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Research Article

Development and Validation of a New BAG-1L–Specific Antibody to Quantify BAG-1L Protein Expression in Advanced Prostate Cancer

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ABSTRACT

BCL-2–associated athanogene-1L (BAG-1L) is a critical co-regulator that binds to and enhances the transactivation function of the androgen receptor, leading to prostate cancer development and progression. Studies investigating the clinical importance of BAG-1L protein expression in advanced prostate cancer have been limited by the paucity of antibodies that specifically recognize the long isoform. In this study, we developed and validated a new BAG-1L–specific antibody using multiple orthogonal methods across several cell lines with and without genomic manipulation of BAG-1L and all BAG-1 isoforms. Following this, we performed exploratory immunohistochemistry to determine BAG-1L protein expression in normal human, matched castration-sensitive prostate cancer (CSPC) and castration-resistant prostate cancer (CRPC), unmatched primary and metastatic CRPC, and early breast cancer tissues. We demonstrated higher BAG-1L protein expression in CRPC metastases than in unmatched, untreated, castration-sensitive prostatectomies from men who remained recurrence-free for 5 years. In contrast, BAG-1L protein expression did not change between matched, same patient, CSPC and CRPC biopsies, suggesting that BAG-1L protein expression may be associated with more aggressive biology and the development of castration resistance. Finally, in a cohort of patients who universally developed CRPC, there was no association between BAG-1L protein expression at diagnosis and time to CRPC or overall survival, and no association between BAG-1L protein expression at CRPC biopsy and clinical outcome from androgen receptor targeting therapies or docetaxel chemotherapy. The limitations of this study include the requirement to validate the reproducibility of the assay developed, the potential influence of pre-analytical factors, timing of CRPC biopsies, relatively small patient numbers, and heterogenous therapies on BAG-1L protein expression, and the clinical outcome analyses performed. We describe a new BAG-1L–specific

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antibody that the research community can further develop to elucidate the biological and clinical significance of BAG-1L protein expression in malignant and nonmalignant diseases.

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy in men and is a leading cause of male mortality.[1] The androgen receptor (AR) remains the main therapeutic target in both castration-sensitive prostate cancer (CSPC) and castration-resistant prostate cancer (CRPC).[2,3] Consistent with this, therapies targeting the AR have improved the outcome for men with advanced disease.[2,3] Despite these developments, resistance to therapies targeting the AR signaling axis inevitably develops.[3] One potential mechanism driving ongoing AR signaling and the development of treatment resistance is the increased expression of key co-regulators that stimulate AR transactivation.[4]

BCL-2–associated athanogene-1 (BAG-1) is a molecular chaperone that binds to a wide range of protein targets to regulate multiple cellular pathways (including apoptosis, proliferation, metastasis, and nuclear hormone receptor transactivation) important for the development and progression of cancer.[5-7] Three major isoforms, BAG-1L (50 kDa), BAG-1M (46 kDa), and BAG-1S (36 kDa), exist in humans and are generated through alternative initiation of translation from a single mRNA.[8] As a consequence, BAG-1L has a unique N-terminus, which contains a nuclear localization sequence and is predominantly localized within the nucleus, where it binds to the AR. In contrast, the other BAG-1 isoforms (BAG-1M and BAG-1S) are found in both the nucleus and cytoplasm.[5-7] BAG-1L binds to the AR N-terminus through its C-terminal BAG domain, leading to receptor transactivation.[9-13] Consistent with this, loss of BAG-1L abrogates AR signaling and reduces prostate cancer growth, suggesting a critical function for BAG-1L in prostate cancer development and progression.[11,14]

Despite these functional data, the clinical qualification of BAG-1L has been challenging due to the lack of a validated BAG-1L–specific antibody. Studies have shown that BAG-1L protein expression is significantly higher in localized hormone-refractory tumors than in untreated primary tumors.[15] This difference has also been shown utilizing an antibody that recognizes all human BAG-1 isoforms (BAG-1L, BAG-1M, and BAG-1S), using nuclear BAG-1 protein expression as a surrogate for BAG-1L protein expression.[16] In the same study, cytoplasmic BAG-1 protein expression, a likely surrogate of BAG-1M and BAG-1S protein expression, was associated with shorter time to progression in patients with localized prostate cancer undergoing radiotherapy.[16] A further study demonstrated that higher nuclear BAG-1 protein expression was associated with reduced benefit from AR targeting therapy in CRPC.[11] The use of antibodies recognizing all 3 human BAG-1 isoforms (BAG-1L, BAG-1M, and BAG-1S), using nuclear BAG-1 protein expression as a surrogate for nuclear BAG-1L protein expression, remains a key limitation of many of these studies.[11,15,16]

To better support the clinical evaluation of BAG-1L protein expression in human tissue, we developed and validated a new BAG-1L–specific antibody. We then performed exploratory immunohistochemistry (IHC) analyses to determine BAG-1L protein expression in normal human, matched CSPC and CRPC, unmatched primary and metastatic CRPC, and early breast cancer tissues. Finally, we determined the association between BAG-1L protein expression and clinical outcome data in patients with CRPC. To our knowledge, this is the only validated BAG-1L–specific antibody and will allow the research community to interrogate BAG-1L biology and its clinical importance in malignant and nonmalignant diseases.

Materials and Methods

Development of a New BAG-1L–Specific Antibody (Clone RM310)

The BAG-1L–specific antibody (RM310) was generated and developed by RevMAb Biosciences using a standard rabbit immunization protocol and its rabbit monoclonal antibody development technology platform. Briefly, rabbits (New Zealand White) were immunized with a human BAG-1L–specific peptide conjugated to keyhole limpet hemocyanin carrier protein. After 4 boosts, blood (30 mL/rabbit) was sampled for memory B-cell isolation using the human BAG-1L peptide. After culturing the B cells for 8 days, supernatants were tested for antibody specificity to the peptide by enzyme-linked immunosorbent assay and screened for expression by western blot with BAG-1L overexpressing LNCaP cell lysates. For the identified B-cell clones suitable for this application, their antibody DNA fragments for the entire light (L) chain and the variable region of heavy (H) chain of rabbit immunoglobulins (IgG) were amplified by PCR with rabbit IgG H and L chain primers and then inserted into mammalian expression vectors with or without built-in constant region of rabbit antibody H chain. Full-length recombinant antibodies were expressed in HEK293 cells (ThermoFisher). The recombinant antibodies were confirmed for their application in western blot and further screened for their application in IHC with formalin-fixed, paraffin-embedded (FFPE) BAG1-L overexpressing LNCaP cells. The finalized recombinant antibody clone (RM310) was purified through a protein A affinity column and confirmed for western blot and IHC applications in collaboration with the Institute of Cancer Research (ICR).

Cell Lines

VCaP (Dulbecco's Modified Eagle Medium/10% fetal calf serum, FCS), 22Rv1 (Roswell Park Memorial Institute 1640/10% FCS), HeLa (Dulbecco's Modified Eagle Medium/10% FCS), and LNCaP/LNCaP derived lines (Roswell Park Memorial Institute 1640/10% FCS) were all purchased from the American Type Culture Collection and grown at 37 °C in 5% CO_2. All media were purchased from ThermoFisher. Cell lines were tested for mycoplasma using the VenorGem One Step PCR Kit (Cambio) and authenticated by short tandem repeat profiled (Eurofins Genomic).

Development of BAG-1L–Specific Transcription Activator-like Effector Nuclease Knockout and BAG-1 Small Hairpin RNA LNCaP Cells

BAG-1L–specific transcription activator-like effector nuclease (TALEN) knockout and TALEN control LNCaP cells were

generated as previously described.[11] BAG-1 short hairpin (sh) RNA and control shRNA LNCaP cells were generated using predesigned BAG1 MISSION shRNA lentiviral transduction particles in pLKO.1; clones NM_004323.2-506s1c1 (clone 506) and NM_004323.2-666s1c1 (clone 666) or pLKO.1 non-silencing control (clone control C2), respectively (Mission, Sigma-Aldrich). Briefly, 1000 LNCaP cells were seeded per well of a 96-well plate, allowed to adhere overnight, and transduced with 1×10^4 TU of viral particles. After 48 hours, the medium was exchanged, and positive mass cultures were selected using 1 μg/mL puromycin.

Western Blotting

Cell lines were lysed with RIPA buffer (Pierce, ThermoFisher), supplemented with protease inhibitor cocktail (Roche, Sigma-Aldrich) and PhosStop phosphatase inhibitor mix (Roche, Sigma-Aldrich). Protein extracts were sonicated and heated for 5 minutes at 95 °C and 25 μg samples were separated on 4% to 12% NuPAGE Bis-Tris gel (Invitrogen) by electrophoresis and subsequently transferred onto Immobilon-P polyvinylidene difluoride membranes of 0.45-μm pore size (Millipore, Merck). Primary antibodies used were rabbit monoclonal anti–BAG-1L (RevMAb Biosciences, RM310), mouse monoclonal anti–BAG-1 (Cell Signaling, 3.10G3E2), and mouse monoclonal anti-vinculin (Sigma-Aldrich, hVIN-1). Chemiluminescence was detected on the Chemidoc Touch imaging system (Bio-Rad).

BAG-1L Immunoprecipitation

Briefly, 22Rv1 cells were plated and left for 48 hours. Cells were resuspended in HMKEN buffer (10 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 142 mM KCl, 2 mM EGTA, 0.2% [v/v] Nonidet P40, 1:100 protease inhibitor cocktail [Sigma Chemical]) by trituration through a 21-gauge needle, lysed on ice for 30 minutes, and clarified by centrifugation (13,000 rpm for 30 minutes). One-thirtieth (50 μL) of the lysate was retained as a whole-cell lysate. The remaining sample was precleared by use of protein A/G magnetic Dynabeads (ThermoFisher) for 30 minutes at 4 °C. Dynabeads were removed by using a DynaMag-2 magnet. Lysate (600 μL) was incubated with 5 μg rabbit monoclonal anti–BAG-1L antibody (clone RM310; RevMAb Biosciences) at 4 °C for 16 hours to analyze the specificity of this antibody for its target. A further lysate (600 μL) was incubated with 5 μg rabbit IgG (Vector Laboratories) to control for nonspecific interactions. The immune complexes were incubated with protein A/G magnetic Dynabeads for 4 to 6 hours and removed using a magnet. The beads were washed 5 times by the use of HMKEN buffer prior to re-suspension in NuPAGE LDS gel electrophoresis sample buffer supplemented with NuPAGE reducing agent (both ThermoFisher) and heated at 95 °C for 5 minutes. Western blotting was performed as described above.

Institute of Cancer Research/Royal Marsden Hospital and University of Washington Tissue Samples

A set of FFPE normal human tissues were established, including prostate, kidney, colon, liver, appendix, ovary, and skin. The ICR/Royal Marsden Hospital (RMH) patient IHC cohort consisted of 43 CSPC and 67 CRPC tissue biopsies from men with CRPC treated at the RMH. The University of Washington (UW) patient IHC cohort consisted of a tissue microarray (TMA), including 30 radical prostatectomies and a TMA, including 30 metastases from the UW Medical Center Rapid Autopsy Program.[17] A cohort of 5 estrogen receptor–positive and human epidermal growth factor receptor 2–negative breast cancer biopsies from women treated at the RMH was established. Human biological samples were sourced ethically, and their research use was in accordance with the terms of the informed consent provided. All tissue blocks were freshly sectioned and were only considered for IHC analyses if adequate material was present.

Immunohistochemistry

For ICR/RMH IHC studies, full-length androgen receptor (AR-FL) and androgen receptor splice variant-7 (AR-V7) IHC was performed as previously described.[18] BAG-1L IHC was performed on a i6000 Automated Staining System (BioGenex) using the rabbit monoclonal anti–BAG-1L (RevMAb Biosciences, RM310) antibody. After deparaffinization and rehydration, antigen retrieval was achieved by microwaving slides in citrate buffer (pH 6.0) for 18 minutes (800 W), and anti–BAG-1L antibody (1:1000) was incubated with tissue for 1 hour at room temperature. After washes, the bound antibody was visualized using EnVision+ Dual Link System-Horseradish Peroxidase (Dako, Agilent Technologies, K4061). Sections were counterstained with hematoxylin. Cell pellets from BAG-1L–specific TALEN knockout and TALEN control LNCaPs, VCaPs, and rabbit IgG, were used as controls for each run. IHC at the UW used the rabbit monoclonal anti–BAG-1L (RevMAb Biosciences, RM310) antibody and was performed by hand. TMAs were first deparaffinized and rehydrated, then heated in Tris-based buffer (Vector Laboratories, H-3301-250) in the pressure cooker to accomplish antigen unmasking before anti–BAG-1L antibody (1:500) was incubated with tissue overnight at 4 °C. After washes, the 3,3′-diaminobenzidine based-chromogenic detection was performed using biotinylated secondary antibodies in conjunction with VECTASTAIN ELITE ABC Horseradish Peroxidase Detection Kit (Vector Laboratories, PK-6100) and ImmPACT DAB Substrate Kit, Peroxidase (horseradish peroxidase) (Vector Laboratories, SK-4105). Sections were counterstained with hematoxylin. Cell pellets from VCaPs and rabbit IgG were used as controls for each run.

Immunohistochemistry Scoring Method

For ICR/RMH and UW IHC studies, BAG-1L protein expression was determined by a pathologist blinded to clinical and molecular data using H-scores ([% of negative staining × 0] + [% of weak staining × 1] + [% of moderate staining × 2] + [% of strong staining × 3]), to determine the overall percentage of positivity across the entire stained samples, yielding a range from 0 to 300.[19]

Study Approvals

All patients treated at the RMH had provided written informed consent and were enrolled in institutional protocols approved by the Royal Marsden National Health Service (NHS)

Foundation Trust Hospital (London) ethics review committee. All procedures involving human subjects at the UW were approved by the University of Washington institutional review board.

Statistical Analyses

Wilcoxon signed-rank test was used to determine the difference between BAG-1L protein expression in matched, same patient, CSPC, and CRPC samples. Mann–Whitney *U* test was used to determine the difference between BAG-1L protein expression in pre- and post-abiraterone acetate (AA) or enzalutamide (E) treatment samples and in unmatched radical prostatectomy and metastatic samples from UW. One-way analysis of variance was used to determine the difference in BAG-1L protein expression between different biopsy sites. Spearman's rank correlation was used to determine the association between BAG-1L protein expression and both nuclear AR-FL protein expression and nuclear AR-V7 protein expression. All clinical outcome data were compared between patients with less than or equal to the median or greater than the median BAG-1L protein expression. Fisher exact tests were used to determine the difference in 12-week prostate specific antigen (PSA) response in response to AR targeted therapy and docetaxel chemotherapy by BAG-1L protein expression. The time to CRPC and overall survival from diagnosis, and time to PSA progression, radiologic/clinical progression, and overall survival on AR targeting therapies and docetaxel chemotherapy, were estimated using the Kaplan–Meier method, and respective hazard ratios were obtained by Cox regression. Statistical analyses were performed with GraphPad Prism version 7 (GraphPad Software). Statistical significance was prespecified at *P* value of $\leq$.05. No adjustment for multiple testing was made.

Results

Development of a New BAG-1L–Specific Antibody (Clone RM310)

To overcome the challenges of BAG-1L protein quantification in advanced prostate cancer, we developed a rabbit monoclonal antibody (RM310) against the unique N-terminus of BAG-1L, in collaboration with RevMAb Biosciences (see Materials and Methods, Fig. 1A). Antibody validation was performed at the ICR/RMH. Western blot analysis of VCaP and 22Rv1 prostate cancer cell lines demonstrated a strong BAG-1L band at 50 kDa (Fig. 1B). In addition, RM310 did not recognize BAG-1M or BAG-1S, with no bands detected at 46 kDa or 36 kDa, confirming specificity for BAG-1L (Fig. 1B). In contrast, 3.10G3E2, raised against recombinant full-length BAG-1S, demonstrated BAG-1L (50 kDa), BAG-1M (46 kDa), and BAG-1S (36 kDa) bands in both VCaP and 22Rv1 prostate cancer cell lines (Fig. 1B). Next, we demonstrated the strong BAG-1L band at 50 kDa to be specific, as it disappeared in our TALEN BAG-1L knockout LNCaP cells and was significantly reduced in our BAG-1 shRNA treated LNCaP cells and BAG-1 siRNA-treated HeLa cells (Fig. 1B).[11] Following initial validation, specificity of RM310 for BAG-1L was confirmed by immunoprecipitation using 22Rv1 prostate cancer cells, demonstrating a strong band at 50 kDa (Fig. 1C). These data demonstrate that RM310 specifically recognizes BAG-1L, with no cross-reactivity with other human BAG-1 isoforms, supporting its development as a new specific primary antibody for detecting BAG-1L protein expression in human tissues, including advanced prostate cancer.

Optimization of a New BAG-1L–Specific Antibody (Clone RM310) for Exploratory Immunohistochemistry Analyses of Tissue Biopsies From Patients With Prostate Cancer

Following confirmation of BAG-1L specificity, RM310 was optimized for IHC using FFPE, cell line pellets. Consistent with our western blot analyses, IHC of VCaP and 22Rv1 prostate cancer cells demonstrated strong BAG-1L staining (Fig. 1D). In addition, TALEN BAG-1L knockout LNCaP cells, BAG-1 shRNA treated LNCaP cells, and BAG-1 siRNA-treated HeLa cells demonstrated no BAG-1L staining, confirming RM310 specificity for BAG-1L, with no cross-reactivity with other human BAG-1 isoforms (Fig. 1D). Having confirmed RM310 specifically recognizes BAG-1L, we performed IHC on a variety of FFPE normal human tissues, including prostate, demonstrating ubiquitous nuclear staining across most tissue types studied (Fig. 2). Next, we performed IHC on FFPE prostate cancer patient tissue biopsies within our study cohorts, demonstrating almost exclusively nuclear staining (Figs. 3A-B and 4A, Supplementary Tables S1-S5). These data demonstrate that RM310 recognizes BAG-1L in normal human tissue and prostate cancer tissue biopsies, providing a research reagent to interrogate BAG-1L protein expression in exploratory IHC analyses.

BAG-1L Protein Expression in Tissue Biopsies From Patients With Prostate Cancer

We next performed BAG-1L–specific IHC to investigate BAG-1L protein expression in same-patient, matched biopsies, as 43 patients progressed from CSPC to CRPC (ICR/RMH patient IHC cohort) (Fig. 3A, Supplementary Table S1). In this cohort, BAG-1L protein expression did not significantly (P = .17, Wilcoxon signed-rank test) increase as patients progressed from CSPC (median H-score, interquartile range [IQR]; 50, 14-90) to CRPC (median H-score 80; IQR, 10-95) (Fig. 4A, B). In addition, BAG-1L protein expression was not significantly different (P = .06, Mann–Whitney *U* test) in CRPC biopsies (n = 67, median H-score 80; IQR, 17-95) taken before AA or E therapy (median H-score 80; IQR, 52.5-102.5) and after AA and/or E therapy (median H-score 70; IQR, 9.75-95) (Fig. 4C). Furthermore, we saw no significant difference (P = .15, one-way analysis of variance) when considering BAG-1L protein expression among different biopsy sites (Fig. 4D). Next, we investigated whether BAG-1L protein expression was associated with nuclear AR-FL protein expression and nuclear AR-V7 protein expression (Fig. 4E). There was no significant association (Spearman's rank correlation test) between BAG-1L protein expression and both nuclear AR-FL protein expression (n = 59, r = 0.20 [−0.07 to 0.44]; P = .13) and AR-V7 protein expression (n = 66, r = 0.22 [−0.03 to 0.44]; P = .08) (Fig. 4F). Finally, utilizing a second clinical cohort (UW patient IHC cohort), we demonstrated that BAG-1L protein expression was significantly higher (P = .01, Mann–Whitney *U* test) when comparing primary localized prostate cancer without recurrence at 5 years (median H-score 0; IQR, 0-50) and (unmatched) metastatic CRPC (median H-score 40; IQR, 3-60) (Figs. 3B and 4H, Supplementary Tables S2 and S3). These exploratory IHC analyses demonstrate that nuclear BAG-1L expression was higher in CRPC metastasis than in unmatched, untreated, castration-sensitive primary localized prostate cancer that had not recurred at 5 years, but not in matched, same patient, CSPC, and CRPC biopsies, in which all men had high-risk disease and progressed to CRPC.

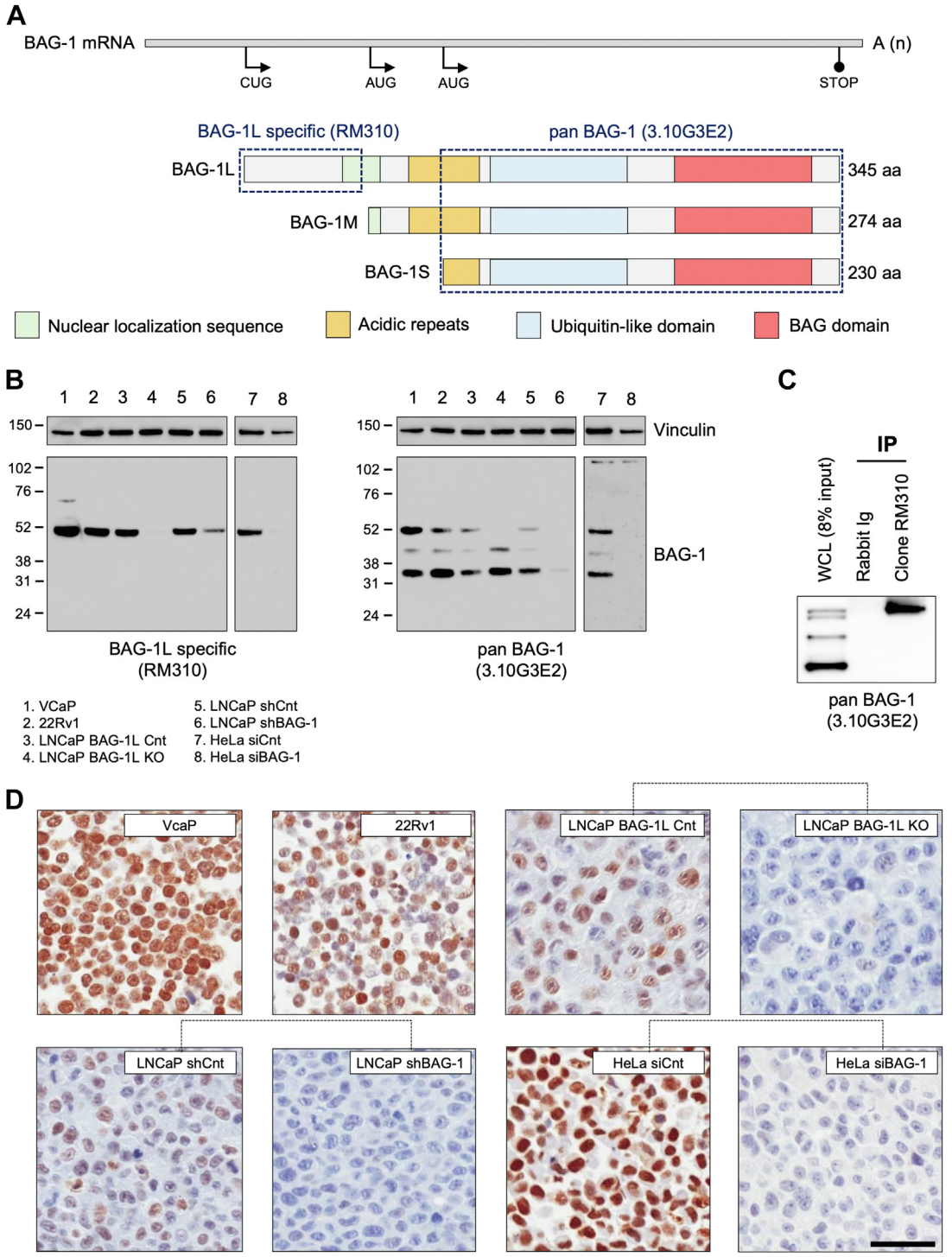

Figure 1.

Specificity of a new BCL-2–associated athanogene-1L (BAG-1L) antibody (RM310) (A) Schematic representation of BAG-1 mRNA showing position of the alternative translation initiation sites that give rise to 3 human BAG-1 isoforms (BAG-1L, BAG-1M, and BAG-1S). The domain structure of the BAG-1 isoforms is shown, including the nuclear localization sequence, acidic repeats, ubiquitin-like domain, and BAG domain. The anti–BAG-1L rabbit monoclonal antibody (RM310) was developed against a peptide corresponding to residues near the N-terminus of human BAG-1L, and the anti–BAG-1 mouse monoclonal antibody (3.10G3E2) developed against recombinant full-length BAG-1S protein are mapped. (B) Western blot of VCaP and 22Rv1 cells, and BAG-1L–specific transcription activator-like effector nuclease (TALEN) knockout LNCaP cells, BAG-1 shRNA treated LNCaP cells (clone 506) and BAG-1 siRNA-treated HeLa cells, compared with control cells, using a new rabbit monoclonal anti–BAG-1L antibody (RM310) and pan BAG-1 antibody (3.10G3E2). Molecular weight is shown in kilodaltons. (C) Immunoprecipitation of BAG-1L from 22Rv1 cells using the BAG-1L antibody (RM310) and western blot performed with pan BAG-1 antibody (3.10G3E2). (D) Micrographs of BAG-1L detection by immunohistochemistry using the BAG-1L antibody (RM310) in cell line pellets from VCaP and 22Rv1 cells, and BAG-1L–specific TALEN knockout LNCaP cells, BAG-1 shRNA-treated LNCaP cells (clone 506) and BAG-1 siRNA-treated HeLa cells, compared with control cells. Scale bar = 50 μm.

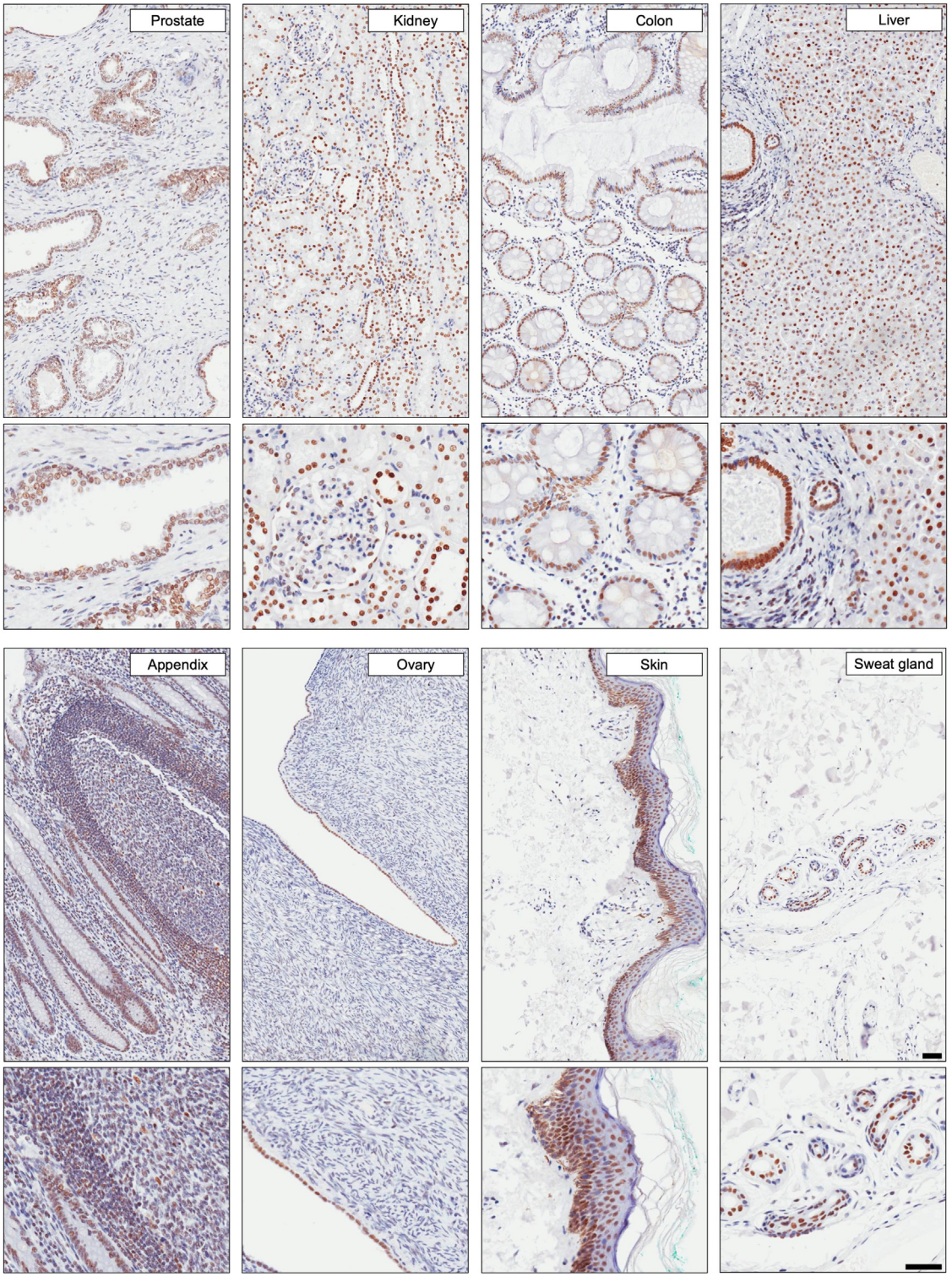

Figure 2.
BCL-2–associated athanogene-1L (BAG-1L) protein staining in normal human tissues. Micrographs of BAG-1L detection by immunohistochemistry using the BAG-1L antibody (RM310) in multiple normal human tissues. Two magnifications are shown. Scale bars = 50 μm.

BAG-1L Protein Expression in Castration-Sensitive Tissue Biopsies and Clinical Outcome in Patients With Prostate Cancer Who Develop CRPC

We next investigated the association of BAG-1L protein expression at diagnosis on clinical outcomes for patients with prostate cancer who universally develop CRPC. Patients with lower BAG-1L protein expression (n = 22, less than or equal to median H-score, ≤50) at CSPC did not show significantly different median time to CRPC (21.1 vs 20.3 months, hazards ratio [HR], 1.28; 95% CI, 0.70-2.34; $P = .40$), or median overall survival (74.8 vs 74.5 months, HR, 0.96; 95% CI, 0.51-1.79; $P = .89$), when compared with patients with higher

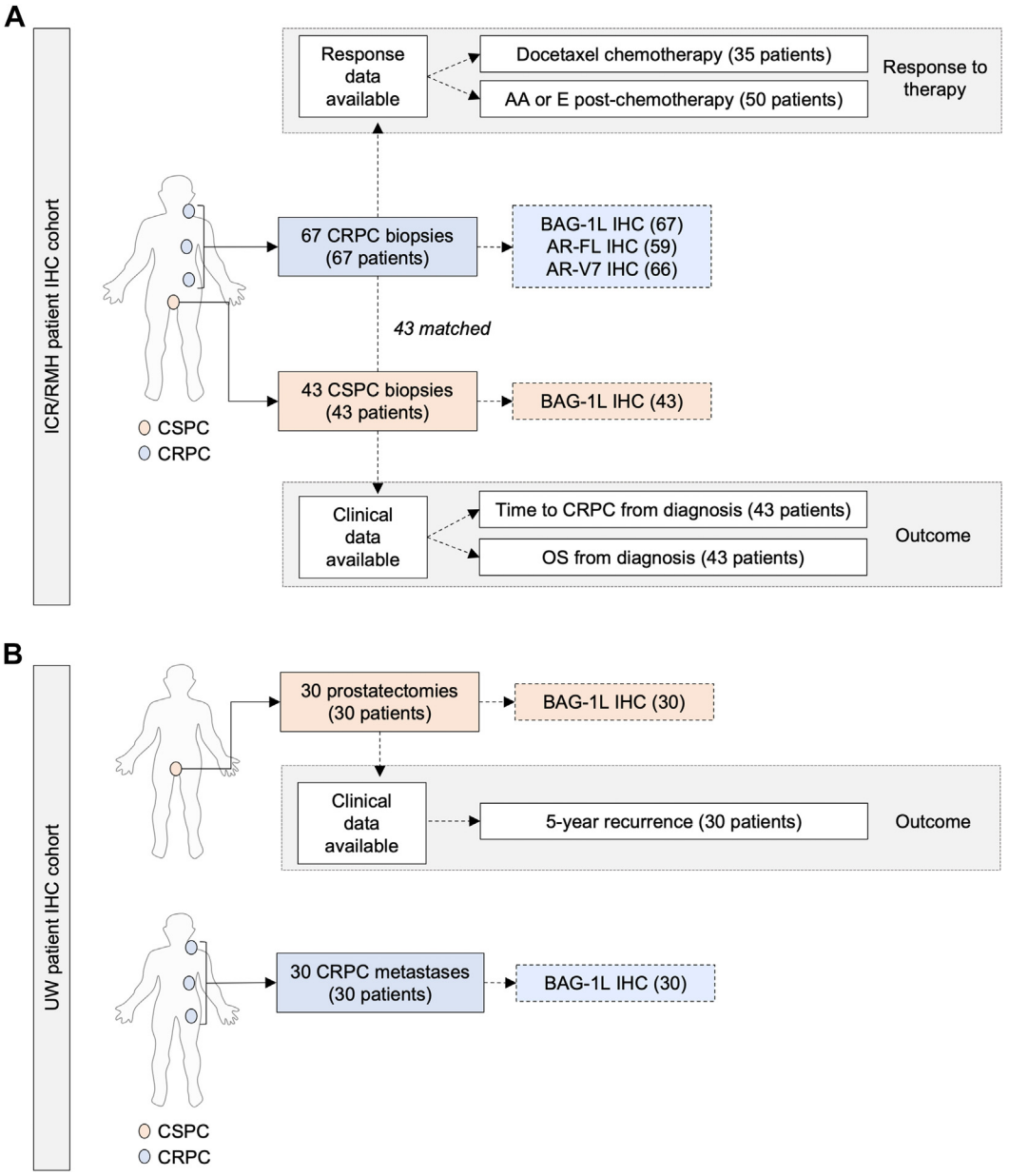

Figure 3.
Overview of the Institute of Cancer Research/Royal Marsden Hospital and the University of Washington prostate cancer immunohistochemistry cohorts. (A) Overview of the Institute of Cancer Research/Royal Marsden Hospital (ICR/RMH) patient immunohistochemistry (IHC) cohort. The ICR/RMH patient IHC cohort included 43 castration-sensitive prostate cancer (CSPC) biopsies and 67 castration-resistant prostate cancer (CRPC) biopsies stained for BCL-2–associated athanogene-1L (BAG-1L) expression. Response data were available for abiraterone acetate (AA) or enzalutamide (E) after chemotherapy in 50 patients and docetaxel chemotherapy in 35 patients (compared with BAG-1L expression at CRPC), and for time to CRPC and overall survival (OS) from diagnosis for 43 patients (compared with BAG-1L expression at CSPC). (B) Overview of the University of Washington (UW) patient IHC cohort. The UW IHC cohort included 30 primary prostatectomies that had not recurred at 5 years and 30 CRPC metastases stained for BAG-1L expression.

BAG-1L protein expression (n = 21, greater than median H-score, >50) (Figs. 3A and 5A-C, Supplementary Table S1). These exploratory data indicate that nuclear BAG-1L expression at diagnosis did not associate with clinical outcomes in men who develop CRPC.

BAG-1L Protein Expression in Castration-Resistant Tissue Biopsies From Patients With Prostate Cancer and Response to AR Targeting Therapies

To investigate the association of BAG-1L protein expression with response to current AR targeting therapies, we determined the response of the ICR/RMH patient IHC cohort to AA or E following chemotherapy in BAG-1L low (n = 26, less than or equal to median H-score, ≤70) and high (n = 24, greater than median H-score, >70) protein-expressing CRPC biopsies (Figs. 3A and 6A, Supplementary Table S4). Patients with lower BAG-1L protein expression did not have significantly different 12-week PSA response rate (35 vs 38%; $P > .99$, Fisher exact test), median time to PSA progression (2.7 vs 3.0 months, HR, 0.90; 95% CI, 0.50-1.57; $P = .68$), median time to radiologic/clinical progression (5.9 vs 4.3 months, HR, 0.68; 95% CI, 0.38-1.19; $P = .15$), or median overall survival (18.5 vs 14.8 months, HR, 0.73; 95% CI, 0.41-1.30; $P = .27$),

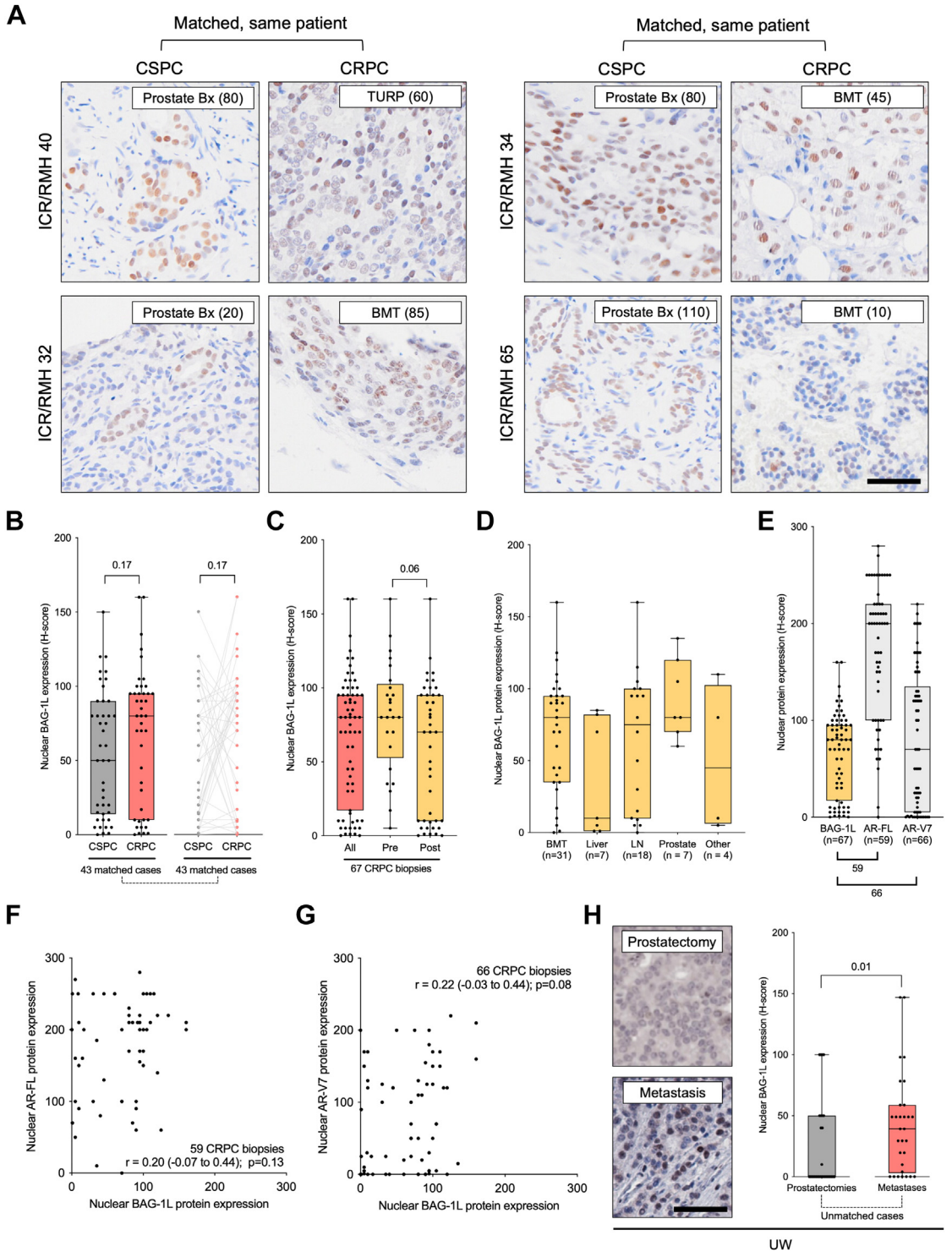

Figure 4.

BCL-2–associated athanogene-1L (BAG-1L) protein staining in tissue biopsies from patients with prostate cancer. (A) Representative micrographs of BAG-1L detection by immunohistochemistry (IHC) in 4 Institute of Cancer Research/Royal Marsden Hospital (ICR/RMH) patients with matched castration-sensitive prostate cancer (CSPC) and castration-resistant prostate cancer (CRPC) biopsies. Nuclear BAG-1L expression (H-score [HS]) is shown for each micrograph. Scale bar = 50 μm. Prostate biopsies (Prostate Bx), transurethral resection of the prostate (TURP), and bone marrow trephine (BMT) biopsies are shown. (B) Nuclear BAG-1L expression in 43 same-patient matched CSPC (gray) and CRPC (red) biopsies from the ICR/RMH cohort is shown. Median HS with interquartile range and smallest and largest value (left), and paired data points (right), are shown. *P* value was calculated for BAG-1L expression at CSPC compared with CRPC using the Wilcoxon signed-rank test. (C) Nuclear BAG-1L expression (HS) in 67 CRPC (red) biopsies and dichotomized (orange) by pre- (25 biopsies) and post- (42 biopsies) abiraterone acetate (AA) or enzalutamide (E) treatment. Median HS with interquartile range, and smallest and largest value, is shown. *P* value was calculated for BAG-1L expression pre- and post-AA or E using the Mann–Whitney *U* test. (D) Nuclear BAG-1L expression (HS) in 67 CRPC biopsies from BMT, liver, lymph node (LN), prostate, and other sites of metastases. Median HS with interquartile range, and smallest and largest value, is shown. There was no difference in nuclear BAG-1L expression between biopsy sites using one-way analysis of variance testing. (E) Nuclear BAG-1L, nuclear full-length androgen receptor (AR-FL), and nuclear AR splice variant-7 (AR-V7) expression (HS) in 67 CRPC biopsies. Median HS with interquartile range, and smallest and largest value, is shown. (F) Expression (HS) of

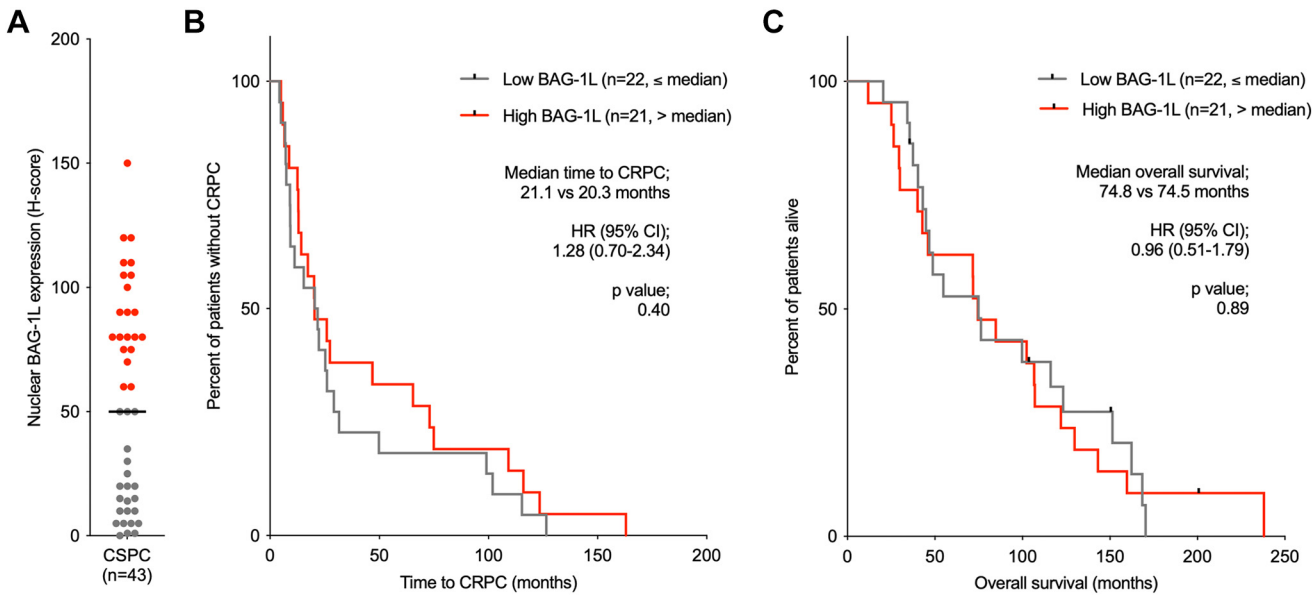

Figure 5.
BCL-2–associated athanogene-1L (BAG-1L) protein expression in castration-sensitive tissue biopsies and clinical outcome in patients with prostate cancer who develop castration-resistant prostate cancer (CRPC). (A) The impact of low (n = 22, H-score less than or equal to median, ≤50, gray) and high (n = 21, H-score greater than median, >50, red) nuclear BAG-1L expression at time of castration-sensitive prostate cancer biopsy on time to CRPC and overall survival was determined for 43 patients. (B, C) Kaplan–Meier curves show time to CRPC progression (B) and overall survival (C) from diagnosis. Hazard ratios with 95% CIs are shown. *P* value was calculated using the univariate Cox proportional hazards model.

when compared with patients with higher BAG-1L protein expression (Figs. 6B-E). Overall, these exploratory analyses indicate that BAG-1L protein expression at the time of CRPC biopsy did not associate with response to AR targeting therapy following taxane chemotherapy in men with advanced prostate cancer.

BAG-1L Protein Expression in Castration-Resistant Tissue Biopsies From Patients With Prostate Cancer and Response to Docetaxel Chemotherapy

Next, to investigate the association of BAG-1L protein expression with response to docetaxel chemotherapy, we determined the response of the ICR/RMH patient IHC cohort to docetaxel chemotherapy in BAG-1L low (n = 18, less than or equal to median H-score, ≤80) and high (n = 17, greater than median H-score, >80) protein-expressing CRPC biopsies (Fig. 3A, Supplementary Fig. S1A, Supplementary Table S5). Patients with lower BAG-1L protein expression had a significantly lower 12-week PSA response rate (17 vs 53%; $P = .04$, Fisher exact test). Despite this, patients with lower BAG-1L protein expression had no significant difference in median time to PSA progression (4.7 vs 5.3 months, HR, 1.04; 95% CI, 0.51-2.10; $P = .91$), median time to radiologic/clinical progression (8.3 vs 8.1 months, HR, 0.75; 95% CI, 0.38-1.47; $P = .37$), or median overall survival (31.4 vs 26.9 months, HR, 0.56; 95% CI, 0.28-1.14; $P = .08$), when compared with patients with higher BAG-1L protein expression (Supplementary Fig. S1B-E). Overall, these exploratory data showed that BAG-1L protein expression at time of CRPC biopsy, although associating with 12-week PSA response rate, did not associate with other clinical outcome measures following docetaxel chemotherapy treatment in men with advanced prostate cancer.

BAG-1L Protein Expression in Tissue Biopsies From Patients With Early Breast Cancer

Considering BAG-1L has been implicated in the development and progression of other hormone-driven cancers, such as breast cancer, we performed BAG-1L–specific IHC on a small cohort of patients with estrogen receptor–positive and human epidermal growth factor receptor 2–negative early breast cancer (Supplementary Fig. S2).[20,21] In this cohort, BAG-1L protein expression remained exclusively nuclear, and varied between individual cases (median H-score, 30; IQR, 10.5-87.0), suggesting that RM310 provides a research reagent to further interrogate the clinical significance of BAG-1L protein expression in larger early breast cancer cohorts (Supplementary Fig. S2).

Discussion

The AR remains the major therapeutic target in advanced prostate cancer with AR targeting therapies improving the overall survival of men with advanced CSPC and CRPC.[2,3] Critical AR co-chaperones, such as BAG-1L that bind and enhance the transactivation function of the AR, are key for prostate cancer

nuclear AR-FL and nuclear BAG-1L in 59 CRPC biopsies is shown. Spearman's rank correlation is shown. (G) Expression (HS) of nuclear AR-V7 and nuclear BAG-1L in 66 CRPC biopsies is shown. Spearman's rank correlation test is shown. (H) Representative micrographs of BAG-1L detection by IHC in 2 University of Washington (UW) patients showing a primary prostatectomy and metastasis. Scale bar = 50 μm. Nuclear BAG-1L expression (HS) in 30 primary (gray) and 30 metastatic (red) prostate cancer biopsies (unmatched) from the UW cohort is shown. Median HS with interquartile range, and smallest and largest value, is shown. *P* value was calculated for BAG-1L expression in primary prostate cancer compared with metastatic prostate cancer using the Mann–Whitney *U* test.

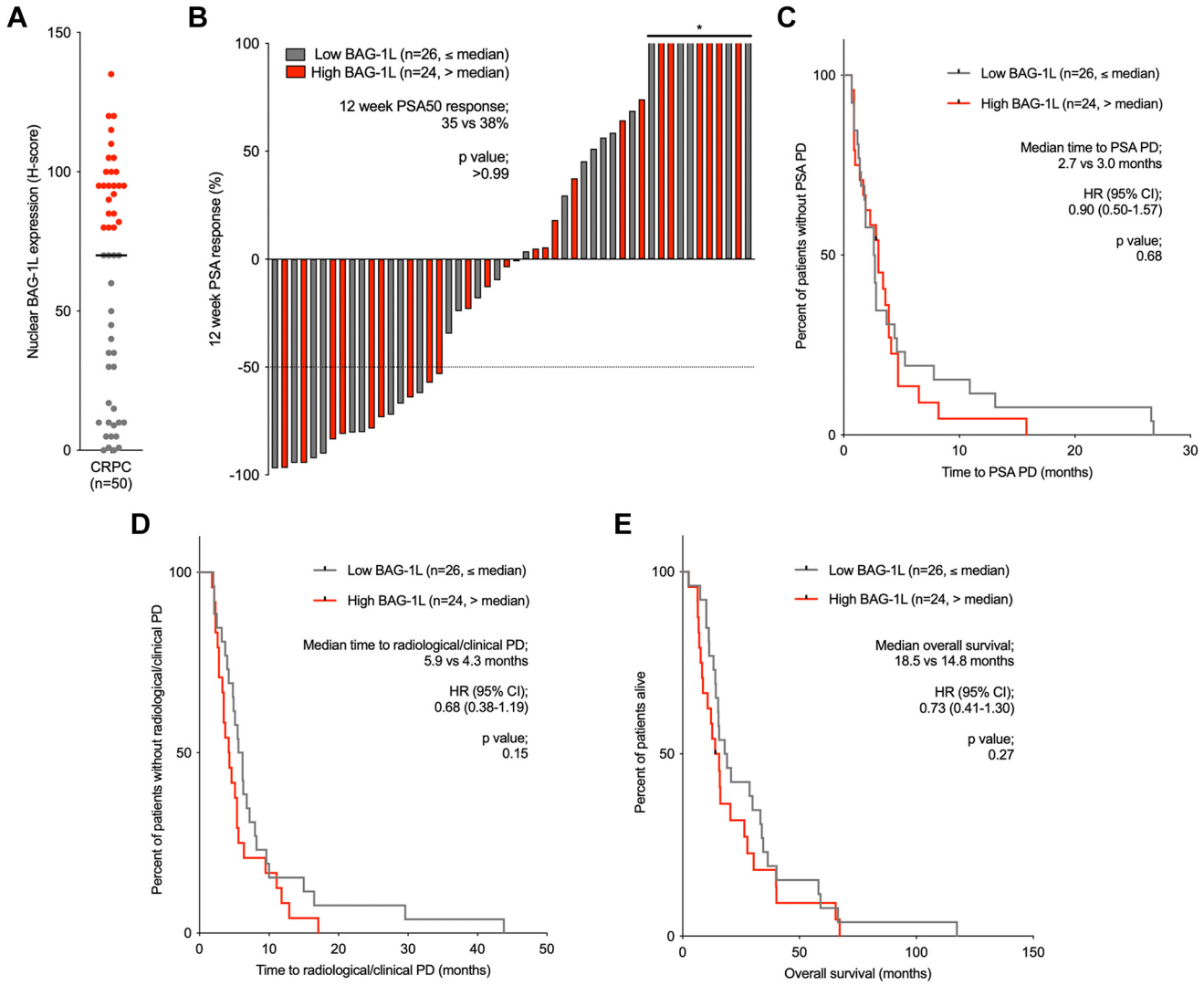

Figure 6.
BCL-2–associated athanogene-1L (BAG-1L) protein staining in castration-resistant tissue biopsies from prostate cancer patients and response to androgen receptor targeting therapies. (A) The impact of low (n = 26, H-score less than or equal to median, ≤70, gray) and high (n = 24, H-score greater than median, >70, red) nuclear BAG-1L expression at time of castration-resistant prostate cancer biopsy on response and clinical benefit to androgen receptor targeting therapies. (B) Percentage 12-week 50% PSA response rate on androgen receptor targeting therapies after chemotherapy for patients with low nuclear BAG-1L (less than or equal to median, gray) and high nuclear BAG-1L (greater than median, red) castration-resistant prostate cancer biopsy protein expression is shown. Twelve-week 50% PSA response rate is shown. *P* value was calculated using Fisher exact test. Kaplan–Meier curves show time to PSA progression (PD) (C), time to clinical/radiologic PD (D), and overall survival (E) from the start of AR targeting therapy. Hazard ratios with 95% CIs are shown. *P* value was calculated using univariate Cox proportional hazards model.

development and progression, and postulated to represent novel therapeutic targets for advanced prostate cancer.[9-14] Despite these promising functional data, clinical qualification of BAG-1L expression in prostate cancer has been challenging due to the lack of validated BAG-1L–specific laboratory reagents.

In this study, we developed and validated a new BAG-1L–specific antibody that demonstrated specific immunoreactivity to BAG-1L, confirming nuclear localization and no cross-reactivity with other human BAG-1 isoforms, using multiple orthogonal methods across multiple cell lines with and without genomic manipulation of BAG-1L and all BAG-1 isoforms. These data provide early proof of antibody specificity for BAG-1L and support the exploratory IHC analyses performed within this study. However, further optimization will need to be considered, and although independent epitope validation will be challenging due to the lack of BAG-1L–specific antibodies, highlighting the importance of this work, confirmation of antibody sensitivity and specificity across different runs, operators, methods, and reagents will be important to support follow on cross-site studies.[22-24]

Following confirmation of antibody specificity, we performed exploratory IHC on a variety of normal human tissues that demonstrated ubiquitous nuclear staining across most tissue types studied consistent with previous studies that utilized antibodies recognizing all 3 BAG-1 isoforms.[25] This is an important finding as BAG-1L has been postulated to be critical for the development and progression of hormone-driven cancers and as a potential therapeutic target for drug discovery efforts.[9-16] Therefore, it may be that BAG-1L, consistent with its function as a molecular chaperone, plays a role through non-oncogenic addiction in cancer progression, and this will be an important consideration if it is to be evaluated as a therapeutic target.[26]

Next, we explored BAG-1L protein expression across a number of prostate cancer tissue cohorts. Our results suggest that BAG-1L protein expression may be higher in CRPC metastasis compared with unmatched, untreated, castration-sensitive prostatectomies that had not recurred at 5 years, consistent with previous studies.[15,16] These data suggest that patients with localized prostate cancer who underwent therapy with curative intent, with no evidence of recurrence at 5 years, had lower BAG-1L protein expression compared with metastases of patients with lethal prostate cancer. However, it is important to note that these were unmatched prostatectomies and CRPC metastases, and the difference observed in BAG-1L protein expression may result from pre-analytical factors, such as cold ischemia, that will need to be further investigated to support further studies of prostatectomies samples specifically.[27]

Following this, we investigated changes in BAG-1L protein expression as patients developed CRPC, using matched, same-patient, CSPC, and CRPC biopsies. Interestingly, BAG-1L protein expression did not significantly change as patients progressed from CSPC to CRPC. In contrast to our comparison between primary localized prostate cancer and metastasis, this suggests that patients who progress to CRPC have higher BAG-1L protein expression at diagnosis, consistent with its role in regulating AR function, although one must acknowledge the potential impact of pre-analytical factors when considering BAG-1L protein expression in prostatectomies.[9-14,27] In contrast, studies utilizing an antibody that recognizes all human BAG-1 isoforms (BAG-1L, BAG-1M, and BAG-1S), using nuclear BAG-1 expression as a surrogate for nuclear BAG-1L expression, demonstrated increased nuclear BAG-1 protein expression as patients progressed from CSPC to CRPC.[11] This, in part, may be due to shorter BAG-1 isoforms (BAG-1M and BAG-1S) localizing to the nucleus under conditions of cellular stress, which is a limitation of studies using this approach and further highlights the importance of the development of this new BAG-1L–specific antibody.[28]

An important consideration for prostate cancer tissues is the ability to quantify the protein of interest in bone biopsies as this is a common site of metastasis. This remains an important challenge to the study of biomarkers by IHC in many cancers.[29,30] Critically, the immunoreactivity of the described BAG-1L IHC does not seem to be negatively impacted when comparing biopsies of the bone to other sites of metastasis. This is a consequence of antibody development, IHC assay optimization, and meticulous attention to pre-analytical preparation required for bone biopsies, all important considerations for IHC assays.[29,31] These important findings demonstrate the potential for this new BAG-1L–specific antibody to be utilized to detect BAG-1L protein expression in biopsies from bone metastasis.

A number of studies that have used antibodies recognizing all human BAG-1 isoforms have demonstrated that increased nuclear BAG-1 protein expression associates with reduced clinical benefit from AR targeting therapies, and cytoplasmic BAG-1 protein expression associates with benefit from radiotherapy in localized disease.[11,15,16] Our current study demonstrates no association between BAG-1L protein expression at diagnosis and time to CRPC or overall survival in patients who develop CRPC, and no association between BAG-1L protein expression at CRPC biopsy and clinical benefit from AR targeting therapies. In addition, although higher BAG-1L protein expression in CRPC was associated with improved PSA response rate to docetaxel chemotherapy, this did not lead to differences in other clinical outcome measures. These differences among studies are unsurprising because analytical validation and clinical qualification of predictive and prognostic biomarkers for prostate and other cancers is challenging, and these studies have multiple variables, including pre-analytical processes, non-isoform specific antibodies, heterogenous patient cohorts, and different quantification strategies.[27,32] Furthermore, the prostate cancer patient cohorts presented in this study are of limited size with CRPC biopsies being performed at various times and patients exposed to heterogenous treatments. Consistent with this, following further optimization of the BAG-1L–specific IHC as discussed above, future studies will require larger patient numbers with homogenous treatments, preferably in prospective clinical studies, to clinically qualify the importance of BAG-1L in lethal prostate cancer.

Finally, as BAG-1L has been shown to play an important role in other common malignancies, including breast cancer, we explored BAG-1L protein expression in a small cohort of patients with estrogen receptor–positive and human epidermal growth factor receptor 2–negative early breast cancer.[20,21] Consistent with our studies of normal and prostate cancer tissues, BAG-1L protein expression was almost exclusively nuclear and varied between cases. Importantly, this antibody can be utilized to further explore the biological and clinical significance of BAG-1L in breast cancer, although to study this comprehensively was beyond the scope of our current study.

In conclusion, we present the development and validation of a new commercially available BAG-1L–specific antibody. Using this antibody for exploratory IHC, we demonstrated that BAG-1L protein expression was lower in localized primary prostate cancer that had not recurred at 5 years when compared with advanced disease. However, BAG-1L protein expression in matched, same patient, CSPC, and CRPC biopsies did not change, suggesting that BAG-1L protein expression may identify patients with aggressive disease at diagnosis. This antibody provides a research reagent that can be further developed to interrogate the biological and clinical significance of BAG-1L in malignant and nonmalignant diseases.

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Author Contributions

A.N., I.F., B.G., D.N.R., R.R., A.F., S.M., and M.C. conducted the experiments. A.N., I.F., B.G., D.N.R., R.R., A.F., S.M., M.C., D.W, M.d.L.D.F.d.L.M, C.G., J.C., R.G., and N.T. acquired the data. All authors designed the studies, analyzed and reviewed the data, wrote, reviewed, and edited the manuscript. A.C.B.C., S.R.P., J.S.d.B., and A.S. supervised and funded the study. A.N. and I.F. contributed as co-first authors of this article. A.S. and J.S.d.B. contributed as co-senior authors of this article.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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Declaration of Competing Interest

J.S. de Bono has served on advisory boards and received fees from many companies, including Amgen, Astra Zeneca, Bayer, Bioxcel Therapeutics, Daiichi, Genentech/Roche, GSK, Merck Serono, Merck Sharp & Dohme, Pfizer, and Sanofi Aventis; he is an employee of the ICR, which has received funding or other support for his research work from AstraZeneca, Astellas, Bayer, CellCentric, Daiichi, Genentech, GSK, Janssen, Merck Serono, MSD, Orion, Sanofi Aventis, Pfizer, and Vertex, and which has a commercial interest in abiraterone, PARP inhibition in DNA repair defective cancers, and PI3K/AKT pathway inhibitors (no personal income); he was named as an inventor, with no financial interest, for patent 8,822,438, submitted by Janssen that covers the use of abiraterone acetate with corticosteroids; and has been the CI/PI of many industry-sponsored clinical trials. A.S is an employee of the ICR, which has a commercial interest in abiraterone, PARP inhibition in DNA repair defective cancers, and PI3K/AKT pathway inhibitors (no personal income). A.S has received travel support from Sanofi, Roche-Genentech, and Nurix, and speaker honoraria from Astellas Pharma and Merck Sharp & Dohme; has served as an advisor to DE Shaw Research and CHARM Therapeutics; and has been the CI/PI of industry-sponsored clinical trials. The remaining authors declare no conflicts of interest.

Ethics Approval and Consent to Participate

All patients treated at the RMH had provided written informed consent and were enrolled in institutional protocols approved by the Royal Marsden NHS Foundation Trust Hospital (London, United Kingdom) ethics review committee. All procedures involving human subjects at the UW were approved by the University of Washington institutional review board.

Supplementary Material

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