Reviewer's report

Title: Diagnosis of lung cancer in individuals with solitary pulmonary nodules by plasma microRNA biomarkers

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Reviewer: Gabriella Sozzi

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The paper reports interesting findings on the analysis of microRNA expression profile in a large series of plasma samples from individuals carrying benign and malignant solitary pulmonary nodules (SPNs) in the lung.

The topic is extremely relevant and timely considering the widespread use spiral-computed tomography (CT) in lung cancer screening trials.

The efficacy of lung cancer screening in heavy smokers by spiral-CT remains a controversial issue even if the recent results of NLS trial showed a 7% reduction in all-cause mortality and -20% lung cancer mortality, compared to annual chest x-rays. However, before CT screening can be offered to millions of individuals worldwide, a number of questions have to be answered, including the overdiagnosis issue. In this respect the development of biomarkers detectable in plasma and their possible use to diagnose malignant nodules versus benign lesions is of major interest.

The manuscript is of interest and represents a useful contribution to the field, clearly written and the study design is properly described.

A major limitation of the paper is the analysis in plasma of only five miRNAs previously selected on the basis of their differential expression in lung tumor tissue. Considering the high tissue-specificity of miRNAs and the critical role of the host and tumor microenvironment in tumor development and progression, other miRNAs could be released and circulate in the bloodstream and be associated with the onset of malignant tumors.

Accordingly, the authors recognize in the last paragraph of the discussion that the accuracy of their test for identification of malignant SPNs is suboptimal for a clinical use and that a more comprehensive and high-throughput miRNA analysis should be performed.

To this purpose they should mention and discuss our recent paper where a widespread miRNA expression profile in lung tissues and plasma samples in CT-detected lung cancer patients and controls, indicated that specific miRNA signatures in plasma could be useful not only for establishing lung cancer diagnosis and prognosis but also to identify the onset of indolent and aggressive disease even before spiral-CT detection (Boeri M. et al., PNAS 2011).

Major Compulsory Revisions
Major weaknesses of the paper are:

- The lack of definition either in the text and in the Tables of the final diagnoses of the benign SNPs. The authors state that final diagnoses of all nodules was confirmed by histopathologic examinations of samples obtained through biopsies, VATS or surgical resection but they do not group and list the benign SNPs analyzed for miRNA expression in none of the two series (training and validation sets).

Clinico-pathological diagnoses were also used as reference standards to decide sensitivity and specificity. Could it be that the observed loss of specificity is related to non specific positive miRNA expression in subsets of benign tumors of inflammatory conditions?

The authors should provide this information.

- The normalization of real-time PCR data is another critical point. The authors performed the experimental work using a sensitive and clinically accessible methodology such as q-real-time PCR. Nevertheless, the normalization of miRNA data in plasma samples still represents a controversial issue and no consensus exists on housekeeping serum/plasma miRNAs. Based on the experience of their previous paper the authors chose mir-16 for data normalization on the basis of its high stability and abundance. However, using for normalization a single and very abundant miRNA, such as mir-16 whose CT amplification values are around 19, to compare miRNAs that are expressed at far lower levels (as the majority of miRNA in plasma, around or over 30 CT values) is not appropriate and it may create artifactual differences. In fact, since the normalization is usually required to adjust for the initial input of RNA, one has to take into account that the kinetics of amplification of the very abundant miRNAs is different from that of low expressed miRNAs. The authors should try a different normalization strategy (i.e. more than one miRNAs or miRNAs with similar range of amplification).

- The results (pag.10) concerning the correlation analysis between the three miRNAs are confusing. In the text the authors say that they used Spearman rank correlation that indicates a low, non-significant correlation and refer to Supp. Table 2. Conversely in Supp. Table 2 a Pearson correlation analysis of coefficients is shown and shows apparently odd results since correlation between the same miRNAs should be always equal to 1, whereas in the Table only mir-21 has value 1 and the other have different values. Thus these results and the conclusion on their “complementarity” should be more appropriately studied, showed and discussed.

Minor points

Results: pag.9, second row: “…miR-486-5p… exhibited higher expression level in patients having malignant SPNs compared to patients with benign SPNs”.

From the values presented in Table 3 and Fig.1 levels of miR-486-5p are lower in patients with malignant compared to benign SNPs.
Fig. 1 Please indicate in the legend what the asterisk and the square symbols stand for.

Suppl. Table 3. miR-126 has a significant p value for association with nodule size, but asterisk indicating statistical significant is missing

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**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' below