Reviewer’s report

Title: ACE gene D/I polymorphism as a modulator of severity of cystic fibrosis

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Reviewer: SALMO RASKIN

Reviewer’s report:

- Major Compulsory Revisions

MATERIALS AND METHODS

Authors say that “Diagnosis of CF was confirmed in patients with two doses of sodium and chloride in the sweat with values of greater than 60 mEq/L and/or by the finding of two mutations in the CFTR gene.” But there is no information in the entire text about patients CFTR genotypes, which is VERY important. The only clue I could find was in Table 2, as authors show that there were 50 patients with only one identified mutation (class I, II or III) in the CFTR gene. I could not find how many from the 180 patients of the cohort had neither of the two CTR mutations identified. In Statistical Analysis, authors say that “All mutations observed in our study were included in classes I to III, thus, patients with two identified mutations, presented no influence of CFTR gene in analysis.” But how many from the 180 patients in the cohort had indeed two identified Class I-III CFTR mutations? It is understandable that due to the rarity of CF patients with the same two mutations genotypes, studies of genes influencing the main locus (CFTR) are very difficult to be done Therefore it is also understandable that many authors are including all patients with Class I, II and II CFTR mutations “as a single group”. Authors of this work say that “All mutations observed in our study were included in classes I to III, thus, patients with two identified mutations, presented no influence of CFTR gene in analysis.”. I would be more careful to make this generalization, but more important, in this work, seems to me that at least 50 patients did not have at least one CFTR mutation identified, and I believe that this patients, as well as patients with no CFTR mutations identified, should not be included in the cohort, because they could have one or two “mild” (Class IV, V or VI) CFTR mutations, and this would introduce a very strong bias to the study. So, in my opinion, authors should inform all patients CFTR genotype in a Table, and exclude from the final cohort the ones with only one CFTR mutation identified as well as patients with no CFTR mutations identified. Analysis should be done only in the patients that are homozygous or compound heterozygous for two CFTR identified Class I-III mutations. I understand that this criteria may reduce the sample size of the cohort up to a point of turning the work unviable, but I think the only way to try to escape of the huge obstacle of the rarity of CF patients with the same two CFTR mutations, is through working with a Consortium of Centers, to get a larger sample of patients with both CFTR mutations identified.
RESULTS AND DISCUSSION

The relative frequencies of polymorphisms of ACE were not tested in local controls without CF and were not compared with published frequencies of these polymorphisms in normal Brazilian populations, to test if there were significant differences between the frequencies of these alleles and genotypes in this CF cohort when compared to control subjects. ACE polymorphism frequencies in 70 controls that live in the same city were the authors and CF patients live, were previously published in the literature and should be used (Yugar-Toledo JC et al. Gene variation in resistant hypertension: multilocus analysis of the angiotensin 1-converting enzyme, angiotensinogen, and endothelial nitric oxide synthase genes. DNA Cell Biol. 2011 Aug;30(8):555-64.)

Authors say that “The ACE gene D/I polymorphism showed a higher frequency for allele D (154 patients) compared with allele I (108 patients). But if there were 180 patients, then there were 360 alleles...What was the frequency of allele D and allele I among the 360 alleles typed? I understand that the genotype frequencies were: 84 (46.4%) (or 46.7%??) D/I, 73 D/D (40.3%) (or 40.5%??) and 24 (13.3%) I/I. But 84+73+24= 181 patients and not 180...Besides, allele and genotypes polymorphism frequencies were not tested to see if they conform to the Hardy–Weinberg equilibrium, to test any possible bias in sampling in this population, such as the presence of sub-structure, or problems with genotyping.

CONCLUSION

Authors conclude that “An association between the D allele in the ACE gene with the severity of CF was found in our study.” In Results, authors show that “There was association of the genotype D/D with early initiation of clinical manifestations (OR: 1.519, CI: 1.074 to 2.146), bacterium Burkholderia cepacia colonization (OR: 3.309, CI: 1.476 to 6.256), and Bhalla score (SB) (p= 0.015). Would this 3 associations, even if true, be enough to conclude that the ACE polymorphism is associated with severity of CF? How to explain that in more then 20 other clinical variables studied, no association was found, including the Kanga and Shwachman-Kulczycki score?

- Minor Essential Revisions

ABSTRACT

In Abstract/Results, authors define Bhalla Score as (SB), but latter use “BS” instead of “SB” in the entire text;

MATERIALS AND METHODS

Authors say that “Two hundred and fifteen patients were selected for the study. Twenty-five patients without clinical data for statistical analysis and those who did not sign the consent were excluded”. So I understood that 190 CF patients were included in the study. But latter in the text, only in RESULTS, authors say that
“From a population of 215 patients attending our Center, 35 were excluded for lack of data and collection of material, resulting in 180 patients analyzed. I suggest that all these exclusions should be explained in Materials and Methods.

Authors should include the full references of the methods used for CFTR genotyping;

Authors say that “Each initial sample with D/D genotype passed to a second PCR reaction. Primers used: Hace 5a, 5'-TGG GAC CAC-AGC GCC CGC CAC TAC-3' and hace 5c, 5'-TCG CCA GCC CTC CCA TGC CCA TAA-3'. What is the exact reference for this new set of primers and why they were not used as first option of the authors, if it seems that with this set of primers and a higher annealing temperature, there was no preferential amplification of allele “D”?

It is not clear in the text how did the authors figure out that there was a preferential amplification of D allele in heterozygous individuals. Did they know this by literature or personal experience before they started to genotype the samples? Or they figure out in the initial genotyping by the different intensity of the two expected bands in heterozygous? In the same context, it is not clear what did authors mean by writing “A 335 bp sequence was amplified in the presence of at least one allele” Were authors trying to say that in homozygous “D/D” patients a single 335bp sequence was amplified? And if this is the case, what was the expected fragment size in heterozygous and homozygous I/I, with the new set of primers?

RESULTS AND DISCUSSION

Authors say that from 180 subjects, Ninety (49.7%) were male,” but 90/180 is exactly half, 50% and not 49.7%;

Authors say that from 180 subjects, 165 (91.7%) were European-Caucasian derived and 15 (7.4 %) were African-derived individuals”. But 15/180 equals 8,3% and not 7,4%. Were there subjects that are not European-Caucasian derived and also not African-derived?

- Discretionary Revisions

RESULTS AND DISCUSSION

Authors say that “The main environmental factor in the clinical variability of CF is the access of patients to treatment”, and we agree. They also say that “treatment is guaranteed by the public health system, which allows equal access for all patients included in the study and it is not an additional factor in the analysis of data”, and then we disagree. As Table 1 shows, there is a 21 months mean delay between age at fist symptoms and age of diagnosis, showing the difficulties of diagnosis of CF in Brazil, and even if access to the Reference Center is free, it is obvious that patients arrive at the Center with different stages of the disease, which is a an additional factor biasing the data.

Authors say that “The ACE gene expressed the ACE protein, which acts on the
inflammatory response [8, 10]. The expression of the ACE gene is influenced by the D/I polymorphism. ACE protein levels in plasma and tissues, including the lung, is determined in part by the D/I polymorphism [12]. This information has been already informed in the “Background” section and therefore can be excluded from DISCUSSION.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

'I declare that I have no competing interests'