

## Case report

### A nucleotide deletion and frame-shift cause analbuminemia in a Turkish family

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#### Abstract

Congenital analbuminemia is an autosomal recessive disorder, in which albumin, the major blood protein, is present only in a minute amount. The condition is a rare allelic heterogeneous defect, only about seventy cases have been reported worldwide. To date, more than twenty different mutations within the albumin gene have been found to cause the trait. In our continuing study of the molecular genetics of congenital analbuminemia, we report here the clinical and biochemical findings and the mutation analysis of the gene in two Turkish infants. For the molecular analysis, we used our strategy, based on the screening of the gene by single-strand conformation polymorphism, heteroduplex analysis and direct DNA sequencing. The results showed that both patients are homozygous for the deletion of a cytosine residue in exon 5, in a stretch of four cytosines starting from nucleotide position 524 and ending at position 527 (NM\_000477.5(ALB):c.527delC). The subsequent frame-shift inserts a stop codon in position 215, markedly reducing the size of the predicted protein product. The parents are both heterozygous for the same mutation, for which we propose the name Erzurum from the city of origin of the family. In conclusion, our results show that in this family congenital analbuminemia is caused by a novel frame-shift/deletion defect, confirm the inheritance of the trait, and contribute to advance our understanding of the molecular basis underlying this condition.

**Key words:** congenital analbuminemia; DNA mutational analysis; single base deletion; frame-shift

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#### Introduction

Human albumin (ALB; UniProt ID: P02768) represents just less than two-thirds of total serum proteins (1) and is encoded by a single gene (ALB; GenBank accession no. NC\_000004.11), mapping at chromosome 4q13.3, which consists of 15 exons (2). Homozygous or compound heterozygous mutations in this gene have been associated with congenital analbuminemia (CAA, OMIM 103600), which results in a significant reduction of the protein in the blood. Heterozygous, or in very rare cases, homozygous mutations may also lead to a circulating genetic variant of ALB (bisalbuminemia or alloalbuminemia, OMIM 103600) (3), which, with

a few exceptions, do not seem to have clinical relevance (4).

Usually, the clinical diagnosis of CAA is based on routine or capillary serum protein electrophoresis, which shows a marked reduction of the ALB concentration and a compensatory increase of the other protein fractions (3,5,6). Of course, physicians should rule out the presence of other more common causes which can lower the concentration of circulating ALB, such as gastrointestinal or renal losses, redistribution into extra-vascular compartments and hepatic dysfunction (3,5-7). In contrast to the first studies on this topic, which de-

defined as "true" analbuminemic subjects only individuals with an ALB concentration  $< 1$  g/L, we have more recently described several cases characterized by ALB concentrations around 10 g/L (7-11). Probably, an overestimation of the real ALB concentration at this low levels by serum protein electrophoresis or by dye-binding bromocresol green reagents (12) may explain this apparent discrepancy. A useful clue to the clinical diagnosis is the presence in the parents of the affected individuals of ALB concentrations significantly lower than normal. In fact, CAA occurs in homozygous or compound heterozygous individuals and heterozygous carriers, due to the presence of only one normal allele, usually show ALB concentrations close to the lower limit of the normal range (1,3,5,6).

The main clinical symptoms in adult analbuminemic individuals are oedema, hypotension, fatigue and, mainly in adult females, lower-body lipodystrophy (1,3,5,6). These symptoms seem relatively mild with respect to the many and important roles of ALB, but its low concentration can be partially compensated for by an increase in the synthesis of other serum proteins (1,3,5,6). The most common biochemical sign is a marked alteration of the lipid profile, with hypercholesterolemia and elevated LDL cholesterol levels (1,3,5,6), which may have been responsible for some cases of premature coronary heart disease (13). Although the absence of a follow-up in most cases makes it difficult to draw conclusions on this point, several authors have recommended treatment with statins (14).

The condition is very rare: only about seventy cases have been so far reported world-wide and are listed in the Register of Analbuminemia Cases (15). However, the prevalence of the causative mutations at the heterozygous state is probably somewhat higher than hitherto believed (less than 1:1.000.000), since the most common among them, Kayseri (c228\_229delAT), has an allele frequency of about 6:100.000 (16). This condition is rare also in consideration that CAA may be a risk factor during the perinatal and the childhood period (17-19). In fact, foetal or neonatal death of siblings has been frequently observed in the families of analbuminemic subjects, pointing to the crucial role of ALB in fetal development (3,5,6).

The clinical diagnosis of CAA needs to be confirmed by screening methods of the ALB aimed at the identification of the defect causing the lack of the protein. This is mandatory in order to differentiate from other secondary causes of hypoalbuminemia and for the genetic counselling of the family. In our continuing study of the molecular basis of CAA, we describe here the clinical and biochemical findings in two analbuminemic Turkish children together with the screening for mutations of the ALB in their family.

## Materials and methods

### Subjects

We observed at the Kanuni Sultan Suleyman Research and Training Hospital, Istanbul, Turkey a consanguineous (first degree cousins) family composed by four offspring from Erzurum, East Anatolia, Turkey. The clinical and biochemical studies were performed from 2013 to 2015. The mother was 27 and the father was 32 years old, and they were both apparently healthy. They have four kids from three pregnancies, two of which are the index cases. The procedures were in accordance with the latest version of the Declaration of Helsinki. We collected blood samples after obtaining signed informed consent from all participants involved in the study. For the four kids the consent was signed by the parents. The molecular analysis was carried out in 2015 at the Department of Molecular Medicine, University of Pavia, Pavia, Italy and at Laboratory on Pathophysiology of Uremia, Istituto Giannina Gaslini IRCCS, Genova, Italy.

### Methods

Serum ALB concentration was quantified by a modified bromocresol purple-binding assay in a Dimension® EXL™ 200 Integrated Chemistry System (Siemens Healthcare GmbH, Erlangen, Germany). Serum protein electrophoresis was performed by the fully-automated V8 Capillary Electrophoresis System (Helena Biosciences Europe, Gateshead, UK), designed to optimise and completely automate electrophoresis testing. Other analytes were assayed by conventional techniques using a fully-

automated ADVIA® 1800 Clinical Chemistry System (Siemens Healthcare GmbH, Erlangen, Germany).

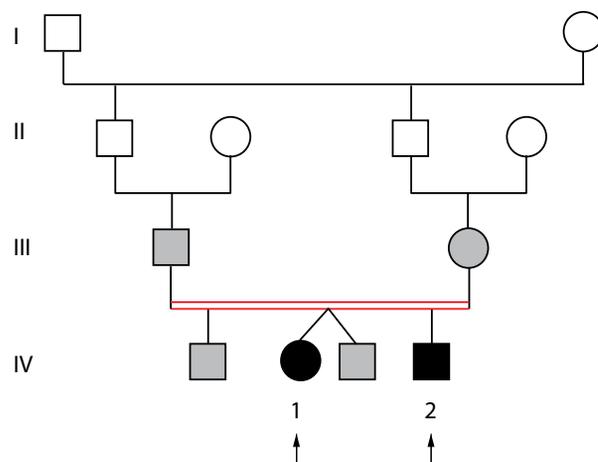
### Mutation analysis

DNA was isolated from EDTA-treated whole blood and sent to Pavia by a Next Day Parcel Delivery. PCR amplification of the 14 segments containing exons and flanking regions of the ALB gene (2) was performed using specific set of primers as described by Watkins *et al.* (18) and the conditions reported by Caridi *et al.* (8). Single strand conformation polymorphism (SSCP) heteroduplex analysis (HA), and the preparation of PCR products for sequence analysis were performed essentially as previously described (8,11,20). Samples were subjected to automated DNA sequencing on an Applied Biosystems™ 3100 xl instrument (Thermo Fisher Scientific, Milano, Italy), and analyzed by Sequencer software (5.0 version, Gene Code Corporation, Ann Arbor MI, USA).

### Case description

Six members compose the family we studied in the present paper. The parents, who are first-degree cousins, have four kids from 3 pregnancies, two of which affected by CAA (Figure 1). There is no history of miscarriage or fetal death.

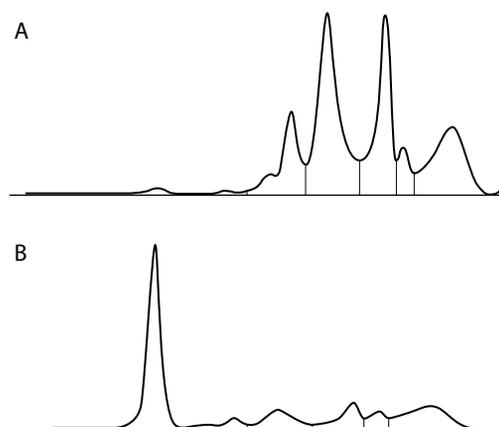
**Sibling 1:** The elder patient is a female, who is now two and half years old (sibling 1 in Figure 1). She was born at 29th week of gestation, from an in vitro fertilization induced twin pregnancy. Her twin brother is healthy. Her birth weight was 1150 g. She stayed for 50 days at neonatal intensive care unit (NICU) because of premature birth and respiratory distress, but the follow-up revealed no history of this problem or of frequent infections. During that period, she had seizures and received anti-epileptic drugs. One year ago her electroencephalography was repeated and the anti-epileptic therapy was stopped. Her renal ultrasonography and calcium excretion were normal, but the blood calcium levels were always low and she consumed vitamin D and calcium supplementation, until her blood vitamin D became too high due to excessive addition. She was diagnosed with hypothyroidism



**FIGURE 1.** Family pedigree.

The analbuminemic patients are represented by black symbols and are indicated by the arrows. Grey symbols denote heterozygous subjects. Circles symbolize females and squares males. Void symbols indicate individuals not examined in the present study.

during neonatal period, and since then she receives thyroid hormone replacement therapy. Upon the detection of low ALB concentrations in her younger brother, we checked her ALB concentration, which was 10 g/L. However, she showed only mild periorbital oedema and had no need for ALB infusion. Capillary electrophoresis of serum proteins showed the presence of minimal ALB concentration associated with a compensatory increase in the non-albumin fractions (Figure 2). Her



**FIGURE 2.** Capillary electrophoresis of serum proteins.

The profiles were obtained via the fully-automated Helena V8 Capillary Electrophoresis System. A: patient 1; B: control. The ALB peak is near absent, whereas all the globulin fractions are increased.

developmental milestones are mildly delayed. She walked at 1.5 years old, and she still talks with a few words. Her growth is also retarded, her weight is at 10th centiles and her height is between 3rd and 10th centiles.

Sibling 2: The second affected subject is the male, last child of the family, who is now one-year-old (sibling 2 in Figure 1). Due to foetal distress, he was born at 32nd week of gestation following a spontaneous conception. His birth weight was 1550 g and he stayed in the NICU for 22 days due to premature birth and respiratory distress. During his hospitalization in NICU, he showed low ALB concentration (8 - 9 g/L), mild generalised oedema and lymphedema of the back of the feet. He re-

ceived ALB infusions. After detailed tests, no gastrointestinal or renal loss of protein was detected. His hepatic and renal functional tests were normal. During follow-up his weight and height stayed below 3rd centiles and his developmental milestones were mildly delayed. He had hypotonia and his head control was achieved after the 3<sup>rd</sup> month of life. He has not walked yet at 1<sup>st</sup> year of age. In contrast to his affected sister, this boy experienced recurrent upper and lower respiratory tract infections and he received ALB infusions during his stays in the hospital. The more recent visit shows that he has neuromotor retardation, severe sensorineural hearing loss, and cannot sit without assistance yet.

**TABLE 1.** Clinical laboratory test results of the analbuminemic patients and of their parents.

Analyte (Unit)	Patient 1*	Patient 2*	Father*	Mother*
Albumin (g/L)	<b>10</b> (38–54)	<b>16</b> (38–54)	40 (39–49)	41 (39–49)
Total protein (g/L)	<b>50</b> (60–80)	<b>49</b> (51–73)	70 (64–83)	76 (64–83)
Albumin (relative) (%) <sup>#</sup>	<b>0.68</b> (54–70)	<b>17.17</b> (54–70)	56.55 (54–70)	<b>48.39</b> (54–70)
α1-globulins (relative) (%) <sup>#</sup>	<b>13.78</b> (2–5)	<b>9.34</b> (2–5)	4.62 (2–5)	4.99 (2–5)
α2-globulins (relative) (%) <sup>#</sup>	<b>35.55</b> (4–11)	<b>37.89</b> (4–11)	10.62 (4–11)	10.73 (4–11)
β1-globulins (relative) (%) <sup>#</sup>	<b>23.49</b> (5–10)	<b>17.27</b> (5–10)	9.34 (5–10)	<b>10.29</b> (5–10)
β2-globulins (relative) (%) <sup>#</sup>	5.25 (2–8)	3.02 (2–8)	2.47 (2–8)	5.85 (2–8)
γ-globulins (relative) (%) <sup>#</sup>	<b>21.26</b> (9–19)	15.31 (9–19)	16.4 (9–19)	<b>19.75</b> (9–19)
Apolipoprotein B (g/L)	<b>1.77</b> (0.60–1.17)	<b>2.34</b> (0.66–1.33)	1.05 (0.66–1.33)	1.12 (0.60–1.17)
Apolipoprotein A-1 (g/L)	1.58 (1.08–2.25)	1.34 (1.04–2.02)	1.44 (1.04–2.02)	1.76 (1.08–2.25)
Total cholesterol (mmol/L)	<b>12.74</b> (2.79–4.99)	<b>9.38</b> (1.96–4.73)	4.99 (2.33–5.17)	<b>5.68</b> (2.33–5.17)
LDL-cholesterol (mmol/L)	<b>9.22</b> (< 2.58)	<b>6.80</b> (< 2.58)	<b>3.05</b> (< 2.58)	<b>2.64</b> (< 2.58)
HDL-cholesterol (mmol/L)	1.55 (1.03–1.55)	1.14 (1.03–1.55)	1.34 (1.03–1.55)	1.42 (1.03–1.55)
Triglycerides (mmol/L)	<b>4.28</b> (< 1.69)	<b>3.14</b> (< 1.69)	1.31 (< 1.69)	<b>3.55</b> (< 1.69)
Calcium (mmol/L)	<b>2.12</b> (2.2–2.7)	<b>2.23</b> (2.25–2.75)	2.38 (2.15–2.5)	2.33 (2.15–2.5)
Phosphate (mmol/L)	1.84 (1.00–1.94)	1.81 (1.13–2.13)	1.04 (1.03–1.45)	1.36 (1.03–1.45)
IgA (g/L)	<b>0.99</b> (0.16–0.98)	0.61 (< 0.81)	1.90 (0.70–4.00)	2.27 (0.70–4.00)
IgM (g/L)	<b>1.52</b> (< 1.22)	1.19 (< 1.22)	0.70 (0.40–2.30)	1.27 (0.40–2.30)
IgG (g/L)	<b>10.02</b> (4.42–8.95)	<b>12.60</b> (4.42–8.95)	12.05 (7.00–16.00)	<b>16.88</b> (7.00–16.00)
Transferrin (g/L)	<b>7.51</b> (2.03–3.60)	<b>6.46</b> (2.03–3.60)	<b>3.83</b> (2.00–3.60)	<b>4.15</b> (2.00–3.60)
Lactate dehydrogenase (U/L 37 °C)	<b>725</b> (< 344)	<b>473</b> (< 451)	198 (< 480)	204 (< 480)

\*Normal values for corresponding age and sex are presented in parenthesis. Values out of the normal reference range are highlighted in bold.

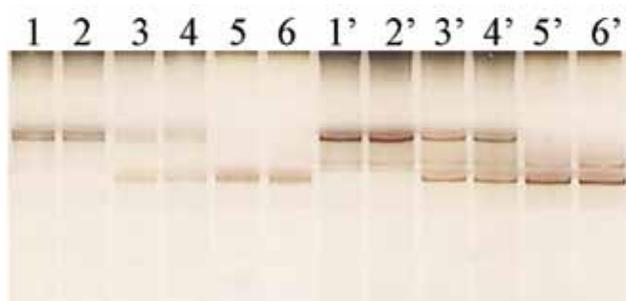
<sup>#</sup>Serum globulin and albumin fractions were detected after automated capillary electrophoresis, which reported their relative values.

The main recent clinical chemistry data of the 2 probands and of their parents are summarized in Table 1. Both siblings show low ALB concentration and low total protein concentration, despite a compensatory increment of all the globulin fractions, and gross hyperlipidaemia, with a marked increment of total and LDL-cholesterol and hypertriglyceridemia. Their total calcium concentrations are both close to the lower limit of the normal range due to absence of or low binding of calcium by ALB.

The other members of the family, the parents (Table 1) and the other 2 children, were tested for ALB, blood lipids, protein fractions and immunoglobulin levels. All had ALB concentrations near the lower limits of the normal range (39 - 41 g/L), suggesting possible carrier status for the CAA trait.

### Mutation analysis

The screening for mutations of the ALB from the DNA of the subjects of this study was carried out as previously reported (8,11,20), in order to confirm the clinical diagnosis of CAA. SSCP analysis allowed us to locate the causative mutation in the 309 bp fragment encompassing exon 5 and the adjacent intron-exon junctions amplified by using PCR primers A09A and A10A (Figure 3). Both the homozygous sample (Figure 3, lanes 1, 2 and 1', 2') revealed the altered conformations of mutated sequences resulting in mobility shifted bands when compared with controls (Figure 3, lanes 5, 6, 5', and 6'). The pattern of the heterozygous parents shows the presence of bands corresponding to those present in the homozygotes and in the controls (Figure 3, lanes 3, 4, 3', and 4'). The amplified fragment was submitted to automated sequence analysis as described (8). The results showed that both probands are homozygous for the deletion of a cytosine residue in a stretch of four cytosines starting from nucleotide position 524 and ending at position 527 (NM\_000477.5(ALB):c.527delC). (Figure 4). Both parents were carrier of the mutation (data not shown). The results of the analysis revealed the genetic origin of CAA, that is caused by a novel single base deletion in the ALB, for which we propose the name Erzurum from the city of origin of the family. The putative effect of the dele-

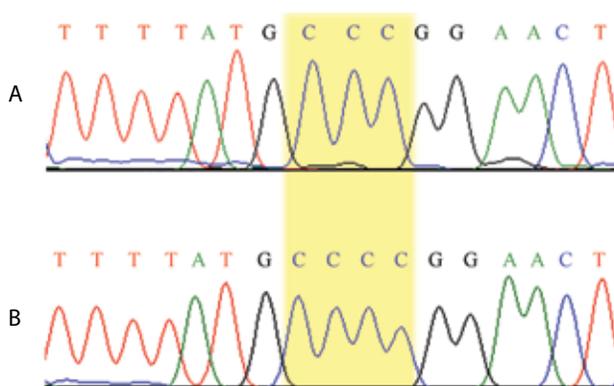


**FIGURE 3.** SSCP analysis of exon 5 from the members of the Turkish family.

The DNA encompassing exon 5 and the exon-intron junction was amplified with primers A09A and A10A and the fragments were electrophoresed onto a non-denaturing polyacrylamide gel: lane 1, patient 1; lane 2, patient 2; lane 3, father; lane 4, mother; lanes 5-6, controls.

The same samples were denatured and cooled before loading: lane 1', patient 1; lane 2', patient 2; lane 3', father; lane 4', mother; lanes 5'-6', controls.

The pattern of the homozygous probands revealed the presence of altered conformations of mutated sequences resulting in mobility shifted bands when compared to controls. In the heterozygous parents are present the bands which characterise both the homozygotes and the controls. Denaturation increases the amount of single-strand PCR products allowing a better detection of the bands.



**FIGURE 4.** Genomic DNA sequence electropherograms of patient 2 (A) and of a control (B).

The region of the mutation is highlighted in yellow. The proband is homozygous for the deletion of a cytosine residue in a stretch of four cytosines starting from nucleotide position 524 and ending at position 527.

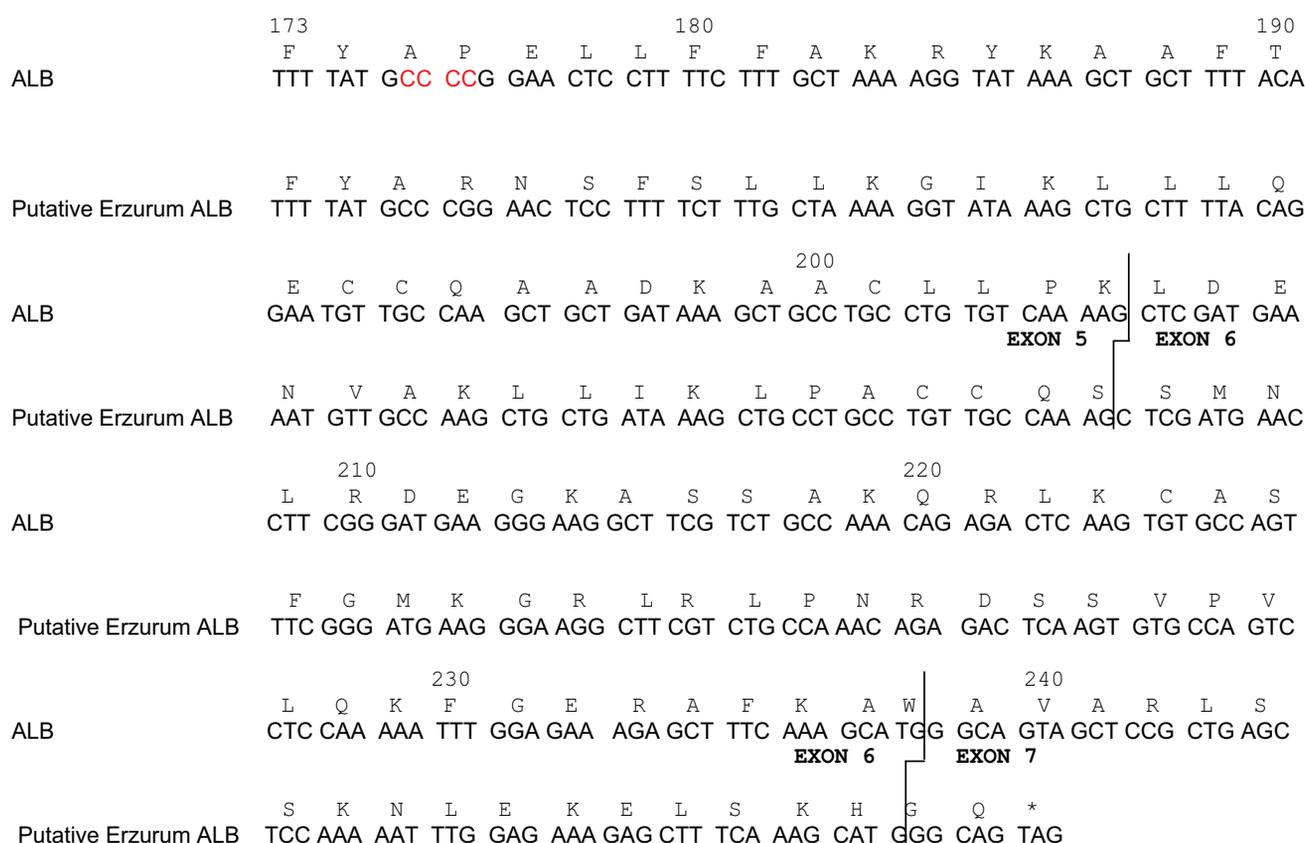
tion at the DNA and at the protein level is summarized in Figure 5. The sequence of four cytosines in which the deleted base is located includes the last

2 nucleotides in the codon for Ala175 and the first 2 bases in the codon for Pro176, respectively, in exon 5. The subsequent frame-shift changes the codon for Pro 176 to Arg, leading to a premature stop codon TGA at position 240, 65 amino acid residues downstream, near the 5' end of exon 7 (p.Pro176Argfs\*65) (Figure 5). Therefore, the predicted translation product from the Erzurum allele would be only 215 amino acids long. This truncation would cause the lack of domain II and III of the mature protein, giving rise to a totally variant sequence of 64 amino acids at its C-terminal end, particularly rich in basic residues (Figure 5), which represents the longest aberrant sequence so far hypothesized in a putative product of a mutation resulting in CAA.

## Discussion

In the past years, most cases of CAA were diagnosed only in adulthood, because of misdiagnosis or delayed diagnosis of the condition (4). Recently, a more detailed knowledge of the trait allows an earlier diagnosis, often in the paediatric age group (21-23), as for the Erzurum mutation. Pre-term birth, low birth weight, respiratory distress with hospital admissions and mild developmental delay are present in the two analbuminemic siblings. This adds supporting evidence to the hypothesis that the lack of the protein may be a risk factor in the perinatal and childhood period (3,5,6).

The Erzurum frame-shift/deletion is the twenty-fourth different mutation identified as a cause of



**FIGURE 5.** Molecular effect of the cytosine deletion on the DNA and amino acid sequence of the Erzurum allele. The numbering above the line refers to the amino acid residue in the ALB precursor. The mutation (c.524\_527delC) would cause a frame-shift, which changes the codon for Pro 176 to Arg, leading to a premature stop codon TGA at position 240, 65 amino acid residues downstream (p.Pro176Argfs\*65). The predicted translation product from the Erzurum allele would consist of 215 amino acid residues instead of the 585 found in the mature protein.

CAA (5,15,23,24). Twenty-two among them cause the analbuminemic trait in the homozygous state: six nonsense mutations, eight mutations affecting splicing, six frame-shift/deletions, one frame-shift/insertion and one mutation in the start codon (5,15, 23,24). Compound heterozygosity for the remaining two molecular defects, a nonsense mutation and a splice site mutation with subsequent reading frame-shift, caused the trait in an Italian man (5,15). Although the variant mRNA could be identified in some cases (5), the putative proteins produced as a consequence of the 23 mutations could never be found in the serum of the affected individuals (5,15). The length of the abnormal polypeptide chains would range from 31 (Codogno, p. Gln56\*) to 532 (Locust Valley, p.Ile537Asnfs\*21) amino acids (5,15). The largely aberrant and truncated molecule predicted as the product of the Erzurum mutation would be only 215 amino acids long, with a theoretical pI and Mw of 8.28 and 27553.75, respectively, instead of 5.67 and 66472.21 of the mature ALB. Due to the age of the probands, we could not get sera to perform two-dimensional electrophoresis, but, on the basis of the above mentioned results, it is likely that the Erzurum variant is also not present in blood. Responsible for this absence may be the partial or complete lack in all these putative translation products of domain III, which plays a crucial role in the ALB binding to the intracellular receptor FcRn (25). This receptor mediates ALB rescue from lysosomal degradation, acting in a pH-dependent way to recycle the protein back to the blood (25). An alternative explanation is that all the truncated transcripts could un-

dergo nonsense-mediated RNA decay with no protein produced (26), since all these mutations occur at least 55 bp upstream of the exon13-intron13 boundary.

The twenty-four mutations identified as cause of CAA are located in nine different exons (1, 3, 4, 5, 7, 8, 10, 11, and 12) and in six different introns (1, 3, 6, 10, 11, and 12) (5,15, 23,24). This scattered distribution throughout the gene seems to suggest that CAA is the result of randomly occurring deleterious mutations (27). However, whereas the Erzurum mutation, as most of the other causative defects, is until now unique, the two bases deletion c.228\_229delAT (analbuminemia Kayseri) (28) is by far the most common cause of the trait, occurring in unrelated individuals in different populations and accounting for about one-third of the cases characterized at the molecular level (5,15,24). Therefore, the sequence c.228–c.230 of exon 3, in which also the mutation of analbuminemia Amasya (c.229\_230 delTG) is located (10), seems to represent the main hypermutable region in the ALB (5,15).

In conclusion, the molecular analysis, performed following our strategy, confirmed the clinical diagnosis of CAA in this family, and revealed that the condition is due to a novel deletion producing a frame/shift defect. The results confirm the inheritance of the trait, and contribute to advance our understanding of the molecular basis underlying CAA.

### Potential conflict of interest

None declared.

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