Genetic analysis of Italian patients with congenital tufting enteropathy

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Background: **Congenital tufting enteropathy (CTE), an inherited autosomal recessive rare disease, is a severe diarrhea of infancy which is clinically characterized by absence of inflammation and presence of intestinal villous atrophy. Mutations in the *EpCAM* gene were identified to cause CTE. Recent cases of syndromic tufting enteropathy harboring the *SPINT2* (19q13.2) mutation were described.**

Methods: **Four CTE Italian patients were clinically and immunohistochemically characterized. Direct DNA sequencing of *EpCAM* and *SPINT2* genes was performed.**

Results: **All patients were of Italian origin. Three different mutations were detected (p.Asp219Metfs*15, Tyr186Phefs*6 and p.Ile146Asn) in the *EpCAM* gene; one of them is novel (p.Ile146Asn). Two patients (P1 and P2) showed compound heterozygosity revealing two mutations in separate alleles. A third patient (P3) was heterozygous for only one novel *EpCAM* missense mutation (p.Ile146Asn). In a syndromic patient (P4), no deleterious *EpCAM* mutation was found. Additional *SPINT2* mutational analysis was performed. P4 showed a homozygous *SPINT2* mutation (p.Y163C). No *SPINT2* mutation was found in P3. *CLDN7* was also evaluated as a candidate gene by mutational screening in P3 but no mutation was identified.**

Conclusions: **This study presented a molecular characterization of CTE Italian patients, and identified three mutations in the *EpCAM* gene and one in the *SPINT2* gene. One of *EpCAM* mutations was novel, therefore increasing the mutational spectrum of allelic variants of the *EpCAM* gene. Molecular analysis of the *SPINT2* gene also allowed us to identify a *SPINT2* substitution mutation (c.488A>G) recently found to be associated with syndromic CTE subjects.**

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Introduction

Congenital tufting enteropathy (CTE, Online Mendelian Inheritance in Man no.613217), is a rare and severe diarrhea that occurs in the first few months of life. CTE, first described in 1994,[1] is characterized by phenotypic heterogeneity including dysmorphic facial feature[2] and choanal atresia,[3] eye-associated abnormalities,[4] and chronic arthritis[5] which have been described in association with epithelial dysplasia. Many patients are subjected to parenteral nutrition and the prognosis is often poor.

Villous atrophy without inflammation and intestinal epithelial cell dysplasia with typical epithelial tufts in the duodenum and jejunum are features of the disease. CTE is an autosomal recessive disease as well evidenced by the high incidence of consanguinity. The prevalence of the disease is higher in the Arabian Peninsula and the island of Malta. The reported incidence of CTE in Western Europe is 1/100 000 live births.[6,7]

Changes in *EpCAM* (epithelial cell adhesion molecule, on chromosome 2p21) were described as responsible to CTE.[8] Different mutations as missense alterations, frame shift mutations, small deletions and splice site changes have been reported in CTE.[4,8-13] Founder effect of two pathogenetic *EpCAM* mutations were detected in seven Arabic family.[11]

Original article

Sivagnanam et al[14] published a case report of syndromic form of CTE carrying *SPINT2* (Kunitz-type serine protease inhibitor; 19q13.2) mutation previously described in congenital sodium diarrhea.[15] The proband showed typical tufts of the intestinal epithelium, abnormal hair, eye problems, blood disorder and choanal atresia. Mutations in *SPINT2* have also been shown to be associated with syndromic congenital diarrhea presenting enterocyte tufting.[16] Taken together, these findings demonstrate a phenotypic overlaps between CTE and *SPINT2* related syndromic congenital sodium diarrhea.

Recently, *SPINT2* previously ascribed to congenital sodium diarrhea has been established as a second gene associated with CTE.[13] The genotype-phenotype correlation and classification of CTE based on molecular, immunohistological and clinical data were suggested. The authors distingished in their patients into three groups. The first group (73% of CTE patients) carrying *EpCAM* mutations mainly displayed an isolated intestinal disease. The second group (21% of the CTE patients) was characterized by mutation in *SPINT2*. The patients with *SPINT2* mutations appeared to be clinically characterized by a syndromic phenotype. The third group (6%) of patients presenting an isolated diarrhea had no mutations in *EpCAM* or *SPINT2*. Duodenal histology in *EpCAM* and *SPINT2* mutations presented tufts and villous atrophy without significant difference in the number of tufts and the level of villous atrophy or inflammatory infiltrate.[13] Thus, mutation screening of *EpCAM* and *SPINT2* may better define differential diagnosis between various congenital diarrheal disorders using genetic test.

Here, we described the molecular study of the *EpCAM* and *SPINT2* genes in four Italian CTE patients and the identification of one *EpCAM* novel mutation associated with CTE.

Methods

Patients

Four Italian patients with CTE were included, after obtainment of written consent, in the screening for *EpCAM* and *SPINT2* mutations. The clinical history and typical histology of biopsy allowed the clinical diagnosis of CTE. Two patients, P1 and P2, were recruited from the same family; DNA of their mother, maternal grandmother and one healthy brother were available for molecular analysis. The other patients (P3 and P4) were enrolled from unrelated families. In order to exclude the presence of this change in a Caucasian population, *EpCAM* novel missense mutation was searched in DNA from 50 healthy subjects. Thousand genomes database searches ruled out a common polymorphic change (http://www.1000genomes.org). PolyPhen-2 v2.2.2r398 and Mutation Taster were queried (http://genetics.bwh.harward.edu and http://www.mutationtaster.org) to predict the pathogenicity of the mutations. Mutational screening was performed according to the World Medical Association Declaration of Helsinki and it was approved by the local ethics committee (Medical School of the University of Foggia).

Nucleotide sequence analysis of *EpCAM*, *SPINT2* and *CLDN7*

DNA samples from CTE patients and their relatives were extracted from 0.2 mL of whole blood (EDTA-treated). Coding regions and splice site of *EpCAM* and *SPINT2* were amplified by polymerase chain reaction (PCR) and the amplicons were purified using the QIA quick PCR Purification Kit (Qiagen, Westburg, Leusden, NL, USA). Direct sequencing was performed using an ABI 3130 automated capillary DNA Sequencer (Applied Biosystem, Foster City, CA, USA). In addition, the *CLDN7* gene including intron-exon boundaries was direct sequenced.

DNA fragment sequences were compared with the *EpCAM*, *SPINT2* and *CLDN7* cDNA sequences, (NM_002354.2, NM_021102.3 and NM_001185022.1 respectively) querying the GenBank database. Current recommendations of the Human Genome Variation Society for nomenclature of variations were used (http://www.hgvs.org/mutnomen/).

Results

The four patients, two of whom were siblings, one male (P1) and one female (P2), showed the characteristic clinical and histological phenotypes of CTE. The features of patients and genetic analysis results are shown in the table.

Patients (P1) and (P2) had a healthy brother. Both were born at term, and diarrhea began at 2 days of life. Parenteral nutrition was started after 48 hours of life. Histological diagnosis was made at 9 (P1) and 6 (P2) months of life showing similar histological features in both patients. The patients (P1 and P2) were transplanted respectively at 10 and 11 years of age, due to reduction of vascular access. Both patients died within three years after they have been transplanted.

P3 was born full-term from non consanguineous parents. Duodenal histology presented tufts and villous atrophy. Clinically, this patient did not show external dysmorphisms or other malformations. The patient is now 11 years old and he receives total parenteral nutrition (TPN).

Table. Clinical and molecular findings of congenital tufting enteropathy patients

Patient identifier	Sex	Age, outcome, current treatment	Malformation and dysmorphological feature	Nucleotide alteration	Coding sequence, protein alteration	Exon/ intron	References
P1	M	Died at 10 y, post intestine transplantation	None	*EpCAM* c.654 DelA c.556-14A>G	p.Asp219Metfs*15 pTyr186Phefs*6	6 5*	13
P2	F	Died at 14 y, post intestine transplantation	None	c.654 DelA c.556-14A>G	p.Asp219Metfs*15 pTyr186Phefs*6	6* 5	13
P3	M	11 y, alive, TPN	None	c.437T>A heterozygous	p.Ile146Asn	4	Novel
P4	M	18 y, alive, intestine transplantation when he was 3 y old	Anal atresia	*SPINT2* c.488A>G	p.Y163C	5	15

TPN: total parenteral nutrition; F: female; M: male. *: Mutation deletes the consensus splice acceptor.

P4 was born full-term and required immediate surgery (first day of life) due to anal atresia. The patient showed no further abnormalities except for keratitis. TPN was started soon after diarrhea began in the second day of life. Standard histologyshowing tufts and villous atrophy at 6 months of life. He received intestinal and liver transplantation because of intestinal and liver failure when he was 3 years old. Now, the patient is 18 years old.

The coding regions of the *EpCAM* gene were analyzed by mutational screening of the four patients. Two patients, P1 and P2, recruited from the same family resulted compound heterozygous for the following mutations: c.654 DelA and c.556-14A>G. The c.654 DelA mutation consists of a deletion of an adenine in exon 6, which determines the alteration of the reading frame and leads to a premature stop codon (p.Asp219Metfs*15). This mutation causes a truncated protein resulting in loss of the transmembrane domain. The c.654 DelA mutation has been previously described.[13] The sequence analysis of P1 and P2 relatives (mother, maternal grandmother and one healthy brother) showed the same heterozygous mutation identified in the siblings. Unfortunately, father's DNA was not available. Furthermore, in P1 and P2, an intronic variant c.556-14A>G was identified in heterozygousity. This variant as recently described[12] introduces a new acceptor site leading to a frame shift with the introduction of six novel amino acids and premature truncation of protein (pTyr186Phefs*6).

EpCAM gene analysis in patient (P3) revealed the presence of only one heterozygous novel missense mutation, a nucleotide substitution of thymine to adenine at position 437 in exon 4 (Fig. A). Mutation c.437T>A predicted the replacement of isoleucine (Ile) to asparagine (Asn) at position 146 (p.Ile146Asn). Studies in healthy controls and 1000 genome database searches ruled out a common polymorphic change. Multiple sequence alignment of amino acid sequences of *EpCAM* orthologs from different species showed that Ile146 was conserved across all the analyzed species (Fig. B) and suggested that this amino acid change might modify *EpCAM* function.

Molecular modeling of mutant was applied using Swiss Pdb Viewer application (http://spdbv.vital.it.ch/) to study the structure of mutant protein.

Comparison of the predicted structures of both wild type and mutant protein showed several conformational changes of secondary structures. In predicted wild type protein structure, Ile146 is located within a loop, while in the predicted mutated protein the amino acid Asn146 is closed to a b-sheets. Then the substitution of isoleucine to asparagine at position 146 in *wild type protein.pdb* file was simulated (Fig. C). The substituted amino acid forms two new hydrogen bonds with the amino acids Tyr 142 and Glu 147. These new bonds seem to confirm the hypothesis that the missense mutation (p.Ile146Asn) introduces a conformational change in the protein structure. In addition, PolyPhen-2 v2.2.2r398 (http://genetics.bwh.harward.edu) and Mutation Taster (http://www.mutationtaster.org) were queried to predict the pathogenicity of the mutation. p.Ile146Asn is predicted to be damaging (score of 0.999 for both applications).

Finally, the direct sequencing of the *EpCAM* gene including flanking intronic sequences and 5' and 3' UTR of P4 allowed to exclude the presence of mutations in the analyzed regions.

P4 showed tufting enteropathy, associated with keratitis and anal atresia. Since *SPINT2* was been established as a second gene associated with CTE,[13] an additional *SPINT2* mutational analysis was performed. *SPINT2* homozygous mutation (c.488AG, p.Y163C) was found.

Additional *SPINT2* mutational screening was also performed in P3 since *EpCAM* missense mutation identified in this patient was not sufficient to cause CTE, an autosomal recessive disease. No *SPINT2* mutations were found. Furthermore, *CLDN7* mutation analysis was performed for this patient. The sequence analysis of all exons of *CLDN7* and the flanking intronic regions showed the absence of mutations in this patient.

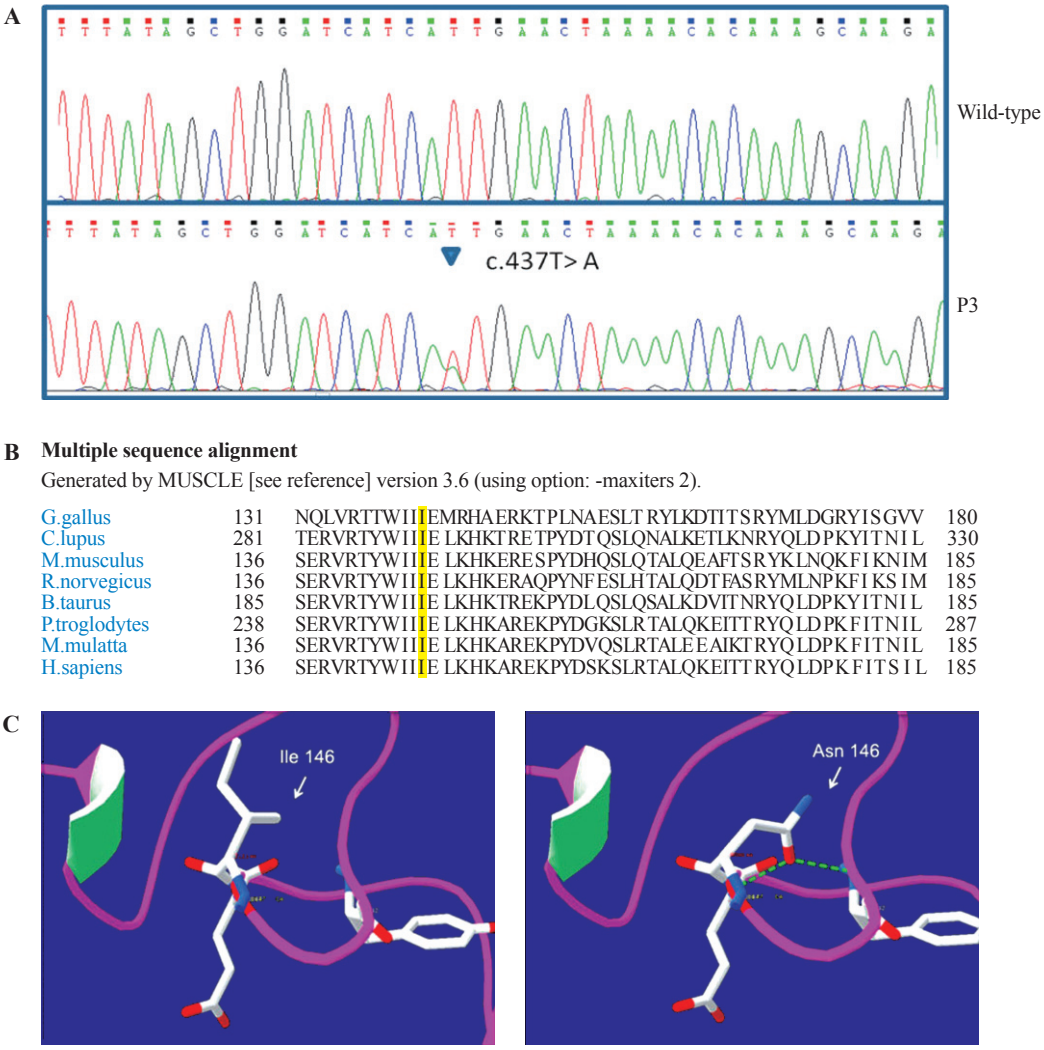

Fig. Identification of the c.437T>A mutation. **A**: Sequence chromatogram revealing heterozygous mutation c.437T>A (arrow heads) identified in patient (P3) with congenital tufting enteropathyl; **B**: Multiple sequence alignment of the amino acid sequences of EpCAM orthologs from different species, showing complete conservation of the isoleucine residue (underlighted); **C**: Comparison of the predicted structures of both wild type and mutant protein: the substitution of isoleucine to asparagine at position 146 forms two new hydrogen bonds with the amino acids Tyr 142 and Glu 147.

Discussion

CTE belongs to a group of congenital diarrheal disorders (CDD) of infancy characterized by severe chronic diarrhea which most frequently represents the prominent clinical feature, although in other cases diarrhea is associated with different clinical signs giving rise to syndromic forms.[17]

The diagnostic process of CDDs is based on the evaluation of clinical history, the laboratory tests and molecular analysis. The first step for the definition of diagnosis is the distinction between CDD with osmotic or secretory mechanism causing diarrhea.[18] Congenital chloride diarrhea or congenital sodium diarrhea are recognizable by the absence of hydramnios and assessment of the electrolytes in blood and stools. In congenital glucose/galactose malabsorption, an osmotic diarrhea, a severe restriction of glucose and galactose followed by complete disappearance of diarrhea helps to establish the diagnosis.

Differential diagnosis among different CDDs characterized by secretive diarrheas, such as CTE and microvillous inclusion disease is based essentially on intestinal biopsy and histological investigation.

At present, we have recognized that the genetic basis of many congenital diarrheas is available for genetic tests that contribute to establishing a clear diagnosis.

Within the group of congenital diarrheas, the diagnosis of CTE has long been difficult because CTE is a heterogeneous condition. Two genes have been associated with autosomal recessive congenital tufting enteropathy: *EpCAM*, epithelial cell adhesion molecule, and *SPINT2*, a Kunitz-type protease

inhibitor.[8,14] With the discovery of the genetic basis of the CTE disease, immunohistochemistry has provided new diagnostic utility.[19] The complete absence of the membranous EpCAM/MOC-31 staining was found in cases with mutations in the *EpCAM* gene. Thus, immunohistochemistry staining with MOC-31/EpCAM antibody is of diagnostic utility in ruling our CTE and excluding others etiologies of protracted diarrhea in infancy. Moreover, EpCAM/MOC-31 antibody staining becomes a very useful tool in cases of tufting morphology, normal pattern of retention of EpCAM/MOC-31 antibody staining and syndromic features, including choanal atresia. Then, further investigation with *SPINT2* mutational analysis may be warranted to better classify these patients.

Different mutations as missense alterations, frame shift mutations, small deletions and splice site changes have been reported.[4,8-13] EpCAM has been also described as a marker for cancer,[20,21] a factor stimulating tumor growth and as morphoregulatory molecule.[22] EpCAM protein interacts physically with CLDN7 at level of the tight junctions.[21] In the intestinal epithelium, *EpCAM* is expressed in both villi (low) and crypts (high).

Here, we reported three different *EpCAM* mutations, one not previously described, in four Italian patients with CTE. Both heterozygous mutations, c.654 DelA and c.556-14A>G, detected in two siblings (P1, P2), determine the shift of the reading frame resulting in the introduction of novel amino acids followed by a premature stop codon (p.Asp219Metfs*15 and Tyr186Phefs*6 respectively) and the loss of the transmembrane domain. Recently, it has been demonstrated that this type of mutations code for proteins that fail to locate at the plasma membrane. Such mutated proteins are not detectable in the medium since they are probably degraded by the proteosome.[12]

P3 showed only one heterozygous novel missense mutation, c.437T>A, that causes the replacement of Ile to Asn at position 146 (p.Ile146Asn). The substituted amino acid forms two new hydrogen bonds with the amino acids Tyr142 and Glu147. The introduction of these new bonds seems to confirm the hypothesis that the missense mutation (p.Ile146Asn) introduces a conformational change in the protein structure that might cause misfolding. Recently, Schnell et al[12] demonstrated by generating all *EpCAM* mutants that the mutant protein is not present in cell membrane probably due to different mechanisms such as secretion, degradation and accumulation of misfolded proteins in the lumen of the endoplasmic reticulum.

SPINT2 homozygous c.488AG (p.Y163C) mutation was found in P4. The tyrosine 163 is highly conserved aminoacid within the catalytic Kunitz domain, in fact the mutated *SPINT2* protein showed a decrease in the ability to inhibit trypsin.[15]

CTE has a pattern consistent with autosomal recessive inheritance. However, in P3 mutation analysis of *EpCAM* and *SPINT2* gene revealed only one *EpCAM* heterozygous mutation (p.Ile146Asn). Exons and flanking introns testing does not exclude all possible aberrations.

We hypothesize that this patient is a compound heterozygote with the other mutation in a gene region not analyzed or in another gene. To test this second hypothesis, we also analyzed the *CLDN7* gene, as a possible candidate gene associated with tufting enteropathy. *CLDN7* codes for Claudin7 protein with similar function and physically interacting with the EpCAM.[20] Moreover, a recent study revealed that *claudin*-7-deficient mice display severe defects in intestinal mucosal architecture.[23] Interestingly, *EpCAM* knock-out pups exhibited phenotypes that shared features with those seen in claudin-7 knock-out mice as well as humans with congenital tufting enteropathy caused by *EpCAM* mutations.[24,25]

CLDN7 mutation analysis in studied patients did not show alterations in the sequenced region of the *CLDN7* gene.

In conclusion, the identification of *SPINT2* mutation (c.488A>G) in our syndromic patient, confirms *SPINT2* ascribed to congenital sodium diarrhea, as the second gene associated with CTE. Our study underlines the important role of molecular screening of both *EpCAM* and *SPINT2* genes in the diagnosis of various congenital diarrheal disorders.

In the present study, we identified a novel *EpCAM* mutation increasing the mutational spectrum of allelic variants associated with this gene and better understanding the pathogenesis of disorders.

Since histological analysis is still not decisive in predicting the outcome of CTE patients,[19] the identification of new mutations contributes to the genotype-phenotype correlation and provides further information about the assessment of the clinical outcome of the patients.

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Ethical approval: Approved by the Institutional Ethics Committee of the Medical School of the University of Foggia. Written informed consent was obtained.

Competing interest: None declared.

Contributors: DM, CA proposed and designed the study. FF, GO, PMM, GP and CA enrolled and managed the patients. DM, PD, GI and GM performed the experiments and analyzed the data. DM, CA wrote draft and revised the manuscript. DM is the guarantor.

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