RGS2 is prognostic for development of castration resistance and cancer-specific survival in castration-resistant prostate cancer

Anna Linder PhD | Karin Larsson PhD | Karin Welén PhD | Jan-Erik Damber MD, PhD

Department of Urology, Sahlgrenska Cancer Center, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Correspondence
Anna Linder, PhD, Department of Urology, Sahlgrenska Cancer Center, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Medicinaregatan 1F, vån 4, Box 425, 405 30 Gothenburg, Sweden. Email: anna.linder@urology.gu.se

Funding information
Västra Götalandsregionen, Grant/Award Number: ALFGBG-138351; Stiftelsen Wilhelm och Martina Lundgrens, Grant/Award Number: vet1-150/2012; Stiftelsen Assar Gabrielssons Fond, Grant/Award Numbers: FB12-59, FB13-48; Cancerfonden, Grant/Award Numbers: CAN 2017/380, CAN 2017/478

Abstract
Background: Regulator of G-protein signaling 2 (RGS2) is a multifaceted protein with a prognostic value in hormone-naïve prostate cancer (PC). It has previously been associated with the development of castration resistance. However, RGS2 expression in clinical specimens of castration-resistant prostate cancer (CRPC) and its clinical relevance has not been explored. In the present study, RGS2 was assessed in CRPC and in relation to the development of castration resistance.

Methods: In the present study, RGS2 expression was evaluated with immunohistochemistry in patient materials of hormone-naïve and castration-resistant primary tumors, also in matched specimens before and after 3 months of androgen deprivation therapy (ADT). Cox regression and Kaplan-Meier curves were used to evaluate the clinical significance of RGS2 expression. RGS2 expression in association to castration-resistant growth was assessed experimentally in an orthotopic xenograft mouse model of CRPC. In vitro, hormone depletion of LNCaP and enzalutamide treatment of LNCaP, 22Rv1, and VCaP was performed to evaluate the association between RGS2 and the androgen receptor (AR). Stable RGS2 knockdown was used to evaluate the impact of RGS2 in association to PC cell growth under hormone-reduced conditions. Gene and protein expression were evaluated with quantitative polymerase chain reaction and Western blot analysis, respectively.

Results: RGS2 expression is increased in CRPC and enriched under ADT. Furthermore, a high RGS2 level is prognostic for poor cancer-specific survival for CRPC patients and significantly reduced failure-free survival (FFS) after an initiated ADT. Additionally, the prognostic value of RGS2 outperforms prostate-specific antigen (PSA) in terms of FFS. The present study furthermore suggests that RGS2 expression is reflective of AR activity. Moreover, low RGS2-expressing cells display hampered growth under hormone-reduced conditions, in line with the poor prognosis associated with high RGS2 expression.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2020 The Authors. The Prostate published by Wiley Periodicals LLC
Conclusions: High levels of RGS2 are associated with aggressive forms of castration-resistant PC. The results demonstrate that a high level of RGS2 is associated with poor prognosis in association with castration-resistant PC growth. RGS2 alone, or in association with PSA, has the potential to identify patients that require additional treatment at an early stage during ADT.

KEYWORDS
androgen receptor, prognostic marker, regulator of G-protein signaling 2

1 | INTRODUCTION

The prevalence of prostate cancer (PC) and high mortality associated with the diagnosis, advocate for new stable biomarkers to guide treatment and diagnosis. This is especially important considering the development of castration-resistant prostate cancer (CRPC), where tumor progression commences despite systemic depletion of androgens in association with maintained androgen receptor (AR) signaling. Despite the introduction of new AR-inhibiting drugs there is no curative treatment for CRPC and relapse commonly occur within a few years of treatment in association to resumed AR signaling.

In the present study, we assess the prognostic value of regulator of G-protein signaling 2 (RGS2) in association to AR signaling, development of castration resistance, and CRPC progression.

We have previously shown that high RGS2 expression in hormone-naïve prostate cancer (HNPC) is associated with a poor prognosis and an aggressive PC phenotype. However, aberrant RGS2 expression has been shown in several types of carcinomas in association to both positive and negative prognosis, suggesting that regulation and action of RGS2 are reflective of the cancer cell type and tumor environment.

Although RGS2 is not generally considered an AR-responsive gene, it has been shown that RGS2 transcription is rapidly increased in response to AR stimulation. Furthermore, low RGS2 expression has been associated with PC acquisition of castration resistance, in association with its attenuating effect on G-protein-associated androgen-independent AR signaling in the absence of androgens.

The current study was conducted to assess the clinical significance of RGS2 in association to AR signaling, development of castration resistance, and CRPC progression.

2 | MATERIALS AND METHODS

2.1 | Patient material

For patient characteristics, see Table 1.

2.1.1 | Cohort I

Cohort I includes archival material of 22 CRPC specimens. CRPC patients were treated with androgen deprivation therapy (ADT) alone.

2.1.2 | Cohort II

Cohort II includes archival material of 28 untreated, HNCP specimens in stage T1b.

| TABLE 1 | Patient characteristics
| Clinico-pathological factor | Cohort I (n = 22) | Cohort II (n = 28) | Cohort III (n = 28) |
|---------------------------|-----------------|-----------------|-----------------|
| Age, y                    | 80 (65-88)      | 78 (60-90)      | 76 (63-86)      |
| Gleason score             | 9 (7-10)        | 7 (5-8)         | 7 (6-9)         |
| PSA                       | 13.5 (normal-200) |                |                 |
| PSA (baseline), ng/mL     |                 | 194 (8.4-5000)  |                 |
| PSA (post.ADT), ng/mL     | 13 (0.1-1200)   |                |                 |
| PSA (nadir), n = 17, ng/mL| 1.4 (0-500)     |                |                 |
| M1                        | n = 13          | n = 5           | n = 17          |
| M0                        | n = 6           | n = 23          | n = 8           |
| T-stage                   | T1b             | T3 (T1c-T4)     |                 |
| Treatment                 | GnRH, TAB, Cast. | Hormone-naïve   | GnRH, TAB, Cast. |

Abbreviations: ADT, androgen deprivation therapy; GnRH, gonadotropin-releasing hormone; PSA, prostate-specific antigen; TAB, total androgen blockade.
The specimens for cohorts I and II were obtained by transurethral resection of the prostate and classified according to Gleason at Sahlgrenska University Hospital, Gothenburg, Sweden.

2.1.3 | Cohort III

Archival material of 28 tumor specimens obtained by needle biopsy from patients at the time of diagnosis and their matched, follow-up specimens obtained approximately 3 months after an initiated ADT. Prostate-specific antigen (PSA) was monitored and values were available at diagnosis, at the time-point for the second biopsy and nadir. Failure-free survival (FFS) was defined as local or distant progress or two consecutive increases of PSA during treatment. The specimens from untreated patients have previously been included as part of a larger study of the expression of RGS2 in HNPC. RGS2 has previously not been assessed in the matched samples collected after ADT. Included patients were diagnosed at the Department of Urology, Sahlgrenska University Hospital, Gothenburg, Sweden. The anonymized materials were handled according to ethical guidelines. Informed consent was obtained from all patients included in the study. The study was approved by the local ethical committee at the Sahlgrenska University Hospital in Gothenburg (reference number for cohorts I and II: 11608 and cohort III: 667-05).

2.2 | Immunohistochemistry

Sections were deparaffinized and rehydrated in graded ethanol. Immunohistochemistry (IHC) for cohorts I and II, was performed as previously described. Incubation with anti-RGS2 (0.8 µg/mL, ab36561; Abcam, Cambridge, UK) antibody was performed overnight at 4°C. Incubation with biotinylated secondary antibody and ABC reagent from the Vectastain ABC Kit (Vector Laboratories Inc, Burlingame, CA) was carried out according to the protocol supplied by the manufacturer. Cohort III was stained using the Dako Autostainer Link 48 instrument (Dako, CA) and EnVision FLEX System (Dako, Glostrup, Denmark). Endogenous peroxide was inhibited using the included Peroxidase-Blocking Reagent followed by incubation with primary antibody anti-RGS2 (1 µg/mL), diluted in supplied antibody diluent, for 1 hour at room temperature. Incubation with biotinylated secondary antibody was followed by EnVision FLEX HRP. For visualization of peroxidase reaction, 3,3-diaminobenzidine (DAB; Liquid DAB + Substrate Chromogen System, Dako) was used. Sections were counterstained with Mayer’s hematoxylin. Negative controls included human RGS2 peptide (5 µg/mL, ab36560; AbCam), rabbit IgG (0.8 µg/mL, Normal Rabbit IgG Control; R&D Systems, Abingdon, UK) and excluded primary antibody.

Microscopic evaluation of the RGS2 protein expression was performed blindly by two investigators and scored with concordant results. The intensity and proportion of epithelial RGS2 expression was taken in consideration and scored as 0 = no detectable staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining; the proportion of positive cells was scored as 0 < 5%, 1 ≤ 33%, 2 ≤ 66%, and 3 > 66% positive cells. The proportion score was multiplied by the intensity value, these values were totaled generating the RGS2 section score.

2.3 | Oncomine: validation of expression

Validation of RGS2 expression in PC and in relation to AR and PSA was conducted in the “Tamura Prostate” data set (GSE6811; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6811). Messenger RNA samples from HNPC (n = 10, primary tumors) and CRPC (n = 25; primary tumors [n = 11], metastases [n = 14]) were extracted from frozen tissue captured with laser microbeam microdissection. Data were retrieved from the Oncomine database on 21 December 2018.

2.4 | Cell culture

LNCaP (ATCC, Manassas, VA), LNCaP clones (ShRGS2 and ShNT, previously characterized) and 22Rv1 (ATCC CRL-2505) were continuously cultured in RPMI-1640 (PAAbb Laboratories, Pasching, Austria) containing stable glutamine, supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (FBS; Gibco, South America). LNCaP-19 (castration-resistant LNCaP sub-line, characterized elsewhere) was cultured with 10% Dextran-Charcoal Stripped FBS (DCC; Gibco). VCaP (ATCC CRL-2876) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Cell line authentication was performed by short tandem repeat profiling according to ATCC’s guidelines. Cell lines were continuously tested for mycoplasma contamination. Cell passages were kept below an upper limit of 18 passages for all cell lines. During hormone depletion experiments, cells were cultured in phenol red-free RPMI-1640 (PAAbb Laboratories) with or without supplementation with 1 nM or 10 nM dihydrotestosterone (DHT; Sigma-Aldrich), for RNA and protein assays, respectively. For enzalutamide experiments, cells were starved for 24 hours before changing to customary growth media supplemented with 10 µM enzalutamide or equivalent amount of dimethyl sulfoxide as vehicle control. For growth curves, cells were seeded and kept in standard culture media overnight until firmly attached. Cells were serum-starved for 24 hours prior changing to a media containing 10% DCC serum, with or without the supplement of 10 nM DHT or equivalent amount of ethanol as control.

2.5 | Animal experiment

Male athymic BALB/c nude mice, 8 weeks old (Charles River Laboratories International Inc., Wilmington, MA), were anesthetized with a mixture of xylazine/ketamine (0.55/110 mg/kg) and kept sedated with isofluorane during surgery. A T-incision was performed in the lower abdomen. Before cell implantation, mice were surgically castrated or sham-operated. One million PC cells, in 7 µL of BD Matrigel (BD Biosciences, Bedford, MA), were injected into the dorsolateral lobe of the prostate using a 30-gauge needle.
Animals were culled 2 months after implantation or when displaying tumor-induced discomfort, which was equivalent to mean tumor growth of 48 (±7) days in the castrated group and 50 (±10) days in the intact group. There was no correlation between tumor weight and time of growth (Spearman’s *r* = −0.316; *P* = .684 and *r* = −0.393; *P* = .384 in the castrated and intact group, respectively). Harvested tumors were weighed, and preserved in RNAlater (Ambion, Austin, TX). For homogeneous groups, in terms of tumor size, one tumor from each group was omitted from analysis, due to the significant effect of oxygenation on RGS2 expression. The use of animals and protocol were approved by the regional animal ethics committee in Gothenburg (reference number: 317-2011).

2.6 | RNA isolation and quantitative real-time polymerase chain reaction

RNA was isolated using the AllPrep cDNA/RNA Mini Kit (Qiagen) according to the provided protocol. RNA integrity number (RIN) was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). One tumor from the intact group was omitted from analysis due to an RIN of less than 6. Reverse-transcription was completed with SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reaction (qPCR) was carried out using TaqMan Fast Universal PCR system (Applied Biosystems, Foster City, CA) with gene-specific TaqMan Gene Expression Assays (Applied Biosystems) (RGS2 Hs00180054_m1, 18S Hs99999901_s1, CASC3 Hs00201226_m1, GUSB Hs00939627_m1, AR Hs00171172_m1, KLK3 Hs02576345_m1, GAPDH Hs02758991). Ct values were defined by ABI 7500 Real-Time PCR System. For in vivo analyses, the expression was calculated relative to the endogenous control human GAPDH or GUSB; CASC3 was used for in vitro experiments. Expression was calculated using the 2^−ΔΔCt method.

2.7 | Western blot analysis

Cells were lysed with CelLytic M Cell Lysis Reagent (Sigma-Aldrich) supplemented with phosphatase (PhosSTOP; Roche Applied Science, Penzberg, Germany) and protease inhibitors (cOmplete Mini; Roche), followed by sonication and centrifugation. A total of 25 µg protein was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Novex, NuPAGE 4–12% Bis–Tris Gels; Invitrogen) and transferred to polyvinylidene fluoride membranes (iblot Transfer Stack; Invitrogen). Antibodies were diluted in 2% Amersham ECL Prime Blocking Agent (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Antibody binding was visualized using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare) and the LAS1000 image-detection system (Fujifilm Life Science, Stamford, CT). Antibodies used were mouse anti-RGS2 (H00005997-M0; Abnova, Taipei, Taiwan), mouse anti-AR (sc-7305; Santa Cruz Biotechnology, Dallas, TX), mouse anti-β actin (A5441; Sigma-Aldrich), rabbit anti-PSA (RB-9056-P; NeoMarkers, Fremont, CA) and rabbit anti-HIF1α (ab51608; AbCam).

2.8 | Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics, version 24. For all tests, *P* ≤ .05, **P** ≤ .01, ***P** ≤ .001 were considered significant. For comparisons of IHC scores, the Mann-Whitney U test was used to identify individual differences between groups. Student’s *t* test was used to calculate the statistical significance of gene expression and cell growth curves. The Spearman rank correlation was used throughout. For analysis of survival and FFS, a Kaplan-Meier chart was created, *χ*² and log-rank (Mantel-Cox) were used to define statistical differences. For Kaplan-Meier charts, RGS2 score was dichotomized according to median score or grouped as described. For Cox regression analysis, 95% confidence interval is specified. A log10 transformation was applied for variables with a significantly skewed distribution (*P* < .01 with Shapiro-Wilk test of normality). When the condition for normality was still not met the median was used as cutoff. Potential interaction between each variable and time was evaluated. The proportion hazard assumption was not violated for any of the variables.

3 | RESULTS

3.1 | Increased RGS2 in CRPC is associated with poor cancer-specific survival

RGS2 expression was evaluated in HNPC and CRPC with IHC. Data showed that in contrast to an overall low cancer cell-associated expression of RGS2 in HNPC, RGS2 expression in CRPC was significantly increased (Figure 1A,B). The clinical relevance of the high RGS2 level in CRPC was demonstrated by the poor cancer-specific survival (CSS) associated with high RGS2 levels in the CRPC group. Median survival time for patients with high expressing tumors were 14 months compared with 24 months in the low expressing group (Figure 1C; n = 21; *χ*², 4.379; log-rank *P* = .036). The result was yet more apparent when only exceedingly advanced cases were included, signified by poorly differentiated histology with a major Gleason grade (GG) of 5. The corresponding median survival times were 8 months in the high RGS2-expressing group compared with 25 months in the low expressing group (Figure 1D; n = 16; *χ*², 4.710; log-rank *P* = .030). Univariable Cox regression showed that an increased RGS2 score was significantly associated with a poor prognosis (Table 2; CRPC, univariable analysis). Additionally, in the analysis together with age and GG, RGS2 was confirmed as an independent prognostic factor (Table 2; CRPC, multivariable analyses 1 and 2). There was no significant increased risk associated with the available clinicopathological factors in the current cohort (GG, M-stage, and age) (Table 2; CRPC, univariable analysis).

Assessment of cancer cell-associated RGS2 expression in the Tamura data set retrieved from the publicly available Oncomine cancer microarray database (Compendia Biosciences; Ann Arbor, MI; www.oncomine.com) confirmed that RGS2 was significantly increased in CRPC compared with HNPC (Figure 1E).
High RGS2 expression after ADT is associated with decreased time to relapse

To evaluate the effects of ADT, RGS2 expression was further assessed in clinical specimens with IHC before (PC HN) and after 3 months of ADT (PCADT). The data showed that RGS2 expression after initiated ADT was prognostic for FFS (Table 2; ADT, univariable analysis). Furthermore, when analyzed together with PSA, RGS2 post ADT was validated as an independent prognostic factor for FFS that outperformed PSA (Table 2; ADT, multivariable analysis). The prognostic value for FFS was exclusive for RGS2 expression post ADT; RGS2 score before ADT was not associated with FFS (Cox regression; hazard ratio [HR], 1.494 [0.757–2.964]; P = .247). The data showed that median RGS2 score was comparable between the paired PC_HN and PC_{ADT} specimens (Figure 2A; n = 28; Wilcoxon rank, P = .538). However, post ADT, 50% (14 out of 28) of the tumors showed an increased RGS2 score, 21% (6 out of 28) an unchanged score, and 29% (8 out of 28) a lower score (Figure 2B).

For assessment of the significance of the expressional variation of RSG2 in association with ADT, patients were grouped according to the change in RGS2 expression. The data showed that patients with decreased RGS2 score had a better prognosis with the increased time between ADT and relapse (median, 25 months), compared to patients with increased or constant RGS2 score (median, 11 months) (Figure 2C; n = 22, log-rank, χ², 4.492; P = .034). We have previously shown that a high RGS2 score is associated with a positive metastatic status for untreated patients in the present material. However, there was no correlation between RGS2 score post treatment and metastatic status (Spearman’s ρ, 0.190; P = .362). Similarly, RGS2 score for the initial biopsy did not correlate with the post ADT score (Spearman’s ρ, 0.085; P = .669).

Although PSA levels showed great variance, a significant decrease confirmed response to ADT (Figure 2D). Additionally, a grouping of patients based on RGS2 level before and after ADT, showed that stable RGS2 expression was associated with a significantly reduced response to ADT, demonstrated by a less significant reduction in PSA (Figure 2E). The level of PSA after ADT was not associated with metastatic status (Figure S1).

RGS2 upregulation in orthotopic CRPC xenografts is reflective of AR activity

To mimic the clinical setting, an orthotopic xenograft mouse model was used to evaluate RGS2 expression in association to the...
The development of castration resistance. Tumor take after orthotopic implantation of androgen-dependent (AD) LNCaP was 90% (9 of 10) in intact and 40% in castrated (4 of 10) mice. One tumor from each group was omitted from further evaluation due to significantly smaller tumor size (less than 10% of the median tumor weight). Additionally, one tumor from the intact group was exceedingly necrotic, thus omitted. The mean tumor weight in the castrated group was 907 ± 455 mg, comparable to the intact group, 888 ± 402 mg.

In accordance with the patient data, increased RGS2 gene expression was shown for CR tumors compared with AD tumors from sham-operated animals (Figure 3A), evaluation of RGS2 protein showed similar levels (Figure 3B,C). Furthermore, increased AR protein level was noticeable in the CR tumors compared with the AD (Figure 3B). PSA expression suggests AR activity, however, somewhat decreased in the CR tumors (Figure 3A-D). Moreover, the correlation between RGS2, AR, and PSA/KLK3 was evaluated in data from the Oncomine database. The data showed that RGS2 gene expression was positively correlated with both AR and KLK3 expression in CRPC (Spearman’s \( \rho \), 0.515; \( P = .008 \) and \( \rho \), 0.524; \( P = .007 \), respectively).

### 3.4 RGS2 expression is reflective of the AR and AR activity

The association between AR activity and RGS2 expression was further assessed in vitro by enzalutamide treatment of androgen-responsive cell lines. The AD LNCaP cell line, displayed increased AR expression in response to enzalutamide treatment. However, substantially decreased PSA expression confirmed adequate AR inhibition; subsequently, RGS2 expression was significantly reduced (Figure 4A, Student’s t test, \( P = .002 \)). The response for the androgen-independent cell line, 22Rv1 was less distinct, a minor decrease in PSA expression suggested insufficient inhibition of AR activity. In line, RGS2 expression showed a minor reduction in the treated group compared with the control (Figure 4B). In addition, the PSA expression for VCaP cells showed no reduction of AR activity in the treated group, represented by equivalent levels of PSA, and notably, expression of AR and RGS2 was increased in the treated group compared with the control (Figure 4C). The general expression of RGS2 in the cell lines suggest that these data would be reflective of clearly visible downregulation (LNCaP), continuously high expression (22Rv1), and fast upregulation in response to AR inhibition (VCaP) (Figure 4D).

### 3.5 RGS2 is downregulated in response to hormone depletion in vitro

Decreased RGS2 expression has previously been associated with the acquisition of castration resistance.\(^8\) To assess the impact of hormone depletion on RGS2 expression, the LNCaP cell line was cultured under hormone-reduced conditions with or without the supplement of DHT. In agreement with the previous study, data

| Cohort I, CRPC | Univariable analysis | Multivariable model 1 |
|----------------|----------------------|----------------------|
| **Variable**   | **HR (95% CI)**      | **P value**          | **HR (95% CI)**      | **P value**          |
| RGS2 (cat.)    | 3.295 (1.020-10.643) | .046*                | 3.860 (1.156-12.891) | .028*                |
| M-stage (cat.) | 1.989 (0.735-5.381)  | .176                 |                      |                      |
| GG (cat.)      | 1.392 (0.443-4.368)  | .571                 | 2.240 (0.777-6.462)  | .136                 |
| Age (cont.)    | 0.998 (0.939-1.062)  | .958                 |                      |                      |
| **Multivariable model 2** | **HR (95% CI)**      | **P value**          | **HR (95% CI)**      | **P value**          |
| RGS2 (cat.)    | 3.446 (1.046-11.346) | .042*                | 3.021 (0.862-10.583) | .084                 |
| M-stage (cat.) |                      |                      | 2.620 (0.905-7.583)  | .076                 |
| GG (cat.)      |                      |                      |                      |                      |
| Age (cont.)    | 0.985 (0.921-1.051)  | .659                 |                      |                      |
| **Cohort III, ADT** | Univariable analysis | Multivariable model 1 |
| **Variable**   | **HR (95% CI)**      | **P value**          | **HR (95% CI)**      | **P value**          |
| RGS2 (cont.)   | 1.313 (1.000-1.725)  | .0501*               | 1.425 (1.054-1.927)  | .021*                |
| PSA, log10     | 1.281 (0.938-1.748)  | .119                 | 1.318 (0.865-2.008)  | .199                 |

Note: Results from Cox regression analysis are presented as HR with 95% CI and P value. RGS2 was evaluated as a continuous (cont.) or categorical (cat.) variable (divided by median). \(* P \leq .05\). Gleason was evaluated based on major GG, 4 vs 5. M-stage, M0 vs M1.

Abbreviations: CI, confidence interval; CRPC, castration-resistant prostate cancer; GG, Gleason grade; HR, hazard ratio; PCD, prostate cancer death.
showed that RGS2 was downregulated under hormone-depleted culture conditions compared with culture supplemented with androgens. Moreover, RGS2 expression corresponded to decreased AR and PSA expression (Figure 5A,B). The effect was sustained after prolonged hormone depletion in association with the acquisition of castration resistance (Figure 5C,D).

Furthermore, before development of castration resistance, hormone-deprived LNCaP cells displayed inhibited cell growth. Cells cultured in DCC without the supplement of DHT did not require passaging during the 1-month frame of the experiment. In comparison, DHT-treated cells required continuous passaging. To test the significance of RGS2 in the low-androgen setting, LNCaP with stable knockdown of RGS2 and the nontarget control were cultured in DCC with or without the supplement of DHT. In the hormone-deprived setting, cells with RGS2 knockdown displayed a significantly reduced growth rate compared with the nontarget control clone (Figure 5E), whereas data showed overlap when androgens were available (Figure 5F). This indicates that although RGS2 is downregulated by androgen depletion, it is still important for proliferation in the primary androgen-deprived setting.

4 | DISCUSSION

Aberrations associated with AR signaling have been recognized as the major mechanism for the development and progression of CRPC. The present study shows that RGS2 expression, in line with its association with poor prognosis and development of castration resistance, is correlated with resumed AR activity.

We have previously shown that high RGS2 expression is associated with advanced PC in association with a poor prognosis. In agreement, the present study showed that RGS2 was significantly higher in CRPC compared with hormone-naïve tumors. Furthermore, data showed that a high RGS2 expression was prognostic for poor CSS in CRPC, suggesting that a high RGS2 level is revealing of a particularly aggressive CRPC. In accordance, PC cells with
continuous RGS2 expression have been shown to be more proliferative than cells with RGS2 knockdown; the present study furthermore emphasized the growth advantage of RGS2-expressing cells under androgen-deprived conditions. Furthermore, in line with decreased proliferation of low RGS2-expressing cells under hormone-depleted conditions, data showed that high RGS2 expression was not only prevalent after ADT initiation, but associated with fast relapse. This suggests that increased RGS2 expression

**FIGURE 3** RGS2 expression is associated with AR signaling. A, Relative gene expression of RGS2 (P = .006), AR (P = .06), and KLK3 in CR compared with AD orthotopic tumors of LNCaP origin evaluated with RT-qPCR. Data represent mean ± SEM. Statistical evaluation was performed with the Student t test. B, Representative WB for expression of CRPC-associated proteins with β-ACTIN for validation of protein loading. C, Analysis of RGS2 protein expression with relative densitometric evaluation of WB normalized to β-ACTIN. Data represent mean ± SD of three separate WB. **P < .01. AR, androgen receptor; AD, androgen-dependent; CR, castration resistant; CRPC, castration-resistant prostate cancer; RT-qPCR, quantitative reverse-transcription polymerase chain reaction; SEM, standard error of the mean; WB, Western blot

**FIGURE 4** RGS2 is downregulated in response to adequate AR inhibition. LNCaP, 22RW1, and VCaP was cultured under standard conditions for 72 hours with a supplement of DMSO (vehicle control, baseline at 1) or 1 µm enzalutamide. A, Relative expression of RGS2, AR, and KLK3 in LNCaP (P < .001, P = .023, and P = .002, respectively). B, Relative expression of stated markers in 22Rv1 (P = .002 and P = .0015 for RGS2 and KLK3, respectively). C, Relative expression of stated markers in VCaP (P < .001 and P = .05 for RGS2 and AR, respectively). D, RGS2 expression in 22RV1 and VCaP relative to LNCaP under standard culture conditions (P < .001). *P ≤ .05, **P < .01, ***P < .001. Data represent mean ± SEM. Relative expression was evaluated with the Student t test. AR, androgen receptor; DMSO, dimethyl sulfoxide; PSA, prostate-specific antigen; SEM, standard error of the mean.
FIGURE 5  RGS2 is downregulated in response to complete hormone depletion. A, Relative gene expression of RGS2, AR, and KLK3 in LNCaP cells cultured under continuous hormone-depleted conditions (DCC) with (+) or without (−) the supplement of DHT (P = .046 and P = .005 for RGS2 and KLK3, respectively). B, Representative WB of complementary protein expression of RGS2, AR, and PSA normalized to β-ACTIN. C, Relative gene expression of stated markers after continuous hormone depletion, comparison between the castration-resistant LNCaP-19 (derived from LNCaP) and LNCaP was assessed with RT-qPCR (P = .002 and P < .001 for RGS2 and KLK3, respectively). D, Representative WB for comparison of protein expression in LNCaP-19 and LNCaP equal loading was verified by β-ACTIN. For evaluation of the impact of RGS2 expression on PC cell growth, wt-LNCaP and LNCaP-derived clones with RGS2 knockdown (ShRGS2) and the nontarget control (ShNT) was submitted to culture in media supplemented with 10% DCC and 10 nM DHT (E), or with DCC alone (F). Cells were serum-starved for 24 hours preceding change into described experiment culture media. The cells were calculated after starvation, and continuously daily for 72 hours. The data represent three independent experiments. Data represent mean ± SEM of three separate experiments. Statistical significance was evaluated with Student's t test. *P ≤ .05, **P < .01, ***P < .001. AR, androgen receptor; DCC, dextran-coated charcoal; DHT, dihydrotestosterone; PSA, prostate-specific antigen; RT-qPCR, quantitative reverse-transcription polymerase chain reaction; SEM, standard error of the mean; wt, wild type; WB, Western blot.
is indicative of mechanisms associated with the development of CRPC, selected under ADT.

The association between PSA and RGS2 expression in response to ADT suggests that for a fraction of patients, both markers have the ability to identify patients that do not respond to treatment, signified by high PSA levels in the group with stable RGS2 expression. Considering the association between RGS2 and the AR, this data may suggest that stable RGS2 expression would be indicative of tumors with innate AR-associated castration resistance mechanisms, such as mutated AR. The data also propose that both markers identify patients with a good treatment response, represented by the low levels of PSA in the group of patients that displayed an RGS2 down-regulation. In addition, RGS2 has the ability to identify impending relapse among patients that seemingly respond well to ADT, signified by the low levels of PSA in the group where RGS2 is upregulated.

In conclusion, these data propose that RGS2 expression is revealing of the future development of CRPC at an early stage during ADT also when the PSA value shows a good treatment response. Additionally, while PSA does not distinguish between indolent and aggressive forms of malignancy, the data suggest that the combined prognostic properties of RGS2 and PSA, with good sensitivity, could identify patients that would benefit from the early inception of additional treatment.

Reflective of CRPC, the orthotopic animal model shows that while the tumor take was significantly reduced in the castrated group compared with the sham-operated group, similar tumor sizes indicate that existing tumors overcame androgen withdrawal and developed unimpeded. In accordance with the data from the clinical specimens, RGS2 expression was upregulated in PC cells that had adapted to growth under androgen-reduced conditions. In line with increased AR expression in response to hormone ablation in clinical specimens and animal models, the CR tumors displayed elevated levels of AR, proposing a mechanism for these tumors to acquire castration resistance. Additionally, LNCaP harbors a promiscuous AR, suggesting that anomalous AR stimulation contributes to resistant tumor growth regardless of the abrogated androgen levels. In agreement, induction of RGS2 expression has been described in association with both traditional ligand and antagonist stimulation of mutated AR. Furthermore, although a small sample number, the data indicated that PSA and RGS2 protein levels in the CR group were regulated together, suggested by sample-by-sample corresponding protein level. This observation was endorsed by the correlation between AR, AR activity, and RGS2 expression in the patient data retrieved from the Oncomine database.

AR inhibition by enzalutamide supports the association between RGS2 expression and maintained AR activity. The adequate inhibition of AR in the AD LNCaP was associated with a significant reduction of RGS2 while the CR cell line, 22Rv1, displayed a minute treatment response in terms of both PSA and RGS2 reduction. However, the VCaP cell line showed an opposing response to treatment under the current conditions. VCaP showed no reduction in AR activity while RGS2 and AR expression was readily increased in response to enzalutamide treatment. This further suggests that RGS2, at an early stage, is reflective of the innate mechanisms by which the PC overcomes AR inhibition.

Upregulation of both full-length and constitutively active AR splice variants in response to enzalutamide and ADT are suggested mechanisms for acquisition of resistance and both 22Rv1 and VCaP has been shown to be unresponsive to enzalutamide treatment at the current concentration. Considering the expression of RGS2 in the specific cell lines, these data are representative of the observed differences in the patient material in response to ADT and may suggest that RGS2 could be a candidate for identification of patients that would develop early resistance to new antiandrogen treatments, thus benefiting from additional treatment.

It has previously been suggested that RGS2 is downregulated in association with the acquisition of castration resistance in vitro. It was furthermore shown that RGS2 could inhibit androgen-independent AR activation in LNCaP in a hormone-depleted milieu, an effect that was abolished in response to AR stimulation. In the present study, data confirmed that RGS2 was significantly downregulated in LNCaP in association with hormone depletion in vitro; however, this is in association with significantly decreased AR activity. This was especially evident after prolonged hormone depletion, where mainly no AR activity was detected for the CR LNCaP-19. This suggests that the RGS2 expression is foremost reflective of the AR activity.

Considering the heterogeneity of PC, this may represent a mechanism in which androgen-responsive PC cells can overcome ADT in the absence of hormone stimulation. However, considering data from the patient material, low levels of RGS2 in CRPC are infrequent. This could be reflective of inadequate ADT and persistent androgen levels in CRPC, that is, resumed AR signaling. Thus, given the consensus of maintained AR signaling in CRPC, and despite continuous ADT treatment, the importance of RGS2 as an inhibitor of androgen-independent AR signaling may be questioned. Additionally, the present data showed that knockdown of RGS2 instantly hampered the cell growth in a complete hormone-depleted setting. This suggests that RGS2-expressing PC cells have a growth advantage in the castrated state, thus proposing a mechanism for the increased RGS2 score after ADT. This stresses that the high RGS2 level seen in PC after initiated ADT is malignant, which is in agreement with the significantly shorter time to relapse observed for these patients.

Today, CRPC patients are monitored by PSA, assessment of performance status, and bone-associated factors (alkaline phosphatase, bone pain, and extent of disease on bone scan). The common drawback of these standard tools is that they are generally detectable first at an already advanced stage. Thus, the present data suggest that RGS2 could be a valuable complement with the ability to identify aggressive PC at an early stage before major deterioration of the patient. However, this needs to be confirmed in a prospective study.

5 | CONCLUSION

In conclusion, increased RGS2 expression in association with ADT and the development of CRPC are both prevalent and associated
with an aggressive cancer phenotype. Furthermore, RGS2 expression is indicative of continuous AR signaling, and may identify PC patients that would benefit from an additional treatment at an early stage.

ACKNOWLEDGMENTS

The authors acknowledge Anita Fae (laboratory technician, Gothenburg University) for assistance with the animals and Andreas Josefsson (PhD, Umeå University), for discussion regarding the IHC scoring. The present work was funded by the Swedish Cancer Society (Grant Numbers: CAN 2017/380 and CAN 2017/478), ALF/Västra Götalandsregionen (Grant Number: ALFGBG-138351), the Assar Gabrielssons Foundation (Grant Numbers: FB 12:59 and FB 13:48) and the Wilhelm and Martina Lundgren Foundation (Grant Number: vet1-150/2012).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Anna Linder http://orcid.org/0000-0002-9444-1346
Karın Welén http://orcid.org/0000-0003-2095-5333

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68(1):7-30.
3. Edwards J, Krishna NS, Grigor KM, Bartlett JM. Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer. 2003;89(3):552-556.
4. Attag G, Parker C, Eeles RA, et al. Prostate cancer. Lancet. 2016;387(10033):70-82.
5. Katsogiannou M, Ziouziou H, Karaki S, Andrieu C, Henry de Villeneuve M, Rocchi P. The hallmarks of castration-resistant prostate cancers. Cancer Treat Rev. 2015;41(7):588-597.
6. Robinson D, Van Allen EM, Wu YM, et al. Integrative clinical genomics of advanced prostate cancer. Cell. 2015;161(5):1215-1228.
7. Beer TM, Armstrong AJ, PREVAIL Investigators, et al. Enzalutamide in metastatic prostate cancer before chemotherapy. N Engl J Med. 2014;371(5):424-433.
8. Watson PA, Arora VK, Sawyers CL. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nat Rev Cancer. 2015;15(12):701-711.
9. Cancer Genome Atlas Research Network. The molecular taxonomy of primary prostate cancer. Cell. 2015;163(4):1011-1025.
10. Antonarakis ES, Lu C, Wang H, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med. 2014;371(11):1028-1038.
11. Linder A, Hagberg Thulin M, Damber JE, Welén K. Analysis of regulator of G-protein signalling 2 (RGS2) expression and function during prostate cancer progression. Sci Rep. 2018;8(1):17259.
12. Jiang Z, Wang Z, Xu Y, Wang B, Huang W, Cai S. Analysis of RGS2 expression and prognostic significance in stage II and III colorectal cancer. Biosci Rep. 2010;30(6):383-390.
13. Smalley MJ, Iravani M, Leao M, et al. Regulator of G-protein signalling 2 mRNA is differentially expressed in mammary epithelial sub-populations and over-expressed in the majority of breast cancers. Breast Cancer Res. 2007;9(4):R85.
14. Lyu JH, Park DW, Huang B, et al. RGS2 suppresses breast cancer cell growth via a MCPIP1-dependent pathway. J Cell Biochem. 2015;116(2):260-267.
15. Sethakorn N, Dulin NO. RGS expression in cancer: oncomining the cancer microarray data. J Recept Sig Transd. 2013;33(3):166-171.
16. Yin H, Wang Y, Chen WP, Zhong SL, Liu Z, Zhao JH. Drug-resistant CXCR4-positive cells have the molecular characteristics of EMT in NSCLC. Gene. 2016;594(1):23-29.
17. Marques RB, Dits NF, Erkens-Schulze S, van Ijcken WF, van Weerden WM, Jenster G. Modulation of androgen receptor signaling in hormonal therapy-resistant prostate cancer cell lines. PLOS One. 2011;6(8):e23144.
18. Cao X, Qin J, Xie Y, et al. Regulator of G-protein signaling 2 (RGS2) inhibits androgen-independent activation of androgen receptor in prostate cancer cells. Oncogene. 2006;25(26):3719-3734.
19. Gustavsson H, Welen K, Damber JE. Transition of an androgen-dependent human prostate cancer cell line into an androgen-independent subline is associated with increased angiogenesis. The Prostate. 2005;62(4):364-373.
20. Quigley DA, Dang HK, Zhao SG, et al. Genomic hallmarks and structural variation in metastatic prostate cancer. Cell. 2018;174(3):758-769.e9.
21. Yuan X, Cai C, Chen S, Chen S, Yu Z, Balk SP. Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. Oncogene. 2014;33(22):2815-2825.
22. Hu R, Dunn TA, Wei S, et al. Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. Cancer Res. 2009;69(1):16-22.
23. Sun SH, Sprenger CCT, Vessella RL, et al. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J Clin Invest. 2010;120(8):2715-2730.
24. Thompson IM, Pauler DK, Goodman PJ, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level ≤ 4.0 ng per milliliter. N Engl J Med. 2004;350(22):2239-2246.
25. Friedrich MJ. Debate continues on use of PSA testing for early detection of prostate cancer. JAMA. 2011;305(22):2273-2275.
26. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med. 2004;10(1):33-39.
27. Veldscholte J, Berrevoets CA, Ris-Stalpers C, et al. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J Steroid Biochem Mol Biol. 1992;41(3-8):665-669.
28. Cai C, Wang H, Xu Y, Chen S, Balk SP. Reactivation of androgen receptor-regulated TMPRSS2:ERG gene expression in castration-resistant prostate cancer. Cancer Res. 2009;69(15):6027-6032.
29. Visakorpi T, Hyytinen E, Koivisto P, et al. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Br J Cancer. 2004;90(2):224-230.
30. Qi W, Morales C, Cooke LS, Johnson B, Somer B, Mahadevan D. Regulation of G-protein signaling 2 mRNA is differentially expressed in mammary epithelial sub-populations and over-expressed in the majority of breast cancers. Breast Cancer Res. 2007;9(4):R85.
34. Ishikawa S, Soloway MS, Van der Zwaag R, Todd B. Prognostic factors in survival free of progression after androgen deprivation therapy for treatment of prostate cancer. J Urol. 1989;141(5):1139-1142.

35. Albala DM. Imaging and treatment recommendations in patients with castrate-resistant prostate cancer. Rev Urol. 2017;19(3):200-202.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Linder A, Larsson K, Welén K, Damber J-E. RGS2 is prognostic for development of castration resistance and cancer-specific survival in castration-resistant prostate cancer. The Prostate. 2020;80:799-810. https://doi.org/10.1002/pros.23994