Author's response to reviews

Title: Autoantibodies to Tumor Associated Antigens as Early Detection Biomarkers for Lung Cancer or Noncalcified Nodules

Authors:

William N Rom MD, MPH (william.rom@nyumc.org)
Judith D Goldberg ScD (jd.goldberg@nyumc.org)
Doreen Addrizzo-Harris MD (doreen.addrizzo@nyumc.org)
Heather N Watson PhD (heather.watson@nyumc.org)
Michael Khilkin DO (michael.khilkin@nyumc.org)
Alissa K Greenberg MD (alissa.greenberg@nyumc.org)
David P Naidich MD (david.naidich@med.nyu.edu)
Bernard Crawford MD (bernard.crawford@nyumc.org)
Ellen Eylers RN (ellen.eylers@nyumc.org)
Daorong Liu MSc (dliu@scripps.edu)
Eng M Tan MD (emtan@scripps.edu)

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Author's response to reviews: see over
Response to Reviewer Caroline Chapman

1. Title- misleading and does not reflect the paper.
Response: The title has been changed to “Identification of an Autoantibody Panel to Separate Lung Cancer from Smokers and Nonsmokers”

2. Abstract- There are words missing and the results stated do not agree with the results in the main body of the paper.
Response: The abstract has been changed to be consistent with the main body of the paper.

3. Introduction is insufficiently referenced. The introduction also states results of the number of trials (without numbers, normals or relevance)- it is disorganized and unfocused. They state NY-ESO abs found in ‘few cancers’ but only reference 1 paper and no number (not Sahin or Chapman). The authors talk a great deal about other cancers and far less about lung cancer- ie put in data that is not relevant to this paper and leave out other. The authors talk at length about MUC1 but it is not used in this study and should be removed. The introduction needs serious restructuring and rewriting. The authors need to focus down on lung cancer and discuss the antigens used in the study.
Response: The introduction has been reorganized, focused on lung cancer, and references to Chapman and colleagues moved forwards. MUC1 has been removed.

4. Study population: The normals tested were younger than the cancers- why?- no information was available and they were likely to be non-smokers so not a suitable match. Why were these normals selected if such a large other group was available; they also need larger numbers.
Response: We agree that the “normals” is confusing and we have used our smoking controls throughout the revised text except when we needed normative values for the ELISAs where we used a group of nonsmokers who were screened to be free of any disease.

5. 10% of the GGO were stated as having developed cancer- when?- did they have before and after bloods? Which samples were positive and which negative (results) Aabs have been described up to 5 years before the cancer- would be good to show if these patients had early aabs.
Response: 5(10.9%) of the individuals with ground glass opacities developed lung cancer on follow-up. The sera tested were from the first visit; the diagnosis of lung cancer was 2.5, 3, 4, 5 and 6.5 years after the tested specimens. We have not been able to test follow-up sera or post-operative sera. We agree with the reviewers that a prospective validation study of autoantibodies needs to be conducted including multiple clinic visits prior to diagnosis, and post-operatively.

6. No details on the lung cancers were given- stage, grade, treatment, type, etc etc. Ranges and SD of all ages need adding.
Response: There were 2 small cell lung cancers (both advanced), 4 squamous (2 stage 1A, 1 stage 2B, 1 stage 4), and 16 adenocarcinoma (13 stage 1 and 3 stage 3). None had received prior chemotherapy or neoadjuvant treatment. There were no differences in autoantibodies by type or stage due to small numbers. Our Biostatisticians preferred medians to describe the demographics.

A) What media and temperatures were the antigens grown at? Were they native preps/denature/inclusion bodies. How pure? How was purity assessed?
B) What negative control protein and positive control antibodies were used?

C) How many times were the samples assessed?

D) What was the reproducibility of the assay? How was it assessed?

E) How was the cut off for positivity determined?

F) How can the authors be sure they were not measuring an age effect?

G) How can they be sure it was specific binding- were any blots performed?

Response: See page 7 Methods third paragraph, “The specificities of the recombinant proteins of cyclin A, cyclin B1, cyclin D1 and CDK2 expressed in the above manner had been validated previously (16) as to expected migration in SDS-PAGE and reactivity to specific polyclonal antibodies. The specificities of the other recombinant proteins, p53, c-myc, survivin, and the three insulin-like growth factor 2 mRNA binding proteins have also been validated in previously reported studies (19, 23). The recombinants were full-length proteins and not polypeptide fragments. Also see page 8 bottom paragraph, “In every microtiter plate, there were a number of internal controls including a blank, a negative control, one high-positive control and one low-positive control to ensure equivalency of results from one plate to another. The cut-off delineating abnormality was the mean of 36 healthy non-smokers plus 3 standard deviations. In this study, we performed only enzyme immunoassay to detect autoantibodies to TAAs since previous studies with these antigens had shown ELIs at this cut-off level were specific and more sensitive than Western blotting (24, 25, 26).

H) 10% of the GGO developed cancer but the data on these was not shown or compared.

Also those that resolved—the data should be shown.

Response: The 5 individuals with GGOs who developed lung cancer did not differ from the remainder in regard to autoantibodies (small numbers) as well as those who resolved (n= 6 GGO n= 5 SN) due to small numbers.

I) Are CT with no nodules normals—why therefore are the ages different?

Response: CT- scan with no nodules with extensive smoking history was considered a comparable group to the lung cancer cases also with extensive smoking history.

J) More samples are needed to be assessed to be sure of the results—the data shown was insufficiently analysed.

Response: We agree with the Reviewer that more samples need to be assessed in a larger prospective validation study (now stated at the end of the Discussion).

K) They need to show a plot of the logistic regression scores for each subject. They also need to show individual sensitivities and specificities of each group as it is not clear from figure 1 how much good ROC curves can be produced.

Response: Box plots for each group are shown for each TAA in Figure 1. Figure 1 shows the ROC curve for cancer vs non cancer groups and the remaining Figures were eliminated.

L/M) The results state varying specificities—and it is hard to see how these were generated. Abstract results stated 24/26 healthy controls identified as non cancer—but state 97% specificity for cancer vs…word missing ( ) (this occurs more than once).

Results state 31/36 controls were correctly classified = 86% but then they state specificity of 97%! Clearly this does not agree re a suitable robust test.
Response: The model, which includes c-myc, cyclin A, cyclin B, cyclin D1, CDK2 and survivin was applied to the healthy non-smoking control group as a test set. We correctly classified 31/36 control patients as non-cancer using a cutoff value of 0.085 which maximized the sensitivity and specificity of the logistic function at 81% and 97% respectively. The abstract has been corrected. A larger sample size in a future validations study is necessary to determine how robust this panel of autoantibodies will be. Chapman and colleagues reported 76% sensitivity in lung cancer to 1/7 in a panel of autoantibodies with a specificity of 92% (22).

8. Table 3 clearly states the test could distinguish between controls and no-nodules—but surely these are normal too. How can this be an accurate test if it can distinguish better between no nodules and normals than no nodules and cancer?

The box plots do not suggest such good specificity—or that such a test could work in another group. The abstract states the ROC curves were also based on age but this is not shown in table 4. Not all data is presented and it is unclear how such good sensitivity and specificity is achieved here, or could be achieved in future studies based on the results presented.

The paper should focus only on the title subject. The paper needs to show all data. The discussion is not sufficient.

Response: We have re-focused the revised paper on the differences between lung cancer and the smoker controls including those with nodules and GGOs. Table 3 is a nonparametric Wilcoxon pairwise test that maximizes differences; the multiple logistic regression models take into account interactions and age for modeling cancer vs. high-risk smokers (no nodules, SN, GGO combined). The box plots demonstrated all of the data consistent with the importance of a panel of biomarkers to show significant differences. We deleted table 5A and Figure 3 that combine cancer/GGO so that all comparisons are cancer vs high risk smokers (no nodules, SN, GGO).

The discussion has been revised. Abbreviations are spelled out in full followed by parentheses.

Response to Reviewer: Iver Petersen

1) Unfortunately there is no data on the specificity of the recombinant proteins that have been generated for the ELISA. This may be determined by performing Western Blot analysis with the commercially available antibodies against these proteins. At least the side of the proteins should be determined for comparison with expected protein size.

Response: See revised Methods pages 7 and 8 and above.

2) There is no data on the concentrations of the autoantibodies in the serum. What are the units provided for the different genes in Figure 1? Are these relative optical densities provided by the ELISA analysis? The values seem to have considerable variability and overlap between the groups. By look at these graphs the high p-values of the statistical analysis are astonishing. The authors should provide a judgment of the amount/concentration of the autoantibodies and relative differences between the sera.

Response: The revised Ms Methods discusses ELISA controls and antibody comparisons; see pages 7 and 8.

3) In addition, they should provide data on the intra-individual variations when several measurements per serum sample are performed. In addition they should analyze or at least comment on the
potential influence of the methodology for serum generation. It is conceivable that this may have a profound impact on the results. The different origin of the sera (New York, Scripps) should be considered in this context.
Response: The revised Ms Methods discusses ELISA controls on pages 7 and 8.

Response to Reviewer: Eckart Meese
1) Throughout the paper the authors claim that the autoantibody reactivity can be used as a biomarker for the early detection of lung cancer. There is, however, no strong evidence to support such a claim.
Response: We have revised the paper carefully so that we are not claiming that the autoantibody reactivity can be used as a biomarker for the early detection of lung cancer. In the abstract, we state that “all pattern of autoantibody reactivity to TAAs may distinguish patients with lung cancer versus smokers with normal CTs, stable solid nodules, ground glass opacities or normal healthy never smokers.” In the conclusion, we call for larger validation studies with increased sample size and prospective collection of samples.

2) Specifically, the authors do not provide information on the kind of lung cancer that was analyzed. Are these advantaged stages in the tumor development? Do the authors analyze NSCLC or SCLC?
Response: See response to #6 Chapman.

3) It is not obvious why the authors do not try performing other separation tasks including the separation of GGO versus non-smoker and the separation between smoker and non-smoker.
Response: Table 3 includes separation of control nonsmoker from GGO (line 3) and smokers versus control non-smoker (lines 1-3).

4) It should be clarified what influence does COPD/emphysema have on the pattern and on the specificity and sensitivity of the separations?
Response: The study population was selected from healthy nonsmokers and smokers. The lung cancer patients had been cleared for surgery. There were only 3 individuals with FEV1/FEV < 0.60 consistent with COPD or emphysema. A subsequent study of a sub-cohort with emphysema could clarify this since we did not have a large enough group to consider this question.

Response to Reviewer: Jianying Zhang
1) Author should give more description about ELISA test. How did you determine the cutoff value?
Response: See revised Methods pages 7 and 8 and above.

2) It is better to add another table showing frequencies of each anti-TAA antibodies in sera from lung cancer patients and controls.
Response: Figure 1 shows box-plots and outliers of each TAA for each of the subgroups: nonsmoking healthy controls, high-risk samples with no nodules, solid nodules, GGOs, and lung cancer.

3) Another minor concern: whether or not all ELISA positive sera have been confirmed by additional assays, such as western blot or slot blot?
Response: See Revised Ms and Methods pages 7, 8, and 9 and above.