Peer Review File

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Reviewer A:

This editorial comment is appropriate for publication. It is written in a very clear manner and helps in understanding the role of metabolism in resistant cancer.

Reply: We thank reviewer A for the positive assessment of our work.

Reviewer B:

To my mind, the editorial commentary is well written, but could outline more precisely why the authors believe that this study is worth writing an editorial commentary.

Reply: We believe that the study is worth highlighting in an editorial commentary as it identifies a novel role for metabolic enzymes in driving enzalutamide resistance in mCRPC; a role not associated with their metabolic functions but rather additional noncanonical functions. In turn, this discovery potentially opens up novel research areas, including characterization of noncanonical functions of metabolic enzymes in prostate cancer pathobiology and possibly development of new treatment approaches.

As suggested by the reviewer, we have now outlined these aspects more explicitly upfront when introducing the study (page 5, line 95-99):

"In a recent study, Li et al. (18) aimed to further address the challenges posed by treatment resistance in mCRPC by systematically identifying genes that, upon knockout, would enhance sensitivity to enzalutamide. In doing so, the authors uncovered a novel mechanism by which a noncanonical function of a metabolic enzyme drives resistance to enzalutamide. This discovery brings attention to previously unrecognized functions of metabolic enzymes in cancer pathobiology and could pave the way for development of new treatment options for mCRPC."

These aspects were also highlighted in the original version (as well as the current version) in our conclusion (page 10, line 216-223):

"To conclude, the work by Li et al. brings attention to the role of noncanonical functions of metabolic enzymes in the development of resistance to anticancer therapies, such as enzalutamide in the treatment of mCRPC, and provides a potential novel therapeutic avenue for overcoming resistance. Further research is however needed to establish the clinical utility of targeting PGAM2 and to better understand the aberrant regulation of noncanonical functions of metabolic enzymes in prostate cancer pathogenesis and in resistance to treatment

more broadly. Ultimately, such work may advance the development of therapeutic approaches to target these moonlighting functions."

The part where the authors discuss the use of CRISPR screening to successfully identify new targets for therapy could also be improved in a way that it is understandable for the readers why this approach should be more successful in comparison to other studies (page 4).

We have now elaborated on the description of pooled CRISPR screens to provide additional background that we hope will make the methodology and its advantages in identification of treatment resistance genes easier to understand (page 4, line 71-84):

"Given the breadth of potential resistance mechanisms, large-scale approaches are needed to effectively identify genetic drivers of resistance that may ultimately be used as predictive biomarkers to guide treatment decisions or as direct therapeutic targets. Since the harnessing of clustered regularly interspaced short palindromic repeats (CRISPR) for human genome editing in 2013 (14,15), the system has been leveraged towards unravelling genotype-phenotype relationships in a high-throughput fashion, employing pooled CRISPR screens. This methodology allows the perturbation of one gene per cell with a barcoded guide (i.e., either knocking out or turning on the expression of the gene), allowing any number of genes to be perturbed in a pooled format. Cells are then exposed to a challenge, such as drug treatment. In the context of resistance, guides that have been enriched for in the final population following drug treatment, compared to control-treated cells, are those conferring resistance, while those depleted may reflect genes that sensitize to treatment. The advantage of CRISPR screening in comparison to other methods such as chronic drug exposure experiments or evaluation of patient samples is primarily the scalability and systematic approach, allowing parallel, unbiased investigation of genotype-phenotype relationships genome-wide (16). Furthermore, compared to similar earlier methods such as RNAi screens, CRISPR-based knockout screens provide higher consistency, complete (rather than partial) knockout of a gene, and less off-target effects (17)."

I would also suggest to omit the general term ARSI, since the study of Li et al only used enzalutamide and, later on, this issue is even criticized.

We thank the reviewer for this suggestion. We believe, however, that it is important to introduce the term ARSI to indicate that there are other androgen receptor signaling inhibitors beyond enzalutamide, especially since cross-resistance between different ARSIs is not uncommon. To clarify this aspect, we have now added the following sentence in the manuscript (page 4, line 64-65):

"Of further note, several of the above-mentioned mechanisms may lead to cross-resistance between ARSIs (e.g., enzalutamide and abiraterone) (4)."

Furthermore, a number of the studies referred to in paragraph 2 of the editorial (page 3-4, line 50-65) have also considered resistance to abiraterone, not solely enzalutamide. We feel therefore that it is more reflective to use the more encompassing term, ARSI, in this discussion. The term ARSI has not been used in describing any of the experiments performed by Li et al., as these were focused solely on enzalutamide, as also noted by the reviewer. Lastly, we believe it is necessary to introduce the term ARSI, to be able to draw attention towards the end of the editorial to our belief that it would also be of relevance to investigate the role of PGAM2 in relation to resistance to other ARSIs, such as abiraterone or apalutamide.

In this regard, it could be discussed, in which way the dosage of 20 µM enzalutamide that was used in the study of Li et al is comparable with regard to drug concentrations that are used in the clinic.

We thank the reviewer for this question. This is a very challenging comparison to make, as the doses employed in the screen are optimized for the particular screen (i.e. cell line, conditions tested, etc.), rather than having something to do with a clinically relevant dose. It is generally recommended that for a negative screen, a dose achieving killing of 25–50% of the perturbed cells, should be employed, while for a positive selection screen a dose killing above 50% of cells is advised (1). It is of course critical to validate screen findings *in vivo* in animal models (which the authors did in Figure 3, discussed on page 6, line 117-121) and ultimately in patient samples (which the authors did in Figures 6 and 7, discussed on page 7, line 142-156) to ensure that the screen results are clinically relevant. We have now acknowledged the importance of validating screen findings in patient samples by adding the following to the manuscript (page 7, line 150-152):

"CRISPR screens are performed in a highly artificial in vitro setting. As such, it is essential to consider the clinical relevance of the candidate resistance genes identified. The authors, thus, investigated a cohort of 41 CRPC patients and found that patients with high PGAM2 expression progressed faster on enzalutamide than patients with low PGAM2 expression. They also investigated a publicly available CRPC cohort consisting of metastatic tumor tissue samples from 31 patients and showed that patients with high PGAM2 expression had shorter overall survival than those with low PGAM2 expression."

References:

1. Bock C, Datlinger P, Chardon F, Coelho MA, Dong MB, Lawson KA, et al. High-content CRISPR screening. Nat Rev Methods Primers. 2022;2(1).