Peer Review File

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Reviewer A

In this study, primary cells cultures were established from pleural fluid samples of three patients with Small-cell lung cancer (SCLC) and metastatic malignant pleural effusion (MPE). Cytokine production by those cells in culture supernatants was compared with cytokine production in corresponding pleural fluid samples from the same patients. According to the authors', "a large range of cytokines of the SCLC cell lines exhibited a relatively higher production compared to the matching MPE". Among them, a high expression of VEGF, ENA-78, PF4/CXCL4, MIP-3α/CCL20, and CXCL9, also known as monokine-induced by interferon-γ (MIG), was observed in supernates of SCLC cells, as compared with samples from pleural fluid in MPE.

The authors conclude that tumor-promoting factors and immunosuppressive mediators of the tumor cells could be separated from effectors supplied by nonmalignant cell populations of the pleural space.

GENERAL COMMENT: The idea of identifying by separate analysis the fraction of mediators provided by the tumor cell populations and the pleural fluids samples themselves in MPE is a plausible one and -as pointed out by the authors- provides a repertoire of cell enriched mediators as potential targets for therapeutic intervention apart from VEGF.

SPECIFIC COMMENTS:

1. POSSIBLE INFLUENCE OF PATIENT´S PREVIOUS TREATMENT ON SCLC PRIMARY CELL CULTURES? According to the authors, three SCLC lines (SCLC26A, S457 and SCLC CTC) cell line BHGc40 had been established at their institution (Section 2.2, line 97). On the other hand, it appears that -according to the manuscript (Section 3.5, lines 159-160)- at least one patient had no treatment prior to pleural fluid cell extraction, but no information is given on patients treatment related to lines S457 and SCLC CTC. In my opinion, specific information on this particular point is essential to make valid conclusions in this study, since previous treatments would likely modify tumor (and possibly non-tumor cells too) behavior.

Reply 1: the pretreatment of the cell line is now supplemented.
Changes in: lines 83-86.

2. INFLUENCE OF TUMOR HETEROGENEITY? Tumor heterogeneity has been associated with poor response to treatment in many cases, especially those with advanced disease. Assessing this aspect appears to be relevant for successful therapeutic strategies in patients with malignant pleural effusion.

I am convinced that MPE could provide a good model for investigation of intratumoral

heterogeneity in lung cancer, but there are several factors that should be considered at the time of taking conclusions in studies involving soluble factors in pleural fluid. Also, there is effusion-to effusion variability in those soluble factors, and data on amount of pleural fluid used, proportion of floating (dead) and alive cells in culture and concentrations of MPE-fluid components should be taken into account (see Basak et al, PLoS One. 2009 Jun 12;4(6):e5884. doi: 10.1371/ journal.pone.0005884).

Together with the above comment on previous treatment, I think that information on details of pleural fluid processing would significantly improve the quality of this manuscript.

Reply 2: the publication of Basak et al. deals with Non-Small Cell Lung Cancer (NSCLC) that has a different course compared to the rapidly growing SCLC tumors. Heterogeneity seems to be no problem, otherwise, three independent MPE samples would show significant differences and the cytokines found in conditioned cell culture media would not have corresponded to their MPEs. Furthermore, the pattern of cytokines detected here is in good agreement with the available literature. The median volume of the MPEs were approximately 100 ml, there were no low or high volume samples.

3. POSSIBLE CANDIDATES FOR FUTURE SPECIFIC TREATMENT? According to the supplementary material provided in the manuscript, a big amount of cytokines was investigated in this manuscript, and a clue on some of the possible candidates for targeted treatment was provided in the text of the manuscript and on Table 5.

As mentioned above, a high expression of VEGF, ENA-78, PF4/CXCL4, MIP-3α/CCL20, and CXCL9, was observed in supernates of SCLC cells, as compared with samples from pleural fluid in MPE. I think that some additional comments and preliminary recommendations from the authors about preferred target candidates would be welcome. For instance, ENA-78 was highly expressed by SCLC cells in this study, but it has been reported that this marked is significantly higher in INFECTIOUS pleural effusions than in MPE, and I wonder if the high expression by SCLC cells in this study was associated to some inflammatory status in those cells. Also, there are some contradictory published reports on the role that PF4 factor plays in prognosis on MPE. A comment on these aspects would also be welcome.

Reply 3. There is still a small fraction of MPE in the tissue culture medium containing the cultured SCLC cells but signs of infection have not been detected that would lead to increased levels of ENA-78. Two references for the potential therapeutic uses of agents directed to ENA78 and PF4 have been added.
Changes: lanes 273-277.

Reviewer B

1. In Fig 1 and 2, the y-axis pertains to the pixel intensity from the human Profiler Array Cytokine XL assay. It was unclear if the authors have calibrated the arrays with controls and, given potential batch-to-batch variability, it remained unclear how to interpret data from Fig 1 and 2 as they appear

to be from different batch of experiments.

Reply 1. The arrays do not yield absolute measurements. All individual membranes used have six calibration spots included that are used for the different analyses.
Changes 1: lines 91-92

2. The authors used the MTT colorimetric assay to determine cell viability. However, it remains unclear whether this is due to changes in cell proliferation vs cell death/apoptosis or both. Additional methods to interrogate mechanisms that alter cell number should be considered to dissect this further,

Reply 2. The MTT test data show an increase in cell numbers in response to added MPE. Therefore it is reasonable to conclude that the test has detected cell proliferation.

3. The authors noted there were 3 patients with advanced small cell lung cancer with malignant pleural effusion samples analyzed. These samples appear to be S996, S1033, and S1035. I don't understand why in Fig 4 there is an additional sample S1088, which was not explained.

Reply 3. Explanation provided. One more independent MPE.
Changes in: line 193-194

4. The number of unique patient MPE samples included here was really small (3), precluding any broadened interpretation of the data.

Reply 4. We have a large number of SCLC MPE samples, that`s not the point. You have MPE in 11 – 30% of SCLC patients but the take rate in tissue culture to establish proliferating short-term cell lines (at least) is low and one needs a large number of patients to obtain three corresponding SCLC cell lines to be compared with their own MPEs. The good agreement of the cytokines found in three independent MPEs and their corresponding cell lines as well as to the available literature point to the validity of the reults.

5. In Tables 1-5, the authors performed pathway analysis and shown some data. However, there was no information included in the method section on how the authors analyzed the data, and whether the authors had taken into account false discovery rate etc given the presence of multiple cytokines interrogated. It was also unclear whether the findings in Table 1-4 referred to average/aggregate dataset from the 3 patients or only one of them.

Reply 5. It has been made clear that all highly expressed cytokines (their protein designations) of all patients have been fed into the Reactome pathway analysis. The FDR rates are now cited.
Changes: lines 123-124, lines 132-133, lines 156-157 and lines 165-166.

Reviewer C

In this manuscript, Rath et al present cytokines data analysis from SCLC MPE samples and supernatants of the corresponding isolated cell lines. Using the Human Profiler Arrays Cytokine XL they uncovered that, multiple cytokines are upregulated in the MPE samples. These cytokines are enriched for the IL4, IL13, IL4, IL18 and IL33 pathways. While the data is interesting, there are multiple aspects of the experimental design and data interpretation that need to be clarified before publication. Major concerns are described below:

1. The Materials and Methods section is very shortly described with details lacking. Some of these details make data interpretation difficult.
2. The Results section is also short and insufficiently developed.
3. What are the controls for cytokine blot arrays?
4. How were the cell lines established, and how long were the cells cultured before cytokines were collected?
5. I find it hard to believe that there is an exact correlation between the highest expression cytokines in MPE samples and supernatants from established cell lines. Again, a description of the procedure would help.
6. The raw data for the cytokines only show one number, yet there are 3 samples for which duplicate experiments were performed.
7. Why is S1088 not included in the cytokine analysis but used for cell proliferation analysis?
8. Figure legends need to be described in more detail.

Reply 1 and 2. Supplemented.
Reply 3. Supplemented, lines 91-92.
Reply4. Supplemented, lines 80-86.
Reply 5. The three cell lines used for the assay of the cytokines in their conditioned media represent M1 metastases of the tumors causative for the corresponding MPEs and therefore related. It is logical the cytokines from primary tumor cells (leaking into MPE) and their M1 metastases are related are in nice agreement with published literature.
Reply 6. Figures 1. and 2. Show mean values ± SD of the samples analyzed.
Reply 7. Because we wanted to add one unrelated further sample for the MTT test.
Reply 8. Supplemented.