Simulating androgen receptor selection in designer yeast

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Abstract

We established a designer yeast assay to simulate natural androgen receptor (AR) selection using AR antagonists. Yeast HIS3 gene transactivation was associated with the ligand-induced recruitment of steroid receptor coactivator-1 (SRC-1) by AR mutants, where yeast growth in histidine-free medium was determined as the outcome. Our simple and efficient assay can enable precise one-pot screening of AR mutants.

Main Text

Defective androgen receptor (AR) mutations are associated with several human diseases, such as androgen insensitivity syndrome (AIS)\(^1\), prostate cancer (PCa)\(^2\), and spinal and bulbar muscular atrophy (SBMA)\(^3\). To date, more than 1000 AR mutations, including point mutations and indels, have been documented in the androgen receptor gene mutations database (ARDB)\(^4\). However, their annotation and potential therapeutic significance in human disease development remain challenging. Synthetic biology in yeast offers a solution to this problem, as yeast can be easily customized to realize various purposes\(^5,6,7,8,9,10,11\).

Here, we developed designer yeast to evaluate the effects of AR mutants by simulating the natural selection of various clinical AR-targeted compounds. AR ligand-binding domain (AR_LBD) and steroid receptor coactivator-1 (SRC-1), a major transcriptional coactivator of nuclear receptors, were used in the yeast 2-hybrid system by separately fusing to the DNA-binding domain (DBD) and transcriptional activation domain (AD) of GAL4, respectively (Fig. 1a). Upon ligand binding, the activated Gal4_DBD-AR_LBD hybrid translocated to the nucleus and bound to the upstream activating sequences of the HIS3 reporter. The recruitment of Gal4_AD-SRC-1 hybrid by AR led to the transactivation of the HIS3 reporter gene, driving the synthesis of histidine (HIS), and ultimately, yeast growth in HIS-free media. Designer yeast integrity requires the co-presence of AR-LBD, SRC-1, and androgen stimulation, as indicated by the quantitative reverse transcription polymerase chain reaction assay, showing that the HIS3 mRNA level was induced ~10-fold upon dihydrotestosterone (DHT) stimulation (Fig. 1b). Tightly regulated HIS3 transactivation contributes to DHT-induced yeast growth without defects compared with growth upon HIS supplementation. The AR response to DHT in designer yeast was dose-dependent (Extended Fig. 1a), and the reliability of this yeast was validated (Extended Fig. 1b). Next, we evaluated a series of AIS-associated AR mutants (L678P, V685I, L701M, G709V, R711T, G725S, M743V) with low androgen-binding activities as negative references\(^4\). Compared with the wild-type (WT) AR_LBD, all these AR mutants exhibited a varying degree of severe growth defects in DHT-supplemented medium, indicating their impaired DHT-responsiveness (Fig. 1c). The extent of loss-of-function was ranked by yeast growth in response to a range of DHT dosages, and the DHT-responsiveness ranking was WT > V685I > R711T > L701M > G725S/M743V > L678P/G709V.

We further streamlined the screening process using designer yeast based on a panel of AR mutants known to be activated by clinical antagonists of AR-ligand binding (termed AR antagonists), providing a
reference for personalized PCa therapy and prescriptions (Fig. 1d). We chose three classic AR antagonists—two non-steroidal AR antagonists enzalutamide (ENZ) and bicalutamide (BIC), and one steroidal AR antagonist cyproterone acetate (CPA). Five known patient-derived AR mutants conferring corresponding resistance to these AR antagonists were assessed—AR-W742L/C (BIC-resistant mutant), AR-T878A (CPA-resistant mutant), AR-F877L (ENZ-resistant mutant), and AR-F877L/T878A double mutant. All five AR mutants were successfully activated by their corresponding AR antagonists in a dose-dependent manner (Fig. 1e and Extended Fig. 2), with real-time and continuous monitoring of the visualized responsiveness using plate culture (Extended Fig. 3). AR-W742L and AR-W742C mutants exhibited a similar resistance level against BIC (Extended Fig. 4a). T878A signifies the antagonistic effects of ENZ on AR-F877L as indicated by the AR-F877L/T878A double mutant exhibiting 3.5-fold greater response to ENZ than the AR-F877L single mutant (Fig. 2a). A dose-response assay on plate culture led to the same conclusion (Fig. 2b), indicating that our designer yeast can determine the combined effects of multiple mutations in AR function. Interestingly, the ENZ-resistant mutant AR-F877L confers partial resistance to CPA, a previously unknown finding (Fig. 1e and Extended Fig. 2). The dose-response assay on plate culture showed that the AR-F877L/T878A double mutant exhibited greater CPA response than the AR-T878A single mutant, followed by the least-CPA-resistant mutant AR-F877L (Fig. 2c). A luciferase assay of AR-negative human liver cancer cell line, Hep3B, was used to further validate the ranking results obtained in the yeast assay and confirm that the AR-F877L/T878A double mutant conferred greater resistance to ENZ/CPA than either F877L or T878A single mutant (Extended Fig. 4b and 4c). Although CPA appeared to exhibit pan-antagonistic activity, it could not activate the BIC-specific AR mutants W742L and W742C in either yeast or luciferase assay, even when AR-WT was already activated by high CPA concentrations (Fig. 2c and Extended Fig. 4d). With the advantages of precision and high-sensitivity, our designer yeast can be used as a reference tool for clinical diagnostics and personalized medicine. For example, a 70-year-old man (in year 2016) with primary tumor of prostate cancer carrying an AR-W742C mutation was referred to the Zhejiang Cancer Hospital, Hangzhou, China. The designer yeast assay results promptly indicated (within a week) the antagonistic effect of BIC (but neither ENZ nor CPA) on the W742C mutant, and ENZ and CPA rather than BIC were recommended.

Furthermore, we propose a one-pot screening assay for the efficient and high-throughput identification of mutant AR libraries taking advantage of yeast auxotrophs (Fig. 2d). A yeast library composed of various AR mutants under the control of AR antagonists was selected. A DNA library was then isolated from yeast pools and subjected to sequencing. This assay enabled us to simulate natural AR selection using AR antagonists. To evaluate this pooled screening method, we constructed an equimolar yeast library composed of all AR mutants with possible single base substitutions at the codon of F877, termed AR-F877-library (Fig. 2e). Initially, the genotypes of all individual mutants from the AR-F877-library were assessed in designer yeast. Only the AR-F877L mutant exhibited resistance to both ENZ and CPA, indicating that the designer yeast was sensitive enough to precisely distinguish the AR amino acid identity in response to ligands. This library was then subjected to pooled screening against a panel of AR antagonists (Fig. 2f). High-throughput Sanger sequencing of the pooled AR-F877-library against DHT
showed mixed chromatogram peaks at the 877 codon when aligned with AR-WT, reflecting minimal selection pressure under DHT. In contrast, the AR-F877L mutant stood out of the AR-F877-library under the selection of both ENZ and CPA, validating the success of the pooled screening method. We demonstrated that designer yeast can simulate natural AR selection and that the process can be extended in developing AR antagonists.

Evans developed a screening assay using a luciferase reporter driven by hormone-responsive mammalian promoters. This assay can provide informative results and is thus often used to investigate the roles of mutant hormone receptors. In contrast, our one-pot screening assay used a HIS auxotroph of yeast and enabled the efficient identification of individual AR mutants from a mixed mutant population by using colony formation and readouts as visual methods that do not require specialized equipment. The ARE-driven ADE2 reporter was previously used for a colorimetric yeast reporter assay, however, our method included the effects of transcriptional coregulators on AR, because the recruitment of coregulators (especially coactivators) by AR plays a significant role in the development and progression of PCa. In addition, our designer yeast provides a clean background, enabling AR mutants to be assessed in a foreign environment and thus allowing us to study the effect of each AR transcriptional coregulator on AR in isolation and in a case-by-case manner. Hence, our method is applicable to the interaction of AR coregulators and AR mutants in general, but not just as exemplified by SRC-1. With the advantages of precision, rapidness, and cost-effectiveness, our designer yeast can be used in AR compound screening, personalized PCa medicine reference, and simulation nature selection of AR mutants.

Methods

Strains, cell line, and materials.

Yeast-2-hybrid (Y2H) strain, YRG-2, was obtained from Stratagene (La Jolla, CA) and grew in YPAD medium (YPD medium with additional 0.1 g/L adenine hemisulfate salt). S. cerevisiae transformants were selected and cultured in synthetic complete (SC) medium without corresponding amino acids. Hep3B obtained from ATCC (American Type Culture Collection) was cultured at 37°C and 5% CO₂ in DMEM (Gibco, USA) supplied with charcoal-stripped fetal bovine serum (FBS, Biological Industries, Cromwell, CT). DHT (HEOWNS, Tianjin, China), CPA (TCI, Shanghai, China), BIC (meilunbio, Dalian, China), and ENZ (Macklin, Shanghai, China) were commercially ordered. Lipofectamine™ 3000 and Dual-Luciferase® Reporter Assay System were purchased from Life Technologies (USA) and Promega (Madison, WI), respectively. All restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA).

Plasmid construction.

Full-length AR (AR-FL) consists of three major functional domains: N-terminal domain (NTD) (residues 1–556), DNA binding domain (DBD) (residues 556–624), and C-terminal Ligand Binding Domain (LBD, residues 666–920), with a flexible hinge region (residues 624–666) in between DBD and LBD. In yeast
system, AR-LBD (ranging from exon4 to exon8 of human AR cDNA) was fused to Gal4-DBD. SRC-1 was fused to Gal4-AD. Y2H plasmids, pBD-GAL4-cam and pGAD424-SRC1, were kindly provided by Dr. Huimin Zhao (University of Illinois at Urbana-Champaign)\textsuperscript{19,20}. Eight wild-type exons of human AR (GenBank: NM_000044) were separately amplified from human H1 genomic DNA and assembled into full-length AR on a digested pRS415 backbone (using \textit{BamH}I and \textit{Pst}I) to generate plasmid pRS415-hAR. AR_LBD (amino acids 629 to 920) was then amplified from pRS415-hAR, and cloned into the multiple cloning site of vector pBD-GAL4-cam with \textit{BamH}I and \textit{Pst}I to generate plasmid pBD-AR_LBD. For luciferase assay, the 2,763-bp full-length AR fragment was digested from plasmid pRS415-hAR by \textit{Sal}I and \textit{Not}I and cloned into pCMV-HA vector to generate plasmid pCMV-hAR. The MMTV promoter was synthesized by Genscript Inc. and then cloned into the pGL3-Basic-LUC vector by \textit{Xho}I and \textit{Hind}III, upstream on luciferase reporter gene, generating pGL3-MMTV-LUC.

\textbf{Site-directed mutagenesis and characterization.}

Site-directed mutagenesis on AR-LBD was performed according to the manual of QuikChange Site-directed mutagenesis Kit (Stratagene). Plasmid pBD-AR_LBD was used as the PCR template.

\textbf{Yeast assay.}

Briefly, pBD-AR_LBD (containing either WT or mutant AR-LBD ) and pGAD424-SRC1 co-transformed YRG2 strain was cultivated to log phase by overnight shaking in liquid SC-Leu-Trp medium (30°C). For liquid yeast assay, overnight cultures were pelleted and washed twice with sterile ddH2O. The washed yeast cells were then inoculated into a 2 mL of SC-Leu-Trp-His medium with a low cell density at a starting OD\textsubscript{600} value of 0.002. Cell density at 600 nm was measured by Thermo Scientific Multiskan FC Microplate Photometer. Dissolved AR antagonists were supplemented into the liquid cultures at indicated concentrations, followed by shaking incubation at 30°C and OD\textsubscript{600} measurement at intervals. For the spotted plate yeast assay, overnight liquid cultures were normalized to an initial OD\textsubscript{600} value of 0.05 and ten-fold serial dilutions of each culture were carried out in a 96-well plate. Five microliters of each dilution were then spotted on the SC-Leu-Trp-His agar plates supplemented with different ligands. After 2–3 days of static incubation at 30°C the plates were photographed.

\textbf{Mammalian cell transfection and luciferase assay.}

This \textit{LUC} reporter system was transiently transfected into Hep3B cells. To measure the reporter activity in a quantitative manner, we normalized the firefly luciferase (FLuc) with the co-transfected \textit{Renilla} luciferase (RLuc), an internal control (plasmid pRLTK) that was read by luminometer at a distinct wavelength. Hep3B cells were cultured in DMEM supplemented with 10% charcoal-stripped FBS (to eliminate interferences of serum hormones) at 37 °C and 5% CO\textsubscript{2}. Approximately 10\textsuperscript{5} cells were subjected to each transfection in a 24-well plate. Vector pCMV-hAR was co-transfected with plasmid pGL3-MMTV-LUC and pRLTK by Lipofectamine 3000 according to the manufacturer’s instructions. After 24h of transfection, the medium was refreshed and additional ligands at indicated concentrations were added. After another 24h
of incubation, cells were harvested. The luciferase assay was performed using the kit of the Dual-Luciferase® Reporter Assay System according to the manufacturer’s instructions.

**Pooled screening of F877-library.**

Briefly, pBD-AR_LBD (containing F877-library mutants) and pGAD424-SRC1 co-transformed YRG2 strain was cultivated to log phase by overnight shaking in liquid SC-Leu-Trp medium (30°C). Overnight cultures were pelleted and washed twice with sterile ddH2O. Cell density at 600 nm was measured. The OD$_{600}$ value of washed yeast were adjusted to 0.05 in 2 mL of SC-Leu-Trp-His medium, followed by the mixing of all these adjusted yeast culture harboring F877-library mutants. The mixed yeast culture of F877-library was used to create ten-fold serial dilutions and five microliters of each dilution were then spotted on the SC-Leu-Trp-His agar plates supplemented with different ligands, followed by static incubation at 30°C. Screened yeast colonies was collected and subjected to the DNA isolation procedure. Isolated yeast DNA mixture were then transformed into *E. coli*. All grown *E. coli* colonies were collected followed by the miniprep. And the isolated plasmid mixture was sent for sequencing.

**The case report on PCa in this study.**

The patient was diagnosed with prostate tumor in 2007 and treated with hormonal therapy: a subcutaneous injection of Leuprolide at a dose of 3.75mg per week; flutamide (Fugerel) was orally took at 250mg three times a day (later changed to bicalutamide at one pill once a day). In 2015, the presented with extreme back pain with a prostate-specific antigen (PSA) level of 65.28 ng/mL and was considered to have developed bone metastasis. In 2016, the patient presented acute urinary retention. Transurethral resection of the prostate was performed on this patient. The paraffin embedded prostate tissue slice were obtained through operation and sent for sequencing. The specimen presented a tumor cell content of 75%. Sequencing results revealed the presence of AR-W742C mutation with a mutant frequency of 1.5%.

**Declarations**

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**Author Contributions**

H.Z., L.Z., and Y.Y. conceived the study and designed experiments; Y.X. and F.L. contributed to the analysis of reported PCa case in this work. S.C. contributed to the design of experiments related ENZ and BIC and provided important insights; H.Z. and L.Z. performed experiments and data analysis. Z.M., M.Y., and B.L. advised on optimizing conditions of dual-luciferase experiments. H.Z and Y.Y. wrote the manuscript.

**Additional information**
Competing interests: there is potential Competing Interest. A patent has been filed for the screening method presented in this study.

Supplementary Information is available for this paper.

Materials & Correspondence: Correspondence and requests for materials should be addressed to Y.Y. (email: yjyuan@tju.edu.cn)

References


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**Figures**
Figure 1

Construction of designer yeast to identify AR mutations. a, Schematic representation of designer yeast. b, Designer yeast integrity requires the presence of AR-LBD and SRC-1, and DHT stimulation ($n = 3$). The $HIS3$ transcript was induced $\times 10$-fold higher upon DHT stimulation ($n = 3$). DHT concentration was 10 nM. c, Loss-of-function AR mutants identified in AIS patients exhibited severely impaired activity in response to DHT in designer yeast ($n = 3$). d, Designer yeast can help streamline personalized PCa
therapy and serve as a prescription reference. e, As delineated in a, tests of a panel of AR-LBD mutants against clinical AR antagonists (ENZ, BIC, and CPA) in yeast ($n = 3$). Ligand conditions: 500 µM CPA; 100 µM ENZ; 200 µM BIC; 100 nM DHT (positive control); Vehicle (dimethyl sulfoxide, negative control). OD$_{600}$ was measured at 36 h for ENZ/CPA/DHT and at 60 h for BIC/Vehicle. Plate pictures were taken at 48 h for CPA/DHT, at 72 h for ENZ, and at 96 h for BIC/Vehicle.

Figure 2
One-pot screening assay to simulate natural AR selection against clinical AR antagonists with high sensitivity. a, An additional T878A mutation amplifies the resistance of the AR-F877L mutant to ENZ \((n = 3)\). Ligand conditions: 500 nM CPA; 50 µM ENZ; 100 µM BIC. b, ENZ dose-response in plate culture. c, Ranking results of the AR-F877L mutant, AR-T878A mutant, and AR-F877L/T878A double mutant against CPA \((n = 3)\). d, Schematic workflow of the pooled screening method in designer yeast. e, Components in the AR-F877-library were first individually assessed in yeast before the library was subjected to the pooled screening method in d. Ligand conditions: 100 nM DHT; 500 nM CPA, 100 µM ENZ; 200 µM BIC. f, High-throughput Sanger sequencing results of the AR-F877-library against DHT, ENZ, and CPA.

**Supplementary Files**

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- [Supplementaryinformation.docx](#)
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