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Anterior Gradient 2 (AGR2): Blood-Based Biomarker Elevated in Metastatic Prostate Cancer Associated With the Neuroendocrine Phenotype

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BACKGROUND. Anterior gradient 2 (AGR2) is associated with metastatic progression in prostate cancer cells as well as other normal and malignant tissues. We investigated AGR2 expression in patients with metastatic prostate cancer.

METHODS. Blood was collected from 44 patients with metastatic prostate cancer separated as: castration sensitive prostate cancer (CSPC, n = 5); castration resistant prostate cancer (CRPC, n = 36); and neuroendocrine-predominate CRPC defined by PSA $\leq$ 1 ng/ml in the presence of wide-spread metastatic disease (NE-CRPC, n = 3). AGR2 mRNA levels were measured with RT-PCR in circulating tumor cell (CTC)-enriched peripheral blood. Plasma AGR2 levels were determined via ELISA assay. AGR2 expression was modulated in prostate cancer cell lines using plasmid and viral vectors.

RESULTS. AGR2 mRNA levels are elevated in CTCs and strongly correlated with CTC enumeration. Plasma AGR2 levels are elevated in all sub-groups. AGR2 levels vary independently to PSA and change in some patients in response to androgen-directed and other therapies. Plasma AGR2 levels are highest in the NE-CRPC sub-group. A correlation between AGR2, chromagranin A (CGA), and neuron-specific enolase (NSE) expression is demonstrated in prostate cancer cell lines.

CONCLUSIONS. We conclude that AGR2 expression is elevated at the mRNA and protein level in patients with metastatic prostate cancer. In particular, we find that AGR2 expression is associated features consistent with neuroendocrine, or anaplastic, prostate cancer, exemplified by an aggressive clinical phenotype without elevation in circulating PSA levels. Further studies are warranted to explore the mechanistic and prognostic implications of AGR2 expression in this patient population. *Prostate 73: 306–315, 2013.*

KEY WORDS: **AGR2; neuroendocrine prostate cancer; metastasis; biomarker**

INTRODUCTION

Prostate cancer is a common disease affecting men in the U.S. with a projected incidence of over 200,000 cases in 2010 [1]. Most patients are diagnosed with localized prostate cancer which is effectively treated with surgery, radiation, or active surveillance. For others, prostate cancer recurs despite primary therapy. The mainstay of therapy for recurrent or metastatic prostate cancer is androgen-deprivation therapy. While most patients initially respond to androgen withdrawal (castration sensitive prostate cancer, CSPC), clinical advancement despite low levels of

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Abbreviations: AGR2, anterior gradient 2; CSPC, castration sensitive prostate cancer; CRPC, castration resistant prostate cancer; NE-CRPC, neuroendocrine-predominate CRPC; PSA, prostate specific antigen; CGA, chromagranin A; NSE, neuron-specific enolase; CTC, circulating tumor cell; ELISA, enzyme-linked immunoassay.

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circulating androgens signals progression to castration resistant prostate cancer (CRPC) which is associated with the majority of the prostate cancer related morbidity and mortality.

Anterior gradient 2 (AGR2) is a member of the protein disulfide isomerase (PDI) family which is expressed in a variety of normal and transformed epithelial tissues [2,3]. Pre-clinical studies have shown that AGR2 expression is associated with increased cell migration and metastatic behavior in a variety of tumor models [4–7]. Other studies show that AGR2 expression is modulated by androgens through ErbB3 binding protein 1 and Fox A transcription factors and confirm an association with a more metastatic phenotype [2,8]. AGR2 expression has also been associated with docetaxel-resistance in the neuroendocrine-like PC3 prostate cancer cell line in vitro [9]. AGR2 is known to be expressed in primary prostate cancer tissues, and AGR2 levels have been explored as a potential urine-based diagnostic marker for prostate cancer [2,10]. At the tissue level, changes in AGR2 expression in primary prostate cancer tissue have been associated with either increased [11] or decreased [12] rates of clinical progression. To our knowledge, no studies have examined expression of AGR2 in blood or tissue in patients with metastatic prostate cancer.

Access to metastatic prostate cancer tissue for molecular characterization is very limited. Metastatic prostate cancer generally develops over many years, and after many therapies from the time diagnostic tissues are usually available. Genetic and other molecular abnormalities present in CRPC may not reflect abnormalities present in an initial diagnostic biopsy obtained many years or even decades earlier. Even if tumor tissue could be directly sampled in the setting of metastatic CRPC, autopsy data highlights the tremendous intra-individual variation in biomarker expression across metastatic tumors sites [13]. Examination of markers in peripheral blood represents a strategy to obtain a more complete representation of all tumors present in an individual.

Circulating tumor cells (CTCs) have been identified in the peripheral blood of cancer patients using a variety of purification and detection techniques [14,15]. The CellSearch system is the first system to receive marketing approval by the US FDA for CTC enumeration as a prognostic marker in patients with metastatic breast, colorectal, and prostate cancer. We have demonstrated that CTC-enriched blood contains androgen receptor mutation profiles similar to those found in metastatic prostate cancer deposits [16]. Other studies have also used the CellSearch system to study gene and protein level changes from patients with metastatic breast and prostate cancer [17–22]. Plasma AGR2 levels have been found to be elevated patients with ovarian and pancreatic cancer [23,24]. Taken together, these studies support our efforts to apply blood-based assays to examine AGR2 expression in patients with metastatic prostate cancer.

MATERIALS AND METHODS

Eligibility and Blood Collection

Patients with histologically diagnosed adenocarcinoma of the prostate with metastatic disease demonstrated by routine clinical imaging studies were approached for participation in a correlative study. Blood collection included an ethylenediaminetetraacetic acid (EDTA) tube processed and stored for plasma. In most cases, parallel tubes (CellSave and CTC profile tubes for CellSearch) were also collected and processed for CTC enumeration and RNA isolation, respectively, as previously described [16,21,22]. All blood was collected with informed consent of subjects under an Institution Review Board approved protocol at all participating institutions. Control plasma from normal male subjects (age >50 years old) was purchased from Bioreclamation, LLC (Westbury, NY).

Enzyme-Linked Immuno-Assay (ELISA)

AGR2 protein levels were quantitatively assessed by a commercial ELISA kit purchased from Uscn Lifesciences, Wahun, China. All samples were maintained at −80°C. Plasma was allowed to thaw on ice, inverted several times, and centrifuged for 1 min at 200*g* prior to immuno-assay. Sample location on the 96-well plate were randomized in order to eliminate plate bias. Each sample was analyzed at multiple dilutions. The final AGR2 concentration was obtained based on the dilution which corresponded to the linear portion of the standard curve.

The AGR2 ELISA was performed according to manufacturer's instructions. Briefly, 100 μl of each sample (diluted with standard diluents provided by Uscn) was added to each well and incubated overnight at 4°C while rocking. Samples were aspirated and 100 μl of detection reagent A was added to each well and incubated for 1 hr at 37°C. Each well was aspirated and washed three times with wash buffer (Uscn). One hundred microliters of detection reagent B was added to each well and incubated for 30 min at 37°C. Samples were aspirated and washed five times. Ninety microliters of the substrate solution was added to each well and incubated for 20 min at 37°C in the dark. Fifty microliters of the stop solution was added to each well and OD at 450 nm was measured. The ELISA kit for CGA was obtained from Abnova

(Walnut, CA) and performed according to manufacturer's instructions. The normal plasma CGA range for this assay is <100 ng/ml according to the manufacturer.

Circulating Tumor Cell (CTC) Enumeration

Seven and a half milliliters of blood for CTC enumeration was drawn into CellSave preservative tubes. Samples were processed using the CellSearch Epithelial Cell Kit (Veridex, LLC; Raritan, NJ) and CTC counts determined on the CellTracks Analyzer (Veridex, LLC) according to manufacturer's instructions.

mRNA Isolation and AGR2 Expression

For gene expression studies, 7.5 ml of blood was drawn into EDTA tubes and processed for CTCs using the CellSearch Profile Kit (Veridex LLC) followed by RNA isolation basically as previously described [16,21,22]. Briefly, RNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen; Valencia, CA) according to manufacturer's instructions. The turnaround time was no more than 36 hr from the blood draw to CTC enrichment and nucleic acid isolation. RNA quality and quantity were assessed with the Agilent Bioanalyzer. All samples were stored at -80°C until further use. First strand cDNA was synthesized from equal amounts of template RNA from CTC-enriched peripheral blood using a High Capacity cDNA archive kit then amplified with the ABI TaqManPreAmp method (Applied Biosystems), which has been shown to reliably and reproducibly amplify mRNA for expression analysis from a single cell [21]. Absolute AGR2 mRNA expression levels were quantified based on the C_T computed on the ABI7900 system (Applied Biosystems, Foster City, CA). The probe sets and conditions were provided by the manufacturer for AGR2 (ABI Assay on demand Probe set: Hs00180702_m1), as described previously [22]. The minimal detection limit was considered to be 40 cycles.CTC-enriched RNA samples from 26 normal individuals were also run as controls.

Cell Culture and Materials

22Rv1, DU145, and PC3 cells were obtained from the ATCC (Manassas, VA) and were cultured in RPMI (22Rv1), DMEM (DU145), and F12K (PC3) containing 10% FBS. AGR2 cDNA and GFP cDNA were purchased from Origene (Rockville, MD) and cloned into the pLVX-cmv-IRES-neo vector purchased from Clonetech (Mountain View, CA). Lentiviral particles containing sequences targeting 3′ UTR of AGR2 (CCGGCCTTGAGACTTGAAACCAGAACTCGAGTTCTGGTTTCAAGTCTCAAGGTTTTTTG) and scrambled shRNA were purchased from Sigma–Aldrich (St. Louis, MO).

Immunoblotting

Prostate cancer cell lines were used to study effects of altered AGR2 levels in prostate cancer models as previously described [25]. Stable cell lines were produced via transfection or lentiviral infection according to manufacturer's instructions, and cell population with altered level of AGR2 expression were obtained following G418 selection (Gemini Biosciences, West Sacramento, CA). Cells were grown in 10 cm plates until 75–85% confluent and lysed with RIPA buffer, homogenized with a 28 gauged syringe, centrifuged at 14,000*g* for 10 minutes. Equal mass of protein lysates was determined by use of Bradford Assay (ThermoScientific, Rockford, IL). Lysates were loaded on a 4–20% acrylamide gel (ThermoScientific) and transferred to nitrocellulose membrane. Antibodies directed to AGR2 (Abnova, Walnut, CA), CGA (Sigma–Aldrich), NSE (Dako USA, Carptenteria, CA), and actin (Sigma–Aldrich, St. Louis, MO) were used according to manufacturers' directions.

Statistical Analysis

AGR2 levels of serum samples were assessed by arithmetic average of measurements across dilutions in which all values were in the linear calibration range and not determined to be outliers. Measurement outliers were detected using either DFFITS >1 in estimating the logarithmic mean value [26] (for $n \geq 5$ replicates) or a value deviating by more than a factor of two from the average of the other replicates (for $n < 5$ replicates, at most one outlier declared per sample); of 316 measurements, 16 outliers (5%) were detected and removed. Parametric testing for significant differences in serum AGR2 between metastatic prostate cancer cohorts and normal controls was performed using ANOVA followed by Dunnett's multiple comparison adjustment; a logarithmic variance normalizing transform was applied prior to significance testing. To accommodate unequal group variances, parametric testing for significant differences in serum CGA was performed using Welch's *t*-test on logarithm-transformed data followed by Bonferroni correction for multiple comparisons. Relative longitudinal values of AGR2 and corresponding 95% confidence intervals were estimated by applying a categorical fixed effects model for logarithmic AGR2 level which included the time point (T0, T1, T2) as a nested factor within the clinical subject factor. All statistical analyses were performed using R version 2.14.0 (www.r-project.org).

RESULTS

Demographics

Patients with metastatic prostate cancer generally exhibit a predictable natural history marked by initial sensitivity to androgen deprivation (castrate sensitive prostate cancer, CSPC) followed by eventual progression in a subset of patients to an androgen-independent state (castrate-resistant prostate cancer, CRPC) [27]. A particularly aggressive variant of metastatic prostate cancer has been described based on the presence of neuroendocrine markers in blood and tissue in association with a conventional adenocarcinoma pattern. Features associated with neuroendocrine-CRPC (NE-CRPC), or anaplastic prostate cancer, include as a usual pattern of early, visceral metastatic spread as well as relatively low levels of serum PSA [28]. Here we conservatively define NE-CRPC based on the presence of wide-spread metastatic deposits (bone, visceral, or both) along with a PSA value $\leq$1.0 ng/ml. Therefore, we divide our cohort of metastatic prostate cancer patients into three groups as CSPC, CRPC, and NE-CRPC.

Baseline demographic characteristics of all 44 subjects with metastatic prostate cancer are summarized in Table I. In the five subjects with CSPC, the median (range) age and serum PSA were 58.1 (51.1–66.6) years and 428.6 (2.4–7,606) ng/ml, respectively. Four of five patients with CSPC had bone metastases (80%) while one subject had metastatic disease only in lymph nodes (20%). In 36 subjects with CRPC, the median (range) for age, serum PSA, and duration of androgen-deprivation therapy (ADT) was 69.4 (57.5–87.7) years, 150 (3.6–2403) ng/ml, and 37.2(5.2–174.2) months, respectively. For patients with imaging studies evaluable within 2 months of study enrollment, the extent of metastatic disease in these patients was typical for this population, as 94% (n = 33) subjects had bone metastases and 29% (n = 28) had lymph node or visceral metastases. Finally, for the three subjects with NE-CRPC, the age, serum PSA, and duration of ADT were 70.7 (68.1–79.2) years, PSA 0.4 (0.1–0.7) ng/ml, and 33.5 (5.2–174.2) months, respectively. Consistent with the typical presentation for NE-CRPC, the distribution of metastatic involvement was distinct from CRPC such that all patients had extensive lymph node and visceral metastases in addition to extensive bone metastases (Table II).

AGR2 mRNA is Expressed in CRPC

Since AGR2 has been found to promote metastatic progression in prostate and other cancer models, we sought to determine if AGR2 was expressed in tissue from patients with metastatic prostate cancer. However, as metastatic tissue is often not available in patients with CRPC, we evaluated AGR2 mRNA expression in CTCs. The feasibility of gene-specific expression analysis based on RNA obtained from CTC-enriched peripheral blood has been previously demonstrated [17,20–22,29]. Here we utilized this basic approach to examine if AGR2 mRNA was expressed in CTCs in patients with metastatic prostate cancer.

Paired blood samples for CTC enumeration along with isolation of CTC-enriched peripheral blood RNA was available from a subset of the overall patient cohort. Specifically 49 samples were identified with $\geq$1 CTC/7.5 ml from patients with CRPC (n = 30), CSPC (n = 2), or NE-CRPC (n = 2). AGR2 mRNA levels, expressed as average $C_T \pm SD$, were significantly elevated in samples from patients with metastatic prostate cancer ($C_T = 33.5 \pm 5.4$) versus 26 samples representing normal controls ($C_T = 37.6 \pm 3.7$) ($P < 0.001$ by Student's *t*-test). The absolute difference in C_T values of -4.1 cycles equates to 17.1-fold absolute increase in AGR2 mRNA in subjects versus controls. Further, we explored the correlation between AGR2 mRNA expression and CTC enumeration in the CRPC subgroup. A strong positive

TABLE I. Summary of Basic Demographic Datain Patients with Metastatic Prostate Cancer

	Average (SD)	Median	Range
CSPC (n = 5)			
Age (years)	57.2 (6.4)	58.1	(51.1–66.6)
Serum PSA (ng/dl)	1978 (3235)	428.6	(2.4–7607)
CRPC (n = 36)			
Age (years)	69.8 (7.6)	69.4	(57.5 87.7)
Serum PSA (ng/dl)	436 (599)	150.0	(3.6–2403)
Duration ADT (months)	49.9 (38.3)	37.2	(5.2–174.2)
NE-CRPC (n = 3)			
Age (years)	72.7 (5.8)	70.7	(68.1–79.2)
Serum PSA (ng/dl)	0.4 (0.3)	0.4	(<0.1–0.7)
Duration ADT (months)	43.6 (41.8)	33.5	(5.2–174.2)

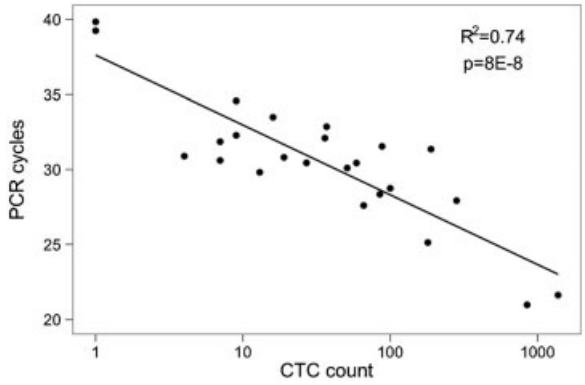

Fig. 1. AGR2 mRNA expression in CTCs. Scatter plot showing AGR2 mRNA level expressed as ΔCT (vertical axis) plotted against CTC enumeration determined by the CellSearch Assay (horizontal axis). The line representing the least squares curve fit is also shown. The coefficient of determination (R^2) is 0.74 ($P < 0.001$).

correlation is observed between CTC enumeration and AGR2 mRNA level ($R^2 = 0.74$, $P < 0.001$, Fig. 1). We conclude that AGR2 mRNA is expressed in CTCs from patients with metastatic CRPC.

Plasma AGR2 Levels are Elevated in Metastatic Prostate Cancer

Having demonstrated CTC-based expression of AGR2 mRNA in CTCs, we next expanded our study to examine plasma AGR2 levels in patients with metastatic prostate cancer. Since AGR2 is known to be an androgen-regulated, secreted protein which is elevated in plasma in ovarian and pancreatic cancer [23,24], we reasoned that plasma AGR2 levels may be elevated and vary in response to therapies in patients with metastatic prostate cancer.

An ELISA assay was used to explore plasma AGR2 and CGA levels in patients with metastatic prostate cancer compared with normal controls (Fig. 2). Consistent with other reports, we observed a median (range) of 25 (6–170) ng/ml AGR2 in normal male subjects (n = 18). However, we found that plasma AGR2 values were significantly elevated in each cohort of patients with metastatic prostate cancer. Specifically, we observed AGR2 values of 105 (20–285), 75 (10–2500), and 945 (50–1,035) ng/ml for patients in the CSPC, CRPC, and NE-CRPC cohorts, respectively ($P < 0.05$ for each comparison). The median (range) of CGA levels in normal subjects was 70 (22–714) ng/ml, which is consistent with the assay's normal value of <100 ng/ml. We observed CGA values of 68 (39–206), 92 (10–551), and 174 (151–235) ng/ml for patients in the CSPC, CRPC, and NE-CRPC cohorts, respectively. Only the NE-CRPC cohort had significantly different CGA levels than normals ($P < 0.05$). We conclude that plasma AGR2 is significantly elevated in patients with metastatic prostate cancer compared with normal male controls.

Plasma AGR2 Levels do not Correlate With PSA

Serum PSA levels are almost uniformly elevated in patients with metastatic prostate cancer, where it is used as a therapeutic biomarker [30]. As both PSA and AGR2 levels are androgen regulated secreted proteins, we explored whether AGR2 levels correlate

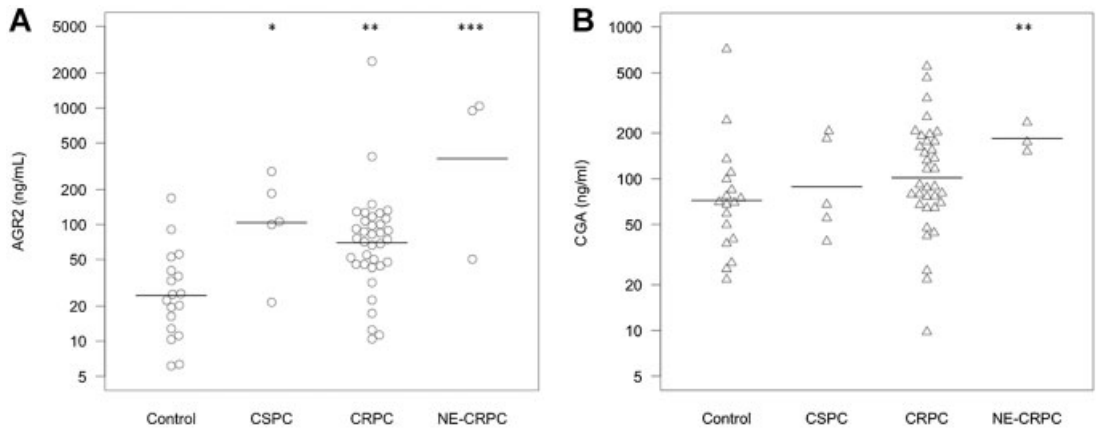

Fig. 2. Plasma AGR2 and CGA values in normal males and metastatic prostate cancer patients. AGR2 (○, **panel A**) and CGA (△, **panel B**) values were measured in plasma by ELISA for subjects representing control (n = 18), CSPC (n = 5), CRPC (n = 36, and NE-CRPC (n = 3) sub-groups. Geometric mean value for each cohort is indicated by a solid horizontal line. Significance in panel A is assessed for each cohort versus control using ANOVA on log-transformed (variance stabilized) AGR2 levels followed by Dunnett's adjustment: CSPC—control $P = 0.016$ (*), CRPC—control $P = 0.0017$ (**), NE-CRPC—control $P = 0.0001$ (***). Significance in panel B is assessed for each cohort versus control using Welch's unequal variance *t*-test on log-transformed CGA levels followed by Bonferroni correction: NE-CRPC—control $P = 0.005$ (**).

with PSA. First, we explored whether plasma AGR2 relates to PSA on a per sample basis and found a weak and non-significant correlation ($R^2 = 0.08$, $P = 0.07$; Supplementary Figure). Next, as changes in PSA values are used as a biomarker associated with response to multiple therapies in CRPC, we explored sequential measurements of AGR2 and PSA. In 10 subjects (CRPC n = 7, CSPC n = 2, NE-CRPC n = 1), sequential samples were available representing baseline and serial measurements at weeks 3–6 and 10–12 in response to standard therapies (Fig. 3). In this preliminary analysis, we observe no clear trend in comparing changes in PSA or AGR2 values in response to standard therapies. In some subjects, a ''discordant'' pattern is observed such that AGR2 levels changed inversely with PSA values (Fig. 3E, H, and I). In other subjects AGR2 levels seemed to closely track declining (Fig. 3A, B, and J) or increasing (Fig. 3C) PSA values. A third pattern with stable AGR2 levels despite decreasing PSA (Fig. 3D, F, and G) was also observed. We conclude that plasma AGR2 and PSA vary independently in relation to treatments used for metastatic prostate cancer.

AGR2 Expression is Associated With a "Neuroendocrine Phenotype"

We noted that the sub-group of patients with the highest median AGR2 expression included three subjects with NE-CRPC. As NE-CRPC remains generally a poorly defined clinical entity, we sought to more precisely describe the clinical and serologic characteristics of these patients (Table II). As expected, patients with NE-CRPC exhibit an unusual pattern of metastatic spread which includes sites such as lung, liver, bone, eye, and brain in addition to the conventional metastatic pattern involving osteoblastic metastases to bone. Chromagranin A (CGA) and other blood based markers are commonly used to help characterize patients with NE-CRPC [31]. We note that CGA is elevated in all subjects with NE-CRPC. Other serum markers such as carcinoembryonic antigen (CEA) and neuron-specific enolase (NSE) are also elevated along with AGR2 in many of these subjects as well. We conclude that plasma AGR2 may be used along with CGA, and other serum markers, to help define NE-CRPC.

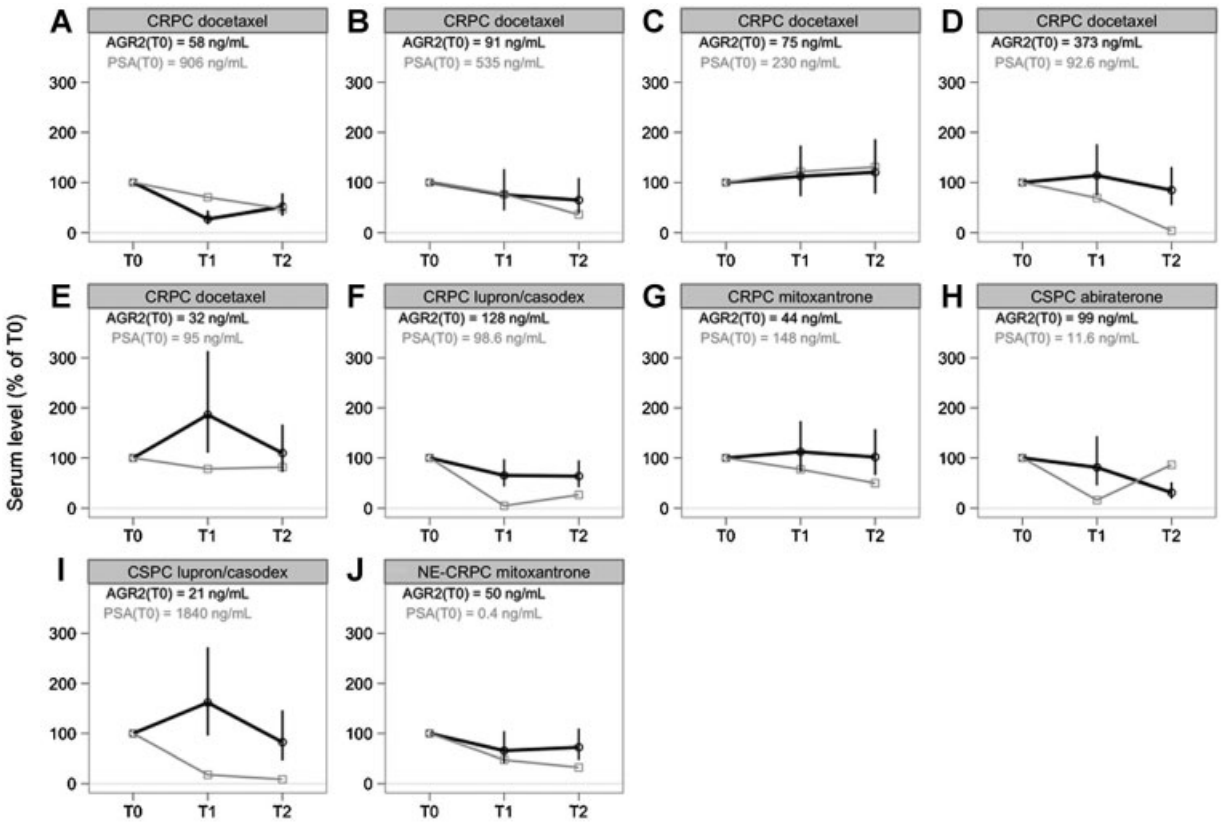

Fig. 3. Serial measurements of plasma AGR2 and PSA values in individual patients in response to therapy. Each panel represents relative changes in AGR2 and PSA values for an individual patient compared to a baseline value (T0 = baseline). Serial measurements were obtained at 3–6 weeks (T1) and 9–12 weeks (T2) following starting the indicated therapy. The relative change of each value is plotted compared to baseline (100 arbitrary units). Error bars represent 95% confidence intervals.

As NE-CRPC represents an uncommon clinical variant of metastatic prostate cancer, we used prostate cancer cell lines to further explore a possible association between AGR2 and neuroendocrine marker expression in prostate cancer cells (Fig. 4). PC3 is a human prostate cancer cell line which is known to express high levels of AGR2 and exhibit other characteristics associated with neuroendocrine prostate cancer [9,10,32]. 22Rv1 is an androgen-responsive human prostate cancer cell line which was also found to expresses AGR2 and is tumorogenic in immune-compromised mice [33,34]. PC3 and 22Rv1 cells were infected with a lentivirus expressing shRNA against AGR2 or a scrambled-shRNA as a control. Down-regulation of AGR2 expression in PC3 and 22Rv1 cells resulted in concordant decreases in CGA levels compared to cells infected with scramble-shRNA control virus (Fig. 4, left and middle panels). DU145 represents and androgen receptor negative human prostate cancer cell line which is generally not tumorogenic and does exhibit appreciable amounts of AGR2. Stable transfectants of DU145 cells were made with ectopic expression of AGR2 or green fluorescent protein (GFP) as a control. In the DU145 cells, we observed that increased expression of AGR2 resulted in a significant increase in CGA expression (Fig. 4, right panel). A similar pattern correlating AGR2 and NSE expression was observed in PC3 and DU145 cells, but was not as pronounced in 22RV1 cells. We conclude that AGR2 expression generally correlates with CGA and NSE expression in prostate cancer cell lines.

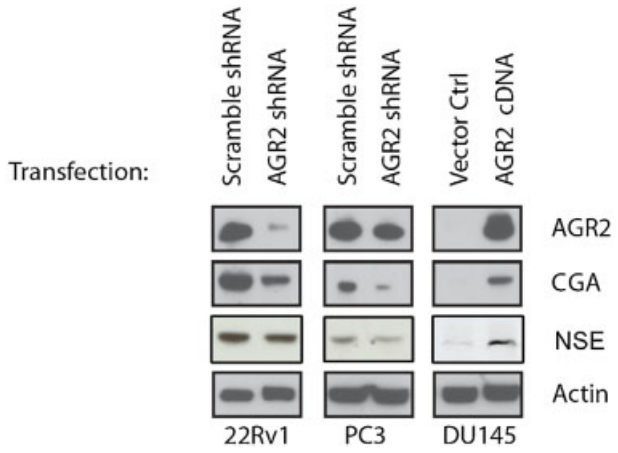

Fig. 4. CGA and NSE levels are modulated by AGR2. Prostate cancer cell lines (22RvI, PC3, and DUI45) were transduced with lentiviral particles to knockdown AGR2 (shRNA in 22RvI and PC3) or induce overexpression (cDNA in DUI45) as compared to control (scrambled) or (GFP) vectors, respectively. AGR2, CGA, NSE, and actin expression in cell lysates were determined by immunoblot.

DISCUSSION

AGR2 is widely expressed in normal mucus-producing and endocrine-responsive cells in lung, stomach, colon, pancrease, breast, and prostate [7]. Microdissection studies show that AGR2 expression is elevated in prostate carcinoma compared to adjacent normal tissue, and urine-based detection of AGR2 has been proposed as a potential diagnostic marker for prostate cancer [10]. Conflicting data exist concerning the potential for tissue-based measurements of AGR2 levels to add to standard prognostic markers used to risk-stratify patients with newly diagnosed prostate cancer [11,12]. Despite strong data supporting a role for AGR2 in promoting a metastatic phenotype in pre-clinical models, expression of AGR2 in metastatic prostate cancer has not been previously explored [5].

Here, we utilized a combination of blood-based approaches to evaluate AGR2 expression in patients with metastatic prostate cancer. First, we used peripheral-blood RNA enriched for the presence of CTCs towards determining if AGR2 mRNA expression was elevated in cancer cells in patients with metastatic prostate cancer. We conclude that AGR2 is expressed in metastatic prostate cancer as evidenced by finding a significant correlation between CTC enumeration and AGR2 mRNA expression. However, as CTCs are not present in all patients and may not accurately reflect AGR2 expression in all tumor deposits, we utilized an AGR2 ELISA assay to quantify plasma AGR2 levels in patients with metastatic prostate cancer. We find that plasma AGR2 levels are significantly elevated in patients with metastatic prostate cancer versus normal male controls. Further, our preliminary results show that plasma AGR2 levels can vary in response to androgen-directed and other therapies used for patients with metastatic prostate cancer. Finally, we noted very high plasma AGR2 levels in patients with a ''neuroendocrine'' or ''anaplastic'' variant of metastatic prostate cancer and suggest that AGR2 expression correlates with expression of neuroendocrine markers (CGA and NSE) in prostate cancer cells.

Despite strong data implicating AGR2 expression in a metastatic phenotype, a mechanistic connection between AGR2 expression and metastatic progression remains elusive. Persson et al. [3] have demonstrated that AGR2 may be a novel type of the protein disulfide isomerase (PDI). Members of the PDI family have roles associated with oxioreduction during protein folding in the endoplasmic reticulum [35]. However, the possible enzymatic effects or down-stream targets of AGR2 expression or activation remains generally unexplored. Induction of AGR2 levels in response to steroid treatment in breast [36] and prostate [2] cancer has been demonstrated. This would suggest that

TABLE II. Summary of Blood and Imaging Evaluation in Patients With Neuroendocrine Phenotype of Metastatic Prostate Cancer

CRPC-NE	Blood markers (normal range)	Imaging studies
Patient #1	PSA: <0.1 (<4) ng/ml; AGR2: 944.2(<25) ng/ml; CGA: 174(<100) ng/ml; NSE: >130 (3.7–8.9) ug/l; CEA: 242.8 (<5) ng/ml	Extensive pulmonary nodules; multiple liver nodules; pelvic mass (prostate bed recurrence); focal osteoblastic bone metastasis (femur)
Patient #2	PSA: 0.7 (<4) ng/ml; AGR2: 1033.8 (<25) ng/ml; CGA: 151 (<100) ng/ml; NSE: 32.7 (3.7–8.9) ug/l; CEA: 142.9 (<5) ng/l; PAP: 14 (<2.8) ng/ml	Multiple pulmonary nodules with mediastinal lymph adenopathy; multiple liver metastases; extensive osteoblastic bone metastases
Patient #3	PSA: 0.4 (<4) ng/ml; AGR2: 50.4 (<25) ng/ml; CGA: 235 (<100) ng/ml; CEA: 4.5 (<5) ng/ml	Bilateral adrenal nodules; eye (choroid); brain (parafalcine mass); retroperiteoneal lymphadenopathy; multiple mixed osteolytic/osteoblastic bone metastases

increasing tissue expression of AGR2 should be correlated with PSA concentration. Our results do not support this hypothesis and instead indicate that serological AGR2 and PSA are decoupled in prostate cancer (Fig. 3). This is further supported by the inverse relationship between AGR2 and PSA levels in NE-CRPC patients (Fig. 2).

NE-CRPC represents an extreme example of the clinical situation where PSA levels are neither elevated nor useful as a therapeutic marker in metastatic prostate cancer. Areas of neuroendocrine differentiation are commonly found in histologic sections representing prostate adenocarcinoma, however the clinical significance of these findings remain controversial [37]. For patients with metastatic prostate cancer, a ''neuroendocrine'' or ''anaplastic'' phenotype has more recently started to be defined in hopes of identifying patients who may benefit from more intensive cytotoxic chemo-hormonal therapy [31,38]. While no clear consensus definition has emerged for NE or ''anaplastic'' CRPC, clinical features used to define this entity often include characteristics such as: (i) the presence of extensive visceral metastases; (ii) low PSA values (typically <10 ng/ml); (iii) osteolytic bone metastases; and (iv) a short duration of response to ADT. Conventional plasma or serum markers often elevated in NE-CRPC include CGA, NSE, and CEA [28,39]. However, not all patients with NE-CRPC can be identified, let alone followed with the available blood-based markers. Our data suggests that plasma AGR2 levels may be used to help define and potentially better direct treatment for NE-CRPC. A limitation of our study is that the clinical association between elevated plasma AGR2 levels and NE-CRPC is based on a small sub-sample ($n = 3$) of our overall cohort. Additional studies involving larger numbers of patients would be required to validate any such association.

Significant effort has been placed on the identification of biomarkers which would enable oncologists to better understand prostate cancer progression. This is especially important with patients with metastatic prostate cancer, as PSA levels do not always correlate with disease progression or therapeutic efficacy. In fact, patients with anaplastic prostate cancer are characterized by low PSA levels and typically have poor prognosis. In summary, we propose that plasma AGR2 levels may be useful to help characterize, monitor, and direct therapies for patients with metastatic prostate cancer. Additional research is needed to better understand potential mechanistic and prognostic implications of AGR2 expression in relation to the underlying tumor biology and disease course in patients with prostate cancer.

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