Author's response to reviews

Title: Fibroblast Phenotypes in Different Lung Diseases

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Author's response to reviews: see over
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Title: Fibroblast Phenotypes in Different Lung Malignancies

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Author's response to reviews: see over
Dr Heng DU et al.

Thank you for reviewing our manuscript submitted to your journal.

We have reviewed the above manuscript according to your reviewer’s comments.

Reviewer # 1 (Dr Fei Xing)

1. Since the author is interested in the TGF-β pathway, it is important to show the smad signaling is activated in the tissues adjacent to the CAFs.

In the present study, the most important thing we wanted to prove was that the fibroblasts had a phenotype conversion in different lung diseases. The purpose we tested the expression of TGF-β was to prove that the EMT (epithelial-mesenchymal transition) might play an critical role in the phenotype conversion of fibroblasts. Yoko Katsuno et al. had proved that TGF-β played a role in EMT and expressing of α-SMA. (Yoko Katsuno, Samy Lamouille, Rik Derynck: TGF-β signaling and epithelial–mesenchymal transition in cancer progression. Curr Opin Oncol 2013, 25:76–84. András Masszí, Caterina Di Ciano, Gábor Sirokmány et al: Central role for Rho in TGF-β-induced α-smooth muscle actin expression during epithelial-mesenchymal transition. Am J Physiol Renal Physiol 2003, 284: F911–F924.) Besides, we planned to research the mechanism and the TGF-β pathway in our next step. So we are sorry that we do not show the smad signaling is activated in the tissues adjacent to the CAFs in our present study.

2. The presentation of Fig 4 is very confusing. Need more description of this fig (number of patients, type of tissues, what does each cycle represent for.)

We have revised in our manuscript and made a detailed explanation (see Page 5):

All the lung cancer samples (including CIS, A and AM, totally 66 samples) expressed different levels of α-SMA, vimentin, E-cadherin, Twist and TGF-β. We performed a correlation analysis between TGF-β and α-SMA, TGF-β and vimentin, TGF-β and E-cadherin, Twist and α-SMA, Twist and E-cadherin and Twist and α-SMA in all the cancer samples.

The figure legends of Fig. 4 was revised according to the reviewer’s reports and number of patients, type of tissues and the meaning of each cycle was supplemented. (see figure legends of Fig. 4 in Page 12):

Correlation analysis between TGF-β and α-SMA, TGF-β and E-cadherin, Twist and α-SMA,
Twist and E-cadherin. Correlation analysis between TGF-β and α-SMA, TGF-β and E-cadherin, Twist and α-SMA and Twist and E-cadherin were performed in all the 66 lung cancer samples (including CIS, A, AM). Each cycle in the figure represented one lung cancer sample. But some cycles were overlapped because these samples had a same score.

3. Chi-square test may be a better way to show the correlation in Fig4

We performed this correlation analysis using a Spearman’s rank test because we thought that the SI score of each sample was fit for this method. Besides, the same method was used in studies performed by Guanghui Wang et. al which named “A comparison of Twist and E-cadherin protein expression in primary non-small-cell lung carcinoma and corresponding metastases” (European Journal of Cardio-thoracic Surgery 2011, 39:1028—1032)

4. In fig3 q, the FAP is expressed in cancer tissues instead of fibroblasts. The author need to discuss this part.

We discussed this novel result in our “Discussion” part as follows: (see Page 7-8).

However, our results showed that FAP was expressed in the cellular matrix of cancer cells rather than in the cellular matrix of CAFs, and no statistically significant differences in its expression were detected among CIS, A, and AM tissues. TGF-β expression was also localized to the matrix of cancer cells, which was not consistent with the results reported by Xing et al. who showed that the secretion of TGF-β by CAFs promotes their proliferation. These two novel phenomena revealed by our findings indicated that cancer cells might have a similar function than CAFs and CAFs may not originate exclusively from normal epithelial cells but may also be derived from mutant epithelial cells, including cancer cells (2).

Besides, we also discussed this part in the figure legend of Fig. 3 as follows: (see Page 12)

This novel result may indicate that some cancer cell could secret FAP just like CAFs or these cancer cells secreting FAP may be a novel origin of CAFs.

5. It is better to discuss the incidence and the probability that the patients with inflammatory pseudotumor atypical and adenomatous hyperplasia develop lung cancer in clinical settings.

We searched PubMed carefully and many studies articles about inflammatory pseudotumor and
atypical and adenomatous hyperplasia were found. After reviewed the relevant studies, we add some message in the discussion part just as follows. (see Page 8)

Both lung inflammatory pseudotumor and atypical adenomatous hyperplasia might have something to do with malignant lung diseases. Lung inflammatory pseudotumor, whose etiology was still unknown, was a relative rare benign lung tumor and account for about 0.7% of primary pulmonary and bronchial tumors. However, this benign lung disease had malignant biological behavior including locally invasive, aggressiveness and unfavorable evolution which needed extensive pulmonary resection to prevent local recurrence. Though no detail data about the incidence and the probability that the patients with inflammatory pseudotumor lung cancer was provided, it was better to have this lesion resected as early as possible. Atypical adenomatous hyperplasia may be the adenoma in an adenoma–carcinoma sequence in the lung periphery, leading to the development of adenocarcinoma. Atypical adenomatous hyperplasia had a low morbidity of 4.4% and 9.6%. The incidence was higher in patients bearing lung cancer especially in those with lung adenocarcinoma (15.6% vs 23.2%). It had been proved that atypical adenomatous hyperplasia might result in lung squamous carcinoma. Whether or not it would result in lung adenocarcinoma still need more research.

Reviewer # 2 (Dr Neta Erez)

1. The presented IHC and figures are of high quality and convincing. However, it would be useful to add arrows to mark the relevant cells in each figure.

   We have added arrows to mark the relevant cells in our figures.

2. Throughout the manuscript, the authors use the term “CAFs” repeatedly to describe activated fibroblasts in inflammatory diseases or in healthy tissue. Per definition, CAFs are Cancer-Associated and do not exist elsewhere.

   We reviewed the manuscript and changed the CAFs used in inflammatory diseases and in healthy tissue into fibroblasts.

3. In the methods section the authors describe the IHC analysis: “200 cells were scored” – it is unclear whether 200 fibroblasts were scored and if so, how were they recognized. The fact that
200 fibroblasts were counted per field in all tissue types seems unlikely. Since this staining is central to the presented work, a more detailed description of the analysis methods is required.

We have already revised the method used in our study just as follows. (see Page 4)

For the markers expressed in cancer cells, high magnification (40 × objective) was used to count the individual cancer cells and a total of 200 cancer cells were scored for each high-power field. For every section, five fields were selected randomly. Normal alveolar epithelial cells stained red by nuclear fast red were selected as negative controls. A staining index (SI, values 0, 1, 2, 3, 4, 6, and 9) was calculated as a product of staining intensity (0–3) and the proportion of positive cells (0% = 0, 1–10% = 1, 11–50% = 2, >50% = 3).

It was not that easy for us to counted fibroblasts or CAFs just like cancer cells, so, for the markers which were expressed in these cells, the SI was calculated as a product of staining intensity (0–3) and the extent of staining (0% = 0, 1–10% = 1, 11–50% = 2, >50% = 3).

4. The first paragraph in the results section describes the staining of α-SMA and Vimentin in different stages. The text goes back and forth between α-SMA and Vimentin, in a repetitive and confusing manner. A more organized description of the results, preferably describing each protein would make this paragraph more understandable.

According to the reviews, we revised the first paragraph in the results and the two proteins, α-SMA and Vimentin, were described in an organized description. (see Page 4)

α-SMA was detected in benign and malignant tissues and its expression was localized to blood vessels and airway ducts in N, I and H. Stromal cells stained positive for α-SMA in the three pathological stages of adenocarcinoma, although with different staining intensity. α-SMA expression was lower in CIS (SI ranged from 1 to 4 and the median was 2) than in A (SI : 1 to 6, median was 4) (P = 0.026), whereas AM (SI: 4 to 9, median was 6) showed higher expression than A (P = 0.009). Vimentin expression was negative in N and H but positive in I. In the cancer samples, Vimentin expression was higher in A (SI: 2 to 6, median was 3.5) than in CIS (SI: 0 to 4, median was 2) (P = 0.017) and it was higher in AM (SI: 3 to 9, median was 6) than in A (P = 0.022). In general, α-SMA and vimentin expression increased gradually from N to AM (Fig. 1).

5. This same paragraph (p. 4) contains an internal contradiction: “α-SMA was detected in benign
and malignant tissues and it’s expression was localized to blood vessels and airway ducts in normal lung tissues and atypical adenomatous hyperplasia” and “α-SMA and Vimentin showed positive expression in stromal cells of benign lung tissue (I) and SI, similar to their expression in CIS”. So which is it? Is α-SMA expressed only around blood vessels and airway ducts in benign lung tissues or was it positive?

α-SMA expressed only around blood vessels and airway ducts in benign lung tissues. We revised the first paragraph just as follows: (see Page 4).

α-SMA was detected in benign and malignant tissues and its expression was localized to blood vessels and airway ducts in N, I and H. Stromal cells stained positive for α-SMA in the three pathological stages of adenocarcinoma, although with different staining intensity. α-SMA expression was lower in CIS (SI ranged from 1 to 4 and the median was 2) than in A (SI : 1 to 6, median was 4) (P = 0.026), whereas AM (SI: 4 to 9, median was 6) showed higher expression than A (P = 0.009). Vimentin expression was negative in N and H but positive in I. In the cancer samples, Vimentin expression was higher in A (SI: 2 to 6, median was 3.5) than in CIS (SI: 0 to 4, median was 2) (P = 0.017) and it was higher in AM (SI: 3 to 9, median was 6) than in A (P = 0.022). In general, α-SMA and vimentin expression increased gradually from N to AM (Fig. 1).

6. The abbreviation “SI” is used in this paragraph for the first time and is not explained in the text or in the list of abbreviations.

We have already explained the abbreviation “SI” for the first time it appeared in the manuscript. (see Page 3 and 4). “SI” was short for “staining index”. Besides, we also explained it in the list of abbreviations. (see Page 9)

7. In section 3.2.2. of the results, FAP expression is analyzed along with TGF-β and twist. FAP was previously described as a marker of both normal fibroblasts and CAFs in other organs and malignancies, but not in cancer cells. This is also stated several times by the authors. Since the data presented here does not agree with previous publications, they should be discussed in more detail in the discussion, in order to put these results in the context of the field.

We discussed this novel result in our “Discussion” part as follows: (see Page 7-8).

However, our results showed that FAP was expressed in the cellular matrix of cancer cells
rather than in the cellular matrix of CAFs, and no statistically significant differences in its expression were detected among CIS, A, and AM tissues. TGF-β expression was also localized to the stroma of cancer cells, which was not consistent with the results reported by Xing et al. who showed that the secretion of TGF-β by CAFs promotes their proliferation. These two novel phenomena revealed by our findings indicated that cancer cells might have a similar function than CAFs and CAFs may not originate exclusively from normal epithelial cells but may also be derived from mutant epithelial cells, including cancer cells (2).

Besides, we also discussed this part in the figure legend of Fig. 3 as follows: (see Page 12) This novel result may indicate that some cancer cell could secret FAP just like CAFs or these cancer cells secreting FAP may be a novel origin of CAFs.

8. In the discussion section (bottom of p.7) the authors state: “our results showed that FAP was expressed in the stroma of cancer cells rather than in CAFs”. This sentence does not make sense, as CAFs are an integral part of the cancer stroma. Please clarify.

We were sorry that we used a wrong word which lead to a misunderstanding of this sentence. In fact, our real intention was that FAP was expressed in the cellular matrix of cancer cells and CAFs did not expressed FAP. So we changed the word “stroma” into “cellular matrix”.

9. There have been several recent studies describing CAFs in lung carcinoma. They should be sited. (Examples: “Transforming growth factor-β1 and α-smooth muscle actin in stromal fibroblasts are associated with a poor prognosis in patients with clinical stage I-III A non-small cell lung cancer after curative resection”. Chen Y. et al. 2014. “Lung cancer-associated myofibroblasts reveal distinctive ultrastructure and function” Karvonen, HM 2014.

We searched the Medline used “lung”, “cancer-associated fibroblasts”, “stromal fibroblasts” and “myofibroblasts”. Several recent articles were found. We totally sited xx studies including the two articles recommended. The added xx articles number were xxxxx in the reference.

Reviewer # 3 (Dr Omar Franco)

In this manuscript entitled “Fibroblast Phenotypes in Different Lung Malignancies” authors presented a an attempt to characterize stromal cells in several lung malignancies by
immunohistochemical analysis of patient samples obtained during surgery. Although the manuscript presented the results of a fair large number of patients, there are several points that were not very clear and need to be addressed before presenting to the scientific community:

Major Compulsory Revisions

1) The manuscript requires “substantial” English language editing.

   According to the reviews suggestion, we invited the company named “Bioedit” (Company no. 04150179 (UK)) to help us edit our manuscript. The reviewers of this company were native English speaker. We believed that our manuscript being edited may make you satisfied.

2) Page 1, Line 3: “The role of CAF in lung cancer has been previously investigated”, This statement is not supported in the main text, there is no reference. Also if the role has been previously studied, please provide the main findings.

   We searched the Medline used “lung”, “cancer-associated fibroblasts” and “stromal fibroblasts”. Several recent articles related were found. We added the main findings about the CAFs in lung tissue in our main text. (see Page 2)

   Fibroblasts in lung tissue were investigated recent years. CAFs in lung cancer promoted lung cancer cells proliferation. Vicent S et. al made a cross-species functional characterization of mouse and human lung CAFs and finally found that CAFs supported the growth of lung cancer cells in vivo by secretion of soluble factors that directly stimulate the growth of tumor cells. They also found that IL-6 (interleukin-6) secreted by CAFs might play roles in promoting cancer cells growth. Besides, CXCL-12/CCR4 axis existed between the cross-talk of CAFs and non-small lung cancer cells also contributed to the proliferation of cancer cells. Some proteins expressed by CAFs such as matrix metalloproteinase (MMP)-2, α-SMA, podoplanin and carbonic anhydrase (CA) IX may also have a role in predicting the prognosis of lung cancer.

3) Page 1, Line 4: “CAFs may also play a role in inflammatory disease”, here there is a fundamental misunderstanding of the term CAF by the authors. CAF as they stated stands for “CARCINOMA associated fibroblasts” emphasizing the presence “only” in the microenvironment of malignant disease. Reactive stroma, activated fibroblasts or myofibroblasts can be seen in inflammatory diseases and in wounds during the process of repair. Please provide a better explanation of the term or use an alternative.
We are sorry that we use a wrong word. We reviewed the manuscript and used the word “fibroblasts” or “activated fibroblasts” instead of “CAFs” in inflammatory diseases and in healthy tissue into fibroblasts.

4) Page 1 second line in the Background section: What does “bad” cancer cells mean? Cancer cells are malignant in nature, perhaps the authors meant cancer cells that will show progression vs. those that will remain localized? Please explain.

The word “bad” we used in the background section meant that cancer cells showed several features such as progression, invasion and distance metastasis. All these features were not friendly to patients and indicated a poor prognosis. We deleted the word “bad” in order to make it easy for readers to understand. (see Page 1)

Background The “seed and soil” hypothesis explains the importance of interactions between tumor cells and their microenvironment. CAFs (Cancer associated fibroblasts) are important components of the tumor microenvironment. The role of CAFs in lung cancer has been investigated previously. Fibroblasts may also play a role in inflammatory disease. We hypothesized that fibroblasts phenotypes may vary among different types of lung disease.

5) Reference 3 by Xing et al is a review in which the authors describe the characteristics of CAF (expression of α-SMA, vimentin, FAP and other markers) and lack of CD31 and epithelial markers without mentioning CK19 loss. Please provide a reference of the lack of CK19 expression by CAF.

We reviewed the reference 3 by Xing et al and found that they mentioned the epithelial marker cytokeratin, so we revised this sentence. Besides, we add a reference 4 by who mentioned the epithelial marker E-cadherin. (see Page 2)

CAFs express high levels of certain proteins, including α-SMA (smooth muscle actin) and vimentin (3,10,11), whereas they show negative expression of the epithelial markers cytokeratin and E-cadherin (3)(4).

Here is the text in the reference 3 by Xing et al:

In most cases, CAFs are negative for epithelial or endothelial markers such as cytokeratin and CD31. It was reported that in breast invasive ductal carcinoma (IDC), alpha SMA +
myofibroblasts are increased in the cancer regions and CD34+ fibrocytes gradually disappear (19).


6) Results, 3.2.1 Detection of CAFs and epithelial markers: In the first sentence the authors state that α-SMA is expressed in blood vessels and airway ducts in normal and in H (atypical adenomatous hyperplasia) tissues, however 6 lines below the state” N and H tissues were negative for α-SMA expression (?) Please explain. In figure 1 α-SMA can be seen expressed in all groups with the highest percentage (optical) and intensity in I>AM>CIS>A>H>N

We are sorry that we could not make it easy to understand for authors. In fact, we meant that in N and H, α-SMA was only expressed in blood vessels and airway ducts. The pulmonary parenchyma cells did not express α-SMA. We have revised this paragraph as follows: (see Page 4-5)

α-SMA was detected in benign and malignant tissues and its expression was localized to blood vessels and airway ducts in N, I and H. Stromal cells stained positive for α-SMA in the three pathological stages of adenocarcinoma, although with different staining intensity. α-SMA expression was lower in CIS (SI ranged from 1 to 4 and the median was 2) than in A (SI: 1 to 6, median was 4) (P = 0.026), whereas AM (SI: 4 to 9, median was 6) showed higher expression than A (P = 0.009). Vimentin expression was negative in N and H but positive in I. In the cancer samples, Vimentin expression was higher in A (SI: 2 to 6, median was 3.5) than in CIS (SI: 0 to 4, median was 2) (P = 0.017) and it was higher in AM (SI: 3 to 9, median was 6) than in A (P = 0.022). In general, α-SMA and vimentin expression increased gradually from N to AM (Fig. 1).

7) The differences between the groups should be presented with a graphical representation using bars or dot plot along with IHC images to see a better stratification of patients within each group.

In order to present the differences between the groups, box plot was used. (see figure 4)

8) Quantitation of the intensity is a somewhat subjective assessment. There are several digital
methods published in the scientific literature that can be used to validate the results. If the authors have access to frozen tissues a quantitative measure by qRT-PCR or protein analysis can be more informative.

We have tried the software named “Imagepro_plus” when we validated our results. We found that this software was very practical. We used the IOD (integrated optical density) recommended by some scientific literature to measure the sections. But the IOD values measured by this software were unsatisfactory for the software mechanically assessed the results. Many factors may had an effect on the IOD. Compared with other kinds of tissues which were very “clean”, lung tissues were always contaminated by the dust in the air. The dust in samples had a great influence on the IOD value measured by software though we tried out best to avoid it. Finally, we had to give up using software to assess the results. We searched many literatures which also used IHC in their studies and finally we decided to use SI (staining index, just as mentioned in our study) to validate the results. We had to acknowledge that quantitation of the intensity was a somewhat subjective assessment. Our sections were from pathology department of our hospital and all the samples were fixed using formalin and embedded by paraffin, so we could not obtained any samples which could be used for frozen tissues and qRT-PCR. We used IHC in order to made a preliminary analysis of the phenotypes conversion of fibroblasts in different lung disease and we planned to collect fresh samples and used qRT-PCR in our next studies.

9) Figure 1, vimentin expression, same concerns as α-SMA

We are sorry that we could not make it easy to understand for authors. In fact, we meant that in N and H, α-SMA was only expressed in blood vessels and airway ducts. The pulmonary parenchyma cells did not express α-SMA. We have revised this paragraph as follows: (see Page 4-5)

α-SMA was detected in benign and malignant tissues and its expression was localized to blood vessels and airway ducts in N, I and H. Stromal cells stained positive for α-SMA in the three pathological stages of adenocarcinoma, although with different staining intensity. α-SMA expression was lower in CIS (SI ranged from 1 to 4 and the median was 2) than in A (SI : 1 to 6, median was 4) (P = 0.026), whereas AM (SI: 4 to 9, median was 6) showed higher expression than A (P = 0.009). Vimentin expression was negative in N and H but positive in I. In the cancer
samples, Vimentin expression was higher in A (SI: 2 to 6, median was 3.5) than in CIS (SI: 0 to 4, median was 2) (P = 0.017) and it was higher in AM (SI: 3 to 9, median was 6) than in A (P = 0.022). In general, α-SMA and vimentin expression increased gradually from N to AM (Fig. 1).

10) Cytokeratin 19 expression in Figure 2; poor tissue in CIS sample, please use consecutive slices of tissues.

As we all know that lung cancer is very easy to progression and metastasis. Unlike other pathological stages of lung cancer, samples of CIS (carcinoma in situ) of lung cancer is relatively hard to found and the size of samples was very small. We could not obtained enough sections from a single patient. As our samples came from pathology department of our hospital, we were very sorry that we could not found any other samples for our study.

11) Figure 3. TGF-β: assessment of TGF-β expression by IHC is very difficult. This secreted protein can be present in the epithelial and stromal compartment, perhaps staining with phosphor-Smad2/3 can show activation of the TGF-β pathway in tumor or stromal cells. Again if frozen tissues are available it can be coupled to the total expression of TG-β by the tissue, but it cannot identify the cell secreting the protein. Sorting cells by flow cytometry (epithelial vs stromal cells) will answer this question.

In the present study, the most important thing we wanted to prove was that the fibroblasts had a phenotype conversion in different lung diseases. The purpose we tested the expression of TGF-β was to prove that the EMT (epithelial-mesenchymal transition) might play an critical role in the phenotype conversion of fibroblasts. Yoko Katsuno et al. had proved that TGF-β played a role in EMT and expressing of α-SMA. (Yoko Katsuno, Samy Lamouille, Rik Derynck: TGF-β signaling and epithelial–mesenchymal transition in cancer progression. Curr Opin Oncol 2013, 25:76–84. András Masszi, Caterina Di Ciano, Gábor Sirokmány et al: Central role for Rho in TGF-β-induced α-smooth muscle actin expression during epithelial-mesenchymal transition. ) Am J Physiol Renal Physiol 2003, 284: F911–F924.) Besides, all the samples were from the pathological department of our hospital, and all tissues were embedded with paraffin. We could not obtain any fresh tissues for frozen. So, we planned to research the mechanism and the TGF-β pathway using frozen tissues and flow cytometry in our next step.
12) It will be useful to have the N, I and H groups in Fig 3 to compare the level of TGF-β, Twist and FAP with the other groups

As mentioned in our results (see Page 5 results 3.2.2), TGF-β, FAP, and Twist expression was detected in the cytoplasm of cancer cells. The three factors were expressed in I, CIS, A, and AM, whereas N, H, and tissues 2 cm away from the tumor focus were negatively stained. As Fig 1 and 2 did not exhibit tissues 2 cm away from the tumor focus and the reason that TGF-β, FAP, and Twist were mainly expressed in lung cancer samples, we showed these samples in Fig 3. N and H did not express these factors.

13) Twist expression seems to be present in the nucleus. Authors claim to be in the cytoplasm.

Explain

Study by Guanghui Wang et al. demonstrated that twist was located in both the cytoplasm and nucleus. (Guanghui Wang, Wei Dong, Hongchang Shen et al: A comparison of Twist and E-cadherin protein expression in primary non-small-cell lung carcinoma and corresponding metastases. European Journal of Cardio-thoracic Surgery 2011, 39:1028—1032.) We found that our results were in accordance with Wang’s and we had revised our results as follows. (see Page 5)

TGF-β and FAP expression was detected in the cytoplasm of cancer cells while twist was located in both cytoplasm and nucleus. The three factors were expressed in I, CIS, A, and AM, whereas N, H, and tissues 2 cm away from the tumor focus were negatively stained (the results of N, I, and H were not included in Fig. 3). TGF-β expression followed a similar pattern than that of α-SMA and vimentin, with positive expression in I, CIS, A, and AM. TGF-β was expressed at higher levels in AM (SI: 3 to 9, median was 6) than in A (SI: 2-9 median was 3.5) (P<0.001). No statistical significance was seen between CIS and A (P = 0.392). On the other hand, Twist expression was seen in A and AM.

14) FAP is considered a stromal marker (PubMed; PMID: 23835897), however the authors found localization in cancer cells with increased expression in CIS compared to the other groups based on the IHC images. Was the quantitation made considering only stromal FAP?
In our results we found that FAP was located in the matrix of cancer cells and the CAFs in the stromal of cancer tissues did not expressed FAP. Though we also have known that FAP was considered to be a stromal marker. We had discussed this novel results in our manuscript as follows. (see Page 8)

However, our results showed that FAP was expressed in the cellular matrix of cancer cells rather than in the cellular matrix of CAFs, and no statistically significant differences in its expression were detected among CIS, A, and AM tissues. TGF-β expression was also localized to the matrix of cancer cells, which was not consistent with the results reported by Xing et al. who showed that the secretion of TGF-β by CAFs promotes their proliferation. These two novel phenomena revealed by our findings indicated that cancer cells might have a similar function than CAFs and CAFs may not originate exclusively from normal epithelial cells but may also be derived from mutant epithelial cells, including cancer cells.

15) The A group show “nuclear” FAP? Authors used a rabbit antibody purchased from Santa Cruz Biotechnology. Santa Cruz sells five antibodies against FAP (three goat, one mouse and one rabbit), none of them are recommended for IHC. The rabbit antibody FAP# (clone H-56) is a protease that can be found in the cytoplasm or ECM. Can the authors provide validation of this antibody for IHC?

The A group showed FAP was located in the matrix of cancer cells.

The antibody of FAP was from Assay Biotech, USA. (FAP antibody #C0188)

16) Several papers have addressed the complex nature of the tumor stroma. Due to the high heterogeneity and the lack of a “single” marker of CAF, attempts have been made to show co-expression at the cellular level of these potential markers. Thus double/triple co-expression of the most abundant primers and categorizing with each group should be presented.

In each group, we detected three CAFs markers which were α-SMA, FAP and vimentin, though we found that in our study FAP was located in cancer cells. Fig 1 showed the results.

17) Figure 4. This graphs are rather confusing. Were the comparisons made for each group or all the groups combined? What do the individual circles represent (patient/s)? Why are there different
number of circles in each graph? Are the X and Y axis representing SI index? If so why the scale is different in some, instead of 10 scale (first graph) up to 4 or 6 in the others? Please show consistency and better labeling. Perhaps a better representation would be to show a table and correlations.

The comparisons were made for all the 3 groups of lung cancer samples (including 3 groups: CIS, A and AM, totally 66 samples). And the individual circles represent patients in this group. But some cycles were overlapped because these samples had a same score. All the four pictures in Fig 4 contained 66 samples, but some patients had the same score, so these cycles were overlapped. The X and Y axis representing SI index. The SI values of the factors we detected had different ranges. The SI of both \(\alpha\)-SMA and TGF-\(\beta\) ranged from 0 to 9, the SI of E-cadherin ranged from 0 to 4, the SI of twist ranged from 0 to 6. So the scale is different in some, instead of 10 scale (first graph) up to 4 or 6 in the others.

We have revised in our manuscript and made a detailed explanation (see Page 5):

All the lung cancer samples (including CIS, A and AM, totally 66 samples) expressed different levels of \(\alpha\)-SMA, vimentin, E-cadherin, Twist and TGF-\(\beta\). We performed a correlation analysis between TGF-\(\beta\) and \(\alpha\)-SMA, TGF-\(\beta\) and vimentin, TGF-\(\beta\) and E-cadherin, Twist and \(\alpha\)-SMA, Twist and E-cadherin and Twist and \(\alpha\)-SMA in all the cancer samples.

The figure legends of Fig. 4 was revised according to the reviewer’s reports and number of patients, type of tissues and the meaning of each cycle was supplemented. (see figure legends of Fig. 4 in Page 12):

Correlation analysis between TGF-\(\beta\) and \(\alpha\)-SMA, TGF-\(\beta\) and E-cadherin, Twist and \(\alpha\)-SMA, Twist and E-cadherin. Correlation analysis between TGF-\(\beta\) and \(\alpha\)-SMA, TGF-\(\beta\) and E-cadherin, Twist and \(\alpha\)-SMA and Twist and E-cadherin were performed in all the 66 lung cancer samples (including CIS, A, AM). Each cycle in the figure represented one lung cancer sample. But some cycles were overlapped because these samples had a same score.

18) The major question(s) of the paper has not been answered. Do the CAF phenotype vary in lung disease, if so, can this variation explain tumor progression?

Our study demonstrated that CAFs phenotype varied in lung disease. But we only used IHC and we did not detected gene expression. We planned to use CAFs phenotype conversion to explain
tumor progression using western blot, qRT-PCR, PCR and animal disease models in our next steps.