SOX2 expression in the primary tumor of castration-naive metastatic prostate adenocarcinoma in association with metastasis extent

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ABSTRACT

BACKGROUND Poor prognosis in patients with metastatic prostate adenocarcinoma (mPCa) may be due to the expression of stem cell-related genes. This study aimed to demonstrate the association between the expression of cancer stem cell markers and metastasis in patients with castration-naive mPCa.

METHODS This cross-sectional, analytical study investigated a formalin-fixed paraffin-embedded prostate specimens from patients diagnosed in Cipto Mangunkusumo Hospital. Patients aged ≥50 years old were grouped based on the extent of metastases (high-volume disease [HVD] and low-volume disease [LVD]). In each case, immunohistochemical staining for CD133, CD44, SOX2, and androgen receptor was performed and analyzed using H-score. All data were recorded and analyzed using SPSS software version 20.0.

RESULTS A total of 61 patients were recruited from 2020 to 2023 and divided into the HVD (n = 38) and LVD (n = 23) groups, with a mean age of 67.9 years. 45 of the patients had International Society of Urological Pathology (ISUP) grade 5 disease, while 16 of them had grade <5. A significant difference of ISUP grade and PSA serum level was observed in the HVD *versus* LVD group ($p = 0.017$ and <0.001, respectively). Additionally, a significant association was found between SOX2 expression and metastatic extent.

CONCLUSIONS The LVD group showed higher SOX2 expression in the primary tumor compared to the HVD group. Different SOX2 expressions in various sites and stages may be due to the cancer cells' systemic network.

KEYWORDS cancer stem cells, metastasis, prostate cancer, prostate-specific antigen, SOX2

After lung cancer, prostate adenocarcinoma (PCa) is the second most frequently diagnosed cancer in men and the fifth leading cause of cancer-related mortality worldwide, including Indonesia, according to the Global Cancer Observatory 2022.[1] The average incidence of PCa in Asia is 7.2 per 100,000 men per year.[2] Recently, the incidence of metastatic PCa (mPCa) has increased in all races and age groups, highlighting the critical need to elucidate the molecular processes beyond mPCa, which is essential for developing effective therapeutic approaches for patients with mPCa.[3] The primary challenge of treating PCa is its tendency to go unnoticed during earlier stages. Over 50% of patients in Indonesia and other low-income countries visit healthcare facilities for the first time with newly diagnosed mPCa, which

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has a worse prognosis than localized PCa.[4,5] Localized PCa has a 5-year relative survival rate of <99%, whereas mPCa has a 5-year relative survival rate of 30.2%.[6] mPCa cases are usually accompanied by a higher International Society of Urological Pathology (ISUP) grading or Gleason score (GS) than PCa. Therapeutic options for mPCa are limited because surgically removing the tumor is ineffective. Pharmacotherapy, or any therapy that interrupts androgen receptor (AR) ligand bonds, may trigger mutations in the tumor and cause resistance.[7,8] Furthermore, several mechanisms, such as AR amplification or mutation,[8] cancer stem cell (CSC) activation, and cell plasticity occur,[9] making therapy ineffective. Cancer cells expressing stem cell markers play an essential role in all PCa stages, and they show distinctive features within the tumor, such as symmetrical cell division and changes in gene expression. Stem cell expression occurs synchronously and may be associated with epithelial–mesenchymal transition (EMT), which eventually leads to metastasis. Bone is the preferred site for mPCa owing to bone morphogenetic protein expression in PCa cells.[10] The Chemohormonal Therapy Versus Androgen Ablation Randomized Trial for Extensive Disease in Prostate Cancer (CHAARTED) trial classified the metastasis extent into high-volume disease (HVD) and low-volume disease (LVD).[10,11] As stem cell markers are usually found in immature cells, cells exhibiting stem cell properties may be considered dedifferentiated cells.[9] However, AR is the hallmark of PCa differentiation, with its expression often reduced in higher-grade, advanced-stage, and dedifferentiated tumors.[12] As AR expression is lower in higher-grade tumors, CSCs are more abundant as the cells become more dedifferentiated and "immature."

Numerous cell surface markers, such as CD24, CD44, CD133, and CD166, and intracellular markers, including BMI1, OCT3/4, and NANOG, sex-determining region-Y-box 2 (SOX2), are used to identify and isolate CSCs.[13] CD44, CD133, and SOX2 are some of the most investigated stem cell markers. SOX2 is a transcription factor crucial for sustaining the survival and pluripotency of undifferentiated stem cells. Additionally, SOX2 plays a role as an epigenetic reprogramming factor and oncogene.[12] Moreover, CD44 and CD133 are transmembrane glycoproteins, while CD44 plays a role in tumorigenesis, metastasis, and resistance to chemotherapy and is associated with patient prognosis.[14] CD133 is essential for the organization of cellular membranes, self-renewal, cell survival, disease aggressiveness, and chemotherapeutic resistance.[15] This study aimed to elucidate the interrelation among CSC markers (SOX2, CD133, and CD44), AR, and metastasis in patients with newly diagnosed castration-naive mPCa.

METHODS

Study design

This study followed a descriptive analytical design and examined patients with newly diagnosed metastatic castration-naive PCa. The inclusion criteria were patients aged ≥50 years, newly diagnosed with mPCa, and therapy-naive. Patients with incomplete medical records, missing slides, paraffin blocks, and those aged <50 years were excluded from the study. All 61 formalin-fixed paraffin-embedded (FFPE) PCa tissue samples were collected from 2020–2023 at the Department of Anatomical Pathology, Cipto Mangunkusumo Hospital. The patients were categorized based on CHAARTED high-volume criteria and divided into the HVD group, defined as the presence of visceral metastases or ≥4 bone lesions with ≥1 outside the vertebral bodies and pelvis, and the LVD group who did not fit the HVD criteria. The research has been approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No: 1074/UN2.F1/ETIK/2017). The gap year between the ethics approval letter and the data collection was due to the lengthy research protocol development process and the emergence of coronavirus disease in 2019, which caused delay in enrolling research subjects.

Based on availability and patient health insurance, metastasis was determined through various radiological examinations, including magnetic resonance imaging, computed tomography, positron emission tomography, and ultrasonography. Data were obtained from the patients' medical records. Clinical data, such as age, GS or ISUP grade group, and prostate-specific antigen (PSA) level, were also collected from electronic medical records to determine the association of clinicopathological characteristics with AR and prostate CSC marker expression. Tissue samples were obtained from the primary site of the prostate through biopsy, transurethral resection of the prostate, or radical prostatectomy with patient consent during the procedure. The GS and ISUP grade groups were reviewed as slides for staining.

Immunohistochemistry and histopathological parameters

Protein expression analysis was performed using immunohistochemical staining. CD44 antibody clone GT462 (GTX628895; GeneTex, USA), CD133 clone 3F10 (NBP2-37741; Novus Biologicals, USA), SOX2 antibody (ab97959; Abcam, UK), and AR antibody clone SP107 (200R-14-RUO; Cell Marque™, USA) with 1:3000, 1:2000, 1:200, and 1:200 dilutions, respectively, were used as primary antibodies. Novolink Polymer 3, 3'-diaminobenzidine (DAB) Detection Kit (Novolink™ Polymer Detection Systems, UK) was used as the secondary antibody. Hematoxylin was used as a counterstain. Immunohistochemical staining was performed using an in-house manual technique. Each FFPE block was cut into sections with 3 µm thickness, dried at 37°C, and heated on a slide warmer at 60°C for 60 min. Deparaffinization was performed in three chambers of xylene for 3 min each. The tissue was rehydrated with absolute, 96%, or 70% alcohol for 3 min in each chamber. The tissue was pretreated with TRIS-EDTA (pH 9.0) in a decloaking chamber at 96°C for 20 min, cooled for 25 min, and rinsed with phosphate-buffered saline (PBS) (pH 7.4) for 3 min. Peroxidase block solution, protein block fluid for 40 min, and diluted primary antibodies were administered to the tissue while rinsing with PBS (pH 7.4) for 3 min before each administration. SOX2 and AR tissues were incubated for 1 hour, whereas CD133 and CD44 tissues were incubated overnight. After incubation, the slides were rinsed with PBS (pH 7.4) for 3 min. The polymer solution was applied for 30 min and washed with PBS (pH 7.4) for 3 min. Finally, the DAB solution was applied for 1–2 min, rinsed in running water for 2 min before drying and covered with a coverslip. Negative controls were used for each staining batch. Normal prostate tissue adjacent to the tumor on the same slide for each batch of staining was used as the positive control.

All stained slides were scanned using a Leica Aperio AT2 Digital Pathology Slide Scanner (Leica Biosystems, Germany) at a 40× objective magnification to assess 300 viable tumor cells. Screenshots of the slides were randomly taken from five different locations, and a grid was installed to divide them into four regions. Negative (0), mild (1), moderate (2), and strong (3) expression intensities were assessed on the membrane of CD44 and CD133 in the nucleus of for AR and SOX2.

Three hundred cells were counted in different areas selected randomly. For the proportion of cells (noted in percentage) that shows strong staining intensity were multiplied by three, percentage of cells with moderate staining multiplied by two, percentage of cells with mild staining multiplied by one. The H-score for quantitative analysis was calculated using equation (3 × % strong tumor staining intensity) + (2 × % moderate tumor staining intensity) + (1 × % mild tumor intensity) + (0 × % negative expression).

The cutoff points of each protein were determined using the receiver operating characteristic method based on ISUP grade <5 *versus* 5 to determine the area under the curve. After the cutoff points were determined, scores less than the cutoff point were considered low expression, and scores higher than the cutoff point were considered high expression. The scoring was performed by an experienced practicing pathologist who became a consultant in uropathology in 2018.

Statistical analyses

Univariate analysis was performed for each parameter to determine means, standard deviations, medians, and ranges. PSA was compared between LVD and HVD and ISUP grade <5 *versus* 5 using the independent *t*-test. ISUP grade and protein expression were compared with the extent of metastasis using the chi-squared test. All analyses were performed using SPSS software version 20 (IBM Corp., USA) for Windows, with $p<0.05$ considered statistically significant.

RESULTS

Patient demographics

This study included 61 patients with a median age of 50 and 86 years in the LVD and HVD groups, respectively. The HVD group had a higher mean PSA levels than the LVD group, and a higher proportion of patients with ISUP grade 5 (74%) than the LVD group (26%) (Table 1).

A significant difference was observed in serum PSA levels between the lower ISUP grades (<5) and the higher ISUP grades.

Immunohistochemical expression of stem cell markers and AR

Figure 1 shows an example of PCa and the immunohistochemical staining results. A crosstab analysis was performed to compare SOX2 expression

Table 1. Characteristics of patients with LVD and HVD

Characteristics	LVD (N = 23)	HVD (N = 38)	Total (N = 61)	*p*
Age (years), mean (SD)	68.7 (5.570)	67.4 (9.243)	67.9 (8.031)	0.523*
PSA (ng/ml), median (range)	57.0 (2.57–288.96)	356.9 (10.64–1,876.21)	122.0 (2.57–1,876.21)	**<0.001**†
ISUP grade, n (%)				**0.017**‡
Grade 2–4	10 (43)	6 (16)	16 (26)	
Grade 5	13 (57)	32 (84)	45 (74)	

HVD=high-volume disease; ISUP=International Society of Urological Pathology; LVD=low-volume disease; PSA=prostate-specific antigen; SD=standard deviation
*Independent *t*-test; †Mann–Whitney *U* test; ‡chi-square test

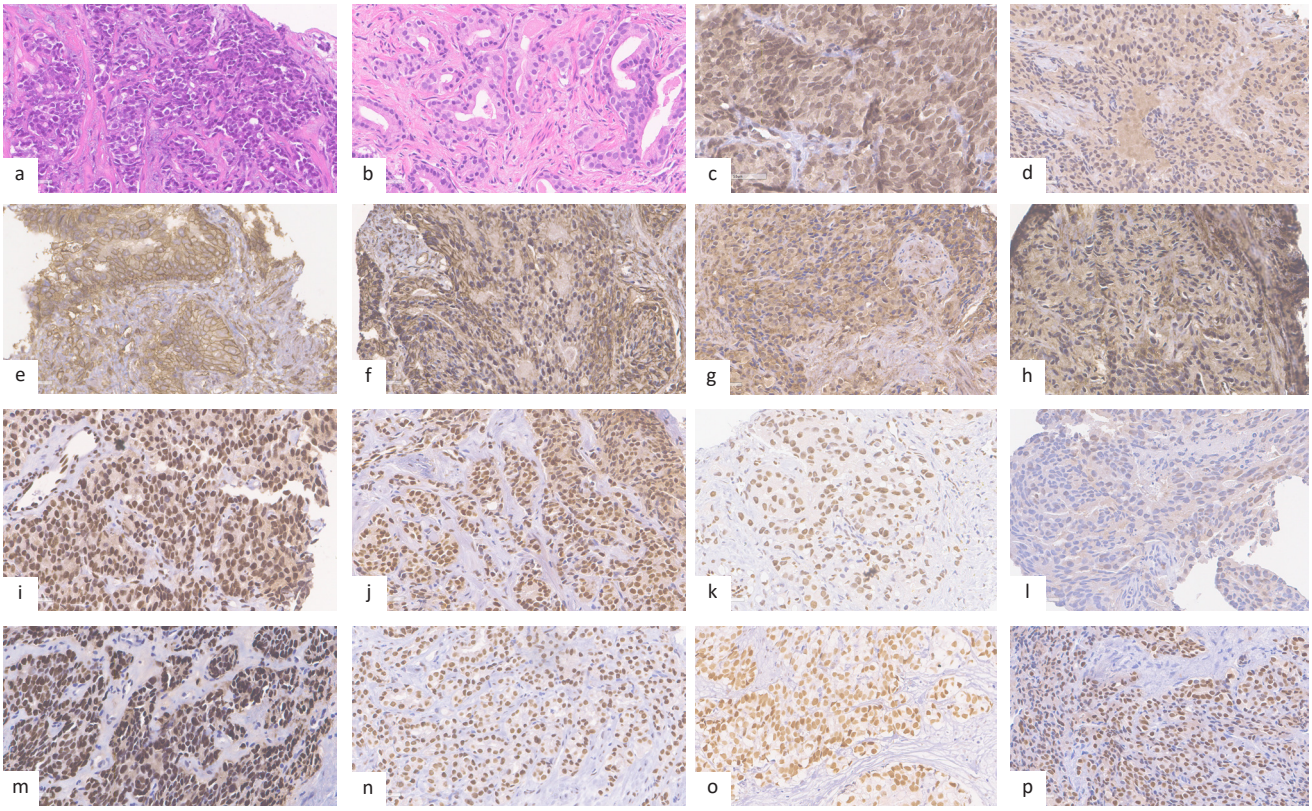


Figure 1. Immunohistochemical staining result. AR and SOX2 expressed in the nuclei, while CD44 and CD133 were expressed in the cytoplasm and/or membrane. (a) PCa ISUP grade 5 (H&E); (b) PCa ISUP grade 2 (H&E); (c) mild CD133 expression; (d) negative CD133 expression; (e) strong CD44 expression; (f) moderate CD44 expression; (g) mild CD44 expression; (h) negative CD44 expression; (i) strong SOX2 expression; (j) moderate SOX2 expression; (k) mild SOX2 expression; (l) negative SOX2 expression; (m) strong AR expression; (n) moderate AR expression; (o) mild AR expression; (p) negative AR expression in some cells. All figures were 400× magnification. AR=androgen receptor; H&E=hematoxylin and eosin; ISUP=International Society of Urological Pathology; PCa=prostate adenocarcinoma; SOX2=sex-determining region-Y-box 2

in the LVD and HVD groups after the cutoff points were determined, and the scores were recorded as low and high expression. A significant difference was observed in SOX2 expression between the LVD and HVD groups but not in the expression of other proteins (Table 2). Primary tumors in patients with LVD had higher SOX2 expression in tissues than those with HVD.

DISCUSSION

This study investigated protein expression and explored the potential association between AR and CSC marker protein expression in FFPE tissue samples obtained from 61 patients recently diagnosed with castration-naive mPCa. No significant difference was observed in baseline age between patients with LVD

Table 2. Comparison of protein expression in LVD and HVD group

Protein expression	LVD (N = 23)	HVD (N = 38)	OR (95% CI)	*p*
AR			0.714 (0.249–2.045)	0.368
Low	6 (26)	14 (37)		
High	17 (74)	24 (63)		
SOX2			0.285 (0.095–0.858)	**0.023**
Low	7 (30)	23 (61)		
High	16 (70)	15 (39)		
CD44			0.559 (0.197–1.592)	0.275
Low	10 (43)	22 (58)		
High	13 (57)	16 (42)		
CD133			0.605 (0.193–1.893)	0.530
Low	9 (39)	18 (47)		
High	14 (61)	20 (53)		

AR=androgen receptor; CI=confidence interval; HVD=high-volume disease; LVD=low-volume disease; OR=odds ratio; SOX2=sex-determining region-Y-box 2

and those with HVD. However, serum PSA levels at the time of diagnosis and the ISUP grade (<5 *versus* 5) were significantly higher in patients with HVD than in those with LVD. A significant difference was also observed in SOX2 expression between the LVD and HVD groups.

SOX2 is a cellular transcription factor involved in maintaining the survival and pluripotency of undifferentiated stem cells.[16] In the prostate, SOX2 is found in the basal epithelial cell layer of normal glandular tissue and prostate tumor cells.[16] Immunohistochemical staining of SOX2 has been used to highlight several stages of prostate tumorigenesis, ranging from benign prostatic hyperplasia and primary PCa to mPCa. However, Alghezi et al[17] reported lower SOX2 expression in mPCa than in primary PCa. SOX2 promotes cellular dedifferentiation and downregulates genes essential for cell differentiation.[18,19] *In vivo* SOX2 overexpression in PCa cells induced a tumor cell quiescence state in a novel model system, reducing proliferation, with growth resuming quickly when SOX2 levels normalize.[20] Elevated SOX2 expression in quiescent cell lines decreases the levels of cyclins and cyclin-dependent kinases (CDK), which regulate the cell cycle and restrict cell proliferation and tumor growth. Conversely, studies using metastatic castrate-resistant prostate cancer cell lines (CWRR1) showed that mitotic inhibitor protein kinase (WEE1) and CDK1 expression increased in cells with elevated SOX2 expression. WEE1 may function as a tumor suppressor by controlling the cell cycle through CDK1/CDK2 deactivation and phosphorylation or as an oncogene under malignant conditions by sustaining genetic instability.[21] SOX2 overexpression in human metastatic prostate cancer (LNCaP) and androgen-independent human prostate cancer cell line (CWR22RV1) results in the downregulation of AR and other prostate differentiation marker genes, namely *NKx3.1* and *PSA* in LNCaP cell lines,[22] supporting its role in tumor dedifferentiation.

mRNA *SOX2* expression was significantly downregulated in the neoplastic epithelium (GS ≤3 and >3) compared to normal epithelium.[23] However, high SOX2 expression has been reported in lymph node metastases (LNM) and primary tumors in node-positive PCa. LNM has different phenotypes from prostate bone metastases.[24] Studies across various cancer types have reported conflicting findings regarding SOX2 expression at different tumor stages. For example, in gastric cancer, SOX2 expression in both primary and metastatic lesions was lower than in matched normal gastric mucosa.[25] In PCa, SOX2 expression has been reported during embryogenesis, normal hyperplasia, and malignancy, both *in vivo* and *in vitro*.[21] However, variations in SOX2 expression across different metastatic sites or stages have not been characterized. In the present study, SOX2 expression in the primary tumor was more prominent during the LVD stage. SOX2 is typically upregulated during critical phases, such as embryogenesis, tumor initiation, metastasis, and treatment response. During HVD, alternative mechanisms and more favorable tumor microconditions can replace SOX2 expression at the primary site. Another possible hypothesis is the possibility of communication among solid tumor cells at multiple sites, causing different expression of various genes at different sites.[26]

The role of SOX2 and its interaction with CSC markers vary across tumor types. Although frequently used as markers for CSCs, SOX2 and CD133 play numerous roles in cellular proliferation, growth, metabolism, and microenvironment modulation. In lung cancer, hypoxic conditions may result in *SOX2* and octamer-binding transcription factor 4 (OCT4) upregulation, which

induces CD133 expression.[27] Characterization of CD133 in melanoma D10 culture cells revealed that CD133+ D10 cells showed *SOX2* downregulation, whereas OCT4 and NANOG levels were increased.[28] These findings highlight the dynamic and context-dependent nature of their expression, which requires further investigation.

In this study, CD133, CD44, and AR were differentially expressed. Metastasis may occur through circulating tumor cells (CTCs), which often express stem cell markers but lack AR expression. The generation of CTCs involves EMT, in which epithelial cells gain motility, separate themselves from the colony, and enter circulation. This process also activates stemness characteristics, such as the expression of CD133 and CD44. The progression of tumors following EMT requires sustained CD133 and CD44 expression, which is regulated through pathways independent of SOX2. EMT may also activate mesenchymal–epithelial transition (MET), promoting cellular plasticity and the generation of differentiated, AR-expressing cells.[29] The dynamic processes of tumor progression and metastasis may explain why its expression is not consistently different between the LVD and HVD groups. Metastasis remains an inefficient process; CTCs do not survive in circulation. However, CTCs with stemness characteristics can evade the immune system, colonize distant sites, manipulate the microenvironment, and establish vascular networks for nutrition and oxygen supply. After the microenvironment is suitable for tumor growth, MET may facilitate further tumor development.[30] The divergent outcomes between mPCa and non-mPCa suggest that cancer cells in the metastatic site may be a subset of the primary tumor with specific gene expression. However, the distinctive differences in gene signatures in metastatic and primary PCa remain unclear.[31] These differences may also be found in cancer cells of HVD and higher ISUP grades than those of LVD and lower ISUP grades, especially in genes involved in therapeutic resistance and tumor cell resilience. Given the absence of significant differences in AR, CD44, and CD133 expression between the LVD and HVD groups, SOX2 may play a more dominant role in tumor plasticity and dedifferentiation.[19,32]

PSA is a protein produced by normal and malignant prostate glands and is widely used for early PCa detection and monitoring. Elevated serum PSA levels in PCa are primarily attributed to structural disruption of the prostate rather than increased PSA synthesis.[33] The significant difference in PSA levels in the LVD and HVD groups is supported by previous studies wherein elevated serum PSA levels exceeding 20 ng/ml demonstrate a positive predictive value of 65% for the presence of metastatic disease and skeletal involvement. This increase in linear predictive accuracy reached 86% when PSA levels surpassed 100 ng/ml. However, high PSA levels of >100 ng/ml do not imply that individuals have metastatic disease at diagnosis.[34] This study also found a significant association between PSA levels and ISUP grade. These results are consistent with those of Spajić et al,[35] who reported that a significant PSA level increase aligned with the ISUP grade group, which is also supported by Okubo et al,[36] who found that PSA levels were notably higher in ISUP grade 5. Similarly, Mahal et al[37] reported that PSA level was generally higher in GS 8–10 than at ≤7, whereas several patients in the higher GS group had low PSA levels (≤2.5 ng/ml) with a worse prognosis of 3.4% compared to 5.1% in the lower group. One patient with ISUP grade 5 had a PSA level of 2.57 ng/ml, whereas none of the patients with ISUP grade <5 in the present study had low serum PSA levels. The range of PSA levels in the ISUP grade 5 group was wider. Mahal et al[37] also found that patients with low PSA levels and high-grade tumors had worse prognoses. This phenomenon explains the transformation of acinar adenocarcinoma of the prostate to neuroendocrine differentiation triggered by chemo hormonal therapy and SOX2 activation, leading to therapy resistance.[12,38]

Histopathological grading may serve as a strong prognosis predictor in PCa when other variables are comparable, thereby guiding treatment decisions.[39] Patients with mPCa often present with higher GS ≥8 or ISUP grades 4 and 5.[40] Yamada et al[41] reported significantly worse cancer-specific and overall survival in patients with GS 9–10 (ISUP grade 5) compared to those with GS 8 (ISUP grade 4). Miyoshi et al[42] proposed a novel prognostic model including HVD, GS of 9–10, and a hemoglobin level of <13 g/dl, with GS 9–10 conferring a hazard ratio of 1.53 compared to GS <8 for predicting resistance to therapy. Kishan et al[43] described transcriptomic heterogeneity in GS 9–10 tumors, characterized by proliferation, metabolic activity, androgen response, and DNA repair pathways, which correlated with reduced time to metastasis. High tumor grade is typically associated with the loss of protein expression that regulates cell maturity and increased proliferative cell expression, along with

self-renewal associated proteins with stem cell-like properties.[44] The association between AR, the protein that regulates cell maturation, and tumor grade, as well as outcome, in PCa has not been conclusive. AR is expressed in almost all primary and metastatic PCa, regardless of its stage or histological grade. Although some studies associated higher AR expression with better outcomes, others report conflicting or null associations.[45] In contrast, CSC marker expression does not exhibit a linear trend across histological grades; some studies report the highest expression in intermediate-grade tumors, while others associate elevated CSC markers with recurrence.[46]

This study had several limitations. Conducted at a central referral hospital, the need for inter-institutional referral was minimal, potentially resulting in a broader patient population. The absence of specimens from patients with benign lesions or non-mPCa reflects the preliminary nature of this study into CSC markers and AR after androgen deprivation therapy. In conclusion, SOX2 expression in primary tumors was associated with the extent of metastasis, with higher expression observed in the LVD group. Given its dynamic expression across tumor stages and sites, further investigation into the regulatory mechanisms of SOX2 and its role in systemic cancer progression is warranted.

Conflict of Interest

Agus Rizal Ardy Hariandy Hamid is the editor-in-chief of this journal but was not involved in the review or decision-making process of the article.

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