REPORT
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GENETIC AND IMMUNE SIGNATURE FOR PREDICTION OF THE RESPONSE TO NEOADJUVANT CHEMOTHERAPY IN PATIENTS WITH BLADDER CANCER

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1. Project summary

Bladder cancer is one of the most common cancers and a leading cause of cancer-related mortality in men. Neoadjuvant chemotherapy with cisplatin (NAC) before subsequent bladder removal (known as radical cystectomy) is the standard treatment for patients with muscle-invasive bladder cancer (MIBC). Unfortunately, a large proportion of patients (more than 50%) do not respond to this treatment. Identifying reliable biomarkers that can classify non-responding patients is crucial to streamline surgery, reduce costs, and decrease morbidity.

The objective of this study is to identify a biomarker that can predict the response to NAC in MIBC patients. To achieve this, we recruited a cohort of patients from whom we obtained fresh peripheral blood and tumor tissue, as well as preserved samples in paraffin. We conducted an in-depth characterization of the immune response in these samples.

2. Results

We recruited 45 patients with a urothelial MIBC T2-4N0M0 and candidates to neoadjuvant chemotherapy with fresh blood and tissue. In addition, we also recruited 20 patients with MIBC T2-4N0M0 and candidates to neoadjuvant chemotherapy with paraffin samples. Controls with no tumor were also recruited.

A multiparametric flow-cytometry analysis was performed in peripheral blood mononuclear cells (PBMCs) and bladder tissue of pT2-T4N0 MIBC patients at pre-treatment (n=17) and post-neoadjuvant treatment (radical cystectomy surgery (RC), n=21). Paired pre/post-treatment samples were available for 8 patients. Seven individuals without cancer were included as controls. Non-response was determined at the cystectomy and patients were classified either as non-responders or responders. We have found that the immune response in responders and non-responders is different.

- The expression of the membrane-bound ATPase CD39 in peripheral blood CD4+ T-cells was differentially expressed in responders and non-responders to neoadjuvant

treatment. The percentage of circulating CD4+ CD39+T cells was significantly increased in patients who non-responded to the neoadjuvant treatment at the time of TURBT, also showing the same trend at RC, compared with responders, suggesting these cells may play a role in tumor resistance. The proportion of those cells in non-responders at the TURBT was also higher than in responder MIBC patients, NMIBC patients, and the control population. In PBMCs, CD39 is particularly enriched in effector memory and central memory CD4+T-cells, and the differences between responders and non-responders were even significantly higher if those central memory and effector cells were compared. CD39-expressing CD4+T-cells have higher expression of the immune checkpoint inhibitor PD-1 and reduced cytolytic enzymes granzyme-1 (GrzB) and perforin, compared to levels found in the CD39- population. In addition, in blood CD4+T-cells CD39 significantly correlated positively with FoxP3+ and TIGIT+, and negatively with GrzB and perforin. These results were also similar in the immune compartment of bladder tissue from MIBC patients, as the frequency of intratumoral CD39+ CD4+T-cells were significantly more abundant in non-responder than responder neoadjuvant-treated patients, and these CD39+ cells also showed a positive correlation with tumor CD4+T-cells expressing TIGIT, ICOS and FoxP3. Therefore, we have identified the expression of CD39 in total and different maturation subsets of CD4+ T-cells as a promising biomarker to predict response to neoadjuvant treatment in MIBC patients. Furthermore, our data suggest that targeting CD39 could provide a new therapeutic strategy to improve clinical outcomes for individuals with MIBC.

- The expression of other markers on CD8+ T cells such as the inhibitory marker NKG2A or PD-1 on the early effector memory cells was also different between responders and non-responders.

- Functional differences were also observed. Cells from patients who respond to the treatment secrete a higher production of IL4 in both CD4+ T cells and CD8+ T cells than patients who do not respond. This suggests an association between the response and the presence of cells with a phenotype Th2. On the other hand, the NK cells of these responding patients produced less IL-21. IL-21 acts by preventing the activation of NK cells, so we could assume that in this case patients with a good response could have a more activated NK compartment. This idea is reinforced by the results observed with the IL-17. Non-responding patients have higher levels of IL17 and it has been described that L-17 promotes tumorigenesis and metastasis constraining NK cell

antitumor and antiviral activity via inhibition of NK cell maturation. Patients with a good response also have a more polyfunctional (secreting more than one cytokine) CD8+ T cells than patients with no response. The immune response was also measured based on T cell activation and surface expression of different receptors. This assay detects cells that are activated as a result of antigen-specific stimulation by upregulation of the activation-induced surface markers CD137, and OX40. These analyses show that non--responding patients have significantly less immune activation than those patients who have a good response in both CD4+ and CD8+ T cells. Overall, the functional analysis indicated that the activation profile of certain blood-immune cell populations could also be used to differentiate the response of MIBC patients to neoadjuvant treatment. Although a deeper insight into the biological mechanisms involved in these differences is warranted, our results seem to indicate that cells from responders tend to have increased immune activation, which might be another factor to explain why these patients obtain better clinical outcomes.

In addition, viable cryopreserved single-cell suspensions from peripheral blood (PBMCs) and tumor samples were stored in liquid N2 and single cell RNA-seq was done in a total of 16 tissue samples (9 samples at the moment of the tumor detection (transurethral resection of bladder tumor (RTU) and 6 matched RTU and cystectomy samples and 1 non-tumoral tissue). In two of these individuals single-cell RNA-seq were done in matched samples of PBMCs (both at the time of RTU and of the cystectomy). Our preliminary data show that:

- After the cell annotation process (using Seurat), we were able to identify a large number of immune populations, both from the innate and adaptive immune systems, T and non-T cells as well as different phenotypes such as exhausted, regulatory or proliferative cells. We have also been able to detect non-immune cells such as endothelial cells, muscle cells and fibroblasts. Using Seurat's cell cycle scoring we were able to calculate cell cycle scores and observe the immune cells that were proliferating. We observed very little proliferation in the epithelial cells due to the low yield obtained with these tumoral cells.

- Differential abundance analysis was done for responders RTU-cystectomy and non-responders RTU-cystectomy. We observed that responders have significant (p. adj<0.1) changes in a number of cell types while no significant changes can be

observed in non-responders. The differences in the different populations are being analyzed in more depth.

- Single-cell RNA-seq and single-cell TCR-seq analysis: In two patients (at the RTU and at the cystectomy) and in tissue and in PBMCs a RNA-seq and TCRseq in single cells was carried out. In these sample we could observe that at least one TCR sequence was detected and as expected, there is a very good overlap between cells with TCR and with cells annotated as T cells. We evaluated the clonality of the TCRs in tissue samples and PBMCs and in the RTU (PRE) and at the Cystectomy (POST) and interestingly for both patients we observed an increase in clonality after treatment. Furthermore, we observed that tumor-infiltrating T cells have a greater change in clonality than peripheral T cells. This is consistent with T cell expansion associated with an antitumour response. If we evaluate the TCR profile in tissue at the RTU and at the cystectomy using a UMAP we can observe that in post-treatment (at the cystectomy) the distribution of clonotypes across cell states changes dramatically in both individuals. What can be observed in both patients is an expansion of CD8 effectors that are already present in the tumor pre-treatment (RTU).

- TCR profile (TCR variable beta chain sequencing) in FFPE samples: Sequencing of the CDR3 regions of human TCRβ chains have been performed using the immunoSEQ® Assay (Adaptive Biotechnologies) from DNA from paraffin obtained from 10 sequential individuals (atRTU and cystectomy) with different treatment response. We are analyzing all the sequences and preliminarily we can observe that: TCR sequences are not shared in high frequencies between individuals in the RTU or cystectomy samples. On the other hand, and as the single cell analysis showed, a high number of TCRs are shared in the same patient between the TUR and cystectomy sample. A deeper analysis is currently in process.

- Spatial transcriptomics in FFPE samples: To complete the analysis of cellular characterization in TUR and cystectomy samples in a more in-depth and specific way, 8 samples from this cohort of patients are being analysed using spatial transcriptomics.

3. Relevance and potential future implications

The clinical implications of the results obtained in the overall project cannot yet be extrapolated as a large amount of data is still being processed as well as the integrated analysis of all of them. However, preliminary results of the project indicate that we will be able to obtain an in-depth characterization of both the composition (phenotype) and the functionality of the immune cells present both in the peripheral blood and in the tissue before and after the treatment with neoadjuvant chemotherapy. This data shows that the immune response (the phenotype and the functionality) of responder and non-responder patients is different. One of the pathways that could be playing an important role in the pathogenesis of bladder cancer and in the treatment response is the CD39/CD73 pathway. This pathway, which is the ATP-adenosine pathway, has emerged in recent years as a promising target for cancer therapy in other types of tumors. In this regard, we have identified the expression of CD39 in total and different maturation subsets of CD4 T-cells as a promising biomarker to predict response to chemotherapy in MIBC patients, which has the potential to be used in clinical practice upon further validation studies. Furthermore, our data suggested CD39 may have a tumor resistance role, and thus CD39 inhibition could provide new therapeutic approaches to target MIBC and their resistance to chemotherapy. Single-cell and spatial transcriptomic and TCR profile data will provide us with corroboration of our cytometry data as well as other cellular pathways that are participating in the response to treatment and that could potentially help in better clinical management of patients. In this regard, we observed that in patients with bladder cancer and in the tumoral tissue there is a T cell expansion, likely associated with an antitumor response and that these responding cell are already presents in the RTU sample. This data suggests that the antitumor responses are not generated de novo after treatment and therefore these infiltrating immune cells could be expanded and used as possible therapeutic strategies.

4. Scientific bibliography generated

Some of the results from this project have been presented in:

- Senserrich J., Buisan, O., Servian, P., Clotet B., Cabrera, C.CD4 T-cell CD39 as a predictive biomarker of response to neoadjuvant chemotherapy in muscle-invasive bladder cancer patients. Senserrich J., Buisan, O., Servian, P., Clotet B., Cabrera, C. ASEICA 40th Anniversary Congress, A Coruña, 14th to 16th November 2023.

- Buisan Rueda, Oscar; Senserrich Velasco, Jordi; Servian Vives, Pol; Sanchez Rodriguez, Maria; Garcia Rodriguez, Elisabet; Pagés Oliveras, Joan; Freixa Sala, Roger; Colomer Gallardo, Anna; García Puche, Mireia; Segura Alabart, Maria; Ferreiro Pareja, Cristina; Vigues Julia, Francesc; Areal Calama, Joan; Clotet Sala, Bonaventura; Bellmunt Molins, Joaquim; Cabrera Navarro, Cecilia. Role of the CD39 pathway in modulating the response to neoadjuvant therapy in muscle invasive bladder cancer patients. XXXVII Reunión Nacional del Grupo de Urología Oncológica, Santander, 25 y 26 de abril, Santander. Awarded with the best poster in bladder.

Part of the results will be presented in the 2024 ASCO meeting:

- Joaquim Bellmunt, Margot Hully, Ilana Epstein, Sonsoles Liria Veiga, Yingtian Xie, Shweta Kukreja, Miguel Munoz, Paula Garoz Martinez, Nuria Fernandez Martinez, Cecilia Cabrera, Toni K Choueiri, Henry Long, Paloma Cejas. Combined high resolution H3K27ac epigenomic and single cell transcriptional profiling identifies a signature predictive of response to neoadjuvant immune checkpoint inhibitors (ICI) in Urothelial Cancer (UC).

An abstract with results has been submitted to the 2024 ESMO meeting:

- O. Buisan, J. Senserrich, P. Servian, M. Sanchez, E. Garcia, J. Pagès, R. Freixa, A. Colomer, M. García, M. Segura, J. Cervera, C. Ferreiro, F. Vigues, J.J. Areal, B. Clotet, J. Bellmunt, C. Cabrera. Role of the CD39 pathway in modulating the response to neoadjuvant therapy in muscle-invasive bladder cancer patients