



Short Communication

## Genotype-phenotype correlation in cystic fibrosis patients bearing [H939R;H949L] allele

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### Abstract

Cystic fibrosis (CF) is caused by CFTR (cystic fibrosis transmembrane conductance regulator) gene mutations. We ascertained five patients with a novel complex CFTR allele, with two mutations, H939R and H949L, inherited *in cis* in the same exon of CFTR gene, and one different mutation per patient inherited *in trans* in a wide population of 289 Caucasian CF subjects from South Italy. The genotype-phenotype relationship in patients bearing this complex allele was investigated. The two associated mutations were related to classical severe CF phenotypes.

*Key words:* CFTR, complex allele, cystic fibrosis, phenotype.

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Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in the Caucasian population and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Ratjen and Döring, 2003; Lommatzsch and Aris, 2009). This disease has a highly variable clinical presentation with the classical form of CF involving multiple organs, including recurrent respiratory infections, elevated sweat chloride levels, early-onset pancreatic insufficiency, and infertility in males; CFTR-related disorders (CFTR-RD) are estimated to account for up to 10% of clinical cases, and symptoms may only affect one organ (Boyle, 2003; Dequeker *et al.*, 2009). The CF phenotype is highly heterogeneous even between siblings carrying identical CFTR mutations, suggesting that the disease severity is affected by the CFTR genotype as well as by environmental and other genetic factors, such as alternative genomic loci containing modifier genes (Rowntree and Harris, 2003). Over 1700 mutations and polymorphisms have been identified throughout the CFTR gene (Cystic Fibrosis Mutation Database), and about one-half are rare mutations leading to an amino acid substitution into the protein (missense mutations). The number of complex CFTR alleles identified, including double-mutant alleles with two mutations inherited *in cis* in the same allele has also been growing (Hojo *et al.*, 1998; Abramowicz *et*

*al.*, 2000; Clain *et al.*, 2001; Rholf *et al.*, 2002; Niel *et al.*, 2006; Krasnov *et al.*, 2008, Taulan *et al.*, 2009; Tümmler *et al.*, 2009; de Prada Merino *et al.*, 2010; Lucarelli *et al.*, 2010). They are thought to affect the expression of the phenotype by modulating the effect of mutations: the combination of two missense mutations on the same allele has been described clinically either to improve (Dork *et al.*, 1991; Duarte *et al.*, 1996) or worsen (Hojo *et al.*, 1998) the phenotype of CF patients with regard to the most common mutation alone. In some cases the double-mutant allele has been related with a severe phenotype (Savov *et al.*, 1995; Duarte *et al.*, 1996), while in other cases complex alleles were not associated with classical form of CF (Brugnon *et al.*, 2004). Thus, further data are needed to clarify their functional role. Our Clinic is the referral Center in the Apulia Region of Italy for the diagnosis and follow-up of CF with more than 250 patients examined yearly (only some new diagnoses) in our Institute. In 2005, we have described for the first time in the CF mutation database, the missense mutation H949L, a nucleotide change of A to T at base pair 2978 in exon 15 of CFTR gene, resulting in a substitution of histidine residue to leucine at codon 949, as potentially disease-associated allele variation. Lately, McGinniss *et al.* reported that a 12 year-old male patient carrying the same mutation with a combined dinucleotide TG repeat (of not specified number of repeats) at the intron 8 – exon 9 junction and a thymidine nucleotide tract of 5 nucleotides (5T), had a clinical phenotype suggestive of CFTR-RD, with sinus problem, and an equivocal sweat chloride test result (McGinniss *et al.*, 2005). Notably, it has been demonstrated that the length of

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this poly-T at exon 9 splice site of CFTR gene correlated with the modulation of the exon 9 skipping and with the full length CFTR RNA expression (Chu *et al.*, 1993), and specifically that the splicing variant 5T allele associated with partial penetrance of CF disease (Rave-Harel *et al.*, 1997). The Cystic Fibrosis Mutation Database lists the H939R missense mutation, a nucleotide substitution of A to G at base pair 2948 in the same exon of the mutation H949L, corresponding to a histidine to arginine amino acid change at codon 939. The Authors also described the clinical phenotype of the patient, a 17 years old male, with the F508del/H939R genotype, mild expression of a chronic lung disease, pancreatic sufficiency, and unequivocally positive sweat chloride test result.

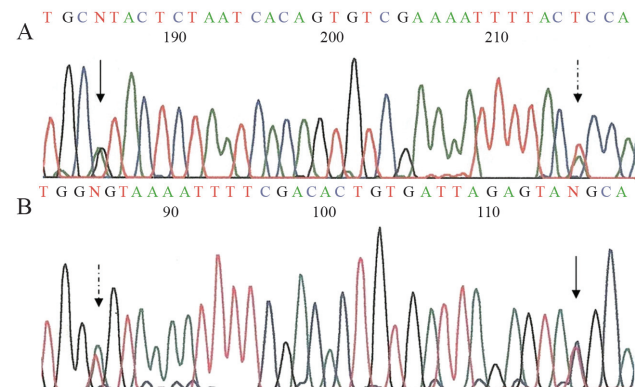
In this study we evaluated the contribution to the phenotype of the two CF-associated allele mutations [H939R;H949L], combined *in cis* in the same exon 15 of CFTR gene, which we observed for the first time in 5 unrelated CF patients.

Case histories referring to 289 Caucasian patients [140 male, 149 female, median age 16 years, (range 1-46)], who attended since 1996 our CF Center at the Paediatric Department of the University of Bari, were examined. The diagnosis of CF was established on the basis of the results of 2 sweat chloride tests (> 60 mEq/L) by Gibson and Cooke procedure, and the identification of two CF-disease causing mutations *in trans* (Rosenstein and Cutting, 1998; Kulczycki *et al.*, 2003; De Boeck *et al.*, 2006; Castellani *et al.*, 2008; Farrel *et al.*, 2008; Dequeker *et al.*, 2009). The following information was collected: gender, age at diagnosis of CF, CFTR genotype, body mass index (BMI), results of pulmonary function tests [median forced expiratory volume in one second (FEV1), expressed as percentages of predicted values], and exocrine pancreatic status. We adopted the Shwachman-Kulczycki score, one of the most widely used scores, to assess health parameters in our CF patients (Shwachman and Kulczycki, 1958). The Brasfield scoring system based on plain film radiographic findings was also recorded (Grum and Lynch, 1992). Sputum swabs were obtained from each patient during routine visits (most patients were regularly examined at 4 month intervals) as well as twice during hospitalization. Chronic airways infection was defined as the presence of *Staphylococcus aureus* and/or *Pseudomonas aeruginosa* and/or *Burkholderia cepacia* in the sputum cultures in three consecutive samples collected in a 6-month period (Brett *et al.*, 1992). Informed consent to collect DNA was obtained from the patients, or, in case of minors, from parents or guardian. Genomic DNA was extracted from peripheral blood leukocytes by standard inorganic methods, using commercial Kit (Genomic DNA Purification Kit -Fermentas Life Sciences, Canada). All CF patients were examined for the most frequent CF mutations using the RDB (Reverse Dot Blot) commercial kit (InnoLipa CFTR 19, InnoLipa CFTR 17+TnUpdate, InnoLipa CFTR-Italian regional-Innogenetics, Ghent, Belgium). A

complete scan of the 27 coding/flanking sequences of the CFTR gene was performed to identify CF alleles carrying unknown mutations, either by denaturing gradient gel electrophoresis (DGGE), using primers and conditions described elsewhere (Fanen *et al.*, 1992; Costes *et al.*, 1993) or by denaturing high performance liquid chromatography (DHPLC) (Le Marechall *et al.*, 2001). Abnormal DGGE and/or DHPLC patterns were followed by automated DNA sequencing using ABI Prism 3100 (Applied Biosystems, Warrington, UK). In most cases, the *cis* versus *trans* status of the alterations was obtained by familial segregation assessment, also by using DGGE and automated sequencing tools. Sputum samples from all patients were mixed with equal volumes of 1% dithiothreitol (Merck, Darmstadt, Germany) before incubation at 37 °C for 30 min, and cultured isolates were identified by the Phoenix (Becton Dickinson, Sparks, MA, USA) automated system (modified protocol from Efthimiadis *et al.*, 2002).

Detailed information on CFTR genotype, clinical, biological, and functional data from enrolled CF patients, excluding those carrying novel complex alleles, will not be provided because of the different focus of the present report; however this information is available upon request.

During the genetic characterization of the 289 enrolled CF patients a new complex allele [H939R;H949L] (Human Genome Variation Society nomenclature c:[2816A>G;2846A>T] <http://www.hgvs.org/mutnomen>) was found in five unrelated patients, in whom the two CF-associated mutations, H939R and H949L, were both carried in the exon 15 on the same allele, as showed in Figure 1. The characteristics of these five patients are summarized in Table 1. The segregation analysis showed that the mother was the carrier of the complex allele [H939R;H949L] in four cases (patients 2, 3, 4 and 5; Table 1), while only in one case (patient 1) the father carried this complex allele (Table 1). We did not find patients bearing the H939R or the H949L mutations alone.



**Figure 1** - Sequence electropherograms showing the complex allele [H939R;H949L] in CFTR exon 15, (A) forward and (B) reverse (Forward primer: TCAGTAAGTAAC TTTGGCTGC; Reverse primer: CCTATTGATGGTGGATCAGC). Continuous arrows show the H939R mutation while the dashed arrows show the H949L mutation.

**Table 1** - Clinical features of five unrelated patients with the complex allele [H939R;H949L].

Patients characteristics*	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Mutation in trans with [H939R;H949L]	R248T	G542X	1259insA	G1349D	F508del
Sex	male	male	male	male	male
Present age (years)	15	15	17	20	25
Age at diagnosis (years)	14	3	0	10	10
Airways colonization	No	SA	SA	SA	PA, BC
Age of first colonization (years)	/	9	6	12	14
BMI (kg/m <sup>2</sup> )	21.9	17.0	15.1	17.6	17.5
FEV1 as % predicted	84.4	114.8	80.9	93.2	53.7
Sweat chloride concentration (mEq/L)	78	100	108	92	95
S-K score	100	70	60	75	40
Brasfield score <sup>‡</sup>	N/A	5	11	7	21
Pancreas status	PS	PI	PI	PI	PI
Diagnosis	CFTR-RD	CF	CF	CF	CF

SA = *Staphylococcus aureus*, PA = *Pseudomonas aeruginosa*, BC = *Burkholderia cepacia*; N/A = not applicable; S-K = Shwachman-Kulczycki: the system is based on four parameters (general activity, physical examination, growth and nutrition and chest radiograph x-ray), and is rated as a) excellent: 86-100 b) good: 71-85, c) mild: 56-70, d) moderate: 41-55, and e) severe: < 40 (Shwachman and Kulczycki, 1958); <sup>‡</sup>scoring system from 3 “mild” to 25 “most severe” (Brett *et al.*, 1992) after x-ray; PS/PI = Pancreatic sufficiency/insufficiency. CF = cystic fibrosis; CFTR-RD = CF transmembrane conductance regulator-related disorder.

\*Patients' enrolment was done based on symptoms.

Patient 1 showed CF-related symptoms and signs restricted to one organ. In fact he presented only hepatopathy with high levels of transaminases combined with elevated sweat chloride concentration. His Shwachman-Kulczycki score was excellent; he showed a good nutritional status and pancreatic sufficiency. The diagnosis of this disease was made relatively late. The other four patients had signs and symptoms consistent with classic CF, including chronic lung and sinus disease, recurrent respiratory infections, failure to thrive and pancreatic insufficiency. They all showed abnormal sweat chloride values. Particularly, patient 3 presented with meconium ileus as a neonate, and patient 5 showed the most severe pulmonary manifestation of CF with generalized hyperinflation and diffuse nodular, cystic and coarse reticular opacities in the lung, with abnormal values on tests of lung function (Table 1).

During our screening analysis we also found thirteen males affected by congenital bilateral absence of vas deferens (CBAVD) bearing the intron 8 (IVS-8) variants TG13-T5 and TG12-T5 in compound heterozygosity with associated CF-causing mutations, and 2 sisters (of 7 and 9 years old respectively) carrying the [R668C;G576A] complex allele in compound heterozygosity with F508del CF mutation, showing a borderline sweat chloride test, recurrent asthmatic bronchitis and pancreatic sufficiency.

Complex disease alleles are rare (we have found only few cases screening a wide population of CF patients from South Italy) and very interesting (the second mutation *in cis* can modulate the effect of the first or viceversa). To our knowledge the complex allele [H939R;H949L] and its correlation to the CF phenotype were not previously described.

In the afore mentioned database the R248T mutation that we found in patient 1 was described as “mild”, occurring in male patients with CBAVD and no other signs or symptoms, even when associated with another severe mutation. The other four patients were compound heterozygotes respectively for G542X, 1259insA, G1349D, F508del and the two associated mutation in exon 15 [H939R;H949L]. The mutations G542X, 1259insA, G1349D, F508del have already been described as severe CF-associated mutation (Casals *et al.*, 1993; Morral *et al.*, 1993; Morral *et al.*, 1994; Kerem *et al.*, 1995; Estivill *et al.*, 1997; Shrimpton *et al.*, 1997; Rowntree and Harris 2003; Bompadre *et al.*, 2007; Castellani *et al.*, 2008). Particularly, 1259insA and G1349D represent with few other mutations, 4382delA, I502T, 852del22, 4016insT, D579G, R1158X and L1077P, almost 20% of the CF alleles found in the Apulian population (Castaldo *et al.*, 2005; Polizzi *et al.*, 2005). The 1259insA results in the increase of a string of four As into five, which leads to the premature termination of product due to the formation of a stop codon, as described by Shrimpton *et al.* (defective protein production, class I mutation) (Shrimpton *et al.*, 1997). Also the G542X prevents the synthesis of full-length, normal CFTR protein due to the creation of a premature termination codon (Rich *et al.*, 1993; Rowntree and Harris, 2003). On the other hand, the F508del mutation, a deletion of three bases encoding a phenylalanine residue at position 508 within the first nucleotide binding domain (NBD), affects CFTR maturation (class II mutations) (Rowntree and Harris, 2003), while the G1349D plays a role in ATP-dependent opening of the chloride channel, resulting in a defective CFTR activation



and regulation (class III) (Bompadre *et al.*, 2007). We speculate that both mutations H939R and H949L might affect the second NBD of CFTR and have a role in altering the conductance of the chloride channel, but to our knowledge there are no reports on their functions.

In our study, the four patients carrying the complex allele [H939R;H949L] associated *in trans* with the severe mutations G542X, 1259insA, G1349D and F508del presented the classic CF phenotype. On the contrary, patient 1 who carried the same complex allele with the R248T mutation showed a CFTR-RD (Table 1). This is likely due to the fact that R248T is a mild mutation (thought to affect CFTR mRNA splicing based on the database: Cystic Fibrosis Mutation Database), and subjects carrying this mutation might have a residual function of the CFTR protein. It seems that the complex allele [H939R;H949L] greatly reduces the residual function of CFTR and, when also on the other allele is present a severe mutation which produces a very low residual function, the combined effect is an overall great reduction of CFTR functionality; on the contrary, when the other allele carries a mild mutation, the overall effect is a cumulative greater CFTR functionality.

We also found in our CF population subjects carrying the variant tracts TG13-T5 and TG12-T5, which have been already described in males with CBAVD in the literature (Castellani *et al.*, 2008; Dequeker *et al.*, 2009), and the [R668C;G576A] complex allele. The R668C in exon 13 is considered a polymorphism (Pignatti *et al.*, 1994) while the G576A, in CFTR exon 12, seems to induce a variable extent of exon skipping that leads to reduced levels of normal CFTR transcripts (Pagani *et al.*, 2003).

The complex alleles and their role in disease pathogenesis still remain a challenge for both researchers and clinicians, thus more information on our newly discovered complex allele [H939R;H949L] or on the H939R and the H949L mutations alone would help to study the effect on the phenotype of these rare mutations.

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## Internet Resources

Cystid Fibrosis Mutation Database: <http://www.genet.sickkids.on.ca/cftr> (July 7, 2010).

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