REPORT
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OVERCOMING RESISTANCE TO IMMUNOTHERAPIES THROUGH MYC INHIBITION IN MUTATED LUNG CANCER IN KRAS WITH DIVERSE MUTATIONAL PROFILES

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1. Summary of the project

Lung cancer is the leading cause of cancer mortality worldwide. Common mutations in this disease activate the KRAS oncogene, which confers poor prognosis and high risk of tumor recurrence. Unfortunately, no personalized therapies are available to target the majority of these mutations. Even immunotherapy, which is now showing efficacy in other indications, displays limited activity in most KRAS-mutated tumors, possibly because the concurrent mutations that often accompany KRAS alterations lead to different immunological properties.

We propose an alternative therapeutic strategy centered on targeting a pivotal downstream node of KRAS crucial in lung cancer pathogenesis: the MYC transcription factor. MYC is deregulated in most, if not all, cancers, and not only promotes tumor progression by inducing cell proliferation but is also responsible for tumor immune evasion, a phenomenon that makes cancer invisible to our immune system. Although there is still no direct MYC inhibitor available in the clinic, the Omomyc mini-protein, an excellent MYC inhibitor in pre-clinical studies, has successfully finished its first clinical trial. We have shown that this mini-protein has a dramatic therapeutic impact in mouse models of KRAS-driven non-small cell lung cancer (NSCLC) and to cause T-cell recruitment to the tumor site. Therefore, our hypothesis is that MYC inhibition by Omomyc in KRAS-driven lung adenocarcinomas bearing the most common co-occurring tumor suppressor gene (TSG) mutations (Trp53, Lkb1 and Keap1) will not only halt tumor progression but also promote a shift from an immunosuppressive microenvironment to a more immune-stimulatory one, fostering an effective antitumor immune response that could overcome, or potentially prevent, the development of immunotherapeutic resistance.

2. Results

To verify our hypothesis, we employed different isogenic cell lines derived from the KRAS-G12D lung cancer (KLA) transgenic mouse model, each previously engineered by Dr. Silvestre Vicent to harbor the most prevalent TSG concomitant mutations: TP53, STK11, and KEAP1. These cell lines provided a robust platform for our investigation. Our primary objective was a comprehensive characterization of these cell lines both in

vitro and in vivo. We observed similar growth kinetics across all KLA cell lines, albeit with subtle deceleration in those carrying STK11 and KEAP1 mutations. Then, we quantified the endogenous levels of MYC within the different cell line variants. Intriguingly, our western blot analyses revealed a notable increase in basal MYC levels across all TSG-mutant cell lines, with particularly significant upregulation observed in the STK11 and KEAP1 mutants.

Furthermore, we conducted transcriptomic analyses to further characterize the different cell lines. In this regard, cells bearing mutations in the TSG displayed increased expression of genes related to cell migration, extracellular matrix re-organization and response to oxidative stress, and presented downregulation of genes related to immune response, the complement cascade and leukocyte migration, in comparison to the parental cell line.

Following our in vitro investigations, we assessed the behavior of the cell lines in an in vivo context. After setting up the proper inoculation conditions, we determined that the KLA-Keap1 cells exhibited accelerated growth in vivo compared to the other cell line variants. We then proceeded to characterize the immune infiltrate in the presence of the different TSG mutations by flow cytometry. To this end, we analyzed different immune cell populations after 3 and 5 weeks of tumor implantation. Results showed that the tumor immune infiltrate undergoes temporal changes, progressively shifting towards a more immunosuppressed phenotype over time, irrespective of the tumor's mutational profile. However, the immunosuppressed phenotype was more profound in the STK11-mutant tumors, mirroring observations made in patients bearing this mutation.

We then went back to the in vitro setting to investigate the response to MYC inhibition by Omomyc of the different cell lines. A standard Omomyc dose resulted in a significant reduction of cell growth across all KLA cell lines compared to untreated controls. Omomyc also changed the cell cycle profile, inducing an intriguing arrest in S phase in all the TSG-mutant cell lines but not the parental one.

Then, to study how MYC inhibition could influence the tumor immune response, genes related to immune evasion and antigen presentation processes were analyzed by qPCR. Results indicated significant alterations in these genes upon Omomyc treatment in all

TSG-deficient cell lines, with more pronounced changes observed in the KLA-P53 model. Notably, each cell line exhibited distinct patterns of gene down- or upregulation in response to Omomyc. Interestingly, further transcriptomic analyses revealed that, in response to Omomyc, all mutant cell lines underwent downregulation of genes related to receptor signaling pathways and upregulation of genes related to the immune response.

Following this, we examined the response to MYC inhibition in vivo and observed that animals treated with Omomyc displayed significantly reduced tumor growth compared to their vehicle counterparts in parental (KLA-NT), P53- and KEAP1-mutant KLA cell lines, with a more pronounced reduction in the p53 mutant model.

Modulation of the tumor immune microenvironment was also observed upon Omomyc treatment. The most profound immune changes were seen in those models where Omomyc was able to better reduce tumor growth, namely NT and P53 mutant models. In contrast, STK11 and KEAP1 mutant models did not show significant changes in the tumor-immune microenvironment.

Based on these findings, we proceeded to in vivo combination of Omomyc with immunotherapy in both NT and P53-mutant models. Such a combination enhanced the median survival obtained with both agents alone, with almost all of the treated mice showing partial (PR) or complete response (CR) during the treatment compared to half of the mice treated only with immunotherapy.

In addition to evaluating the combination of Omomyc with immune checkpoint inhibitors, this project provided an opportunity to explore Omomyc's synergy with other anticancer treatments commonly employed for KRAS-mutant NSCLC, specifically KRAS and MEK1/2 inhibitors. Our findings underscored the remarkable therapeutic efficacy of combining these inhibitors with Omomyc, thus paving the way for further investigation in this promising research line.

3. Relevance with possible future implications

Current cancer drugs target degenerate and redundant functions of cancer cells, which evolve and compensate for the blocked function, leading to disease recurrence and resistance. Moreover, they often cause undesirable side-effects and toxicity. In this project funded by La Marató we propose an innovative approach to attack MYC, a non-redundant function controlling cell division, immune cell recognition and resistance to therapy. OMO-103 (Omomyc) is a first-in-class direct MYC inhibitor that has recently successfully completed a first-in-human (FIH) clinical trial in patients with advanced solid tumors. Excitingly, the results of the trial support excellent safety, dose-dependent target engagement in patients and clinical activity. The potential clinical impact of this approach is unprecedented as an efficient first-in-class MYC inhibitor could serve to treat several, if not all, types of cancer.

In this project we have focused on KRAS-mutant NSCLC presenting concurrent mutations in the most common tumor suppressor genes (TP53, KEAP1 and STK11). Patients with KRAS mutations are the most underserved patient segment since no effective targeted drugs are available for them, hence they are recurrently treated with highly toxic and ineffective chemotherapies. In fact, most targeted therapies for KRAS-driven lung cancer have failed due to the emergence of compensatory or adaptive resistance mechanisms. This is a clear limitation, which demands the development of novel therapeutic approaches to cover the whole spectrum of KRAS mutations. To address this unmet need, in this project we have demonstrated that MYC inhibition is an effective therapeutic strategy to treat KRAS-mutant tumors, even in the presence of different concomitant TSG mutations, demonstrating the highest efficacy in the tumors with combined KRAS and P53 mutations, followed by the ones with only mutations in KRAS.

In addition, we have observed a profound modulation of the antitumor immune response after Omomyc treatment, with the immunosuppressive microenvironment switching towards a more immune-stimulatory state. With these results, we have provided the proof of concept that MYC inhibition is beneficial for patients with KRAS-driven NSCLC with different mutational profiles, fact that could influence the clinical decision to add these patients to clinical trials assessing an anti- MYC therapy. Immunotherapy has recently been approved as first-line monotherapy or in

combination with standard chemotherapy for the treatment of advanced NSCLC patients. However, despite showing remarkable responses in some patients, immune checkpoint inhibitors (ICIs) do not display efficacy against the vast majority of KRAS-mutant tumors, due to innate or acquired resistance. This may be explained by the presence of different patterns of immune system engagement ignited by concomitant mutations in tumor suppressor genes like P53, STK11 and KEAP1. The results derived from this project sustain this hypothesis, since we have demonstrated that the tumor-immune microenvironment is different among the tumors presenting these mutational profiles and that it turns into a more immunosuppressed microenvironment as the tumors evolve. This degree of immunosuppression depends on the mutational profile, tumors with KRAS and P53 mutations or with KRAS mutation only being less immunosuppressed. Interestingly, these two mutational profiles were the ones showing a more profound immune engagement after Omomyc treatment.

Based on these findings, we conducted experiments to evaluate the synergy between MYC inhibition and two clinically available immunotherapies. Our results demonstrated the remarkable therapeutic synergy achieved through these dual combination treatments. Identifying combination strategies with synergistic anti-tumor activity, designed to counteract immunotherapeutic resistance, could offer first-in-class therapies for NSCLC patients, personalizing cancer treatments for the different patient subgroups and thus unveiling new interventional strategies for the treatment of this underserved patient group. Importantly, since Omomyc is currently being tested in clinical trials. The results from this project can have significant clinical impact, providing a strong rationale for additional arms in phase I/II studies for KRAS-mutant patients with diverse mutational profiles. There, Omomyc could be used as a combinatorial therapy to potentially eradicate tumors currently considered immunotherapy-resistant. Since, to date, there are no effective therapeutic options for KRAS-mutant tumors, this project provides the proof of concept for novel drug combinations that could provide lung cancer patients with more effective and less toxic therapies, substantially reducing current health care costs while also improving patients' quality of life. Importantly, given that KRAS mutations are predominant in pancreatic and colorectal cancers as well, the potential application of these combination treatments to these highly prevalent tumor types holds the promise of an even more substantial clinical impact.

In conclusion, this La Marató project has pioneered another new line of research poised for rapid and substantial clinical advancement. In this context, we are also actively exploring the therapeutic synergy achieved by pairing Omomyc with recently approved KRAS inhibitors, a pursuit that has already provided very promising outcomes.

4. Scientific bibliography generated

The outcomes of this project have been showcased at numerous national and international conferences through both poster and oral presentations:

- Íñigo González-Larreategui *et al.* MYC ***inhibition by an Omomyc-based therapy abrogates tumor progression and induces immune cell recruitment in models of KRAS-driven NSCLC.*** I Congress of Naïve Immunologists from Catalan Society of Immunology, Barcelona, September 2022. **Poster presentation**

- Íñigo González-Larreategui *et al.* ***Characterization of KRAS-driven NSCLC cell lines with diverse mutational landscape and their response to MYC inhibition.*** Summer School in Translational Cancer Research (CancerCore Europe), Algarve (Portugal), October 2022. **Poster presentation**

- Íñigo González-Larreategui *et al.* ***Characterization of KRAS-driven NSCLC cell lines with diverse mutational landscape and their response to MYC inhibition.*** Molecular Targets and Cancer Therapeutics Symposium (EORTC-NCI-AACR), Barcelona, October 2022. **Poster presentation**

- Sílvia Casacuberta-Serra *et al. MYC* ***inhibition by OMO-103 induces immune cell recruitment in preclinical models of NSCLC and modulates the cytokine and chemokine profiles of Phase I patients showing stable disease***. XVI Congress of the Catalan Society of Immunology (SCI) Advanced Immunotherapies. Barcelona, November 2022. **Oral Communication**.

- Íñigo González-Larreategui *et al.* ***Characterization of KRAS-driven NSCLC cell lines with diverse mutational landscape and their response to MYC inhibition.*** XVI Jornada Científica Vall d'Hebron, Barcelona, December 2022. **Poster presentation**

- ***A new generation of miniproteins for cancer treatment.*** Cancer Biology module at Brunel University, London (UK), February 2023. **Invited speaker**

- Laura Soucek *et al.* ***Identification of potential biomarkers of response to OMO-103, a first- in-modality pan-MYC inhibitor, in patients with advanced solid tumors.*** Presented at the Biomarkers of Response in Novel Molecular Therapeutics Minisymposium in the AACR2023 Annual Meeting, Orlando (USA), April 2023. **Selected Speaker**

- Íñigo González-Larreategui *et al.* ***Characterization of KRAS-driven NSCLC cell lines with diverse mutational landscape and their response to MYC inhibition.*** Defence is the Best Attack: Immuno-Oncology Breakthroughs, organized by the European Association for Cancer Research (EACR), Barcelona, May 2023. **Poster presentation**

- Laura Soucek *et al.* ***Progress in the development of a clinically viable MYC inhibitor***. EACR 2023 Congress: Innovative Cancer Science, Turin (Italy), June 2023. **Poster presentation**

- Íñigo González-Larreategui *et al.* **Therapeutic impact and immune modulation by MYC inhibition in KRAS-driven NSCLC with diverse mutational landscapes.** XVII Annual Scientific Congress of the Catalan Society of Immunology (SCI), Barcelona, November 2023. **Oral communication.**

- Íñigo González-Larreategui *et al.* **Therapeutic impact and immune modulation by MYC inhibition in KRAS-driven NSCLC with diverse mutational landscapes.** 17th Vall d'Hebron Scientific Meeting, Barcelona, November 2023. **Poster presentation.**

The development of this project has also led to the following scientific publications:

- Sílvia Casacuberta-Serra, Íñigo González-Larreategui, Daniel Capitán-Leo, Laura Soucek. MYC *and KRAS Cooperation: From Historical Challenges to Therapeutic Opportunities in Cancer*. In second revision in Signal Transduction and Targeted Therapy (IF 39.2).

- Casacuberta-Serra S, Gonzalez-Larreategui I, Soucek L. *eIF4 A dependency: the hidden key to unlock KRAS mutant non-small cell lung cancer's vulnerability.* Transl Lung Cancer Res. 2023 (Editorial comment).

Additional scientific publications encompassing all the results derived from this project are currently in preparation for submission to high impact journals.