Author's response to reviews

Title: Serum p53 antibody detection in patients with impaired lung function

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Version: 4 Date: 12 November 2012

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Answers to specific questions

Reviewer 1

Major comments:

1- (Table 1) The authors reported that no difference in p53 Ab was observed between group 1 (399 normal people) and group 2 (275 patients with impaired lung function), since in group 2, three groups have been determined (including 2% with mild impairment). However, a difference in sex/smoking habits distribution between the 2 groups can be observed. Is this difference significant? If a significant difference is observed, it would be interesting to adjust p53 Abs detection to sex and/or smoking habits to determine whether a statistical difference exist between normal and cases group in term of p53 Abs detection (results section, paragraph 2, lines 2-4).

We have inserted the following sentences:

“Based on the univariate analysis, sex was a statistically significant predictor, but not the smoking habit. Furthermore, based on the multivariate analysis, lung function test impairment was a statistically significant predictor of serum p53Ab detection, but not others parameters, such as age, sex or smoking habit.”, at the end of the second paragraph of the Results section (page 8, line 7 from bottom).

2- a) In addition to statistical analysis of p53 Abs detection on the series of 675 subjects, the authors should investigate whether there is any difference of p53 Abs detection for the 2 different groups (399 normal peoples and the 275 cases) in regard to age, sex, number of cigarettes per days and pack-year. As suggested, we have included the following paragraph:

“We also investigated whether there were differences in the presence of serum p53 Abs between people with normal lung function tests and cases with regard to age, sex, number of cigarettes per day and pack-year (Table 3). No difference was found by age and sex; on the other hand, there was a significant correlation of serum p53Abs with cigarettes smoked per day (p=0.001) and a trend of increase with pack-year (p=0.081) in normal lung function group, while no correlation was observed in cases.”, after the second paragraph of the Results section (page 8, second-last line). In addition, we have included Table 3.

We have also added the following sentences:

“We also considered the difference in serum p53Abs between subjects with normal lung function and patients with altered lung function; we found a significant correlation of p53Abs with cigarettes smoked per day and a trend of increase with pack-year in normal lung function group, while no correlation was observed in cases.”, in the Discussion section (page 10, line 3).

b) In addition to compare “negative” vs “positive” p53 Abs detection (ie. qualitative detection), it would be important to compare the median p53 Abs levels for each group presented in Table 2, to determine whether quantitative p53 Abs levels is correlated to smoking status and/or lung impairment.

As suggested, we have inserted the following sentences:

“Further, median serum p53Ab levels were calculated to evaluate whether there was any correlation with clinicopathologic features. As reported in Table 4, no correlation was found between median p53Ab levels and clinicopathologic features, such as age, sex, smoking status and lung function...
impairment.”, after the third paragraph of the Results section (page 9, line 5). In addition, we have included Table 4.

3- The ex-smokers have been defined as people that had quit smoking at least 6 months before lung function tests. Is there any benefit of quitting (ie. reduction of p53 Ab throughout time?). If so, the analyses based on number of cigarettes smoked per days and pack-year should also be performed in ‘current smokers only’ or ‘current smokers and recent ex-smokers’.
We have added the following sentences:
“With regard to quit smoking, we could not observe a significant difference in p53Ab levels throughout time, due to the small number of positive subjects; in fact, we found four positive subjects out of 72 ex-smokers, who had quit smoking since < 10 years, and three positive subjects out of 54 ex-smokers, who had quit since > 10 years, with a median serum p53Ab level of 4.1 and 3.9 units/ml, respectively.”, at the end of the fourth paragraph of the Results section (page 9, line 8).

Minor comments:

1- Methods complement: a) Does the cut-off of p53 Abs levels used to determine the positivity, correspond to commercial cut-off or to authors’ experienced-based cut-off? Is this cut-off accurate to other studies? The authors should give this information.
As suggested, we have given the following information:
“according to the manufacturer’s suggestion; this cut-off was accurate to other studies [24].”, at the end of the first paragraph of the Serum p53Ab assay subsection of the Methods section (page 6, line 2). Reference 24 has been added to the reference list.
Furthermore, we have substituted the term “p53Abs” with “p53Ab” in the same subsection (page 6, line 2).

b) The authors should also indicate that ELISA has a low sensibility for serum antibody detection and that novel and more sensitive methods have been developed.
As suggested, we have inserted the following sentences:
“Furthermore, ELISA shows a high specificity, but a low sensitivity for serum antibody detection and novel and more sensitive methods have been developed, such as the particle agglutination assay [25]. However, ELISA still retains its value for diagnostic accuracy and easy performance in routine diagnostic procedures [26].”, at the end of the second paragraph of the Serum p53Ab assay subsection of the Methods section (page 6, line 8). References 25 and 26 have been added to the reference list.

2- In ELISA detection assays, human recombinant WTP53 protein was used to evaluate p53 Abs in human serum produce by patients over-expressing MT p53 protein, as explained by the authors (background section, lines 5-7). This method is a conventional method for such analysis, however is it possible that p53 Abs produced by patients can only recognise MT p53 conformation and not the one of WT p53, thus introducing false-negative results? Has this method been evaluated by testing conformational p53 antibodies in ELISA assays (ie. PAb 240 specific of MTp53 conformation and PAb 1620 specific of WTP53 conformation)?
We have added the following sentences:
“Usually, p53Abs recognize epitopes in the amino and carboxy termini of p53 protein, outside the DNA-binding domain where most mutations occur, thus identifying both wild type and mutant p53 proteins; however, p53Abs to certain types of mutant p53 proteins might not bind to wild type
recombinant p53 protein used as antigen in our assay, with the possibility of false negative results.

3- Methods complement: a) In which circumstances, and where, subjects have been recruited (ie. hospital, physicians, normal consultation...). This might determine in which extent the 675 subjects come from normal or case population.

We have inserted the following sentences:

“All people were recruited at the Respiratory Physiopathology Unit of the Regina Elena National Cancer Institute, Rome, Italy, between June 2004 and March 2009. They were enrolled either by a voluntary pulmonary visit at the Respiratory Physiopathology Unit or coming from the Tumour Prevention Centre of the same Institute, because of increased risk of lung cancer. We included regular smokers, ex-smokers and non smokers, without age limit, with no history of previous malignant diseases. The number of non smokers was small compared to smokers, because they were only recruited at the Respiratory Physiopathology Unit together with smokers and ex-smokers, while from the Tumour Prevention Centre came exclusively smokers and ex-smokers, associated with lung cancer risk.”, after the term “(Table 1)” in the Patients subsection of the Methods section (page 4, second-last line).

Furthermore, in the same subsection, the term “cases” has been substituted with “people” (page 4, line 7 from bottom), the sentence “received routine physical examination and” has been inserted after “enrolled” (page 5, line 10), the term “lung function tests and” has been added before “low” (page 5, line 11), the term “to” after “complied” has been substituted with “with the” (page 5, line 12) and finally the term “until June 2011” has been included after “follow-up” (page 5, line 12).

b) For the follow-up, nothing is said about development of cancer development different from lung cancer.

According to the reviewer, we have added the sentences:

i) “specialists or other instrumental examinations suitable for monitoring the development of lung cancer or other cancer types, such as breast, prostate or colon cancers.”, after the term “scans,” in the Patients subsection of the Methods section (page 5, line 12 from bottom);

ii) “or other specific examinations, carried on to verify the development of lung cancer or other cancers, such as breast, prostate or colon cancers.”, at the end of the last paragraph of the Results section (page 9, line 10 from bottom);

iii) “Finally, none of the followed-up p53Ab positive subjects showed development of lung cancer or other cancers, such as breast, prostate and colon cancers.”, at the end of the first paragraph of the Discussion section (page 10, line 7).

Furthermore, in the Results subsection of the Abstract section, the term “checked” has been substituted with “assessed” (page 2, line 12) and the term “by CT examinations of the chest” has been omitted (page 2, line 13).

3- Table 1: percentage should be given. This table can be clarified by placing side by side the 2 distinct groups in columns. There are some missing data (Normal LFT – no of cigarettes smoked per days and pack-years = 355 subjects / 357 current and ex-smokers; Impaired LFT – no of cigarettes smoked per days and pack-years = 276 cases / 277 current and ex-smokers), however that did not affect the statistical analyses (Same in table 2, age).

As suggested, we have given percentage in Table 1. Furthermore, this table has been clarified by placing side by side the 2 distinct groups in columns. Then, we have added the numbers of missing data in Table 1 and 2.

4- Table 2: percentage should be given. Statistical tests used to obtain p-value
should be indicated at the bottom for each individual analysis.

As suggested, we have given percentage in Table 2. We have also indicated the statistical test used to obtain p-value for each individual analysis at the bottom of Table 2.

5- The authors showed that 2 of 44 non-smokers (4.6%) patients with impaired tests are positive for p53 Abs expression. The authors said that p53 mutation, due to smoke exposure, promotes over-expression of p53 leading to increase in p53 Abs production in serum. How the authors explain that non-smokers are positive for p53 Abs?

We have inserted the following sentences:

“Airway inflammation has a part also in lung function decline independent of smoking, as observed in asthma and pulmonary fibrosis. Through oxidative DNA damage, this inflammation can promote p53 mutation and overexpression in the surrounding lung cells, with subsequent induction of serum p53Abs; this might be the reason why we found two p53Ab positive patients in non smokers with impaired lung function.”, after the term “function.” in the first paragraph of the Results section (page 8, line 5).

6- It would have been interesting to analysed other well-defined markers of lung cancer, such as K-Ras or EGF-R expression by ELISA, or at least mention them in the discussion.

According to the reviewer, we have mentioned K-Ras and EGF-R in the second-last (page 11, line 12) and last paragraph (page 11, line 5 from bottom), respectively, of the Discussion section:

“Other well defined markers of lung cancer are Kirsten rat sarcoma viral oncogene homolog (KRAS) and Epidermal growth factor receptor (EGFR). KRAS is involved in several signaling pathways and mutations in this gene may lead to cancer development. In fact, KRAS mutations are present up to 30% in non-small cell lung cancers (NSCLC). They are found prevalently on codon 12 and appear early in cancer development; furthermore, in some tumors they have been detected in blood before clinical diagnosis [32]. In NSCLC, KRAS mutations correlated with progression and poorer survival and an association between the presences of KRAS mutations in tumor tissues and in circulating DNA was reported [33]. In several studies, KRAS mutations in the blood have been correlated with poor survival and response to therapy [34, 35]; however, other studies have not found any prognostic relevance [36].” References 32-36 have been added to the reference list.

“On the other hand, EGFR is highly expressed in various cancers, including lung cancer. EGFR is a member of the family of EGF tyrosine kinase receptors and upon ligand binding activates several intracellular pathways, including Ras-MAPK, PI3K-Akt and STAT pathways, thus affecting cell proliferation, differentiation, survival, angiogenesis and migration. In NSCLC cells, EGFR is overexpressed up to 80%, but the prognostic value of EGFR expression remains controversial and the results of a meta-analysis demonstrated that EGFR overexpression was not a statistically significant prognostic factor in NSCLC [37]. A soluble fragment of the EGFR extracellular ligand domain can be detected in the blood of cancer patients, including NSCLC patients. The prognostic role of soluble EGFR in NSCLC has been investigated and an increasing serum EGFR level after gefitinib therapy was reported to be a significant indicator of disease progression and shorter progression-free survival [38]; furthermore, in a recent study involving a large cohort of NSCLC patients, lower levels of baseline plasma EGFR, detected by ELISA, were correlated with a poor survival [39].” References 37-39 have been added to the reference list.

Furthermore, “KRAS, Kirsten rat sarcoma viral oncogene homolog; EGFR, Epidermal growth factor receptor; NSCLC, Non-small cell lung cancer.” have been added to the List of abbreviations section (page12, line 4 from bottom).
Reviewer 2

Comments:

This study has a number of limitations: the number of non-smokers is very small compared to smokers; the criteria for inclusion are poorly described;
As in number 3- Methods complement: a) of “Minor comments” of the Reviewer 1.

it is not indicated whether the individual included have been monitored for other diseases (p53 Ab antibodies have been linked to other cancer types);
As in number 3- Methods complement: b) of “Minor comments” of the Reviewer 1.

there are no data on lung cancer occurrence, thus the presence of these serum p53Ab cannot be correlated with cancer risk. The design of the study is thus questionable.
In conclusion, the study is poorly designed and thus cannot answer the question addressed by the authors: can p53 serum Ab have a role as biomarkers for the early detection of lung cancer. Although smoking and lung function impairment are associated with increased cancer risk, they cannot be taken as surrogate for cancer risk.

We have added the following sentences:
a) “However, since in our study we have not observed lung cancer occurrence in the followed-up positive subjects, we cannot directly correlate the presence of serum p53Abs with lung cancer risk.”, at the end of the Conclusion subsection of the Abstract section (page 2, line 16);
b) “However, since in our study, due to the small number of p53Ab positive subjects who complied with the follow-up, we have not observed lung cancer occurrence, we cannot correlate the presence of serum p53Abs with lung cancer risk.”, at the end of the third paragraph of the Discussion section (page 11, line 9);
c) “However, in our study, no correlation was observed between serum p53Abs and lung cancer risk.”, at the end of the Conclusions section (page 12, line 12).

Furthermore, we have inserted the term “possible” before “role” in the Methods subsection of the Abstract section (page 2, line 5) and in the Background section (page 4, line 16).
We have also substituted the term “may identify” with “associate with” and the term “with an” after “patients” with “at” in the Discussion section (page 11, line 2).