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Genetic basis of cystinosis in Tunisian patients: Identification of novel mutation in CTNS gene

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ABSTRACT

Nephropathic cystinosis (NC) is an autosomal recessive disorder characterized by defective transport of cystine across the lysosomal membrane and resulting in renal, ophthalmic, and other organ abnormalities. Mutations in the *CTNS* gene cause a deficiency of the transport protein, cystinosin. This study was performed to investigate mutations of the CTNS gene in three Tunisian families with NC.

Polymerase chain reaction (PCR), ARMS multiplex PCR and direct sequencing were performed for molecular characterization of the *CTNS* gene in 3 unrelated Tunisian patients and their parents. Based on family history, prenatal diagnosis (PND) was performed in fetal DNA isolated from chorionic villi obtained at 10–12 weeks of gestation.

None of the patients showed the most common 57-kb deletion in heterozygous or homozygous status. One patient was homozygous for the previously reported mutation c.1515G > A (p.G308R). One patient presented the novel gross deletion of 20,327 bp. One was homozygote for the previously reported mutation c.771_793del (p.Gly258Serfs*30). In addition, eight polymorphisms were identified in the 3 patients and their parents. The prenatal diagnosis in one family showed that the fetus DNA was heterozygous for the c.771_793del (p.Gly258Serfs*30) mutation.

This study expands the mutational and population spectrum of NC, representing the first molecular diagnosis of NC in Tunisian population. The mutation screening of the *CTNS* gene was used for prenatal diagnosis to prevent and/or limit this inheritable disease in our country where the families are particularly large and have a high rate of consanguinity.

1. Introduction

Nephropathic cystinosis (MIM ≠ 219,800) is an autosomal recessive lysosomal storage disorder with an incidence rate of 1 per 100.000-200.000 of the word population (Gahl et al., 2002). In Tunisia the prevalence of this pathology is unknown; this report represents the first molecular analysis of NC in Tunisian population. NC is due to deficient transport of the disulfide amino acid cystine across lysosomal membrane (Jonas et al., 1982). Although NC is a monogenic disease, three clinical forms of cystinosis are distinguished: nephropathic infantile form (OMIM ≠ 219,900); nephropathic juvenile form (OMIM ≠ 219,750) and non-nephropathic adult form (OMIM ≠ 219,750). Affected patients are normal at birth, but typically exhibit failure to thrive, acidosis, dehydration. These symptoms reflect renal tubular Fanconi syndrome but renal glomerular damage soon supervenes, leading to kidney failure at approximately 10 years of age (Gahl et al., 2002).

CTNS gene, (OMIM 606272; GenBank NM_004937.2) is located on the short arm of chromosome 17 (13p) and contains 12 exons that are distributed across ~23 kb of genomic DNA (McDowell et al., 1995). *CTNS* gene codes for cystinosin, a 367 amino-acid peptide, predicted to contain seven transmembrane domains and it is sorted via a classic tyrosine-based GYDQL lysosomal sorting motif in its C-terminal tail (Cherqui et al., 2001). Since the cloning of *CTNS* in 1998, over 90 mutations have been reported. The most common mutation accounting for approximately 75% of the affected alleles in Northern Europe is a 57-kb deletion, affecting the first 10 exons of *CTNS* (Town et al., 1998).

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The aim of this investigation was to search for the common known most frequent Northern European 57-kb deletion and also to screen the coding regions of the *CTNS* gene for novel mutations. This report represents the first prenatal and molecular diagnosis of NC in the Tunisian population.

2. Materials and methods

Three patients (TN1, TN3, TN6) from three families from Central and Southern Tunisia were previously diagnosed by their characteristic clinical findings (Table 1). The patients were all products of consanguineous matings (Fig. 1), and there were no known relationships among the families who lived 120–140 km apart. Prenatal diagnosis (PND) has been done in family 2 with a reported NC case.

2.1. Ethics statement

All procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 and approved by the Ethics Committees of the respective Tunisian hospitals. Informed consent was obtained from all patients and their families for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

2.2. Molecular analysis

Blood samples were collected from all patients and their parents; genomic DNA was extracted from leukocytes and also from chorionic villus cells in the PND, as previously described (Sambrook and Maniatis, 1989).

The polymerase chain reaction (PCR) was carried out by a set of self designed primers using Net primer software (http://www.premierbiosoft.com/netprimer/) in one hand for 57-kb deletion, in the other hand for all the exons (1–12) of *CTNS* gene and also for the novel large deletion. All the exons and flanking intron/exon junctions of the *CTNS* gene were amplified (Table 2).

2.3. Detection of the common 57-kb deletion

The 57-kb deletion was tested using the method ARMS multiplex PCR not previously described for the screening of *CTNS* gene; firstly to identify subjects that were either heterozygote or did not carry the deletion, and secondly to investigate the whole coding regions and exon–introns junctions (Table 2). We chose two pairs of primers; the first pair encompasses the 57-kb deletion as below: the 5′ primer, 57-kb deletion forward, matched the sequence of *TRPV1* gene and the 3′ primer, 57-kb deletion reverse was located in intron 10 (IVS10) of *CTNS* gene; since the resulting PCR products generated a 387 base pair (bp) amplicon length. We chose the second pair of primers in IVS10 of *CTNS* gene. Using these primers, the PCR products generated had to differ in size by ~90 bp. The 989 bp amplicon across the 57-kb deletion was generated using the 5′ primer, 57-kb deletion forward, and the 3′ primer, IVS10 reverse (Fig. 2).

Table 1
Clinical and laboratory finding of the three NC patients.

Family	Family 1	Family 2	Family 3
Origin	Mahdia	Kairouan	Kairouan
Patients	TN1	TN3	TN6
Age	6 years	5 years	4 years
Sex (M/F)	M	M	F
Age at diagnosis	15 months	15 months	18 months
Parental consanguinity	1st cousins	1st cousins	1st cousins
Fonconi syndrome	+	+	−
Growth retardation	+	+	+
Corneal crystals	+	−	−
Leukocyte cystine (nmol half-cystine/mg protein)	4.0 (control = 0.1)	3.8 (control = 0.1)	3.2 (control = 0.1)
Treatment via cysteamine	None	None	Yes
Phenotype	Infantile nephropathic cystinosis	Infantile nephropathic cystinosis	Infantile nephropathic cystinosis

2.4. Mutation screening by direct sequencing

Each of the 12 exons and flanking intron–exon junctions of *CTNS* gene were amplified by the polymerase chain reaction (PCR) from genomic DNAs isolated from the patients TN3, TN6's both parents and patient TN1's mother. All PCR amplified products were directly sequenced using the Big Dye 3 Terminator chemistry (Applied Biosystems) employing an ABI 3130xl genetic analysis (Applied Biosystems).

2.5. Prenatal diagnosis

Based on family history the parents of family 2 who represented a couple at risk were selected for PND. The fetal DNA was isolated from chorionic villi obtained at approximately 10–12 weeks gestation.

Genetic analysis of NC mutation was performed by direct sequencing of exon 10 of *CTNS* gene (morbid loci). We conducted a study of microsatellites by capillary electrophoresis to verify the absence of contamination of fetal DNA from maternal cells.

3. Results

3.1. Identification of CTNS mutations

Before sequencing, all patients were tested for the 57-kb deletion. We could not find this deletion in any of them (Fig. 2).

Analysis of the entire CTNS gene in all patients and their parents revealed the presence of three mutations (Table 3): Two of these mutations were previously described c.1515G > A (p.G308R) in exon 11 of *CTNS* gene in patient TN6 of family 3 (Fig. 3A) and c.771_793del (p.Gly258Serfs*30) in exon 10 of *CTNS* gene in patient TN3 of family 2 (Fig. 3B) and one was previously unreported deletion of 20,327 bp in patient TN1 of family 1 (Fig. 3C).

3.2. Identification of 20-kb deletion

In family 1, the novel mutation was a large deletion removing not only the first 5 exons of the *CTNS* gene (exon 1 to 5) but also the part of intron 1 of the adjacent gene *CARKL*. To identify this mutation, we amplified the entire *CTNS* gene in addition to the promoter region and part of intron 1 of gene *CARKL* (the adjacent *CTNS* gene), then we analyzed all PCR products of the patient and of his mother (the father refusing to give a blood sample). No PCR product was obtained of the first 5 exons of *CTNS* gene and the promoter region and part of intron 1 of the *CARKL* gene in patient. However we could get an amplicon for the mother. Genomic DNA PCR encompassing the exon 6 of the *CTNS* gene and the intron 1 of the *CARKL* gene revealed the amplification of an abnormal fragment of approximately 540 bp in the affected patient and in his mother (Fig. 3C).

In addition to the mutations, eight apparent polymorphisms were identified in coding exons and junctions exons–introns of the *CTNS* gene. One of these are novel, IVS5 + 100 T > A (Table 3).

3.3. Prenatal diagnosis

The prenatal diagnosis in family 2 identified the fetus DNA to be heterozygous for the c.771_793del (p.Gly258Serfs*30) mutation similarly to his parents.

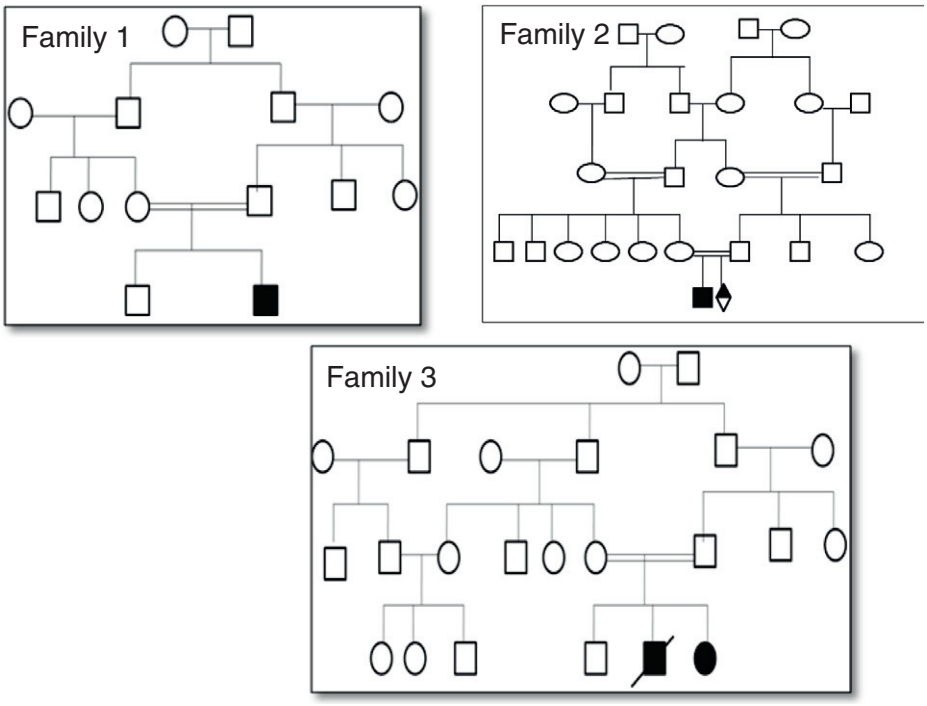

Fig. 1. Pedigrees of the three investigated cystinosis Tunisian families. Squares and circles indicate male and female members, respectively. Shaded symbols indicate affected individuals. Double lines indicate consanguineous matings, lozenge indicate heterozygous fetus who was implicated in the PND.

4. Discussion

Three patients from three unrelated families were evaluated. Patients' ages ranged from 2 to 9 years; and age at diagnosis was 1 month to 5 years. Consanguinity was reported for all the investigated families (Fig. 1). One patient receives cysteamine treatment since diagnosis.

Table 2
Primer for multiplex and PCR amplifications.

	Name	Sequence 5′ > 3′
	del57–kbF1	CCTGGCTGCTCTCCTCTTCTTGCCGTTC
	del57–kbR1	TCCCTGTGGCTTTCCGTCCTGTTTCCTC
Intron10	Intron10F CTNS	GCCCTCACCACTGCCTCCATTTCC
	Intron10R CTNS	GCTGGCCCTCCTGAGCTCACAGAA
CTNS Promotor	Promotor 1F	GATTTCCGCCCAATGGAGGG
	Promotor 1R	CTGCTGTGCCTCAAGGGGTC
Exon 2	2FCTNS	GTTCCCAGCCCCTTACCTTC
	2RCTNS	GAGGTTGCAGTGAGCCGAGA
Exon 3	3FCTNS	CAGATTGTCTACAGGGAGCT
	3RCTNS	CAAGAGGCAGACAGAGACAT
Exon 4	4FCTNS	TCTCAGAGCCTGTCATCG
	4RCTNS	GCCAGGGAAAGTGAGGAC
Exon 5	5FCTNS	GCATTTCCTAAGCCTAACTG
	5RCTNS	CAGATTTCTAGGTTGATGGA
Exon 6	6FCTNS	GATTGAACCTCAGTCTTCC
	7RCTNS	AAGGGTAGAGGGGACGTTG
Exon 8 + 9	8FCTNS	CCCTGCCCTGTCTTGTCC
	9RCTNS	GCTCTGCCGTGTCTTCTGTC
Exon 10	10FCTNS	CTTGCAGGGGCTCCTTCAAG
	10RCTNS	CCTGTGGCTTTCCGTCCTGT
Exon 11	11FCTNS	CCGCCTCTGCTGGAGCTGT
	11RCTNS	GGGGCTGGATTGGCTTGG
Exon 12	12FCTNS	CCTTCGTAGCTGGAGGCTTT
	12RCTNS	GACGAAGGCAGGCTATTG
3′UTR	3′UTRF	AGGCTTCAGGCAGCGCGC
	3′UTRR	GCTAATCTGAGAAGGTGG
Intron 1	Intron 1F CARKL	CACCCCACCAGGGTCAGAGCC
	Intron 1R CARKL	GATTGGCAGGCGACTCCCCG

This report represents the first description of the genetic basis of cystinosis in Tunisian patients. Our data reveal that the Tunisian cystinosis population has novel genetic characteristics. We did not detect the most common 57-kb deletion in any studied patient. However, a large number of NC Tunisian patients should be investigated to confirm the absence of this mutation in Tunisia. These findings were approved by recent reports from Saudi Arabia and Turkey in which the 57-kb deletion was not founded (Shahkarami et al., 2013), whereas the 57-kb deletion seems to be specific to 50–60% of Northern European and North American patients (Topaloglu et al., 2012).

In contrast, three different variants were identified in Tunisian patients: two were previously reported: c.1515G > A (p.G308R) and c.771_793del (p.Gly258Serfs*30) and one novel mutation, deletion of 20,327 bp.

Patient TN6, with classical NC, was found homozygous for a previously reported missense mutation in *CTNS* gene, c.1515G > A (p.G308R) (Attard et al., 1999). Her parents were heterozygous for this same mutation. Interestingly, two missense mutations at the 308 codon in exon 11 of *CTNS* gene, have been reported (Kiehntopf et al., 2002) in accordance with our findings. Recently a transitional mutation with the same consequence regarding the amino acid level has been found (Shahkarami et al., 2013). By using a cystinosin mutant lacking the C-terminal tyrosine based motif (cystinosin-ΔGYDQL) for studying cystine transport at the plasma membrane of COS cells, it has been demonstrated that the p.G308R mutation completely abolishes cystine transport (Kiehntopf et al., 2002). Therefore, we expect that our patient carries a mutation on the both alleles which would permit the completely loss of functional protein and which could consequently account for the severe phenotype.

Patient TN3 was found homozygous for a previously reported mutation c.771_793del (p.Gly258Serfs*30) which most likely occurs in European population (Attard et al., 1999). His parents were heterozygous for this described mutation. This genetic anomaly was a small deletion of 23 nucleotides in exon 10 of *CTNS* gene which induces a frameshift mutation and presumably leads to a truncated protein due to a premature stop codon, 30 amino acids downstream of the stop

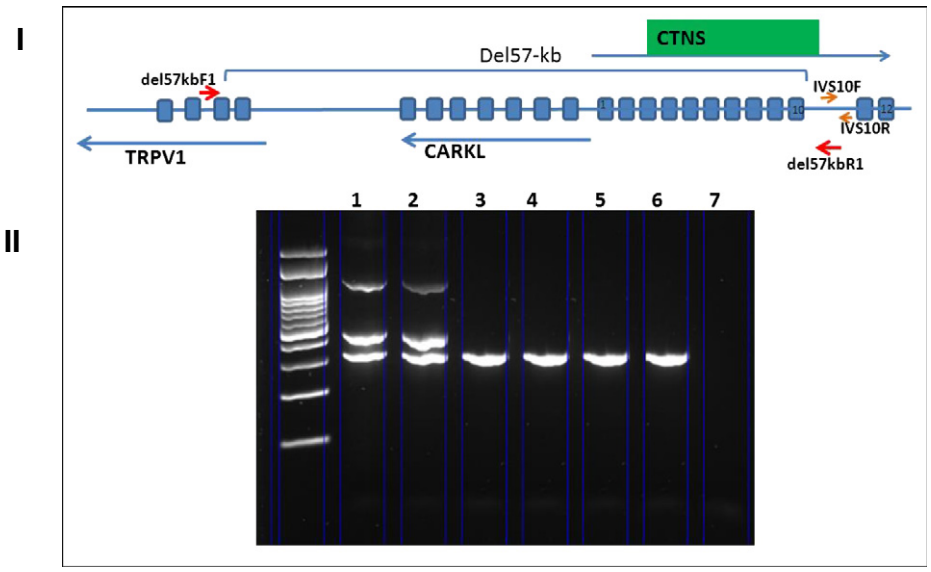

Fig. 2. Schematic illustration showing primers in the proposed multiplex PCR assay method to detect del57-kb. (I) Exons are represented as blue boxes while introns are represented by single line. (II) Agarose gel electrophoretogram. Lane 1: PCR products using DNA from a patient homozygote for del57-kb, three bands were detected. Lane 2: PCR products using DNA from a patient heterozygous for del57-kb, three bands were detected. Lanes 3, 4 and 5: PCR products using DNA from Tunisian patients TN1 (family 1), TN3 (family 2) and TN6 (family 3) respectively, one band was detected. Lane 6: PCR products using DNA from a healthy Tunisian subject, one band was detected. Lane 7: control.

codon terminator (Attard et al., 1999). As a result, this mutation is predicted to cause complete loss of all cystinosin transmembrane domains and thus should be associated with the severe infantile nephropathic form of the disease. This is in accordance with the clinical phenotype of the studied patient who presented with growth retardation, renal tubular Fanconi syndrome, polyuria, polydipsia, hypophosphatemic rickets, corneal cystine crystals and elevated leukocyte cystine levels.

Prenatal diagnosis has been done in family 2 which has an index case (patient TN3). We have found that the fetus DNA was heterozygous for the c.771_793del (p.Gly258Serfs*30) mutation similarly to his parents and the family made the decision regarding pregnancy maintenance.

Within TN1 patient, the mutation was a large deletion removing not only the first 5 exons of the *CTNS* gene (exons 1 to 5) but also a part of intron 1 of the *CARKL* gene. This new gross deletion is most probably a disease-causing mutation in the studied Tunisian patient who was diagnosed with NC by clinical and laboratory findings at the age of 15 months. The patient was hospitalized several times because of variable symptoms including growth retardation, corneal crystals, and hypothyroidism.

This novel large deletion extends into *CARKL* gene which plays a role in sedoheptulose phosphorylation. This may predict to cause complete loss of cystinosin protein and thus should be associated with the severe infantile nephropathic form of the disease. Further functional studies will confirm this hypothesis.

Our findings support a correlation between *CTNS* mutations and clinical severity in NC (Anikster et al., 1999; Attard et al., 1999). We have identified a *CTNS* mutation that is, to date, novel and specific to Tunisian families.

Table 3
Summary of all detected mutations and SNPs in the individuals being diagnosed with NC.

Patients	Mutations	Amino acid change	Status	SNP	Position	Status
TN1	20-kb deletion	Deleted intron 1 *CARKL*–intron 6 *CTNS*	Homozygous	rs161400C > T	Intron 10	Homozygous
				rs459613C > G	Intron 6	Homozygous
				rs467277G > A	Intron 7	Homozygous
				rs199950876C > T	Intron 7	Homozygous
				rs1800528G > A	Exon 9	Homozygous
Parents (mother)	20-kb deletion	Deleted intron 1 *CARKL*–intron 6 *CTNS*	Heterozygous	rs11299981delT	Exon 2	Homozygous
				rs467277G > A	Intron 7	Heterozygous
				rs459613C > G	Intron 6	Homozygous
				rs457419G > A	Intron 7	Heterozygous
				rs199950876C > T	Intron 7	Heterozygous
				rs1800528G > A	Exon 9	Heterozygous
TN3	c.771_793del	Gly258Serfs*30	Homozygous	rs459613C > G	Intron 6	Homozygous
				IVS5 + 100 T > A	Intron 5	Homozygous
Parents	c.771_793del	Gly258Serfs*30	Heterozygous	rs459613C > G	Intron 6	Heterozygous
				IVS5 + 100 T > A	Intron 5	Heterozygous
TN6	c.1515G > A	G308R	Homozygous	rs459613C > G	Intron 6	Homozygous
				rs161400C > T	Intron 10	Homozygous
				IVS5 + 100 T > A	Intron 5	Heterozygous
				rs457419G > A	Intron 7	Heterozygous
Parents	c.1515G > A	G308R	Heterozygous	rs459613C > G	Intron 6	Heterozygous
				rs161400C > T	Intron 10	Heterozygous
				IVS5 + 100 T > A	Intron 5	Heterozygous
				rs457419G > A	Intron 7	Heterozygous

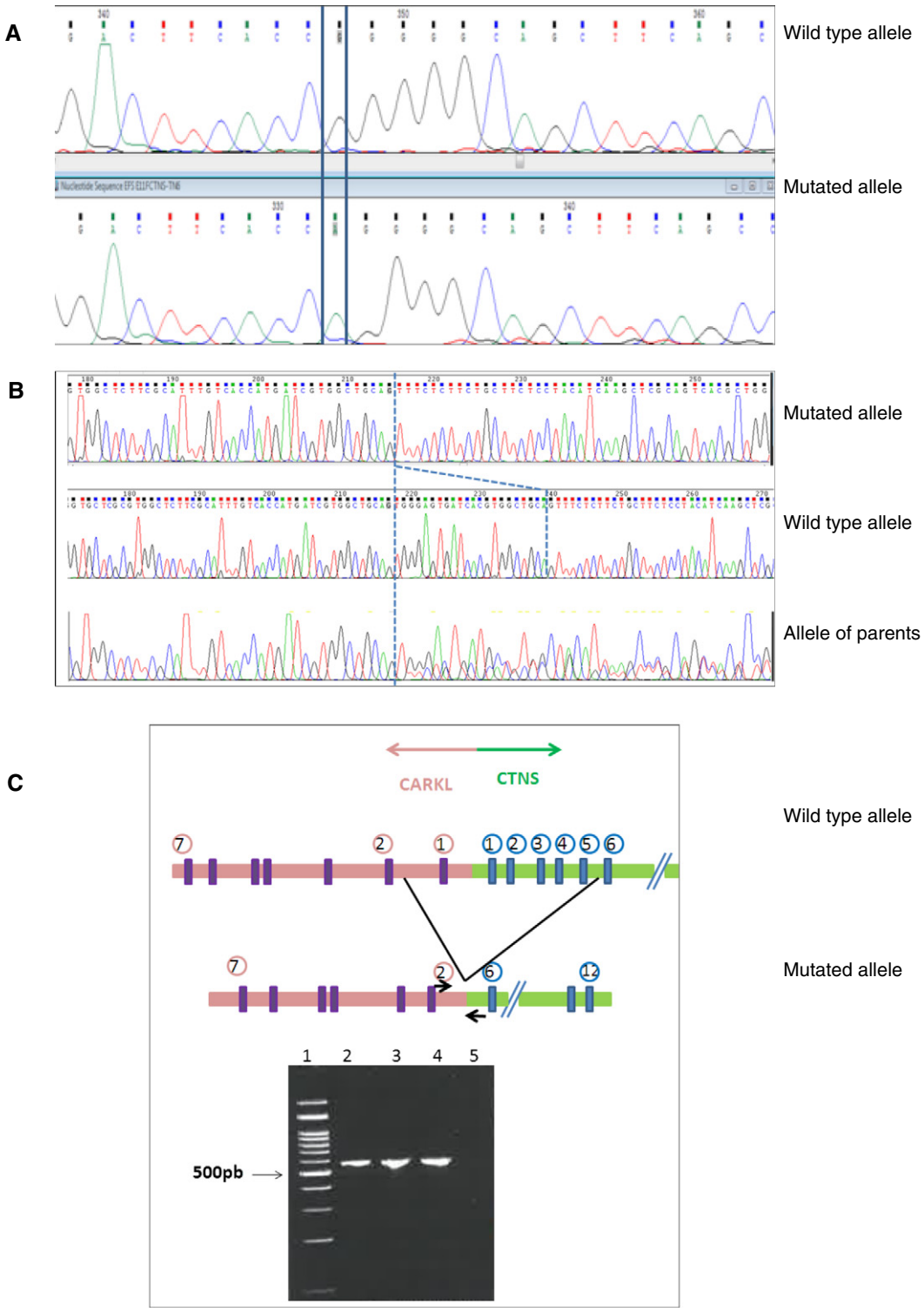

Fig. 3. Direct sequencing of *CTNS* gene in Tunisian patients. A: Sequence electropherograms of exon 11 of the *CTNS* gene for c.1515G > A (p.G308R) mutation. B: Sequence electropherograms of exon 10 of the *CTNS* gene c.771_793del (p.Gly258Serfs*30) mutation. C: Characterization of the large rearrangement removing exons 1 to 5 of the *CTNS* gene and a part of the intron 1 of *CARKL* gene. 1C: Schematic representation of the *CTNS* and *CARKL* genes rearrangement. Blue boxes represent SGSH exons and purple boxes represent *CARLK* exons. The 20,327 bp deleted region is delineated. The black arrows correspond to the primers (IVS1F_CARKL/IVS6R_CTNS) used to amplify the junction fragment. PCR of the deletion breakpoint. Lane 1: DNA 100-bp increment ladder; Lane 2: patient; Lane 3: patient; Lane 4: patient's mother; and Lane 5: control. The amplification showed a 540 bp product corresponding to the junction fragment in the patients and her mother. This product was not present in the control DNA sample.

5. Conclusion

To conclude, we have suggested that Tunisian NC patients have different disease-causing variants of *CTNS* gene compared with European and North American patients. The mutation spectrum of the *CTNS* gene was used for prenatal diagnosis to prevent and/or limit this inheritable disease in our country where the families are particularly large and have a high rate of consanguinity.

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