Peer Review File

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Reviewer A

The authors present a study investigating the impact of alpha anti-trypsin deficiency alleles on lung cancer survival. The study concludes that patients with alpha antitrypsin deficiency alleles has a higher probability of death within the first six months compared with cancer patients with wildtype genotype.

Comments:

1) A limitation of this study is the small sample size particularly of the deficiency group, Pi*≠MM with n = 28. This would imply that there is not sufficient power to pick up differences between the two groups. This could be the explanation why no significant difference was detected in any features between the groups.

- We agree with the reviewers that the low sample size may influence the results. The vast majority of deficient subjects are carriers of the S allele (26), with little representation of the severely deficient alleles. Being aware that the most frequent deficient genotype was Pi*MS would justify that there were no significant differences between the two groups in terms of their basal characteristics. On the other hand, some studies suggest that being a heterozygous carrier of an S allele is associated with an increased risk of squamous cell lung cancer, without finding a relationship with other histological types (Topic A.S., Jelic-Ivanovic Z.D., Spasojevic-Kalimanovska V.V. Association of moderate alpha-1 antitrypsin deficiency with lung cancer in the Serbian population. Arch. Med. Res. 2006;37(7):866–870.). Given our small sample size, we are not able to analyze whether there are differences between the histological type and the deficiency allele. We must highlight that our results in relation to the ATT concentration coincide with the study by Li et al., where a higher concentration of α1AT was significantly associated with worse survival.

2) A second concern is with regards to the statistical test performed on the features with multiple subcategories e.g. stage I - IV; and perhaps histologic diagnosis. My impression is that the t-test may be inappropriate for these categories. Lumping the subcategories into a single category for testing is also inappropriate if the features are not balanced in the two groups. Authors need to justify their analysis.

- In Table 1, different tests were applied according to the nature of the variable being compared, as described in the statistical analysis section. For continuous variables, Student's t-test was applied while the chi-square test was applied for categorial variables, using Fisher's test when necessary. The TNM staging variable is a categorical variable, therefore we contrasted whether the proportions for each of the categories of a group were similar to the proportions of the other group, there was no category grouping.

3) Authors only show the kaplan meier survival curve up to the first six months. Though they state that average length of follow up was 13 months. The whole data needs to be shown in the survival curve.

- The graph has been changed to show the entire patient follow-up and the results of the survival analysis have been added considering the entire follow-up period (Table 1a,b). The test is no longer significant after including the entire follow-up, it should be kept in mind that after the sixth month, there are only 10 living patients in the group with the genotype, making the statistical power insufficient to detect differences between the two groups and resulting in less robust results. We have tried to reflect this situation in the results section.

4) Authors need to show the data of the Cox proportional hazard regression models even if results are negative perhaps as supplementary data.

- The coefficients for the Cox regression model are included for the 6 months and for the entire follow-up period in the results section. The sample size is presented in a graphic for each 6 months throughout follow-up. In the methods section, we have also added the result of the proportional hazard test necessary to evaluate the assumptions of using Cox regression which, by not being significant, verifies that the assumption is met.

5) It is important to point out that nonsmall cell and small cell lung cancer are two entirely different diseases that could significantly impact the results. However authors include both in the analysis though the literature suggests that AAT impacts nonsmall cell carcinoma development. My concern is that this could confound their analysis if there is not a balance representation in the two groups. They need to justify why this was done.

- Patients with small cell tumors are balanced in the two groups, with no significant differences between them in the proportion of this characteristic. 3 patients (10.7%) in the deficient genotype group were diagnosed with a small cell tumor while 17 cases (12.5%) were detected in the non-deficient group, with a p value = 0.793. However, a subanalysis was done excluding patients with a small cell line (Table 1).

6) The study shows that plasma levels of AAT was not significantly different between the two groups unlike what is in the literature. They propose an novel mechanism for carcinogenesis but the references they give are not sufficient justification since the English language reference refers to its effect on a bleeding disorder not on cancer. A better explanation is required.

- No significant differences were found between AAT levels since the majority of the deficient subjects were carriers of an S allele (mild-very mild deficiency), which could initially go unnoticed in a basal determination. On the other hand, the high levels detected (195.3 ± 56.1 mg/dl) may be related to the proinflammatory situation of these subjects (Kueppers F. Genetically determined differences in the response of alpha-antitrypsin levels in human serum to typhoid vaccine. Humangenetik. 1968;6(3):207–214. PMID:5709080), levels that would not be significantly influenced in carriers of a deficient S allele.

Regarding the second point, cancer progression and prognosis:
In clinical reports, increased neutrophil elastase has been detected in many different types of cancer, and the concentration of neutrophil elastase is associated with cancer stage, grade, and survival. Yamashita et al. tested both free and 1-antitrypsin-combined forms of neutrophil elastase in 144 non-

small-cell lung cancers. Neutrophil elastase was detected in 115 of 144 tumor extracts, ranging from 0.21 to 23.25 microg/100 mg protein. The concentration of detected neutrophil elastase was significantly higher in those with clinical T4 disease than in those with clinical T1, T2, and T3 disease. The tumors with aortic invasion had a significantly higher neutrophil elastase concentration than those with involvement of other sites. In their early small-sample-size study, the authors reported that 34 of 40 lung cancer tissues had increased neutrophil elastase. It was significantly higher in stage IIIB than in stages I, II, IIIA, and higher in stage IIIA than in stage I. Larger tumors (T3 or T4) had a higher concentration of neutrophil elastase than smaller tumors (T1 or T2). The patients with a higher concentration of neutrophil elastase had shorter survival than those with low-level neutrophil elastase. Neutrophil elastase was a significant prognostic factor. Sun Z, Yang P. Role of imbalance between neutrophil elastase and alpha 1-antitrypsin in cancer development and progression. Lancet Oncol. 2004 Mar;5(3):182-90. Topic A, Ljujic M, Nikolic A, Petrovic-Stanojevic N, Dopudja-Pantic V, Mitic-Milikic M, Radojkovic D. Alpha-1-antitrypsin phenotypes and neutrophil elastase gene promoter polymorphisms in lung cancer. Pathol Oncol Res. 2011 Mar;17(1):75-80.

Reviewer B

1) Commas cannot be used as decimal points. Please revise Figure 1.

- Done

2) Add the age unit in Table 1 and 2.

- Done

3) Move p-value to the correct column.

Smoking status, *n* (%)				
Former smoker	58 (40.3)	44 (37)	14 (56)	0.204
Current smoker	73 (50.7)	64 (53.8)	9 (36)	
Lung cancer histology, n (%)				
Adenocarcinoma	67 (46.5)	53 (44.5)	14 (56)	
Squamous	42 (29.2)	35 (29.4)	7 (28)	0.483
Other	35 (24.3)	31 (26.1)	4 (16)	

- Done

4) Numbers in Table 2 do not add up.

- Done

5) Check if this should be changed to "Hospital Universitario Nuestra Señora de Candelaria".

46 approved by the Ethics Committee of the Hospital Nuestra Señora de Candelaria
47 (*Code PI 75-17)*. and informed consent was taken from all the patients.↵

- Done