Secreted Indicators of Androgen Receptor Activity in Breast Cancer Pre-Clinical Models.

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Abstract

**Purpose.** Accumulating evidence has attracted attention to the androgen receptor (AR) as a biomarker and therapeutic target in breast cancer. We hypothesized that AR activity within the tumor has clinical implications and investigated whether androgen responsive serum factors might serve as a minimally invasive indicator of tumor AR activity.

**Methods.** Based on a comprehensive gene expression analysis of an AR-positive breast cancer patient-derived xenograft (PDX) model, 163 dihydrotestosterone (DHT)-responsive genes were defined as an androgen responsive gene set. Among them, we focused on genes that were DHT-responsive that encode secreted proteins, namely *KLK3, AZGP1* and *PIP*, that encode the secreted factors prostate specific antigen (PSA), zinc-alpha-2-glycoprotein (ZAG) and prolactin induced protein (PIP), respectively. Using AR-positive breast cancer cell lines, expression of candidate factors was assessed in response to agonist DHT and antagonist enzalutamide. Gene set enrichment analysis (GSEA) was performed on publically available gene expression datasets from breast cancer patients to analyze the relationship between genes encoding the secreted factors and other androgen responsive gene sets.

**Results.** Anti-androgen treatment decreased proliferation in all cell lines tested representing various tumor subtypes. Expression of the secreted factors was regulated by AR activation in the majority of breast cancer cell lines. In GSEA, the candidate genes were positively correlated with an androgen responsive gene set across breast cancer subtypes.

**Conclusion.** *KLK3, AZGP1* and *PIP* are AR regulated and reflect tumor AR activity. Further investigations are needed to examine the potential efficacy of these factors as serum biomarkers.

Introduction

Among women worldwide, breast cancer is the most frequently diagnosed cancer and one of the major causes of cancer-related mortality (1), and although much more rare (< 1% of all breast cancer cases), it can afflict men as well (2). Systemic therapy plays a major role in the treatment of both early- and late-stage disease. Therapies are selected based on the biological characteristics of the tumor, namely subtypes classified by the expression of estrogen receptor alpha (ER), progesterone receptor (PR), and amplification of the gene encoding the human epidermal growth factor receptor 2 (HER2) (3–6). While tumor biology-based selection of systemic therapy has significantly improved the prognosis of breast cancer in the past decade, relapse is still not uncommon, regardless of subtype (3,5). Since tumor biological characteristics can change as a result of treatment, for optimal subsequent treatment selection, it is critical that less invasive biomarkers be identified that reflect tumor biology that can be evaluated over time during disease treatment and progression (7).

Androgen receptor (AR) is a transcription factor activated by androgens, such as dihydrotestosterone (DHT) in women and testosterone in men (8) and is expressed at various levels across all breast cancer subtypes (9). While AR is associated with an overall favorable prognosis among women with ER-positive
tumors (10,11), AR, like ER itself, can support breast cancer progression. For example, we previously found that proliferation of MCF-7 depends on AR signal and AR antagonist enzalutamide (Enza) inhibits tumor growth of MCF7 xenografts (12). Also, AR contributes to ER-targeted therapy resistance mechanisms (13,14), and switching from ER-dependence to AR-dependence is hypothesized to be a mechanism of resistance to ER-directed endocrine therapies such as tamoxifen and aromatase inhibitors (7,12,15–18). AR also plays a role in anchorage-independent cell survival and cancer stem cell-like characteristics including tumor initiation in-vivo (18–22). Based on these findings, both ongoing and completed clinical trial have been initiated to test the efficacy of AR-targeted therapies, such as bicalutamide and Enza, alone and in combination with other agents in both ER-positive and -negative breast cancer (15,23). Although there is no established predictive marker for response to AR-targeted therapy in breast cancer, Enza plus the aromatase inhibitor exemestane was shown to be effective in patients with ER-positive advanced breast cancers with a gene signature-based biomarker indicating AR activation (24), and both bicalutamide and enzalutamide showed efficacy in metastatic AR + triple-negative breast cancer (TNBC) (25,26). In the ER-negative subset, cell lines corresponding to the luminal AR (LAR) subtype, a TNBC subtype characterized by an AR-regulated gene signature, respond to anti-androgen therapy (21). In a phase II trial testing the efficacy of Enza in advanced AR-positive TNBC, clinical outcomes appeared superior in patients who had an androgen-driven gene signature (27). AR expression is associated with poor response to neoadjuvant chemotherapy in ER-positive breast cancer (28), and patients with AR-positive or LAR breast cancer also have poor pathological clinical response to neoadjuvant chemotherapy when compared to other TNBC (29,30). Thus, being able to predict AR activity within the tumor via a minimally invasive method may enlighten therapeutic selection (8,31). In this study, we explored candidate serum factors that reflect tumor AR activity to support development of noninvasive serum biomarkers of AR activity in breast cancer.

Materials And Methods

Reagents

DHT (Sigma-Aldrich Corporation, St. Louis, MO, USA) was diluted in 100% ethanol. The AR antagonist enzalutamide (Enza) was provided by Medivation, Inc. (San Francisco, CA, USA) and diluted in dimethyl sulfoxide (DMSO).

Patient derived xenograft (PDX) study

In our previous study (32), we identified genes regulated by AR in an AR-positive, androgen responsive TNBC PDX model. Using these data, in the present study, 163 genes up-regulated by DHT (p < 0.05; fold change ≥ 1.5) were defined as the androgen responsive gene set and used in Gene Set Enrichment Analysis. Briefly, mice bearing ER-negative, AR-positive HCI-009 PDX tumors (33) were treated with DHT or vehicle control. Then gene expression profiling of HCI-009 tumors was assessed by RNA-sequencing (RNA-seq), and these data are available in the Gene Expression Omnibus (GEO) database as GSE152246 (32).
Chromatin immunoprecipitation sequencing

We expanded our data from chromatin immunoprecipitation sequencing (ChIP-seq) previously conducted. These data are available in the Gene Expression Omnibus (GEO) database as GSE157862 and reported by the Richer lab (34). Briefly, serum starved MDA-MB-453 cells were treated for 4 hours with vehicle control, DHT (10nM) or DHT (10nM) plus Enza (10 µM) followed by chromatin immunoprecipitation using AR antibody. Libraries were sequenced by a next gen-sequencer, read data were processed as described in the previous paper (34). In the present study, read depth was visualized using Integrative Genomics Viewer software with Human hg19 set as a reference genome.

qRT-PCR assay

Total RNA from MDA-MB-453 cells cultured for 4-days with vehicle control, DHT (1nM) alone, DHT (1nM) plus Enza (20 µM) alone was extracted using RNeasy Plus Mini Kit (QIAGEN, Germantown, MD, USA). cDNA was synthesized from 1 µg total RNA using qScript cDNA SuperMix (Quanta BioSciences, Inc., Gaithersburg, MD, USA). SYBR Green quantitative gene expression analyses were performed using 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) according to the manufacturer’s standard protocol. Primer sequences used in this study are listed in Supplementary Table 1. Expression of target genes was quantified using the comparative cycle threshold method normalized to 18SrRNA. All PCRs were performed in biological and technical triplicates.

Cell culture and cellular assays

Information about culture medium, authentication of each cell line is summarized in Supplementary Table 2. For specified cellular assays, steroid-depleted media was prepared for each cell line using phenol red-free basal medium with dextran-coated charcoal (DCC)stripped serum. Cell line subtyping (ie. ER and HER2 status) was defined based on the reports from Holliday, D. L et al, and Ai, J et al (21,35). For all cellular assays using DHT, cells were cultured in media with the DCC stripped sera for 72 hours prior to the assay. For crystal violet assays, cells were plated in 96-well plates and grown for 4-days in indicated conditions. Cells were then fixed in 10% formalin, rinsed in PBS, and stained with 5% crystal violet. Cell bound crystal violet was then dissolved in 10% acetic acid and absorbance was measured at 540 nm. Data were normalized to the mean absorbance of vehicle-treated cells. IC_{50} values for Enza were calculated with GraphPad Prism ver. 8.2.0. The proliferative response of each cell line to DHT was determined as “promotional” or “suppressive” when a significant change was observed at any DHT concentration compared to the control in same cell line. When neither a promotional or suppressive effect was significant, results were designated as “no response”.

Immunoblotting

Whole-cell lysate and centrifuged conditioned media, consisting of 50 µg protein were denatured, separated on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. After blocking in 5% milk in Tris-buffered saline–Tween, membranes were probed overnight at 4°C. Primary antibodies include AR (PG-21, 1:500 dilution; EMD Millipore), PSA (A0562, 1:1000 dilution; DAKO), FKBP5 (#8245, 1:1000 dilution; Cell Signaling Technology).
dilution; Cell Signaling), ZAG (sc-13585, 1:1000 dilution; Santa Cruz), GCDFP 15 corresponding to PIP (ab62363, 1:1000 dilution; abcam) and GAPDH (G8795, 1:10000 dilution; Sigma-Aldrich). For secondary antibodies, IRDye 800 goat anti-rabbit and IRDye 680 goat anti-mouse (LI-COR, Lincoln, NE, USA) were used at 1:10000. Bands were visualized and quantitated with the Odyssey® CLx Imaging System (LI-COR, Lincoln, NE, USA). Ponceau S staining of the membranes was used as loading controls for western blots of conditioned media.

Analysis of gene expression profile data set.

Two publically available gene expression profile data sets, obtained by RNA-seq from breast cancer patient samples, were utilized for analysis in this study: The Cancer Genome Atlas breast (36,37) (TCGA) cohort (n = 1,100) and the Sweden Cancerome Analysis Network-Breast (38) (SCAN-B) cohort (n = 3,273). Clinical and Gene expression data were downloaded from cBioportal (https://www.cbioportal.org/) and Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/), respectively. All mRNA expression values were log2 transformed prior to analysis. Definitions of clinicopathological characteristics are described in their original papers [33, 34]. Subtypes are classified as follows based on receptor status assessed by immunohistochemistry; ER+ / HER2- as Luminal, ER+ / HER2+ as Luminal HER2, ER- / HER2+ as HER2, ER- / HER2- as TNBC. Cases showing ER- / PgR+ (progesterone receptor-positive) were defined as unknown subtype. Gene set enrichment analysis (GSEA) (39,40) was performed to compare specified gene sets using GSEA software v3.0 with the number of permutations set at 1000 and permutation type set as phenotype. Expression values of indicated genes or subtype were used as phenotype labels and Pearson's correlation or Signal-2-Noise was set as Metrics for Ranking Genes. Thresholds for nominal p-value and FDR q-value was set at < 0.05 and < 0.25, respectively. Using the TNBC type online subtyping tool (http://cbc.mc.vanderbilt.edu/tnbc/) (41), IHC defined TNBC samples (SCAN-B; n = 143, TCGA; n = 123) were pre-processed by excluding possible ER-positive samples at the transcriptome level (SCAN-B; n = 15, TNBC; n = 8), then classified according to Lehmann's molecular subtypes (21). Subtypes other than LAR were defined as non-LAR, followed by various analysis in LAR vs non-LAR.

**Statistical analysis**

All statistical analyses were performed using the GraphPad Prism ver. 8.2.0 software. For comparison of multiple groups in *in-vitro* studies including proliferative response to different doses of DHT, statistical significance was tested by one-way ANOVA with Dunnett's multiple comparisons test. For comparison of dose-response curves for cell viability between cell lines, Mixed-effect analysis with Tukey's multiple comparisons test was used. Correlation between protein expression levels and Enza IC$_{50}$ were analyzed by Spearman's rank correlation coefficient. Correlation between genes was analyzed by Pearson's correlation coefficient. For comparison of gene expression of two groups, unpaired t-test was used. The TNBC subtype classification performance by candidate gene expression level was evaluated by receiver operating characteristic (ROC) analysis.

**Results**
DHT-responsive genes encoding secreted proteins in AR-positive PDX breast cancer model.

As described above, in a previous report (32), we explored AR activity in TNBC PDX model HCI-009 that grows in response to DHT in mice and 163 genes up-regulated by DHT were identified. Among these genes, seven encode secreted proteins (Supplementary Table 3, (42)) (Fig. 1a). We focused our study on three genes that were most highly upregulated following DHT treatment, specifically, *KLK3*, *AZGP1* and *PIP* that encode the secreted proteins prostate specific antigen (PSA), zinc-Alpha-2-glycoprotein (ZAG) and prolactin induced protein (PIP) (Fig. 1b).

Gene expression of *KLK3*, *AZGP1* and *PIP* is directly regulated by AR in MDA-MB-453 cells.

MDA-MB-453 breast cancer cells highly express AR (43). AR-ChIP-seq analysis in MDA-MB-453 cells showed multiple AR binding peaks induced by DHT and/or suppressed by Enza in the gene loci of *KLK3*, *AZGP1* and *PIP*. These included known enhancers containing the AR binding motif (*KLK3* at ~4.2 kb (44), *AZGP1* at ~ + 0.2 kb (45), *PIP* at ~ + 11kb (46)) (Fig. 2a). qRT-PCR showed that expression of these genes was induced by DHT and suppressed by Enza (Fig. 2b), suggesting that *KLK3*, *AZGP1* and *PIP* may be direct transcriptional targets of AR.

Protein expression of *PSA*, *ZAG* and *PIP* are regulated by AR in a wide variety of breast cancer cell lines.

In order to investigate whether protein expression of the candidate genes was also AR regulated, nine AR expressing breast cancer cell lines were analyzed for PSA, ZAG and PIP protein in whole cell lysates and conditioned media. AR and FKBP5, a known AR-regulated protein, were assessed as well (Supplementary Fig. 1). While all cells, responded to Enza treatment in a dose-dependent manner, proliferative response to DHT treatment was highly variable (Supplementary Fig. 2a-d, Supplementary Table 4–6). We did not find any significant correlation between Enza IC₅₀ levels and levels of AR or AR-regulated proteins, perhaps due to small sample size (Supplementary Fig. 2e). PSA in cell lysates was induced by DHT and suppressed by Enza in all cell lines examined (Fig. 3a, Supplementary Fig. 3a). Although ZAG and PIP levels were more variable, ZAG was induced by DHT and suppressed by Enza in 6 out of 9 cell lines (Fig. 3b, Supplementary Fig. 3b), while PIP was induced by DHT and suppressed by Enza in 4 out of 9 cell lines (Fig. 3c, Supplementary Fig. 3c). On the other hand, analysis of conditioned media showed that while PSA was secreted only in DHT-treated BT-474 cells, ZAG was secreted in all ZAG-expressing cell lines and was induced by DHT and suppressed by Enza in the majority of cell lines and secretion of PIP was obvious in 7 out of 9 cell lines and was induced by DHT and suppressed by Enza in the majority of cell lines (Fig. 4, Supplementary Fig. 4).

Candidate genes are positively correlated with a gene expression profile representative of tumor AR activity.

The correlation between the expression level of candidate genes and that of *AR* was analyzed in the SCANB and TCGA datasets. While *PIP* expression was positively correlated with *AR* expression in all subtypes, *KLK3* and *AZGP1* seemed to be most highly correlated with AR only in the luminal and TNBC
subtypes (Supplementary Fig. 5a-c). GSEA was then performed on the SCAN-B and TCGA datasets to analyze the relationship between candidate gene expression and the whole androgen responsive gene set defined in HCl009 PDX tumors (Fig. 1a). Expression of \textit{KLK3}, \textit{AZGP1} and \textit{PIP} were shown to be positively correlated with the androgen responsive gene signature in both the SCAN-B and TCGA cohorts across all subtypes (Fig. 5a-c). As a control comparison, when GSEA is performed using two gene sets representative of estrogen response in breast cancer cells (47), the candidate genes showed no correlation or tended towards being negatively correlated with estrogen responsive genes (Supplementary Fig. 6a-f), suggesting that they are indicative of AR activity, but not ER activity.

According to Lehmann’s molecular subtypes of TNBC, IHC defined samples were classified into LAR (n = 15 for SCAN-B, n = 13 for TCGA) and non-LAR (n = 113 for SCAN-B, n = 102 for TCGA) (Fig. 6a) (21,41). When GSEA was performed on LAR vs non-LAR to examine the relation between TNBC subtype and the androgen responsive gene set, the androgen responsive gene set was shown to be enriched in the LAR samples, as would be expected (Fig. 6b), corresponding to Lehmann’s finding that LAR have up-regulated AR activity. We next examined expression of \textit{AR} and candidate genes in the TNBC LAR subtype and \textit{AR}, \textit{AZGP1} and \textit{PIP} were significantly higher in LAR compared to non-LAR TNBC (Fig. 6c). Similarly, A ROC analysis was performed to examine whether these candidate genes can predict the LAR phenotype among TNBC subtypes. Area under the curve (AUC) values showed that \textit{PIP} was as effective as \textit{AR} in distinguishing the LAR TNBC subtype (Fig. 6d).

Discussion

Recent findings regarding the role of AR in breast cancer (7,12–17) suggest that estimating AR activity may have some clinical utility since trials with both Enzalutamide and Enobosarm (48) in metastatic ER + disease resistant to other endocrine and chemotherapy treatments. Here we identified PSA, ZAG and PIP as candidate biomarkers reflecting tumor AR activity in breast cancer. These factors are all secreted proteins with potential to serve as serum biomarkers (49–53).

We selected three genes encoding secreted proteins most responsive to DHT \textit{in vivo} analysis: \textit{KLK3}, \textit{AZGP1} and \textit{PIP} in an AR + PDX. In AR-ChiP-seq analysis of MDA-MB-453 cells, AR binding was demonstrated at androgen response elements upstream of these genes. Other candidate genes among seven androgen responsive genes included \textit{FASN} (fatty acid synthase), \textit{CD9} (CD9) and \textit{THBS1} (thrombospondin 1), which also showed significant AR binding at their gene loci (data not shown). The majority of AR-regulated genes expression data are from prostate cancer cell lines and PSA is a well-known AR-regulated protein (44,54,55). In breast cancer, previous studies show that DHT can induce PSA expression in cell lines (56–58). Also, there is evidence in the literature supporting our determination of ZAG (59,60) and PIP (46,61,62) as androgen responsive in breast cancer. In this study, candidate protein expressions were analyzed in 9 cell lines, showing that some are more responsive to androgen and some are less so (Fig. 3, 4). GSEA analysis (Fig. 5a-c) suggests that these candidate factors correlate with AR in a considerable number of breast cancer patients.
In the analysis of anti-proliferative responses of cell lines to Enza, it effectively inhibited growth in all cell lines tested, and IC50 for each cell line averaged 43.8 ± 12.9 µM which is a clinically achievable concentration (20). These results suggest that AR + breast cancer cells rely on AR function for survival, to some extent, regardless of subtype or proliferative response to DHT (Supplementary Fig. 2a-d). These observations are consistent with previous reports showing the pro-tumor roles of AR in multiple breast cancer subtypes (12–14,16,18–22,63,64). We theorized that high expression of candidate proteins indicates a higher androgen dependence and responsiveness to Enza. We analyzed correlations between Enza IC50 and expression of AR or the candidate proteins in in vitro models (Supplementary Fig. 2e), and while statistical significance was not achieved, it is a future direction to test this correlation in clinical trials using AR-targeted therapy and would be particularly useful if PSA, AZGP1 or PIP in serum correlated with AR dependency/response to AR targeting agents.

As shown above, with regard to KLK3 and AZGP1, the response in MDA-MB-453 to DHT and Enza is consistent in the individual cells where they are expressed at all. MDA-MB-453 has such an abundance of PIP protein that the reactivity to DHT and Enza cannot be evaluated properly, but previous reports from other groups using breast cancer cell lines showed that gene and protein expression are linked, with regard to PSA and PIP (46,58,65). We therefore examined the significance of these candidate genes in publically available data sets. KLK3 and AZGP1 expression were not strongly correlated with AR expression in all breast cancer subtypes (Supplementary Fig. 5a, b). However, AR activity cannot be measured solely by AR expression, and it is known that activity can be regulated by ligand binding and competing ER (66–70). However, consistent with our hypothesis, the results of GSEA using the androgen responsive gene set demonstrated that the expression of candidate genes accurately reflects a larger gene signature representing tumor AR activity in all breast cancer subtypes (Fig. 5a-c). Since the candidate genes were identified from the androgen response genes in HCI-009 PDX and GSEA was performed using this androgen response gene set, it is not surprising that candidate genes correlate with this androgen-responsive gene set. However, we also validated this analysis using another androgen-responsive gene set derived from an AR-positive breast cancer cell line MDA-MB-453 (63). With the exception in KLK3 expression in some subtypes, candidate genes showed a significant positive correlation with this androgen-responsive gene set (Supplementary Fig. 7). When the same analysis was performed on the less strongly expressed -candidate genes, (FASN, CFH, CD9 and THBS1), only FASN showed a correlation with the androgen responsive gene set (data not shown). Interestingly, AZGP1 and PIP were particularly high in the LAR TNBC subtype (Fig. 6c); thus, serum levels may be useful as markers for predicting LAR TNBC AR dependency with high accuracy.

Although it is difficult to accurately quantify the protein levels in conditioned medium because there are no good internal standards, relative levels suggest that the secreted candidate proteins in the conditioned medium are regulated by AR in many of the cell lines (Fig. 4). Serum PSA is an established biomarker of tumor burden in prostate cancer (71). Although there is some controversy regarding the differences in PSA expression between normal and breast cancer tissues (72), several groups, including us, have reported that serum PSA levels are higher in breast cancer patients than in healthy women, indicating that
tumor derived PSA is detectable in serum of breast cancer patients (52,55,73,74). Recently, we found that serum PSA levels positively correlated with AR expression in primary tumors (52). Combined with the present findings, the future clinical use of serum PSA as a biomarker for tumor AR activity in breast cancer is promising. However, in this study, PSA could not be detected in the conditioned medium of most cell lines examined (Fig. 4). In our previous analysis, the positive rate of serum PSA was 36.1% for metastatic breast cancer and 13.3% for early-stage breast cancer. (52), suggesting considerably low levels of serum PSA in most breast cancer cases. Therefore, establishing a more sensitive assay for PSA quantification is necessary for full clinical translation. ZAG expression in breast cancer has been documented and is considered as a potential biomarker for breast carcinoma (50,75–78) because its expression was detected exclusively in patients with ductal carcinoma when compared to the normal breast tissue of healthy women. Serum ZAG is also significantly higher in breast cancer patients than healthy control patients as well as correlating with disease burden in breast cancer patients (50). PIP, also known as gross cystic disease fluid protein 15 (GCDFP-15), is commonly used in the clinic as a breast cancer biomarker (79–82) to assist in characterizing metastases of unknown origin. There has also been controversy over the difference in PIP expression between normal and breast cancer tissues. In early studies, PIP was shown to be absent in normal breast epithelium, whereas in breast cancers PIP is frequently expressed (80,81). Others have shown that PIP is frequently present in uninvolved breast tissue (83). However, since highly increased levels of PIP have been detected in the peripheral plasma from patients with primary and metastatic breast cancer were detected in comparison to normal subjects, its significance as a potential serum marker in breast cancer is promising (51,81,84). Taken together, serum levels of these candidate protein may reflect tumor AR activity. Thus, further studies are needed to determine the relationship between serum levels of these factors and tumor biology. It is possible that these proteins might not have a predictive advantage over the general AR gene signature of tumor tissue itself. However, breast cancer cells change their biological properties as they develop resistance to treatment (reviewed in T. Hanamura et al (7)). Clinically, in the advanced or metastatic setting, systemic therapy is thought to induce alterations to tumor biology as well (85). Because gene signature monitoring requires tissue collection and cannot be done easily or often, these candidate secreted proteins are suggested to be useful as less invasive biomarkers that can be assessed at any time point to help monitor drug response or resistance.

Assessments of tumor AR signaling by liquid biopsy have been reported by other groups. Both AR mRNA and protein in circulating tumor cells was investigated and shown to be evaluable in blood samples (86–88). In recent years, deep-sequencing techniques applied to blood samples have shown that AR pathways are activated in circulating tumor cells from bone-predominant breast cancer (89). Other groups found that 47% of all \( AR \) variants in cell-free DNA of breast cancer patients were pathogenic or likely pathogenic (90). Although, as our three candidate proteins, the predictive value of these potential biomarkers remains to be evaluated in further trials.

AR signals are known to have immunosuppressive effects in \textit{in-vivo} models of various autoimmune diseases, follicular thyroid cancer and colon cancer (91). ZAG has structural similarities to MHC class I, and analysis of various disease models has shown that it may suppress immune response, but its
function in the cancer microenvironment is not clear (78). PIP plays multiple roles in biology, including fertility, immuno-regulation, anti-microbial activity, and tumor progression (92). Interestingly, ZAG and PIP can form a complex with each other, suggesting a cooperative role between these two proteins (93), although their biological significance/activity in breast cancer remains to be determined. Thus, we are currently conducting further pre-clinical and clinical evaluations of these proteins and their immune-modulatory potential in breast cancer.

**Conclusion**

We identified PSA, ZAG and PIP as candidate biomarkers reflecting tumor AR activity in breast cancer. The expression levels of candidate factors are closely correlated with a gene signature representing tumor AR activity in all breast cancer subtypes. However, the responsiveness to DHT and Enza in cell lines is highly variable. Further verification of the biological significance of these candidate factors will be needed in the future. To establish the potential utility of these secreted proteins as serum-derived indicators of tumor AR activity, further studies are needed to determine the relationship between serum levels of these factors and tumor biology, specifically how they correlate with response to AR targeting agents in clinical trials. These secreted factors may be particularly useful in breast cancer patients with metastatic disease resistant to traditional therapies, where AR targeting drugs are being evaluated in multiple ongoing clinical trials.

**Abbreviations**

AR: androgen receptor

DHT: dihydrotestosterone

Enza: enzalutamide

PDX: patient derived xenograft

*KLK3*: kallikrein related peptidase 3

*AZGP1*: Alpha-2-Glycoprotein 1, Zinc-Binding

*PIP*: Prolactin Induced Protein

PSA: prostate specific antigen

ZAG: Zinc-alpha-2-glycoprotein

PIP: Prolactin Induced Protein

GSEA: gene set enrichment analysis
ER: estrogen receptor alpha

HER2; human epidermal growth factor receptor 2

LAR; luminal AR

TNBC; triple negative breast cancer

RNA-seq; RNA-sequencing

ChIP-seq: chromatin immunoprecipitation sequencing

NSE; normalized enrichment score

GCDFP-15; Gross Cystic Disease Fluid Protein 15

Declarations

Ethical Approval and Consent to participate

In-vivo experiments were approved by the University of Colorado Institutional Animal Care and Use Committee [IACUC protocol #00141] and were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. Because no clinical sample were used in this study, consent to participate is not applicable.

Consent for publication

All authors read the manuscript and agreed to the publication of the manuscript.

Availability of data and materials

Request for additional details of the data used in the manuscript can be directed to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions
TH developed concepts, designed and performed the experiments, interpreted the data, and wrote the manuscript. JKR and JLC interpreted the data and wrote the manuscript. JLC, KIO, ER and MMW performed the experiments. All of the authors read and approved the final version of this manuscript.

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Figures
Figure 1

(a) Gene expression profile of HCI-009 PDX tumor in mice supplemented with cellulose, as a control, or DHT, assessed by RNA-seq. Gene set X consists of 163 genes significantly up-regulated (fold change $>1.5$, $q<0.05$) in HCI-009 PDX tumors from mice treated with DHT compared with controls. Gene set Y consists of genes encoding proteins listed in secretome of breast cancer cell lines reported by Ziegler YS, et al (2016).

(b) Fold change expression of top 163 DHT-responsive genes

KLK3, AZGP1 and PIP are the most DHT-responsive genes that also encode secreted proteins. (a) Gene expression profile of HCI-009 PDX tumor in mice supplemented with cellulose, as a control, or DHT, assessed by RNA-seq. Gene set X consists of 163 genes significantly up-regulated (fold change $>1.5$, $q<0.05$) in HCI-009 PDX tumors from mice treated with DHT compared with controls and is defined as the androgen responsive gene set. Gene set Y consists of genes encoding proteins listed in secretome of breast cancer cell lines reported by Ziegler YS, et al (2016). (b) Fold change expression of genes in DHT treated mice relative to control mice in descending order. Each dot represents a gene, and white circles represent genes encoding secreted proteins and are accompanied by official gene symbols.
Gene expression of KLK3, AZGP1 and PIP is regulated by AR in MDA-MB-453 cells. (a) AR binding on gene loci was assessed by AR-ChIP-seq in MDA-MB-453 breast cancer cells. Gene locus for each candidate gene and read depth for each binding are shown. Red arrows indicate significant AR-binding peaks detected, and black arrows indicate known enhancer regions containing the AR binding motif. (b) Expression of each candidate gene in MDA-MB-453 cells was examined by qRT-PCR after 4-days cultured in indicated conditions. Expression levels were normalized to 18SrRNA; mean ± standard deviation, *** p<0.001.
Expression of PSA, ZAG and PIP is regulated by AR in breast cancer cell lines. Protein extracts of whole cell lysates were examined by western blot after 4-days cultured with vehicle control, DHT (10nM), DHT (10nM) plus Enza (20 μM) or Enza (20 μM). Each band was quantified by densitometry and the expression levels of PSA (a), ZAG (b) and PIP (c) relative to GAPDH normalized to vehicle control are
shown for representative cell lines. Data for the remaining cell lines are shown in Supplementary Figures 3a-c. (d) AR-dependent regulation of candidate protein expression in all tested cell lines are summarized.

**Figure 4**

AR-regulated secreted proteins in conditioned media from nine breast cancer cell lines. Conditioned medium (s, secreted proteins) from each cell line was examined by western blot after 4-days cultures with vehicle control, DHT (10nM), DHT (10nM) plus Enza (20 μM) or Enza (20 μM). Ponceau S staining of the membranes as loading controls for western blots of conditioned medium are shown in Supplementary Figure 4.
Figure 5

Expression of candidate genes is correlated with a gene expression profile representative of tumor-AR activity. (a, b, c) Gene expression profile data from SCAN-B and TCGA cohorts was applied to GSEA to analyze the relationship between candidate gene expression and the androgen responsive gene set defined in Figure 1a. Enrichment plots with normal enrichment scores (NES) and p-values for each gene and breast cancer subtype combination are shown.
Predictive value of candidate gene expression in TNBC subtypes. (a) TNBC samples from the SCAN-B and TCGA cohorts were classified into Lehmann’s molecular subtypes, with samples characterized as being either LAR (luminal AR TNBC subtype) or non-LAR TNBC. The distribution of TNBC subtypes in each cohort is indicated. (b) GSEA was performed on LAR vs non-LAR to examine the relationship between TNBC subtype and the androgen responsive gene set. Enrichment plots with normal enrichment.
scores (NES) and p-values for each cohort. (c) AR and candidate gene expression in LAR vs non-LAR are shown. (d) ROC curves of a given gene as a classifier for LAR with area under the ROC curve (AUC) analyses. The diagonal line denotes the ROC curve of a random classifier of AUC=0.5. P-value tests the null hypothesis that the AUC really equals 0.50.

**Supplementary Files**

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