Editorial Commentary

Metabolic enzymes moonlighting to drive enzalutamide resistance in prostate cancer

Maria Rusan[1,2,3#], Simone Weiss[1,3#], Karina Dalsgaard Sørensen[1,3]

[1]Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark; [2]Department of Clinical Pharmacology, Aarhus University Hospital, Aarhus, Denmark; [3]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

[#]These authors contributed equally to this work.

Correspondence to: Karina Dalsgaard Sørensen, MSc, PhD. Professor, Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark; Department of Clinical Medicine, Aarhus University, Palle Juul-Jensens Boulevard 99, 8200 Aarhus N, Denmark. Email: kdso@clin.au.dk.

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The catalogue of life-prolonging treatment options for patients with metastatic castration resistant prostate cancer (mCRPC) has expanded significantly in recent years. Among these treatments are androgen receptor signaling inhibitors (ARSIs), such as enzalutamide. Unfortunately, a significant proportion (25–30%) of mCRPC patients present with primary ARSI resistance (1) and acquired resistance inevitably develops in the remaining patients (2), drastically limiting life expectancy. Drug resistance thereby poses a major challenge in the clinical management of advanced prostate cancer.

Several recent studies have characterized resistance to ARSIs, employing preclinical approaches (3-6), as well as characterization of clinical samples from prostate cancer patients that have progressed on treatment (7,8). These have described re-activation of the androgen receptor (AR) by mutation or amplification of the *AR* gene, and via alternative splice variants of AR lacking the ligand binding domain, as well as by increased intra-tumoral production of androgens, as drivers of enzalutamide resistance (7,9). Additionally, activation of AR-target genes by bypass activation of the glucocorticoid receptor or the Wnt/β-catenin pathway has been noted (8,9). AR-pathway independent mechanisms have also been identified, such as combined TP53/RB1 loss driving lineage plasticity from adenocarcinoma to a neuroendocrine phenotype (9,10), autocrine activation via FGFR/MAPK signaling (3) or IL-6/JAK/STAT signaling (4), and activation of MAPK signaling via gain-of-function BRAF mutations (5). Recently, tumor myeloid infiltration has also been demonstrated to mediate resistance to ARSIs in a paracrine fashion, possibly via reactivation of AR signaling, highlighting the role of the tumor microenvironment in resistance development (11). Of further note, several of the above-mentioned mechanisms may lead to cross-resistance between ARSIs (e.g., enzalutamide and abiraterone) (1).

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 (12), 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 unbiased investigation of genotype-phenotype relationships genome-wide (13). 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 (14). In relation to prostate cancer, this has led to the identification of several novel genes involved in enzalutamide resistance, employing CRISPR knockout or activation screens (5,6,15,16). While CRISPR screening is a powerful tool to investigate the functions of thousands of genes in parallel, the limitation of many published CRISPR screens is in-depth functional validation of top candidate genes in pre-clinical models, and ultimately the translation of findings into the clinic. To fully exploit the potential of CRISPR screening, greater emphasis must be placed on the clinical validation of these findings.

In a recent study, Li *et al.* (15) 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. To consider enzalutamide sensitivity, the authors performed a pooled genome-wide CRISPR-Cas9 knockout screen, in the C4-2 cell line, which has been derived from a patient with mCRPC (15). The authors primarily focused on negative hits from the screen, i.e., genes that upon knockout resulted in increased cell death during enzalutamide treatment. The validity of the screen was confirmed by the identification of several genes known to mediate resistance to enzalutamide in prostate cancer, such as *AR*, *BIRC6*, and *ID1* (1,17,18). However, the top negative hit in the screen was the *PGAM2* gene, which encodes the glycolytic enzyme, phosphoglycerate mutase 2, that has not previously been associated with responsiveness to enzalutamide.

To validate this novel "hit", the authors carried out an array of elegant follow-up studies in multiple preclinical mCRPC models. First, they performed cell proliferation (CCK-8) and colony formation assays using the two enzalutamide-resistant cell lines C4-2R and 22Rv1, demonstrating that enzalutamide treatment combined with PGAM2 inhibition decreased cell proliferation and colony formation. Immunofluorescent staining of caspase 3/7 and flow cytometry analysis of Annexin V-FITC/PI-stained C4-2R and 22Rv1 cells further demonstrated that combined PGAM2 inhibition and enzalutamide treatment increased apoptosis. By western blotting, the authors also demonstrated that increased apoptosis levels were mediated by downregulation of anti-apoptotic BCL2 signaling. Findings were then validated *in vivo* in a mouse xenograft model employing C4-2R cells. Here, combined enzalutamide treatment and PGAM2 inhibition reduced xenograft tumor growth and weight. Immunohistochemistry of the xenograft tumor tissues also confirmed that combined PGAM2 inhibition and enzalutamide treatment was associated with downregulated BCL2 signaling.

Interestingly, overexpression of an enzymatically inactive version of PGAM2 in C4-2R and 22Rv1 cells did not enhance enzalutamide sensitivity in proliferation, colony formation, and apoptosis assays, as opposed to direct inhibition of endogenous (wildtype) PGAM2 expression. This suggested that the effect noted was not mediated by the canonical enzymatic activity of PGAM2. Diving more into the mechanism of PGAM2-mediated enzalutamide resistance, the authors overexpressed Flag-PGAM2 in C4-2R cells followed by anti-Flag affinity purification and mass spectrometry analysis, identifying 225 interaction partners of PGAM2. Focusing on proteins previously reported to be apoptosis-related, the authors narrowed in on 14-3-3ζ as a potential critical interaction partner in mediating resistance. Indeed, mutation of 14-3-3ζ at the site predicted to interact with PGAM2, along with enzalutamide treatment, phenocopied the effects seen upon silencing of PGAM2 expression using shRNA. Given that c-Jun N-terminal kinase (JNK) is known to phosphorylate 14-3-3ζ leading to activation of pro-apoptotic signaling, the authors then explored whether PGAM2 influenced phosphorylation of 14-3-3ζ and found that PGAM2, by binding to 14-3-3ζ, prevented its phosphorylation, thereby hindering apoptosis initiation. This negative regulation was demonstrated even in the presence of a JNK activator (anisomycin) (19). Cumulatively, these results indicate a novel non-canonical role for PGAM2 as a negative regulator of 14-3-3ζ-driven apoptosis.

The authors then analyzed prostate cancer tissue samples from 14 patients before and after treatment with an ARSI,

and observed downregulation of AR and PGAM2 post-treatment, suggesting a potential link between the two proteins. To explore this, they treated C4-2R cells with the AR agonist, dihydrotestosterone, and observed increased expression of PGAM2. Using public ChIP-seq data, they identified an AR peak near the *PGAM2* promoter in prostate cancer cells and then confirmed the interaction between AR and the promoter of *PGAM2* in C4-2R cells using ChIP-PCR, demonstrating that *PGAM2* is an AR target gene.

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.

Collectively, the above results led the authors to propose PGAM2 as a promising therapeutic target in enzalutamide-resistant prostate cancer (15). Although Li *et al.* performed extensive preclinical validation *in vitro*, which was supported by additional *in vivo* data, it remains to be seen how broadly generalizable the mechanism presented is. Given that AR upregulates *PGAM2* transcription, PGAM2 targeting is likely to be of relevance primarily in AR-dependent enzalutamide resistance, and less likely in AR-independent resistance mechanisms, such as reprogramming to a neuroendocrine phenotype. As it previously has been shown that <70% of mCRPC tumors maintain AR-dependence (3), PGAM2-targeting may not be beneficial in a significant subset of mCRPC patients. Additional clinical validation is necessary, including consideration of whether PGAM2 expression is upregulated at the time of acquired enzalutamide resistance, as well as additional *in vivo* or *ex vivo* studies to consider the potential of PGAM2-targeting in overcoming enzalutamide resistance in AR-driven and AR-indifferent contexts. It would also be of relevance to investigate the role of PGAM2 in relation to responsiveness to other ARSIs, such as abiraterone or apalutamide. Finally, the relevance of these results will need to be considered in the rapidly changing treatment landscape of mCRPC, where double and triple combination therapy (androgen deprivation therapy + ARSI +/– docetaxel) is shifting to the upfront metastatic hormone-sensitive prostate cancer setting (20,21).

No inhibitors of PGAM2 are currently available to test the utility of directly targeting this protein in patients. However, Li *et al.* speculated that inhibition of JNK may be an alternative approach (15). JNK is known to phosphorylate the PGAM2-interaction partner 14-3-3ζ and the pro-apoptotic 14-3-3ζ-sequestering partner BCL2 associated agonist of cell death (BAD), thereby promoting apoptosis (19). Thus, the proposal to use a JNK inhibitor to overcome enzalutamide resistance contrasts with the current knowledge about the functional role of JNK as a mediator of apoptosis. It further contradicts the data presented by Li *et al.* themselves, showing enhanced phosphorylation of 14-3-3-ζ and enhanced apoptosis upon addition of the JNK activator anisomycin. Accordingly, the logical conclusion would therefore be that JNK activation, rather than inhibition, may have therapeutic potential. However, another study (22) did indeed previously show that JNK inhibition with Bentamapimod (AS602801) synergized with enzalutamide in prostate cancer cellular models. Surprisingly, the authors of that study observed that JNK was actually activated upon treatment with the AS602801 JNK inhibitor, suggesting regulation via a negative feedback loop, and potentially explaining the paradoxical finding that both JNK activation and JNK inhibition may potentially promote apoptosis (22). Overall, the mechanism behind JNK activation/inhibition enhancing enzalutamide-induced apoptosis in prostate cancer is still speculative and needs more thorough investigation before being mature for clinical testing.

As mentioned, Li *et al.* interestingly found that the role of PGAM2 in driving enzalutamide resistance is independent of its metabolic function in glycolysis (15). Metabolic reprogramming in tumor progression is well described, however there is increasing evidence that metabolic enzymes also serve noncanonical functions ("moonlighting" functions), and that these can have aberrant activities contributing to cancer development (23), and possibly to treatment resistance (24). Metabolic enzymes have been shown to be able to alter chromatin structure via a broad range of epigenetic modifications, to modulate the activity of transcription factors and their coactivators, and to alter mRNA stability, thereby regulating gene expression. Additional roles have been described in cell-cycle progression, homologous recombination repair and non-homologous end joining DNA repair, and in the regulation of a number of key proliferation and survival pathways (i.e., PI3K-AKT-mTOR, MAPK, NF-κB, and TGF-β), as well

as in autophagy and apoptosis (11,25). Lastly, noncanonical functions of metabolic enzymes have been implicated in modulation of cytoskeleton dynamics (25) and remodeling of the tumor microenvironment via exosomes and ectosomes (23). These processes have been implicated in tumorigenesis and metastasis in a multitude of cancer types, including non-small cell lung cancer, breast cancer, cervical cancer, glioblastoma, pancreatic cancer, and prostate cancer (11,25). The study by Li *et al.* expands on this novel role of metabolic enzymes, demonstrating that they also can moonlight to drive treatment resistance to enzalutamide in advanced prostate cancer.

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.

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