RESEARCH NOTE

Angiotensin-converting enzyme insertion/deletion gene polymorphism in cystic fibrosis patients

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Introduction

Cystic fibrosis (CF) is the most common recessive autosomal disease in Caucasian population, caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Bremer *et al.* 2008). CF affects a number of organs but its effects on the lung constitute the major cause of morbidity and early mortality. Disease variability expression in patients bearing the same combination of mutations emphasizes the role of genetic background (modifier gene) and environment (Cutting 2005).

The angiotensin-converting enzyme (*ACE*) gene was selected as a possible modifier gene for CF because of the proinflammatory activity of the ACE protein (Marson *et al.* 2012). The ACE enzyme is an important vasoconstrictor and stimulant of aldosterone; it catalyzes the transformation of angiotensin I to angiotensin II peptide and is involved in the blood pressure control, and the electrolyte balance of blood (Arkwright *et al.* 2003). The *ACE* gene is located in the 17q23.3 region of intron 16, a polymorphism based on the insertion or deletion of a 287-bp ALU repeat sequence resulting in three genotypes: DD and II homozygous and ID heterozygous (Marson *et al.* 2012).

The aim of this work was to study the role of the *ACE* gene I/D polymorphism in the severity of the clinical expression of cystic fibrosis in a Tunisian CF population.

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Materials and methods

Patients

This study included 115 Tunisian CF patients aged between three days and 17 years with a median of six months. The CF patients analysed were 49 males (42.60%) and 66 females (57.39%) with 0.74 as sex ratio. The selection was based on typical clinical manifestations of lung or/and gastrointestinal disease, and high levels of sweat chloride concentration (higher than 60 mmol/L). The sweat test was conducted by pilocarpine iontophoresis (Exsudose method). A control group consisting of 80 healthy subjects aged between one month and 15 years was also studied.

The parents of minor patients and all patients signed informed consents. The work was carried out in accordance with the Ethical Guidelines of the World Medical Association Declaration of Helsinki and was approved by the Ethical Committee of the Children's Hospital of Tunis.

Genomic DNA was extracted from peripheral blood with the classical salting-out protocol (Miller *et al.* 1988).

Determination of mutations in the CFTR gene

CFTR mutations were previously identified in the biochemistry and research laboratory of Children's Hospital (Tunis, Tunisia) using denaturing high performance liquid chromatography (DHPLC), denaturing gradient gel electrophoresis (DGGE) and direct sequencing (Messaoud *et al.* 2005; Fredj *et al.* 2009; Boudaya *et al.* 2013). Different CFTR genotypes were determined: 91 patients (79.13%)

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with two identified mutations (46.08% F508del/F508del, 10.43% E1104X/E1104X, 2.60% 711+1G → T/711 + 1G → T, 3.47% W1282X/W1282X, 2.60% N1303K/N1303K, 1.73% W19X/W19X, 0.86% 4268 + 2T → G/4268 + 2T → G, 1.73% G542X/G542X, 0.86% E1104X/3729 delAinsTCT, 3.47% F508del/N1303K, 3.47% F508del/G542X, 1.73% F508del/1104X and 0.86% F508del/R785X), eight patients (6.95%) with one identified mutation (4.34% F508del/undetermined, 1.73% E1104X/undetermined and 0.86% W19X/undetermined). In 16 cases (13.91%), the CFTR mutations remained unidentified.

ACE gene I/D polymorphism analysis

Genotyping of the insertion/deletion polymorphism in angiotensin I-converting enzyme gene (ID: rs1799752) was performed using polymerase chain reaction (PCR) with the following primers: Hace3s/5′-gccctgcaggtgtctgcagcatgt-3′ and Hace3as/5′-ggatggctctccccgccttgtctc-3′ (Castellano *et al.* 1995). PCR amplification was carried out with 200 ng of template DNA, 0.166 μM of each oligonucleotide primer, 0.33 mM dNTPs, 2.5 mM $MgCl_2$, 0.625 U *Taq* polymerase and 1× buffer PCR. DNA was amplified in 30 PCR cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 30 s, followed by final extension at 72°C for 7 min. PCR products were separated by agarose gel electrophoresis (2%). The insertion of Alu repeat, allele (I), was detected as a 597 bp and the deletion, allele (D), was visualized as a 319 bp band (genotypes described as II-597 bp, ID-597 + 319 bp and DD − 319 bp).

To avoid a possible mistyping of the ID heterozygous as DD homozygous, all DD genotypes were reamplified using an insertion-specific primer pair, Hace5c/5′-tcgcgagccctcc catgcccataa-3′ and Hace5a/5′-tgggaccacagcgcccgccactac-3′ (Shanmugam *et al.* 1993).

PCR conditions were similar to those used for I/D detection. PCR generated a 335 bp fragment in the presence of I allele.

Statistical analysis

Onset of manifestations, pulmonary and digestive symptoms, pancreatic insufficiency and meconium ileus (MI) were analysed. Statistical analysis was performed using ver. 20.0 of the statistical package for the social sciences software: SPSS (SPSS, Chicago, USA). Data were presented as means, medians for variables with a skewed distribution. The differences between the two groups were evaluated by the non-parametric Mann–Whitney test. Qualitative variables were assessed with Pearson's χ^2 test. Allele frequencies, genotype frequencies, odds ratio (OR) and 95% confidence interval (CI) were all estimated. Hardy–Weinberg equilibrium (HWE) was calculated. The analysis was carried out at 5% level of signification and value of $P < 0.05$ was considered to be significant.

Results and discussion

Genotypes and alleles frequencies of the I/D polymorphism in CF patients and controls are shown in table 1. The D allele frequency was higher compared with I allele in both groups (68.70% versus 31.30% in CF patients and 65% versus 35% in controls), whereas there were no significant difference in genotypes ($\chi^2 = 0.730$; $P = 0.694$) and alleles ($\chi^2 = 0.584$; $P = 0.444$) frequencies between the two groups were observed (table 1). This finding is in accordance with a previous study conducted in a Tunisian population of healthy subjects and patients with diabetes type 2 and others with coronary artery disease. Similar results were also reported in cystic fibrosis in various English and Tunisian studies (Arkwright *et al.* 2003; Mehri *et al.* 2010; Abdelhedi *et al.* 2013; Sahli *et al.* 2014). The distribution of the *ACE* I/D genotype was in HWE in both the groups ($\chi^2 = 0.563$, $P = 0.453$ in CF patients and $\chi^2 = 0.009$, $P = 0.921$ in controls). The distribution of ACE I/D genotype in patients with unidentified CFTR mutation ($n = 16$) was similar to that found in the total patients: 62.50% DD, 31.25% ID and 6.25% II. This result compared with the control group shows no significant difference in genotype distribution with: $\chi^2 = 2.208$; $P = 0.331$. No statistical difference was observed between the 16 patients and healthy subjects in allelic distribution $\chi^2 = 2.083$; $P = 0.148$.

According to the CF genotype, we classified the CF population into three groups . The first group ($n = 53$) made up of patients homozygous for the most frequent mutation F508del. The second group composed of 16 patients with compound heterozygous F508del/other mutation. The last group of subjects ($n = 46$) includes homozygous or compound heterozygous for mutations other than F508del.

Table 1. Genotype and allele frequencies of the *ACE* I/D polymorphism in CF patients and healthy controls.

ACE I/D polymorphism	CF patient	Healthy controls	ORs	95% Cl	*P* value
DD%	48.70	42.5	1.284	0.722–2.281	0.393
ID%	40	45	0.814	0.457–1.451	0.486
II%	11.30	12.5	0.892	0.376–2.148	0.799
Statistics		$\chi^2 = 0.730$, $P = 0.694$*			
D%	68.70	65	1.181	0.770–1.812	0.444
I%	31.30	35	0.846	0.551–1.298	0.444
Statistics		$\chi^2 = 0.584$, $P = 0.444$*			

OR, odds ratio; CF, cystic fibrosis; CI, confidence interval.

The distribution of CF mutations, *ACE* gene I/D polymorphism and clinical variables among groups of CF patients are shown in table 2. Our results revealed that the D allele prevalence was higher than the I allele in both CF patients and control groups. These data are in accordance with similar studies in Brazilian and UK CF patients (Arkwright *et al.* 2003; Marson *et al.* 2012). Similarly, our study revealed no significant difference in ACE genotype distribution compared to those of Brazilian and UK CF patients (Arkwright *et al.* 2003; Marson *et al.* 2012). We found that patients with DD genotype were associated with an earlier onset of symptoms ($P = < 0.001$) and an early inflammation and deterioration of the lung ($\chi^2 = 32.98$, $P < 0.001$, OR = 9.88) (table 2). These data could be explained by the higher gene expression associated with the DD genotype. This expression would promote a higher inflammatory response, leading to the early age of symptoms. This observation suggests the role of the *ACE* gene as a modifier gene of cystic fibrosis.

On the other hand, for the first time, we noticed in our series that the MI was present only in patients with heterozygous ID genotype. Therefore, it seems that this genotype is a risk factor for developing MI ($\chi^2 = 4.96$ $P = 0.025$). In our study, four new-borns had MI. We did not identify any mutations in these patients. The remaining newborns had mutations of classes I and II (F508del/F508del, F508del/G542X, E1104X/E1104X and W1282X/W1282X). This may suggest that the predisposition to MI could be associated with severe CF mutations when inherited with ID genotype. This new observation is difficult to explain at this stage. It could be due to the few research works interested in the analysis of the involvement of the *ACE* gene in the development of IM in CF patients. Further studies including a higher number of CF patients with MI may, therefore, determine the role of *ACE* gene as a modifier gene to MI in CF.

ACE I/D polymorphism does not appear to modulate digestive and pancreatic symptoms in CF patients, with no significant difference observed (table 2). We found that DD genotype associated with F508del/F508del mutation was responsible for early occurrence of disease and lung symptoms, respectively ($P = < 0.001$ and $P = 0.003$, OR=5.70; table 2). Moreover, significantly high prevalence of lung disease was observed with DD ACE polymorphism in both the other groups of CF patients. The same results for the pulmonary symptoms were found in the group of patients with unidentified mutations ($P = 0.017$).

In our study, we focussed on the implication of I/D polymorphism in the severity of cystic fibrosis. We first showed that CF patients with DD genotype had a higher risk for the deterioration of the lung function accompanied with an early onset of symptoms. Second, we noticed that patients with ID genotype had more risk to develop MI. Based on these results and those of previous studies, further work is needed to determine whether ACE inhibitors may alleviate pulmonary symptoms in patients with CF and verify the

Table 2. Association of *ACE* gene D/I polymorphism with clinical symptoms of patients in consideration of CFTR mutations.

CF mutations	ACE genotype	Onset of manifestations (months)				Lung symptoms				Digestive symptoms				PI				MI			
		Median	Min.	Max.	*P*	P*	A	*P*	OR; CI 95%	P*	A	*P*	OR; CI 95%	P*	A	*P*	OR; CI 95%	P*	A	*P*	
F508del /F508del	DD	3.300	1	18	< **0.001**	19	6	**0.003**	5.70; 1.71-18.92	12	13	0.364	1.66; 0.55-4.99	9	16	0.767	1.18; 0.38-3.70	0	25	0.340	
	ID+II	31.139	2	120		10	18		0.17; 0.05-0.58	10	18		0.60; 0.20-1.81	9	19		0.84; 0.26-2.62	1	27		
F508del /other mutations	DD	4	2	8	0.277	6	1	**0.002**	48; 2.46-932.90	2	5	0.374	3.20; 0.22-45.19	4	3	0.614	1.66; 0.22-12.22	0	7	0.362	
	ID+II	38	11	108		1	8		0.02; 0-0.40	1	8		0.31; 0.02-4.41	4	5		0.60; 0.08-4.39	1	8		
Other mutations/ other mutations	DD	11.160	1	84	0.504	21	3	< **0.001**	18.66; 4.03-86.28	7	17	0.602	0.72; 0.20-2.48	7	17	0.845	0.88; 0.25-3.10	0	24	0.061	
	ID+II	17	0.3	53		6	16		0.05; 0.01-0.24	8	14		1.38; 0.40-4.77	7	15		1.13; 0.32-3.98	3	19		
Total	DD	9.151	1	84	< **0.001**	46	10	< **0.001**	9.88; 4.04-24.13	21	35	0.551	1.26; 0.58-2.72	20	36	0.838	1.08; 0.50-2.33	0	56	**0.025**	
	ID+II	22.300	0.3	120		17	42		0.10; 0.04-0.24	19	40		0.79; 0.36-1.70	20	39		0.92; 0.42-1.98	5	54		
Unidentified mutation/ unidentified mutation	DD	5	1	84	0.827	9	2	**0.017**	18 ; 1.24-260.93	4	7	0.119	–	2	9	0.350	0.33; 0.03-3.51	0	11	0.125	
	ID+II	18	1	42		1	4		0.05; 0-0.80	0	5		–	2	3		3; 0.28-31.63	1	4		

P*, presence; A, absence; CF, cystic fibrosis; PI, pancreatic insufficiency; MI, meconium ileus; Significant *P* values are in bold.

proinflammatory activity in a larger CF population with homogenous CFTR mutations.

References

Abdelhedi R., Kharrat N., Abdelmoula B. N., Abid L., Abdelmouleh W., Trabelsi Sahnoun I. *et al.* 2013 Lack of association of NOS3 and ACE gene polymorphisms with coronary artery disease in southern Tunisia. *Biochem. Genet.* **51**, 92–100.

Arkwright P. D., Pravica V., Geraghty P. J., Super M., Webb A. K., Schwarz M. *et al.* 2003 End-organ dysfunction in cystic fibrosis: association with angiotensin I converting enzyme and cytokine gene polymorphisms. *Am. J. Respir. Crit. Care Med.* **167**, 384–389.

Boudaya M., Fredj S. H., Siala H., Bibi A. and Messaoud T. 2013 Identification of a cystic fibrosis mutation W19X in Tunisia. *Ann. Biol. Clin.* **71**, 223–226.

Bremer L. A., Blackman S. M., Vanscoy L. L., McDougal K. E., Bowers A., Naughton K. M. *et al.* 2008 Interaction between a novel TGFB1 haplotype and CFTR genotype is associated with improved lung function in cystic fibrosis. *Hum. Mol. Genet.* **17**, 2228–2237.

Castellano M., Muiesan M. L., Rizzoni D., Beschi M., Pasini G., Cinelli A. *et al.* 1995 Angiotensin-converting enzyme *I*/*D* polymorphism and arterial wall thickness in a general population. *Circulation* **91**, 2721–2724.

Cutting G. R. 2005 Modifier genetics: cystic fibrosis. *Annu. Rev. Genomics Hum. Genet.* **6**, 237–260.

Fredj S. H., Messaoud T., Templin C., des Georges M., Fattoum S. and Claustres M. 2009 Cystic fibrosis transmembrane conductance regulator mutation spectrum in patients with cystic fibrosis in Tunisia. *Genet. Test. Mol. Biomarkers* **3**, 577–781.

Marson F. A. L., Bertuzzo C. S., Hortencio T. D. R., Ribeiro J. D., Bonadia L. C. and Ribeiro A. F. 2012 The ACE gene D/I polymorphism as a modulator of severity of cystic fibrosis. *BMC Pulm. Med.* **12**, 41–49.

Mehri S., Koubaa N., Hammami S., Mahjoub S., Chaaba R., Nakbi A. *et al.* 2010 Genotypic interactions of renin–angiotensin system genes with diabetes type 2 in a Tunisian population. *Life Sci* **87**, 49–54.

Messaoud T., Bel Haj Fredj S., Bibi A., Elion J., Férec C. and Fattoum S. 2005 Molecular epidemiology of cystic fibrosis in Tunisia. *Ann. Biol. Clin.* **63**, 627–630.

Miller S. A., Dykes D. D. and Polesky H. F. 1988 A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16**, 1215.

Sahli C., Fredj S. H., Siala H., Bibi A. and Messaoud T. 2014 First study of angiotensin converting enzyme in cystic fibrosis Tunisian patients. *Clin. Chem. Lab. Med.* **52,(Suppl.)**, 211–215.

Shanmugam V., Sell K. W. and Saha B. K. 1993 Mistyping ACE heterozygotes. *PCR Methods Appl* **3**, 120–121.

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