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Analysis of the Doctorate thesis presented by **mgr Anna Muchlińska**

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The work by: **mgr Anna Muchlińska**
Entitled:

"**Znaczenie biologiczne i kliniczne fibroblastów związanych z nowotworem oraz krążących fibroblastów w progresji raka piersi"**

Focuses on an important aspect of the mechanism of cancer progression which is linked to the cooperation of the microenvironment with the tumor cells. In the particular instance the subject is devoted to the role of the main population of cells that, in addition to the cancer cells, are building the cancer organ i.e. the fibroblasts. Upon participating to the cancer site microenvironment, the fibroblasts phenotype and activity change into the cancer activated fibroblast phenotype (CAF).

The latter is highly significant for the tumor evolution and aggressiveness, for this reason it is very important to define physically, biologically and molecularly the reasons of the recruitment and roles of these cells.

The work here is mainly devoted to the possibility to use the characteristics of the CAFs to the diagnotic aspect: is there a direct relation between the presence and properties of the CAFs and the type and properties of the developing cancer.

Here the focus is turned to the breast cancer (BC) which is especially difficult due to its diversity in terms of histopathology as well as properties and evolution. In consequence, the finding of new characters that would help its better definition will considerably help curing it.

Consequently, the strategy of the present doctoral work was to examine the properties of the CAFs in the tumor site as well as taking advantage of their presence in the circulation to find out whether such circulating CAFs: cCAFs, can be a criterion in the diagnosis. This is approached in conjunction with the circulating tumor cells: cCTCs and analyzed in the context of the previously and classically characterized breast cancer cases, lumA, lumB, their markers ER+, PgR+, HER2+ as well as TNBC….

Considering the sampling of the study, it involves sufficiently large numbers of patients that permit to validate the in vitro research by in vivo data. In addition, this allowed to produce primary tumor cell lines that were of very high significance in terms of expression of critical molecules and most precisely the alpha smooth muscle actin a-SMA, which is a typical marker of CAFs and which is useful for further isolation of the concerned cells.

Moreover, this marker is sufficiently specific since it allows to isolate the CAF cells by its expression on the surface and distinguishes them from the tumor cells as very nicely demonstrated here.

The work, shows in addition that one can take advantage of the level of expression of the a-SMA by the CAFs and show its significance as far as the tumor evolution is concerned. It shows that the populations of CAFs are not equal in their expression of the protein. The high expression of a-SMA by the CAFs corresponds to a cell population that is able to cooperate to the tumor cells growth and expansion. The works shows this point but does not identifies totally (although it shows a role for osteopontin) which molecule(s) produced by the CAFs, would be responsible for the tumor cells stimulation of the growth.

Similarly, it appears necessary to have shown the role of direct cell to cell contacts, i.e. whether or not this cell-to-cell contact would have a better effect than the use of the conditioned media. This is very significant in the tumor site where cells can be both in direct contact or distant, separated by a matrix as mimicked in the present experimental setting by the Matrigel.

The work examines the growth of the tumor cells under the influence of the conditioned medium from the CAFs and this is performed in conditions that are called 3D because the cells are in Matrigel.

It is true that the MCF7 cells are thus making colonies but the 3D structures are not defined. The work does not describe a real 3D structure as we do not have data on the volume of the colonies (only 2D pictures). This being remarked and provided the fact that the dilutions correspond to the exactly same number of cells and are standardized, the data are clear that the conditioned media from CAFs-a-SMA+ are responsible for the activation of tumor cells growth.

It is understandable that the hypothesis is based on the possibility that circulating CAFs are able to condition distant (pre)metastatic niches, this is probably why the experiment was limited to testing the soluble molecules

The best molecular candidate for the effects mediated by the cCAFs is suggested here to be attributable to osteopontin.

The work nicely shows that among the differently expressed genes between the CAFs primary lines, that express high levels of a-SMA, and the lines that display a poor expression of the molecule, a set of over expressed genes are related to: controlling the ECM, the regulation of the ERK1 and 2, production of IL8 and the cell-to-cell interactions. No further evidence concerned the ERK1 and 2 phosphorylation.

Moreover, the differential display of genes on a large analysis of the high and low a-SMA expressing lines, pointed to 29 upregulated DEG and 25 downregulated genes.

The validation on *in vivo* biopsies indicate very clearly that the most engaged gene as well as identified protein is OPN (Fig, 12 and Fig. 13).

The work concerning OPN is clear and well conducted too. The growth effect on tumor cells of OPN, coming from the CAFs that are a-SMA+ is mainly, proven by the direct and specific inhibition by the use of neutralizing antibodies. It needs to be remarked that the direct conclusions about the Ki-67+ cells seems overinterpreted (Fig 14 E) and needs to be more documented.

The next steps attempt to uncover and characterize the circulating fibroblasts linked to cancer in relation to the phenotype of the circulating tumor cells.

Using the image stream machine (Amnis) it was possible to analyze first the characters of the tumor cell lines LumA, LumB, HER2+, TNBC and observe some differences on the basis on their side scatter, pan-K labelling, and Vimentin intensiveness.

Remark: the picture Fig. 15, indicates CD43/CD31 labelling while the text and caption to figure speaks of CD45/CD31. Please explain the discrepancy.

This is corrected in the Fig. 16 which shows very clearly the characterization of the cCAFs distinguishable from the cCTCs by their a-SMA expression. None of the tumor cells do express it.

Further is a thorough analysis of the possible correlations between cCTCs populations and the cCAFs, together with the significance for the possible diagnostic.

This interesting work leads to the conclusion that by analyzing the characteristics and the proportions of the circulating tumor derived fibroblasts in association with the circulating tumor cells, it is possible to conclude about the metastatic potential and validate the prognostic of survival especially in relation with the EMT.

Remarks:

The 10 pages introduction are very clear and the aim of the work is well expressed. Remark concerns the explanatory figures, which are very nice, they should be annotated in term of their origin (for example page 17), and if original, the name of the program used should be indicated. This is true for the whole manuscript.

Page 16: the description of the microenvironment is by far too light. There are fundamental mechanisms that are not even cited (angiogenesis for example).

It is surprising and should be justified. In particular, the angiogenesis is announced in the introduction, the discussion and the conclusions although absolutely nowhere else is there any data about it, despite the fact that the OPN and IL8 are among the main actors of the thesis. Please explain.

In the MATERIAL AND METHODS SECTION

Page 20: it is true that the positive selection has positive aspect but it should not be forgotten that it is dangerous if the selected cells must be used after the selection procedure.

Pages 25 and 26: in the description of the antibodies the type of immunoglobulin is important. It is absolutely not enough to say: « antibody ».

Page 36: is it possible to make a better description of the 3D and compare them to spheroids.

RESULTS

Is it possible to comment a population that would be EpCAM+ SNAIL+ would they be all a-SMA+ ?

It is possible to suggest other candidate pathways than AKT and ERK phosphorylation ?

Page 58: Fig 14 could the E-Ki67 data be more commented? Is it significant?

In General

The work is clearly explained, the experiments well conducted and analyzed with distance and critical view. This is very important.

The main conclusion ***"*** *wykazano, ze CAFs a-SMA high koreluja z gorszym rokowaniem w luminalnym raku piersi i moga byc zwiazane z bardziej agresywnym fenotypemkomórek raka piersi.*

Wydzielanie OPN przez te Komórki moze byc jednym z mechanizmów wyjasniajacych to zjawisko. Dodatkowo pokazano pierwsze badanie wykazujace zdolnosc imFC do wspólwykrywania cCAFs i CTCs we krwi obwodowej pobranej od chorych z rakiem piersi. cCAFs, wspólwykrywane z CTCs o róznym fenotypie,moga wskazywac na bardziej zaawansowana chorobe, co zasluguje na dalsze badaniaw wiekszych kohortach chorych."

Is strictly valid and the whole work is largely documented. It appears that the candidate is very familiar with her subject and highly competent.

Magister Anna Muchlinska is the first author of 2 publications in the last 2 years, one of them with a high impact factor, thus:

In conclusion:

W podsumowaniu stwierdzam ze rozprawa doktorska mgr Anna Muchlińska pt. :

„Znaczenie biologiczne i kliniczne fibroblastów związanych z nowotworem oraz krążących fibroblastów w progresji raka piersi", ma oryginalny charakter, swiaczy umiejetnocsi samodzielnego wprowadzenia badan naukowych i zasluguguje na bardzo wysoko ocene.
Rozprawa doktorska spelnia warunki okreslone w art.187 Ustawy Prawo o szkolnictwie wyszym i nauce (t. j. Dz. U. z 2021r poz. 478 ze zm.). W zwiazku z powyszym, zwracam sie do Wysokiej Rady Dyscypliny Nauk Medycznych Gdanskiego Unywersytet Medycznego o dopuszczenie Magister Anna Muchlinska do dalszych etapow przewodu doktorskiego.
Jednoczesnie wnioskuje o odpowiednie wyroznienie rozprawy doktorskiej Magister Anny Muchlinska.

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