and around the mouth persisted. Later zinc assays were repeated in France after the start of the therapy, which were normal. We do not know the time frame between the patient's return from France to Abu-

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Dhabi. When the parents visited his primary care physician for a regular checkup in Abu-Dhabi, a repeat zinc level was near zero, which we assume was incorrect since life is incompatible at this level. His physician increased the zinc dose to 150 mg twice a day but he started having diarrhea and the dose of zinc was reduced to 75 mg twice a day. At the time of admission to CHM, he was consuming oral 75 mg chelated zinc twice a day and was asymptomatic.

Past history indicated that the baby had frequent ear infections. He was never breast fed. For the first year of his life he drank Similac with iron and at the age of 7-8 months, solid foods were introduced which as the mother recalls did not seem to flare up the rash. He was now drinking milk.

Development for his age was appropriate, determined by height and weight and compared to standard growth charts. The parents are consanguineous and are first cousins. No relative is reported to exhibit AE symptoms. The patient is an only child with no allergies and having received routine immunizations.

Physical Examination: The child was playful and there was no apparent distress. The weight was 12.5 kg (50th percentile), and height was 85 cm (50th to 75th percentile)

Only pertinent positive finding on examination was slight erythema around mouth, which extended to the malar area. Hyper pigmentation in the diaper area, especially on medial side of his thigh, was observed. Routine hematological laboratory tests, (white blood cell count, differential count, hemoglobin, hematocrit, red cell indices and platelet count) were within normal limits. The plasma zinc assayed by atomic absorption spectrophotometry was 58.7µg/dl. The plasma zinc of the father and mother were 93µg/dl and 98µg/dl, respectfully. The normal levels for plasma zinc in our laboratory are (mean ± SD) 100 ± 10 µg/dl.

The genetic analysis of ZIP 4 showed a mutation in exon 7, Gly 409 → Arg, identified by sequencing and confirmed by RE analysis. The patient is homozygous for this mutation and both parents are heterozygous for this mutation. During his stay at the Children's hospital, he developed ROTA virus diarrhea, which was treated successfully. The patient was advised to take elemental zinc 25 mg (as acetate) twice daily. He was also advised to take one multivitamin pill containing 1 mg copper daily. The patient was discharged and returned to UAE for followed-up by his physician.

Case 2

An eleven-year-old Caucasian female from Turkey presented with rashes around mucocutaneous junctions, periorbital and perianal areas and the extremities. The clinical signs and symptoms of AE began at three and a half months old. The parents were also consanguineous-(second cousins). A sibling male also had AE, was deceased when he was three years old. Other siblings, brother and sister, were healthy but exhibited decrease in plasma zinc levels (Table 1).

On examination the patient showed extreme growth retardation, partial alopecia, skin eruptions around orifices, dermatitis which was widespread, skin rashes and pustular lesions on her knees and hands, dystrophic changes of the fingers and the nails and had abnormal taste acuity. Zinc assays of plasma, erythrocytes and hair showed decreased levels (Table 1).

Table 1: Zinc Levels in Plasma, Erythrocytes and Hair (Case 2).			
	Plasma Zn µg/dl	Erythrocyte Zn µg/ml	Hair Zn µg/g
Normal	88.5 ± 17.4	12.1 ± 1.8	192.9 ± 12.5
Patient	20	5.7	116.66
Mother	72	13.05	276.78
Father	80	11.55	175
Brother	76	8.7	211.36
Sister	72	11.55	145

Zinc was assayed by AAAS (Atomic Absorption Spectrophotometry). The patient was severely deficient in zinc, whereas her parents were normal. Her brother showed a mild decrease in plasm and erythrocyte zinc and the sister showed a slight decrease in plasma zinc and decreased hair zinc level.

The patient responded to oral zinc sulfate (2 mg zinc/kg body weight daily) and all the symptoms were corrected promptly (unfortunately, the exact time frame is unknown). The genetic mutation in ZIP4 was in exon7, Leu 415→ Pro, identified by sequencing and confirmed by RE analysis. This patient is homozygous for this mutation.

Case 3

This patient was a four-year-old Caucasian male who was first seen at CHM in 1971. His clinical features were typical for AE.

He was first treated with diodoquin but in 1975 he was started on zinc supplementation which was highly effective in the management of all symptoms and signs related to AE. We received blood samples for genetic analysis of AE gene (*SLC39A4)* from this patient when he was 27 y old, his father was 53 y and his mother was 52 y in 1998. The parents were non-consanguineous.

We identified a mutation in exon 6 which consisted of a single nucleotide substitution G→A (c.1120 G>A, p.Gly-374-Arg). The patient was homozygous and the parents were heterozygous for this mutation.

Case 4

This patient was an eight year old Caucasian male when he was presented in CHM for failure to thrive and diarrhea. Upon admission, his plasma zinc was 64µg/dl. He was diagnosed with AE and was treated with zinc supplementation with success (we do not know how much zinc was administered). The patient and his father were both heterozygous for a mutation consisting of a single nucleotide substitution of G→A in exon 5 (c.900 G>A, p.Glu 284Lys). Blood samples from the mother and or siblings of the patient were unavailable.
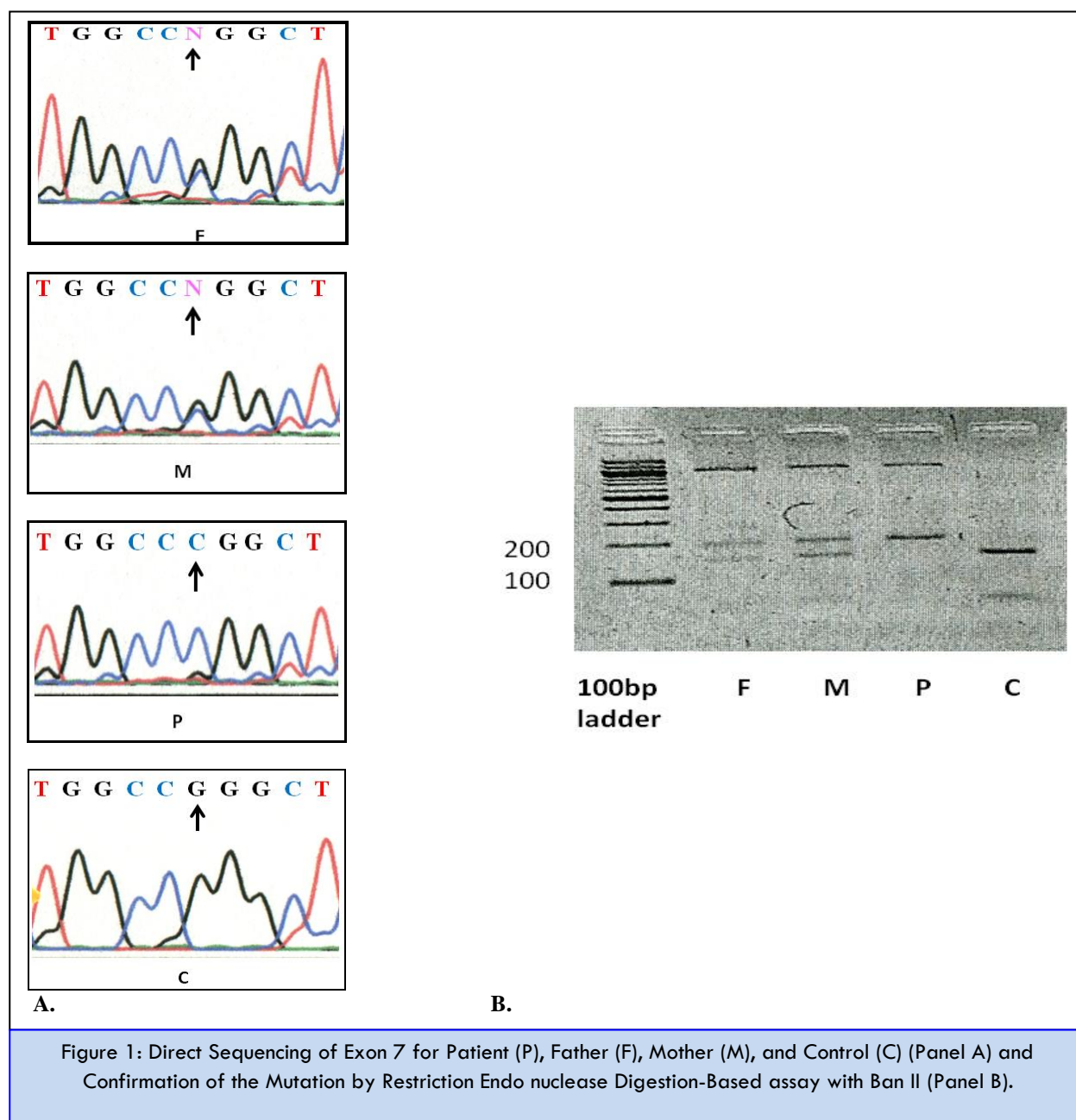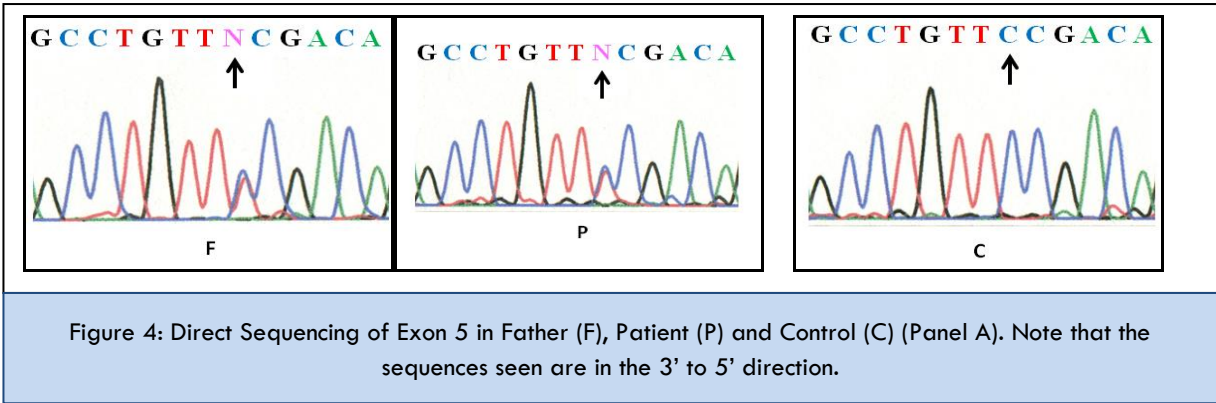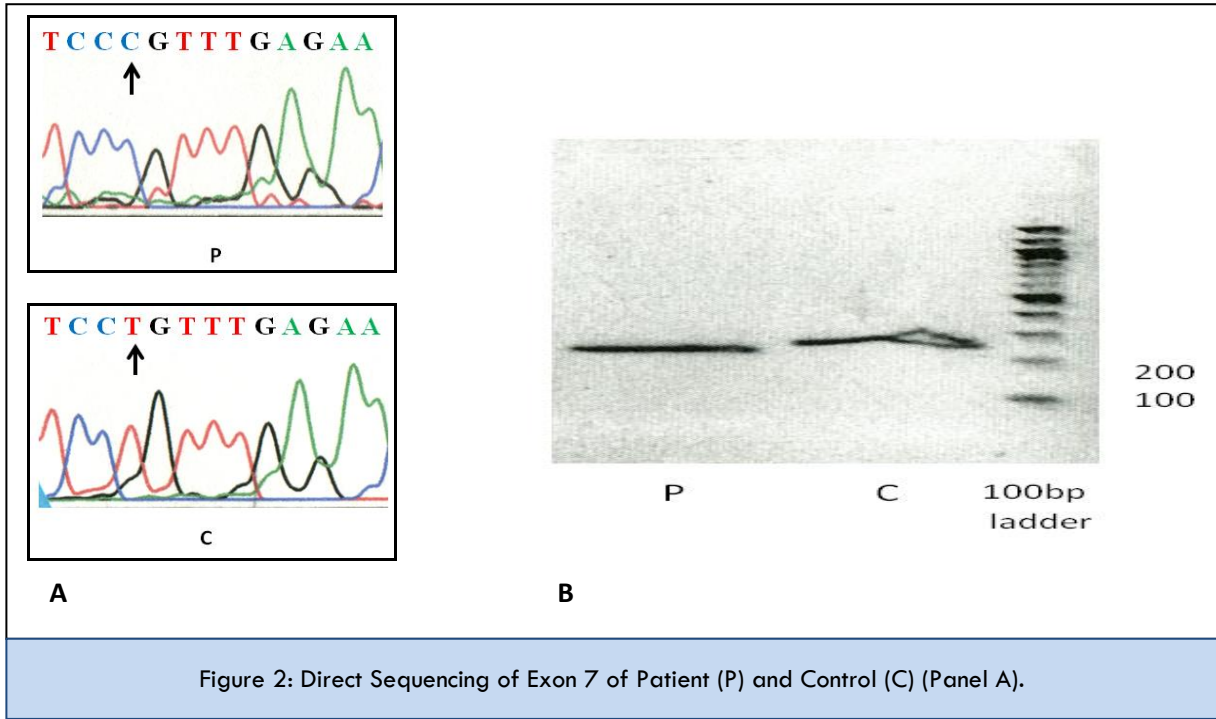

Figure 1: Direct Sequencing of Exon 7 for Patient (P), Father (F), Mother (M), and Control (C) (Panel A) and Confirmation of the Mutation by Restriction Endo nuclease Digestion-Based assay with Ban II (Panel B).

To confirm the presence of the mutation, a RE digestion-based assay was designed. The primers used for this assay are listed in Table 1. Ban II is unable to cut the mutant allele, but cleaves the PCR products from the parents' and the control DNA.

Figure 2: Direct Sequencing of Exon 7 of Patient (P) and Control (C) (Panel A).

Figure 4: Direct Sequencing of Exon 5 in Father (F), Patient (P) and Control (C) (Panel A). Note that the sequences seen are in the 3' to 5' direction.
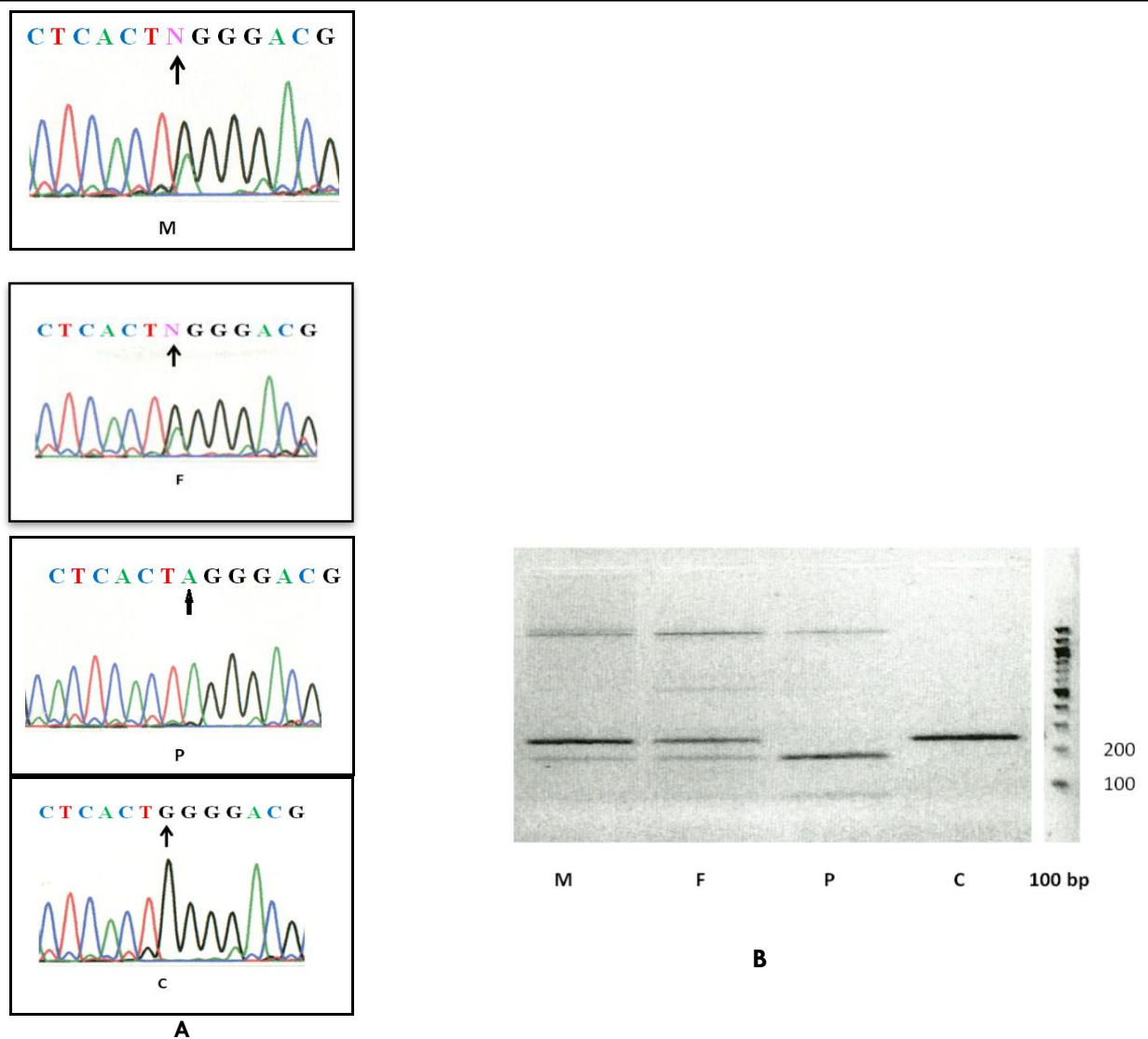
Figure 3: Direct Sequencing of Exon 6 in Mother (M), Father (F), Patient (P), and Control (C) (Panel A) and Confirmation of the Mutation by Restriction Endo nuclease Digestion-Based Assay with Bfa I (Panel B).

To confirm the presence of the mutation, a restriction Endo nuclease digestion-based assay was designed. The primers used for this assay are listed in Table 2. Bfa I is able to digest the mutant allele, creating two fragments.

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RESULTS

In this study 4 mutations were found, 2 novel mutations and 2 previously reported mutations. The first mutation was a novel mutation in a family which originated from the UAE. This mutation was a single nucleotide substitution G→C in exon 7 (c.1225 G > C, p.-Gly409Arg) (Figure 1). The parents of the affected child were heterozygous for this mutation and the child was homozygous. A RE assay with Ban II confirmed the mutation (Figure1).

The second novel mutation was in the Turkish patient. This mutation was also identified in exon 7 and consisted of a single nucleotide substitution T→C (c.1244 T>C, p.-Leu415Pro) (Figure 2). The patient was homozygous for this mutation, which was confirmed using a RE assay with Aci I (not shown).

The 2 other mutations identified in this study were previously reported from outside the USA; however we observed these mutations in patients from the USA. One of these mutation was identified in exon 6 and consisted of a single nucleotide substitution G→A (c.1120 G>A, p.Gly374Arg) (Figure 3). The patient was homozygous for this mutation and the parents are heterozygous. This was confirmed by a RE assay using Bfa-I (Figure 3).

The other also previously reported mutation was a single nucleotide substitution of G→A in exon 5 (c.900 G>A, p.Glu284Lys) (Figure 4). The affected patient and his father were both heterozygous for this mutation. This was confirmed by a RE assay using Taqa–I. (not shown).

DISCUSSION

AE is a rare autosomal recessive disorder occurring during early infancy, at birth or at weaning and is characterized by severe nutritional deficiency of zinc [1,4-8]. Characteristic features are symmetrical acral and circumorifical dermatitis, alopecia and diarrhea. Other manifestations include ophthalmologic disorders, growth retardation, hypogonadism; cell mediated immune dysfunctions, increased incidence of infections and neuropsychiatric disorders. Supplementation by 75 mg zinc twice a day ameliorates the clinical symptoms. Defective zinc uptake in duodenum and jejunum have been demonstrated in these patients [1,4-8].

The molecular basis of this disorder was first reported by Wang et al., [4] and Kury et al., [5]. A novel human gene,

belonging to a solute carrier family, *SLC39A4* (ZIP 4) was shown to be mutated in several AE families coming from Europe, North Africa, or Middle East by Kury et al., [4] and Wang et al., [5]. Intestinal zinc uptake function of *SLC39A4*, together with the perfect co-segregation between *SLC39A4* mutations and phenotype of AE subjects were strong arguments in favor of *SLC39A4* as the gene for AE. Covering about 4.5 kb of chromosomal region 8-q-24.3, the human *SLC39A4* gene is composed of 12 exons ranging from 55 bp (exon 9) to 292 bp (exon 1) in size, and 11 introns ranging from 76 bp (intron 7) to 506 bp (intron 1). The *SLC39A4* gene encodes a 647-amino acid protein of about 68 kDa. This protein which is designated as ZIP4, belongs to the family of 14 members of specific ZIP zinc transporters (for zinc/iron regulated transporter like protein), which facilitate zinc influx from outside the cell or cellular compartments into the cytoplasm [4-9]. The maintenance of intracellular zinc homeostasis is critical and multiple genes modulate the efflux and uptake of this element in the cell [10]. Two super-families of mammalian zinc transporters have been identified that belong to the solute carrier (SLC), SLC39A and SLC30A. Fourteen members of the SLC39A family (ZIP) function in the uptake of zinc and other metals in the cell. Ten SLC30a (ZNT) function in zinc efflux and compartmentalization of zinc intracellularly. Many of these zinc transporters are expressed in a tissue-specific manner and are located in specific areas intracellularly.

The expression of the AE gene is regulated by zinc in the intestine and visceral endoderm in mice [9,11] and the mouse *SLC39A4*gene is essential for early embryonic development. Homozygous knockout embryos die during morphogenesis [9,11,12] and *SLC39A4* gene expression in the pre-implantation mouse embryo is active in the visceral endoderm at the egg cylinder stage which demonstrates an essential role of *SLC39A4* gene in the uptake of zinc into the mouse concept soon after implantation. *SLC39A4* heterozygosity is also teratogenic and embryo toxic in mice and maternal *SLC39A4* heterozygosity renders mice hyper-sensitive to zinc deficiency during pregnancy. The number of mutations or unclassified variants identified until 2007 in the *SLC39A4* gene was 31. Since 2009 [6], a few more mutations in SLC39A4 have been published [13-16]. These 31 variants are evenly distributed in

the12 exons [6]. Several different mutation types are represented: missense and nonsense mutations, small and large deletions or insertions with frame shift, and RNA splice-site changes have been reported. Among these 31 variants, 26 were homozygous or compound heterozygous in at least 51 AE patients from 30 independent families, and five variants were observed in heterozygous state. Three of the 26 variants were found in a heterozygous state, with no other variant on the second allele in patients studied by Schmidt et al., [6]. Schmidt et al., [6] also reported in a Spanish family a heterozygous deletion of p. Lleu 256. Serfs x16 (c.766 del c) which was present in two siblings with AE, however, the father had the same deletion but he was clinically normal. Our case 4 showed heterozygous mutation consisting of a single nucleotide substitution of G→A in exon 5 (c.900 G>A, p.-Glu-284-Lys) who was diagnosed to have AE but his father who had the same heterozygous mutation was clinically unaffected.

Several hypotheses have been proposed to explain the pathogenesis of AE in heterozygous carriers [6]. First the effect of the mono alleles *SLC39A4* mutation could be amplified by a dysregulation of *SLC39A4* transcription resulting from the loss of MREs in the regulating region of the gene [6]. Secondly, it has been shown in the mouse model that heterozygosity of mutation of this gene may confer hypersensitivity to zinc deficiency [12,13]. Thus it is possible that the manifestation of AE symptoms in heterozygotes could be due to an insufficient intake of zinc or to poor bioavailability of zinc in the diet. A third possibility may be that another gene may also be involved in cellular zinc transport or homeostasis which could cause AE and not related to heterozygosity of AE gene.

ACKNOWLEDGEMENTS

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