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Original Research Article

Simulating androgen receptor selection in designer yeast

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ABSTRACT

Androgen receptor (AR) mutation is closely associated with prostate cancer (PCa) and is one of the mechanisms of resistance to PCa therapies such as AR antagonists. Although sequencing technologies like next-generation sequencing (NGS) contributes to the high-throughput and precise detection of AR mutations carried by PCa patients, the lack of interpretations of these clinical genetic variants has still been a roadblock for PCa-targeted precision medicine. Here, we established a designer yeast reporter 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. This assay is applicable to determine a wide range of clinical AR mutants including those with loss of function relating to androgen insensitivity syndrome (AIS), and those associated with PCa conferring resistance to AR antagonists such as enzalutamide (ENZ), bicalutamide (BIC), and cyproterone acetate (CPA). One clinical AR mutant previously reported to confer ENZ-resistance, F877L, was found to confer partial resistance to CPA as well using designer yeast. Our simple and efficient assay can enable precise one-pot screening of AR mutants, providing a reference for tailored medicine.

1. Introduction

Androgen receptor (AR) is a ligand-dependent transcription factor that regulates the expression of target genes in response to endogenous androgens such as testosterone (T) and dihydrotestosterone (DHT), playing a critical role in the physiology of both males and females [1] [–] [3]. Ligand-activation induces AR translocation from cytoplasm into the nucleus, where AR homodimers recognize and bind to androgen response elements (ARE) in the promoter regions of target genes, followed by the recruitment of general transcription machinery and co-regulatory proteins (such as the cAMP-response element binding protein and p160 family of coactivators) to modulate the transactivation in a cooperation manner. Importantly, defective AR mutations are associated with a number of human diseases, such as androgen insensitivity syndrome (AIS) [4,5], spinal and bulbar muscular atrophy (SBMA) [6,7], and especially prostate cancer (PCa) [8,9].

AR mutations is one of the mechanisms of resistance to PCa therapies [10] [–] [12]. Androgen deprivation therapy (ADT) is a standard treatment for PCa patients because androgenic stimulation contributes to the progression and development of PCa [13,14]. As PCa patients on ADT often inevitably develop castration-resistant symptoms [15,16], pharmacological antagonists of AR-ligand binding (termed AR antagonists) are clinically used to suppress the tumor growth for patients with castration-resistant prostate cancer (CRPC) [17–19]. AR antagonists bind to AR through a higher affinity to block the effects of androgens and thus inhibit AR activities [20]. However, AR mutations can even confer resistance to AR antagonists by broadening its agonistic spectrum and turning antagonists to agonists [11]. Hence, the identification of AR mutations conferring resistance to AR antagonist contributes to the precision medicine for PCa patients and provides genetic resources for

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clinical prescriptions [21,22]. The development of sequencing technologies such as next-generation sequencing (NGS) helps us to easily detect mutant ARs in patient specimens [23,24]. To date, more than 1000 patient-derived AR mutations, including point mutations and indels, have been documented in the androgen receptor gene mutations database (ARDB) [25]. However, the annotation and potential therapeutic significance of these clinical genetic resources in PCa development remain challenging. And the handbook of personalized therapies for AR-mutated PCa patients is still missing. Synthetic biology in yeast offers a solution to this problem, as yeast can be easily customized to realize various purposes [26] [–] [32]. Yeast Two-Hybrid (Y2H) is commonly used to study binary protein interactions. In addition to advantages of yeast as a general model organism such as fast growth, easy manipulation and low cost, yeast genetic background is clear and suited for the study of specific interaction between human AR and its transcriptional cofactor without interference of other human gene players. Success of the applications of Y2H method in nuclear hormone receptor superfamily members is precedential. For instance, it has been used as a readout for directed evolution of glucocorticoid receptor [33] and estrogen receptor [34,35].

In this study, we developed a designer yeast to simulate natural androgen receptor (AR) selection using AR antagonists. The identification of resistance-conferring AR mutations against clinical AR antagonists is based on the interaction between AR ligand binding domain (LBD), a hot spot of mutational region in AR, and a major transcriptional co-activator of AR, steroid receptor co-activator 1 (SRC-1) [36]. Yeast *HIS3* gene transactivation was associated with the ligand-induced recruitment of SRC-1 by AR mutants, where yeast growth in histidine-free medium was determined as the outcome. In addition, we developed a simple and efficient assay for high-throughput identification of mutant AR libraries by precise one-pot screening of AR mutants. We envisioned that the designer yeast model developed in this study to effectively assess the functionality of AR mutations will contribute not only to precision medicine for PCa patients, but also to other fields such as drug development of AR antagonists.

2. Materials and Methods

2.1. 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). *Saccharomyces cerevisiae* (*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% CO_2 in DMEM (Gibco, USA) supplied with charcoal-stripped fetal bovine serum (FBS, Biological Industries, Cromwell, CT). Dihydrotestosterone (HEOWNS, Tianjin, China), cyproterone acetate (TCI, Shanghai, China), bicalutamide (meilunbio, Dalian, China), and enzalutamide (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).

2.2. 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) [34,35]. 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 *BamH*I and *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 *BamH*I and *Pst*I to generate plasmid pBD-AR_LBD. For luciferase assay, the 2763-bp full-length AR fragment was digested from plasmid pRS415-hAR by *Sal*I and *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 *Xho*I and *Hind*III, upstream on luciferase reporter gene, generating pGL3-MMTV-LUC.

2.3. 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.

2.4. 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 ddH_2O. The washed overnight yeast cultures were subjected to measurement of optical density at the wavelength of 600 nm using a 96-well plate reader (Thermo Scientific Multiskan FC Microplate Photometer) and then diluted with a corresponding fresh medium (SC-Leu-Trp-His) to a specified cell density roughly at 4×10^4 cells per milliliter. Dissolved AR antagonists were supplemented into the liquid cultures at indicated concentrations, followed by shaking incubation at 30 °C and OD_{600} measurement at intervals. For the spotted plate yeast assay, overnight liquid cultures were normalized to an initial cell density roughly at 10^6 cells per milliliter 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.

2.5. Mammalian cell transfection and luciferase assay

This *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 *Renilla* luciferase (RLuc), an internal control (plasmid pRLTK) that was read by luminometer at a distinct wave-length. *Hep3B* cells were cultured in DMEM supplemented with 10% charcoal-stripped FBS (to eliminate interferences of serum hormones) at 37 °C and 5% CO_2. Approximately 10^5 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 24 h of transfection, the medium was refreshed and additional ligands at indicated concentrations were added. After another 24 h of incubation, cells were harvested. The luciferase assay was performed using the kit of the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions.

2.6. 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 5 μL 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.

2.7. 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 3.75 mg per week; flutamide (Fugerel) was orally took at 250 mg

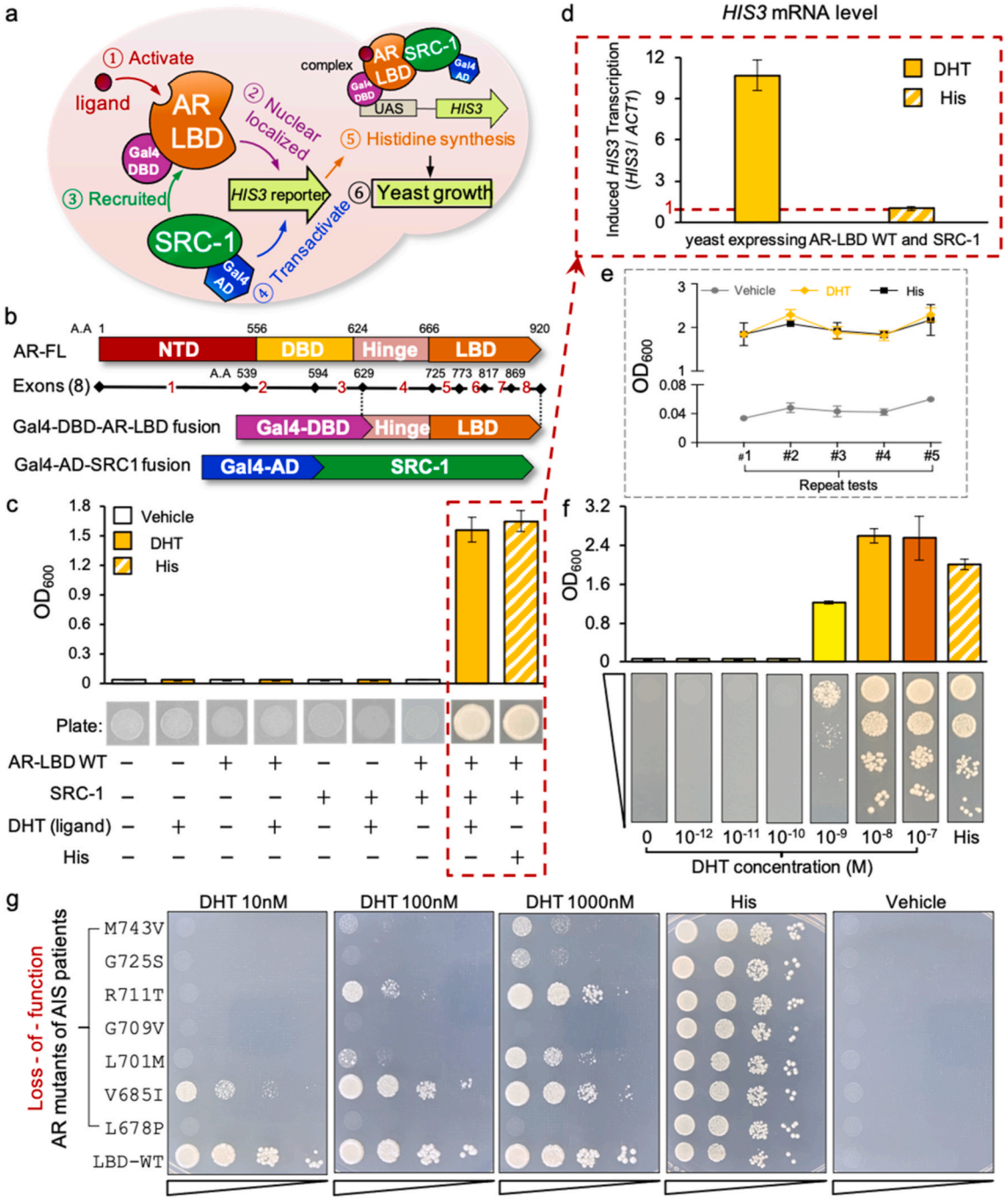

Fig. 1. Construction of designer yeast to identify AR mutations.
a-b, Schematic representation of designer yeast. **c,** Designer yeast integrity requires the presence of AR-LBD and SRC-1, and DHT stimulation (n = 3). Bars indicate mean ± s.d. **d,** The HIS3 transcript was induced ~10-fold higher upon DHT stimulation (n = 3). DHT concentration was 10 nM. Bars indicate mean ± s.d. **e,** Readouts from five independent tests validate the reliability of designer yeast (n = 3, 10 nM DHT). Bars indicate mean ± s.d. **f,** DHT dose-response assay in designer yeast using both liquid and solid cultures (n = 3). Bars indicate mean ± s.d. **g,** Loss-of-function AR mutants identified in AIS patients exhibited severely impaired activity in response to DHT in designer yeast.

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%.

3. Results

3.1. Construction of designer yeast to identify AR mutations

Here, we developed designer yeast to evaluate the effects of AR mutants by simulating the natural selection of various clinical AR-targeted compounds. AR_LBD (ranging from exon4 to exon8 of human AR cDNA) and SRC-1 were used in the yeast 2-hybrid system [37] by separately fusing to the DNA-binding domain (DBD) and transcriptional activation domain (AD) of *GAL4*, respectively (Fig. 1a and Fig. 1b). SRC-1 is used in the yeast model given its important roles in interacting with basal transcriptional machinery and its histone acetyltransferase (HAT) activity that contributes to chromatin remodeling [38,39]. Upon ligand binding, the activated Gal4_DBD-AR_LBD hybrid translocated to the nucleus and bound to the *cis*-acting element (upstream activating sequences, UAS) of the *HIS3* reporter encoding Imidazoleglycerol-phosphate dehydratase, a protein that catalyzes the sixth step of the histidine biosynthesis pathway in yeast. 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. We first validated yeast growth as an indicator of *HIS3* reporter transactivation that relates to the ligand-induced activity of AR. Yeast growth was measured by optical density at 600 nm wavelength (OD_{600}) of liquid yeast cultures, or visually determined by growth size of yeast colonies on culture plates. The integrity of designer yeast requires the co-presence of AR-LBD, SRC-1, and androgen stimulation (Fig. 1c), demonstrating its high stringency. In addition, tightly regulated *HIS3* transactivation contributes to DHT-induced yeast growth without fitness defects compared with growth upon HIS supplementation that compensates the lack of *HIS3* reporter transactivation without DHT stimulation. As indicated by the quantitative reverse transcription polymerase chain reaction assay (RT-qPCR), *HIS3* mRNA level was induced ~10-fold upon DHT stimulation (Fig. 1d), reflecting the direct relationship between yeast growth in HIS-free media and the transactivation of *HIS3* reporter. The reliability of this yeast was further validated through five repeatability tests (Fig. 1e), and the AR response to DHT in designer yeast reporter assay was dose-dependent (Fig. 1f).

In addition, the yeast reporter assay enabled us to identify loss-of-function AR mutations against DHT. AIS is a X-linked recessive disorder due to the loss-of-function mutations of the AR gene in 46, XY individuals. Depending on the dysfunction degree of AR mutants, the phenotypic diversity of AIS ranges from complete AIS (CAIS, characterized by phenotypically normal female external genitalia), to partial or mild AIS (PAIS and MAIS, patients with undervirilized male external genitalia). Through site-directed mutagenesis, we evaluated a series of AIS-associated AR mutants (L678P, V685I, L701M, G709V, R711T, G725S, M743V) with low androgen-binding activities as negative references [25]. 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. 1g). Surprisingly, the varying severity of these AR mutants towards DHT stimulation could be clearly distinguished through their growth defects under the treatment of a series of DHT concentrations, taking advantage of plate culture allowing real-time observations of yeast growth. As Fig. 1g shown, 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.

3.2. The identification of AR mutants conferring resistance to clinical antagonists

We streamlined the screening process using designer yeast based on a panel of resistance-conferring AR mutants known to be activated by clinical AR antagonists. Sequenced clinical AR mutations are subjected into our yeast reporter assay to assess their resistance-conferring phenotypes against a series of AR antagonists, providing a reference for personalized PCa therapy and prescriptions (Fig. 2a). We chose three classic AR antagonists—two non-steroidal AR antagonists enzalutamide (ENZ [40,41]) and bicalutamide (BIC [42,43]), and one steroidal AR antagonist cyproterone acetate (CPA [44,45]). Five known PCa-derived AR mutants conferring corresponding resistance to these AR antagonists were assessed along with AR_LBD WT serving as a negative control—AR-W742L/C (BIC-resistant AR mutant [46–48]), AR-T878A (CPA-resistant AR mutant [49–51], AR-F877L (ENZ-resistant AR mutant [52,53]), and AR-F877L/T878A double mutant [24]. All five AR mutants mentioned above were able to be activated by DHT but not vehicle conditions (Fig. 2b), which indicated no fitness defects were caused by these AR mutations in designer yeast. All five AR mutants mentioned above were successfully activated by their corresponding resistant AR antagonists, as indicated by the antagonist-induced yeast growth, further proving the precision of this yeast reporter assay. In addition, the static plate culture helps to alleviate the leaky expression of *HIS3* compared with liquid culture that requires vigorous shaking. In particular, AR F877L/T878A double mutant responds actively to both ENZ and CPA, as its phenotypic resistance is inherited from both of AR F877L and T878A single mutant. Similar to DHT-response, AR mutations responses to AR antagonists in a dose-dependent manner as well, which further revealed the precise cause-effect relationship of induced activities of mutant ARs by resisting antagonists in designer yeast (Fig. 2c and Fig. S1). Importantly, with the advantage and convenience using yeast plate culture, we achieved a real-time and continuous monitoring of the visualized resistance-responsiveness against AR antagonists conferred by various AR mutations (Fig. S2).

The high-sensitivity of yeast reporter assay enabled us to differentiate the resistance levels conferred by different AR mutants against the same AR antagonist via a dose-response assay. For instance, 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. 3a). A dose-response assay on plate culture led to the same conclusion based on the visually-measured colony size grown on plates (Fig. 3b), which further indicates 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. 3c). The dose-response assay using either liquid culture (Fig. 3d) or plate culture (Fig. 3e) revealed 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, as AR mutations conferring the greater antagonist-resistance were able to be activated upon lower dosages of AR antagonist. 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 (Fig. S3a and Fig. S3b). Although CPA appeared to exhibit pan-antagonistic activity on numerous AR mutations [54], 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. 3e and Fig. S3c). AR-W742L and AR-W742C mutants exhibited a similar resistance level against BIC (Fig. 3f). Unlike ENZ and CPA that activated corresponding AR mutations after 36 h

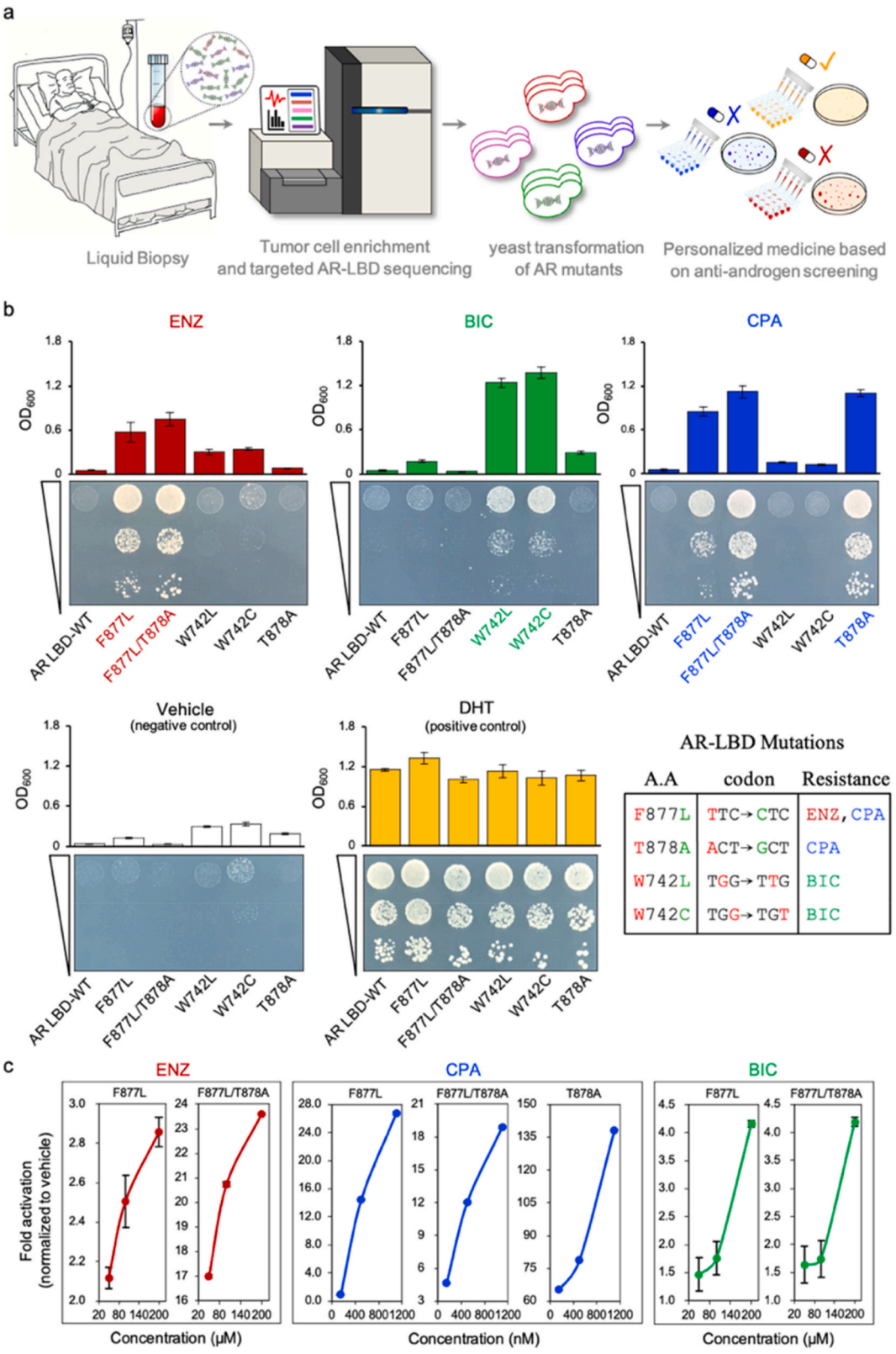

Fig. 2. The identification of AR mutants conferring resistance to clinical antagonists.
a, Designer yeast can help streamline personalized PCa therapy and serve as a prescription reference. **b,** As delineated in a, tests of a panel of AR-LBD mutants against clinical AR antagonists (ENZ, BIC, and CPA) 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). OD600 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. Bars indicate mean ± s.d. **c,** Dose-response assay of AR antagonists in designer yeast (n = 3). Bars indicate mean ± s.d.

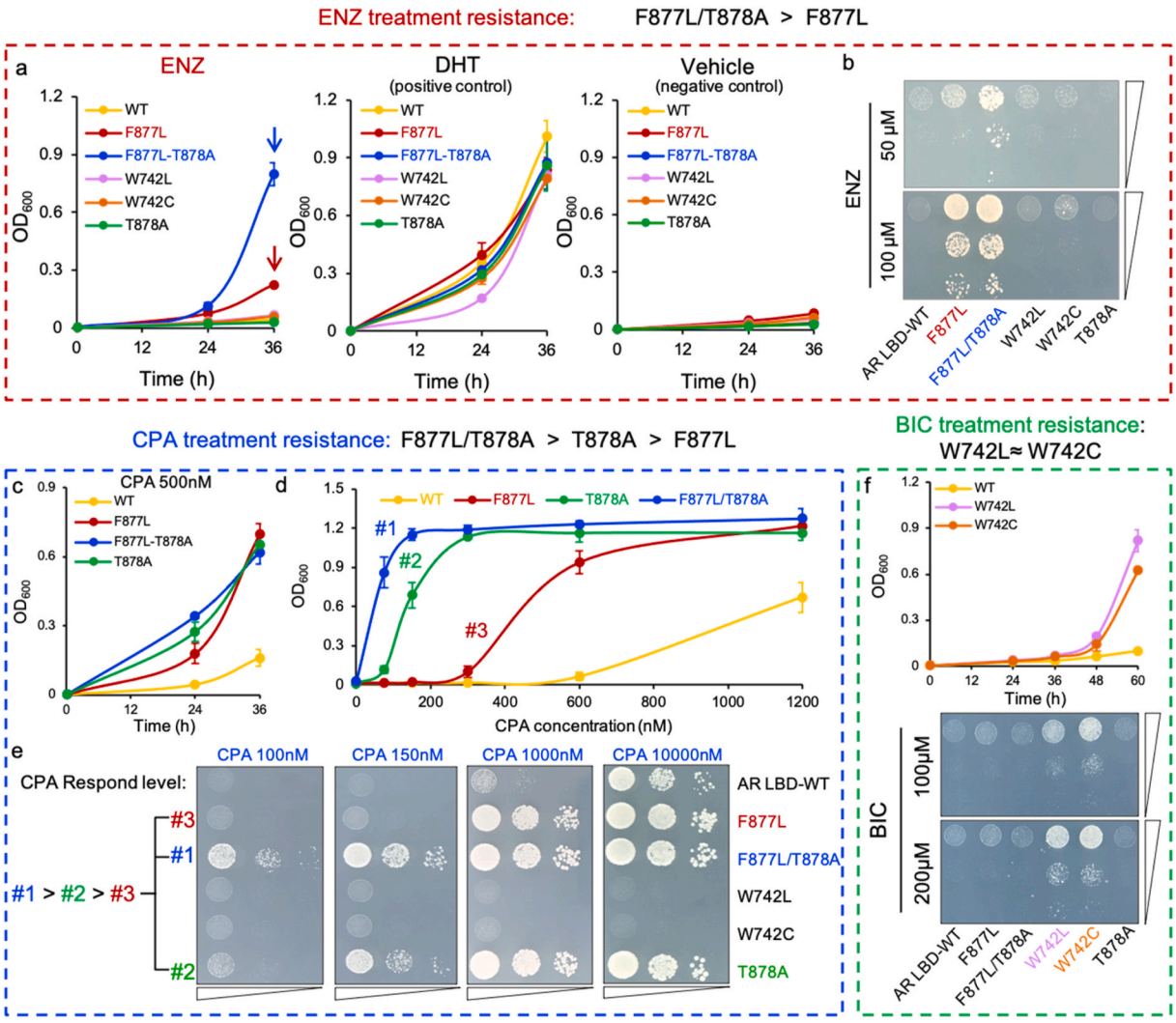

Fig. 3. The identification of AR mutants conferring resistance to clinical antagonists.
a, An additional T878A mutation amplifies the resistance of the AR-F877L mutant to ENZ (n = 3). Ligand conditions: 500 nM CPA; 50 μM ENZ; 100 μM BIC. Bars indicate mean ± s.d. **b,** ENZ dose-response assay in plate culture. **c,** Growth curve of indicated AR mutation upon treatment of 500 nM CPA (n = 3). Bars indicate mean ± s.d. **d,** Ranking results of the AR-F877L mutant, AR-T878A mutant, and AR-F877L/T878A double mutant against CPA using liquid culture (n = 3). Bars indicate mean ± s.d. **e,** Ranking results of the AR-F877L mutant, AR-T878A mutant, and AR-F877L/T878A double mutant against CPA using plate culture. **f,** AR mutations, W742L and W742C, exhibited similar resistance level against BIC in designer yeast (n = 3). Bars indicate mean ± s.d.

of liquid culture (Fig. 3a and c), it took longer for BIC (60 h) to activate AR-W742L and AR-W742C mutants in designer yeast (Fig. 3f), which indicated the differences of pharmacological action time among various AR antagonists on AR mutations.

With the advantages of precision and high-sensitivity demonstrated above, 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 (described in Materials and Methods) was referred to the Zhejiang Cancer Hospital, Hangzhou, China. According to the designer yeast assay results that promptly indicates the antagonistic effect of BIC (but neither ENZ nor CPA) on the W742C mutant, ENZ and CPA rather than BIC should be recommended for better treatment effects.

In addition, all these five AR mutants were tested for their abilities of conferring resistance to the recently FDA approved, structurally distinct non-steroidal AR antagonist, darolutamide [55]. As results shown in Fig. S4, none of the five AR mutants (F877L, T878A, W742L, W742C, F877L/T878A) was able to switch darolutamide from an antagonist to an agonist, consistent with previous studies [56,57] that demonstrated the inhibitory effects of darolutamide on the transcriptional activities of AR mutants F877L and F877L/T878A.

3.3. *One-pot screening assay to simulate natural AR selection against clinical AR antagonists with high sensitivity*

Furthermore, we propose a one-pot screening assay for the efficient and high-throughput identification of mutant AR libraries taking advantage of the auxotrophic selection marker, *HIS3*, of yeast (Fig. 4a). A yeast library composed of various AR mutants under the pressure of AR antagonists was selected. A DNA library was then isolated from yeast pools and subjected to sequencing for resistance-conferring AR mutations against the corresponding pressure of AR antagonist. 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. 4b). Initially, the genotypes of all individual mutants from the AR-F877-

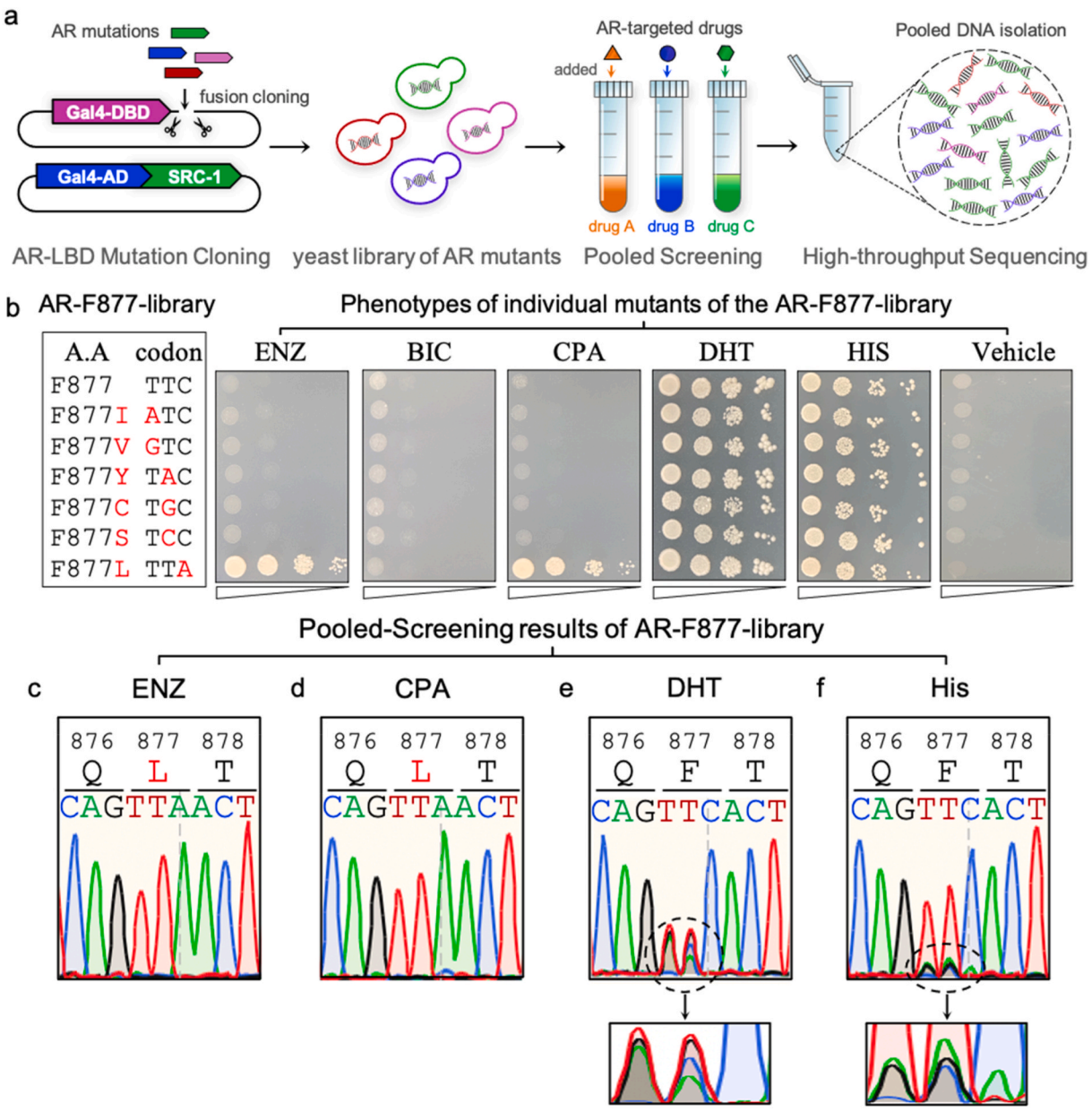

Fig. 4. One-pot screening assay to simulate natural AR selection against clinical AR antagonists with high sensitivity.
a, Schematic workflow of the pooled screening method in designer yeast. **b**, Components in the AR-F877-library were first individually assessed in yeast before the library was subjected to the pooled screening method in **a**. Ligand conditions: 100 nM DHT; 500 nM CPA, 100 μM ENZ; 200 μM BIC. **c**, High-throughput Sanger sequencing results of the AR-F877-library against DHT, ENZ, and CPA.

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 identity differences of AR mutations on the same amino acid in response to ligands. This library was then subjected to pooled screening against a panel of AR antagonists. High-throughput Sanger sequencing (Fig. 4c) 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.

4. Discussion

AR-LBD is the major therapeutic target and its mutation is considered as one mechanism of castration-resistance in advanced PCa diseases as AR is involved in the progression of PCa. Despite the fact that next-generation sequencing have opened up new possibilities for clinical diagnostics and personalized treatment of cancer by high-throughput detecting numerous clinical genetic mutations, the development of precision therapy may still be hampered by a lack of functional annotations for a large number of sequenced genetic variants. In ARDB, over one thousand AR mutations have been discovered in human diseases such as prostate cancer and breast cancer [25]. The functional significance of the majority of sequenced AR mutations found in patient samples has yet to been determined.

Here we establish a *Saccharomyces cerevisiae*-based assay that provides readiness, simplicity, and robustness in high-throughput assessment of the functional impacts of AR mutants in response to a variety of AR-targeted ligands, particularly in the assessment of loss-of-function AR mutations in relation to AIS, and PCa-associated AR mutants conferring resistance to clinical AR antagonists as exemplified by ENZ, BIC and CPA in this study. The success of this yeast model that is based on AR interaction with SRC-1 in response to AR antagonists suggested that AR transcriptional co-regulators could be relevant therapeutic targets for PCa. In addition, we discovered that F877L, a well-studied AR mutant that was previously proved to be resistant to ENZ, imparts additional resistance to CPA using our designer yeast. The designer yeast had tightly controlled reporter gene expression and excellent sensitivity for assessing the functional responses of AR mutations to clinical AR-targeted compounds. As we demonstrated in this paper, the high sensitivity of this designer yeast allows us to distinguish between the levels of resistance conferred by different AR mutants against the same ligand, as evidenced by the results showing that F877L/T878A double mutant confers greater resistance against both ENZ and CPA than the F877L single mutant. Although most AR mutants investigated in this work are single-nucleotide variants, the designer yeast has proven to be capable of determining the combined effects of multiple mutations on AR function as demonstrated by the functional assessment of F877L/T878A double mutant, which has greater resistance than either the F877L or T878A single mutant.

Studies on AR in mammalian cells generally depend on methods like fluorescence resonance energy transfer (FRET) [58] or reporters like chloramphenicol acetyltransferase (CAT) [59–61], and green fluorescent protein (GFP) [52]. In particular, Evans [62] 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 [46,48,50,53,63,64]. Compared to luciferase and FRET assays, which rely on specialized fluorescence equipment, our yeast reporter system can be used to assess AR antagonist resistance by a simple absorbance spectrometer or even eyeballs, which suffice to distinguish HIS auxotrophic growth rates. Furthermore, our system allows one-pot screening assay of a mixed population containing different AR mutants, which is not doable with the luciferase or FRET assay. Computer deep learning of the data from luciferase assays provided insightful information of the relevance between AR mutants and clinical outcomes of drug treatment [64]. It is anticipated that with our method giant dataset can be easily accumulated and aid in machine learning. An *ARE*-driven *ADE2* reporter was previously used for a colorimetric yeast reporter assay [65]; 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 [66–69]. In addition, compared with all the other methods mentioned above, 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. In the current proof-of-concept study, all tested AR mutants including F877L and T878A are well-studied and confer known resistance to some AR antagonists. However, taking advantage of the one-pot screening assay developed here, it's reasonable to envision the further application of our designer yeast in identifying unknown AR mutations. 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.

CRediT authorship contribution statement

Haoran Zhang: Formal analysis, Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Lu Zhang:** Formal analysis, Investigation, Methodology. **Yipeng Xu:** Resources. **Shaoyong Chen:** Writing – review & editing. **Zhenyi Ma:** Resources. **Mingdong Yao:** Reviewing. **Fangyin Li:** Resources. **Bo Li:** Reviewing. **Yingjin Yuan:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

There is potential Competing Interest. A patent has been filed for the screening method presented in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.07.005.

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