

Shh Overexpression Is Correlated with GRP78 and AR Expression in Primary Prostate Cancer: Clinicopathological Features and Outcomes in a Chinese Cohort

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Xiangyu Zhang^{1,*}
Yanmin Zhang^{2,*}
Fanzhong Lin¹
Xin Shi¹
Longquan Xiang¹
Liang Li¹

¹Department of Pathology, Jining First People's Hospital, Jining Medical University, Jining 272000, People's Republic of China; ²Department of Pathology, Gaomi People's Hospital, Gaomi 261500, People's Republic of China

*These authors contributed equally to this work

Introduction: Shh plays an important role in prostate cancer progression, but its correlation with GRP78 and AR is elusive.

Methods: The study included 539 patients in total, of which 443 had primary prostate carcinoma and 96 patients had benign prostatic hyperplasia (BPH). The clinicopathologic features, histologic scores of protein expression, and correlations between protein and disease state were studied in this cohort. Kaplan–Meier and Pearson correlation analyses were used to compare measures between groups. We performed immunohistochemistry to evaluate the expression of the Shh protein in benign prostatic hyperplasia (n=96) and prostate cancer (Gleason scores ≤ 6 [n=399] or ≥ 7 [n=44]). We quantified the expression of Shh, AR, and GRP78 using the weighted histoscore method, studied the correlation between Shh expression and AR and GRP78, and evaluated the impact of Shh protein expression on patient survival.

Results: Shh expression was significantly higher in prostate cancer with Gleason scores ≥ 7 than in cancer with lower Gleason scores or benign hyperplasia and was much higher in AR-positive cancer than in AR-negative cancer. Shh is overexpressed in high-grade prostate cancer and is positively correlated with the expression of both GRP78 and AR.

Conclusion: Therefore, Shh may be a useful prognostic marker and therapeutic target for prostate cancer.

Keywords: prostate cancer, GRP78, Shh, AR, survival analysis

Introduction

Prostate cancer is one of the most commonly diagnosed cancers both in China and worldwide and is the second leading cause of cancer-related deaths in the USA.^{1,2} The increasing prevalence of prostate cancer has made it a growing public health concern in China. Early diagnosis and effective treatment of prostate cancer have largely increased the five-year survival rates for prostate cancer.³ The widespread use of prostate-specific antigen (PSA) testing enables some men to be diagnosed with very early-stage prostate cancer; therefore, clinical use of this testing in screening, diagnosis, prognostic stratification increases the survival rate of prostate cancer patients.^{4,5} However, benign prostate hyperplasia and other urologic diseases also lead to serum PSA changes. Histopathological diagnosis is the “golden standard” for prostate cancer diagnosis. Gleason score is the most widely used grading system for prostate cancer, it can

Correspondence: Longquan Xiang; Liang Li
Department of Pathology, Jining First People's Hospital, Jining Medical University, No. 6 Jiansong Road, Jining 272000, People's Republic of China
Tel/Fax +86 537 6051547
Email xianglongquan01@163.com; liliang011@126.com

effectively predict the clinical outcome of patients. Gleason scoring on a prostate-cancer histopathological examination is also very important for predicting patient survival and is one of the prognostic indicator for prostate cancer patients.^{6,7} However, the Gleason grading system is based on subjective judgments, which may lead to varied results among different observers. Further, due to the limitations of biopsy sampling, biopsy grading may be inconsistent with the prostatectomy specimen.^{8,9} Therefore, prostate cancer patients with apparently identical morphologies may have different survival rates, partly due to variable subjective observations. However, generally, patients with higher Gleason scores usually have worse prognoses than those with low scores.¹⁰

In recent years, immunohistochemistry (IHC) plays an important role in cancer clinical diagnosis. IHC has brought revolutionary changes to traditional clinical pathology and has become a powerful tool for clinical pathological diagnostics.^{11,12} Several important biomarkers such as AR, Shh, GRP78, PSA, P63, P504S were usually detected using the IHC method for clinical pathologic diagnosis. The prostate is composed of epithelial glands and fibromuscular stroma, and its main function is to secrete seminal fluid. And prostate adenocarcinoma usually arises from tissues of the epithelial gland. Epithelial cells in the prostate secrete prostatic fluid and PSA in an androgen-dependent manner. Epithelial cells usually express the androgen receptor (AR), and their growth can be stimulated by binding with androgen. Similar to normal prostate epithelial cells, prostate cancer cells also require androgen to grow and survive. Therefore, orchiectomies, which lead to androgen deprivation, can retard prostate cancer growth. However, androgen receptor variants V7 and V9 have been shown to be associated with drug resistance.^{13,14} In a normal prostate, the stroma is also androgen-responsive. Fibroblasts, smooth muscle cells, endothelial cells, and infiltrating cells can produce growth factors that provide paracrine signals to the prostate epithelial cells. However, in prostate cancer, the stroma is altered and the extracellular matrix is remodeled, increasing angiogenesis and alters inflammatory cells.¹⁵ Thus, the stroma is reactivated, and bidirectional signaling between cancer and stromal cells can further accelerate the progression of prostate cancer.^{16,17} The Sonic hedgehog (Shh) protein probably plays some essential roles in this bidirectional signaling.

Shh protein is an important component of the Shh signaling pathway and participates in maintaining stem cell populations and repairing tissue damage. The Shh protein is also expressed by cancer cells and is secreted into the tumor

microenvironment, thus stimulating the stromal cells to express more growth factors by activating the Shh signaling cascade and, in turn, stimulating cancer cell growth.^{18,19} Prostate cancer cells express more levels of Shh compared with normal prostatic epithelia, and Shh potentiates cancer cell growth.²⁰ Shh signaling pathway components express more typically expressed at much higher levels in advanced prostate cancer (Gleason scores 8–10) compared with low-grade prostate cancer (Gleason scores 3–6).²¹ In addition, blockade of the Shh signaling pathway has been shown to lead to cancer shrinkage and remission in a xenograft model of prostate cancer.²² Recently, one study showed that a deficiency in Shh could lead to ER stress of Paneth cells in the intestines.²³ Further, another recent study has suggested that ER stress could affect medulloblastoma tumorigenesis via Ptch1, a component of the Shh signaling pathway.²⁴ Prostate cancer cells usually require increased endoplasmic reticulum capacity and function to synthesize more proteins in the endoplasmic reticulum (ER). ER chaperones play important roles in maintaining ER homeostasis. The 78 kDa glucose-regulated protein (GRP78)/heat-shock protein 5 (HSPA5) is one of the most important ER chaperones and is involved in tumorigenesis and drug resistance in various cancers.^{25,26} We hypothesized that AR regulates the expression of Shh and GRP78 and that GRP78 could interact with Shh to regulate its expression.

In this study, we focused on three important proteins – AR, Shh, and GRP78 – to evaluate their prognostic value in prostate cancer with different Gleason scores. Further, we investigated possible relationships between AR, Shh, and GRP78. This information could be of value in diagnosing prostate cancer patients and evaluating prognoses.

Materials and Methods

Ethical Standards

The ethics committee of Jining First Peoples' Hospital, China, approved this study, and informed consent was written by all patients before being included in the study. The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

Patients

The study included 443 patients with primary prostate carcinoma and 96 patients with benign prostatic hyperplasia (BPH). Prostate cancer (PCa) tissues were obtained during radical prostatectomies performed between 2000 and 2015

at the Jining No. 1 Peoples' Hospital, China. All tissues were taken from prostate cancer patients at the time of transurethral prostate resection and were immediately fixed in 20% formalin. Two independent consultant pathologists determined the Gleason scores for all samples. [Table 1](#) summarizes the clinicopathological data for the patient cohorts.

Immunohistochemistry

Paraffin-embedded tissue blocks were sectioned into 4- μ m slices, deparaffinized, and mounted on glass slides. Antigens were demasked in EDTA buffer (pH 8.4). The primary antibodies used were specific for Shh (Cell Signaling; No. 2207; 1:50 dilution; we used a microwave and citrate buffer-based method for antigen retrieval), GRP78 (Santa Cruz Biotechnology H-129; sc-13539; 1:100 dilution citrate buffer and water-bath antigen retrieval), or AR (polyclonal rabbit Ig; Abcam, Cambridge, MA, USA). After incubation with the secondary antibody, the presence of antigen was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB), and the slides were counterstained with hematoxylin. To exclude nonspecific staining from the secondary antibodies, we tested negative controls that lacked the primary antibody.

Table 1 Clinicopathologic Characteristics of Patients Included in the Study

Variable	No. Patients
Malignant tissue	443
Benign tissue	96
Median age (range), year	68 (62–73)
Follow-up time (range), months	117 (43–265)
Median PSA at diagnosis, median (range), ng/mL	8.4 (4.1–47)
Gleason score	No.
2–6	399 (90.2%)
$\geq 3+4$	44 (9.8%)
Clinical stage	
T1	341 (77.0%)
T2	89 (20.1%)
T3	13 (2.9%)
CRPC	
Yes	146 (41%)
No	292 (34%)
Not available	5 (1.1%)
Overall death	
Yes	138 (31.1%)
No	291 (65.6%)
Not available	14 (3.1%)

Expression Analysis

Histoscores were applied to interpret the results. Briefly, staining intensity was graded as negative (0), weak (1), moderate (2) or strong (3), then the score was multiplied by the percentage of tumor cells within each category.²⁷ The final histoscores ranged from 0 to 300. Two observers, who were blinded to all outcome data, graded the GRP78/BiP, AR, and Shh staining.

Statistics

Statistical analyses were performed using GraphPad Prism V (GraphPad Software, San Diego, CA, USA). Statistical data were evaluated using the Mann–Whitney test to compare differences in GRP78 expression among the different cancer samples. Disease-specific survival rates were generated using the Kaplan–Meier method. The survival curves were compared using the Log-rank test and correlations were calculated using the Pearson correlation coefficient. A result with $P < 0.05$ was considered statistically significant.

Results

PCa Patient Cohort Characteristics

[Table 1](#) shows the patient and prostate cancer characteristics of the 443 PCa patients. The median age at the time of surgery was 61 years (range, 45–71 years). More than two-thirds of patients had a clinical T1 stage, and only a few patients had a pathological Gleason score (GS) ≥ 8 . Median follow-up time was 117 months (range, 43–265 months), and 23% of patients developed biochemical recurrence (BCR) of PCa following radical surgery, which was defined as a confirmed postoperative PSA level > 4 ng/mL. Shh expression was evaluated in both the benign and cancer tissue samples, in AR (+) and AR (–) cases, and in GRP78 (+) and GRP78 (–) cases. The individual patients' Gleason scores and relevant protein expression levels of Shh, AR, and GRP78 were listed in [Table S1](#).

Protein Expression

Shh, GRP78, and AR expressions were evaluated in the 443 cases of primary PCa and the 96 control benign prostatic hyperplasia (BPH) samples. Shh was mainly expressed in the extracellular matrix, with low signal in the nucleus or cell membrane. GRP78 was also mainly expressed in the cytoplasm but not in the cell membrane. AR was mainly expressed in the cell membrane, with low signal in the nucleus and cytoplasm ([Figure 1](#)). Shh signals in the PCa

tissue ($GS \geq 7$) were much stronger than in the PCa tissue ($GS \leq 6$) and BPH samples ($P < 0.0001$). The median histoscore was 58 for benign tissue, 172.8 for the malignant PCa

tissue with $GS \geq 7$, and 147.5 for the malignant PCa tissue with $GS \leq 6$ (Figure 2A). PCa patients were divided into AR^+ and AR^- groups based on AR expression, and Shh

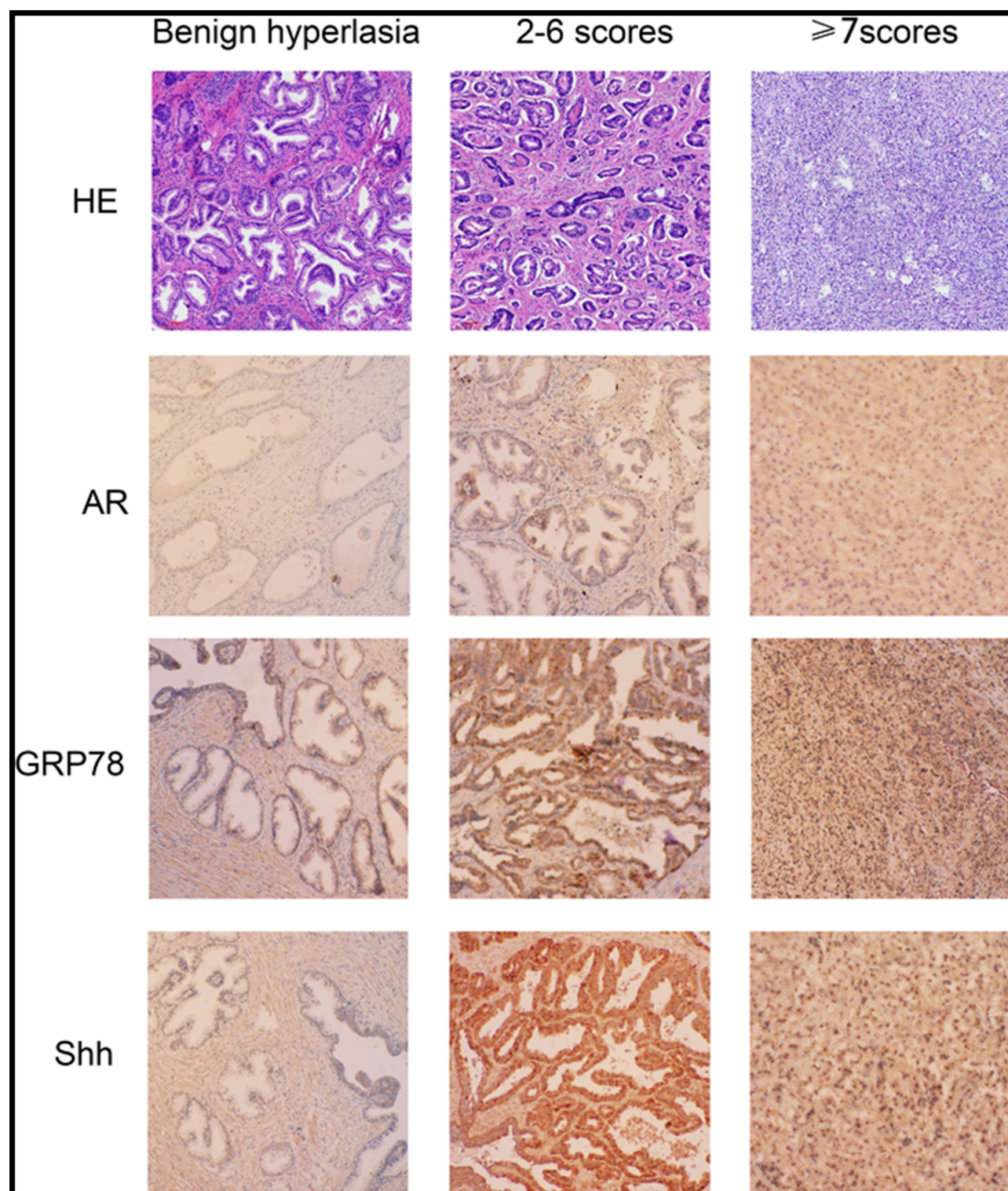


Figure 1 Shh, GRP78, and AR protein expression levels in benign prostate hyperplasia and prostate cancer (Gleason scores of 2–6 or 7–10) were evaluated via immunohistochemistry.

expression was found to be much higher in the AR⁺ group than in the AR⁻ group, with histoscores of 154.8 and 88.0, respectively (Figure 2B). Similarly, the PCa patients were divided into GRP78⁺ and GRP78⁻ groups, and Shh expression in the GRP78⁺ group was found to be much higher than in the GRP78⁻ group, with histoscores of 168.5 and 102.7, respectively (Figure 2C). Therefore, elevated Shh expression was associated with increased expression of both AR and GRP78. Cytoplasmic GRP78 expression and membrane AR expression, in particular, were associated with Shh

expression, with correlation coefficients of 0.227 and 0.334, respectively (Table 2).

To evaluate the prognostic value of Shh, we conducted a survival analysis in this patient cohort. Shh overexpression in all PCa patients was associated with shorter disease-specific survival in patients with AR⁺ tumors (Figure 2D). In the AR⁺ subgroup, the median survival time for patients with tumors that had overexpression of Shh was 6.9 years compared with 8.1 years for patients with low GRP78-expressing tumors, which was statistically significant

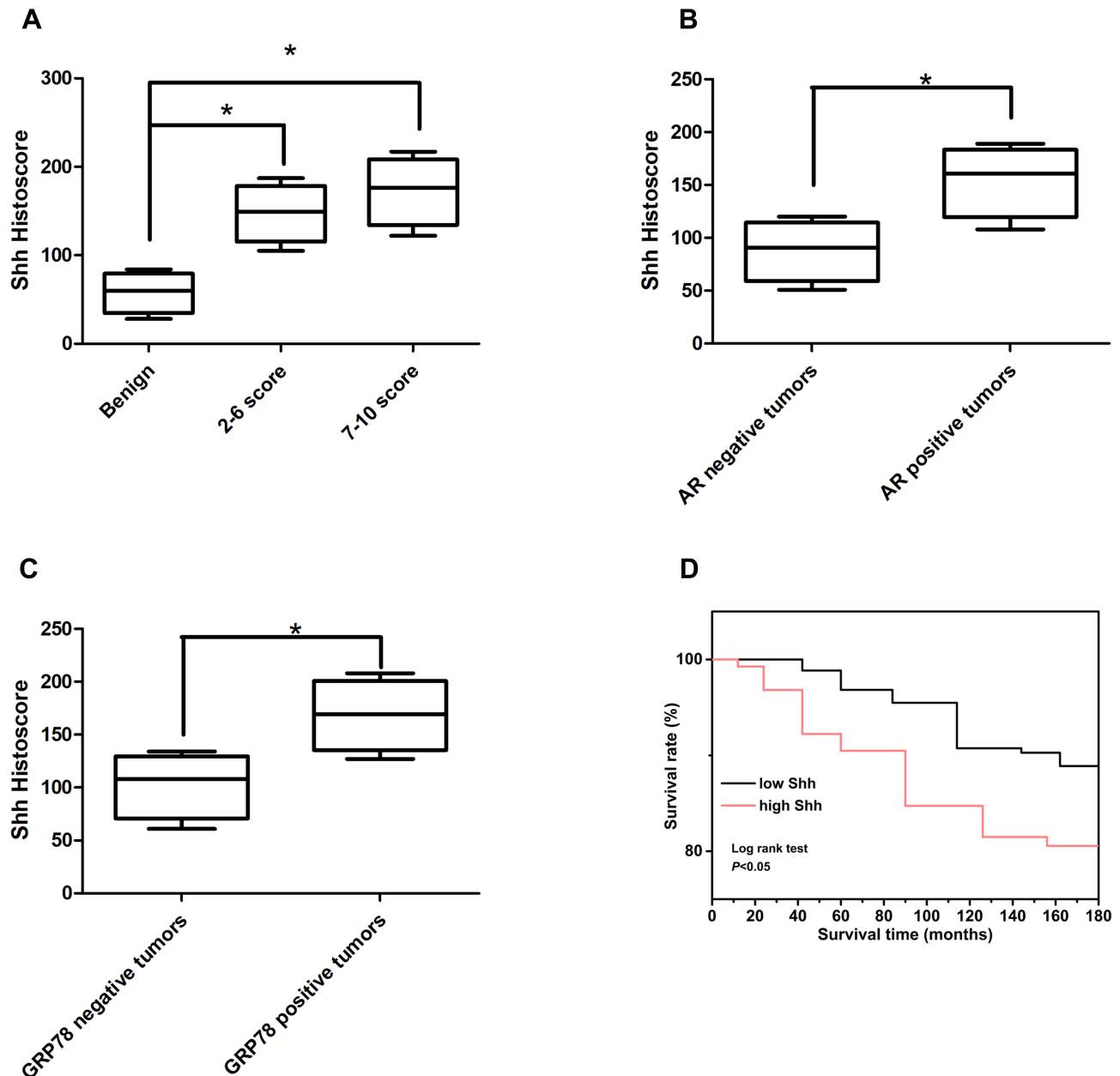


Figure 2 Shh expression levels in benign prostate hyperplasia and prostate cancer (Gleason scores of 2–6 or 7–10) (A). Difference in Shh expression between the AR-positive and AR-negative groups (B) and in the GRP78-positive and GRP78-negative groups (C). Disease-free survival analysis for AR-positive patients with Shh overexpression or with no expression (D). * $P < 0.05$.

Table 2 Association of Shh Expression with GRP78 and AR

	Correlation to Shh Expression	
	Correlation Coefficients	Significance <i>p</i> values
GRP78	0.227	0.001
AR	0.334	0.003

($P < 0.05$). This correlation was not seen in AR[−] PCa patients ($P > 0.05$) (Figure 3).

Prostate Adenocarcinoma Data Analysis Using TCGA Database

There was no significant difference in the expression of AR in normal or tumor samples ($p > 0.05$); however, higher AR expression was associated with a shorter survival time, compared to low expression of AR ($p > 0.05$). Across cancer subgroups (Gleason scores=6, 7, 8, 9, 10), in the high AR expression group, the group with Gleason score=10 had the shortest survival. The low/medium AR expression group had similar results, those with Gleason score=10 had the shortest survival, when compared with other groups ($p < 0.05$) (Figure 4).

The relative expression of GRP78/HSPA5 in normal versus tumor samples was not significantly different ($p > 0.05$); however, higher GRP78/HSPA5 expression was associated with a shorter survival time compared with patients with low GRP78/HSPA5 expression ($p > 0.05$). Across different cancer subgroups (Gleason scores=6, 7, 8, 9, 10), in patients with high GRP78/HSPA5 expression, those with Gleason score=6 had the shortest survival, in the GRP78/HSPA5 low/medium expression group, the

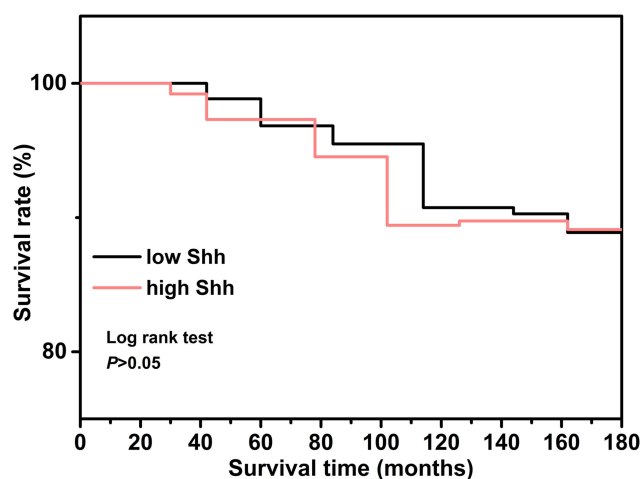


Figure 3 Disease-free survival analysis for AR-negative patients with Shh overexpression or no expression.

group of Gleason score=10 had the shortest survival, when compared with other groups ($p < 0.05$) (Figure 5).

The relative expression of Shh in normal versus tumor samples was not significantly different ($p > 0.05$), however higher Shh expression was associated with a shorter survival compared with the low Shh expression ($p > 0.05$); while across different cancer subgroups (Gleason scores=6, 7, 8, 9, 10), in the Shh high expression group, the group of Gleason score=10 had the shortest survival, in the Shh low/medium expression group, the group of Gleason score=9 had the shortest survival when compared with other groups ($p < 0.05$) (Figure 6).

Discussion

Initially, one study found that Hedgehog (Hh) was important in embryonic cell development.²⁸ Recently, several studies have shown that an aberrant Hh signaling pathway contributes to cancer cell growth directly or indirectly via the tumor microenvironment.^{18,29} The regulation processes of the Shh-Gli signaling pathway are listed here: Shh is an Hh signaling pathway ligand, and when it binds to the Patched (Ptch) receptor expressed on the cell membrane, it attenuates the inhibitory effect on Smoothened (SMO), thus activating the Gli proteins. Gli proteins are important transcriptional factors that translocate to the nucleus after activation in the cytoplasm, and they regulate the expression of target genes, including Cyclin D1, Bcl-2, IL6, and PTHrP.³⁰ Actually, Shh-Gli signaling pathway plays important roles in various types of cancer. Therefore, some targeted therapies have been developed to inhibit the Gli's transcriptional activity to treat cancer.³¹ Moreover, the Shh-Gli signaling pathway also exerts effects in cancer metastases and drug resistance, and downregulation of Shh could inhibit bone metastasis and partially reverse paclitaxel resistance in prostate cancer. And one study showed that inhibiting the Shh signaling pathway promotes docetaxel anti-cancer effects in prostate cancer.³² Cancer cells face a tough growth environment and are usually under chronic stress. Therefore, ER stress usually occurred in various types of cancer, including prostate cancer. However, to manage these difficulties, prostate cancer cells overexpress GRP78, an endoplasmic reticulum chaperone that enables cancer cells to survive and grow.^{25,26}

In this study, the cancer patient cohort contained 443 patients. Of these, 399 patients had Gleason scores ranging from 2 to 6, and 44 had Gleason scores ranging from 7 to 10. The 44 patients with higher Gleason scores had the highest Shh expression levels (mean histoscore, 172.8),

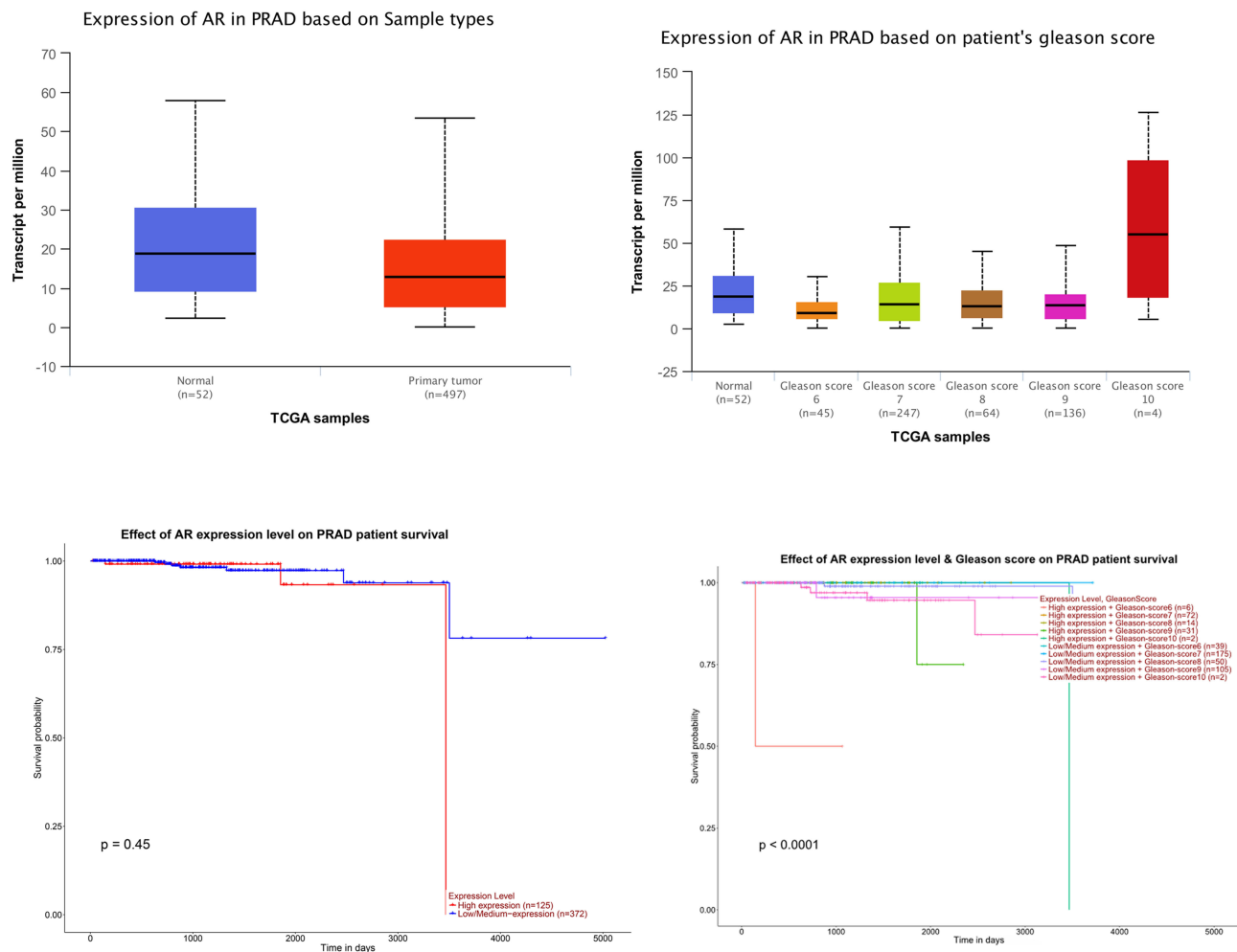


Figure 4 TCGA data comparing the relative expression of AR in normal tissues versus tumor tissues, and survival analysis showed the association of gene expression levels with patients' overall survival.

while those with scores of 2–6 had a mean histoscore of 147.5, and the control group with benign prostate hyperplasia had a mean histoscore of 58. It seems that Shh expression was positively correlated with the Gleason scores of prostate cancer patients. High Shh expression may be an indicator of poor prognosis, as the survival analysis indicated that PCa patients had shorter survival than patients with lower Shh expression ($P < 0.05$). We also noted that Shh was mainly located in the cell membrane and cytoplasm, maybe because that Shh could be secreted out of cancer cells. This result was consistent with a previous study, which reported that Shh was detected in cultured cancer cell media.³³

We also studied the relationship between the expression of Shh and AR. First, we divided cancer patients into the AR-positive (AR⁺) and AR-negative (AR⁻) groups. The mean histoscores of Shh in the AR-

negative and AR-positive groups were 88 and 154.8, respectively. The correlation coefficient between Shh and AR expression was 0.334, and the difference between the AR-negative and AR-positive groups was significant ($P < 0.05$). This indicated that Shh expression may be influenced by AR directly or indirectly. Prior work has suggested that AR was negatively regulated by SHH signaling. AR has been reported to activate the Gli transcriptional factor and enhance the expression of the Shh signaling pathway. Further, it has been shown that interaction between AR and Gli promotes prostate cancer progression to the CRPC stage,³⁴ but the exact mechanism of this interaction requires further study.

GRP78 was usually considered as a poor prognostic indicator for prostate cancer. Moreover, we studied the clinical correlation between GRP78 and Shh. When

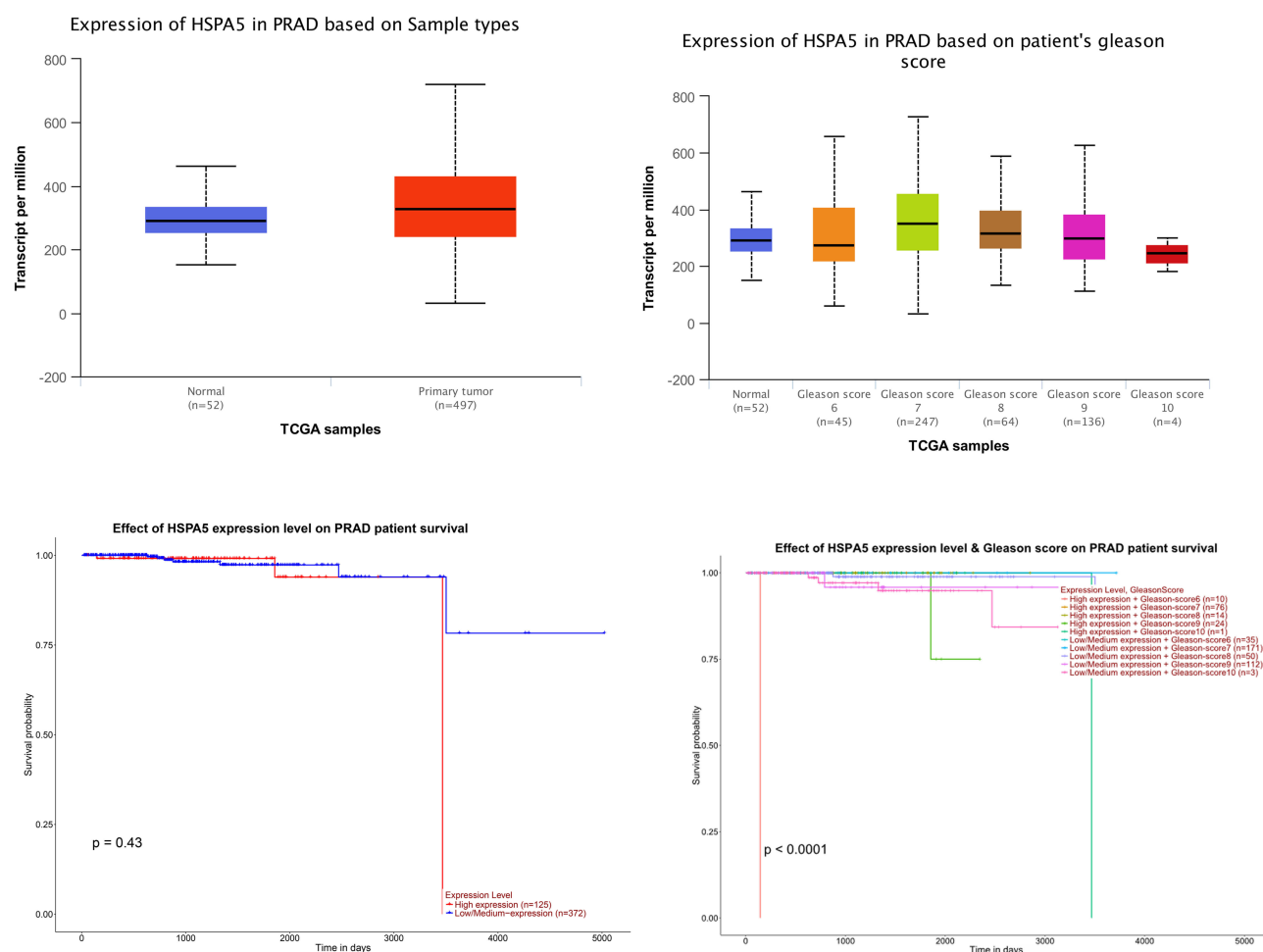


Figure 5 TCGA data comparing the relative expression of GRP78 in normal tissues versus tumor tissues, and survival analysis showed the association of gene expression levels with patients' overall survival.

prostate cancer cells express high levels of GRP78 after chemotherapy, it indicates that chemotherapeutic treatment may be ineffective.³⁵ We also observed that GRP78 was expressed in both the cytoplasm and the cancer cell membrane, which is consistent with previous studies.²⁶ The mean histoscores of the Shh protein in the GRP78-negative and GRP78-positive groups were 102.7 and 168.5, respectively. The correlation coefficient between Shh and AR expression was 0.227, and the AR-negative and AR-positive groups differed significantly in Shh expression ($P < 0.05$). The relationship between the expression of GRP78 and AR in prostate cancer is an interesting problem, and we plan to study the possible regulatory mechanisms in our future research. This may be related to AR, as one report has suggested that AR can regulate the expression of GRP78. This study focused on the three proteins Shh, AR, and GRP78 in prostate cancer patients using IHC methods, and the

concise mechanism of their interactions was not clear. Future work to further elucidate their interactions could provide valuable new information.

As Gleason score is an important indicator in prostate cancer treatment, it can determine which treatment strategies are suitable for a patient. By using the TCGA database, we analyzed the expression of AR, GRP78, Shh in different Gleason score groups, and analyzed the patient survival of different Gleason score groups. The protein expression level was different in the different Gleason score groups, and the patient's survival was also different.

In conclusion, Shh plays multiple important roles in prostate cancer progression and may be regulated by AR. Shh is also closely related to poor survival rates among prostate cancer patients. However, the mechanism underlying its correlation with AR and GRP78 requires further study. This work provides support for future investigation

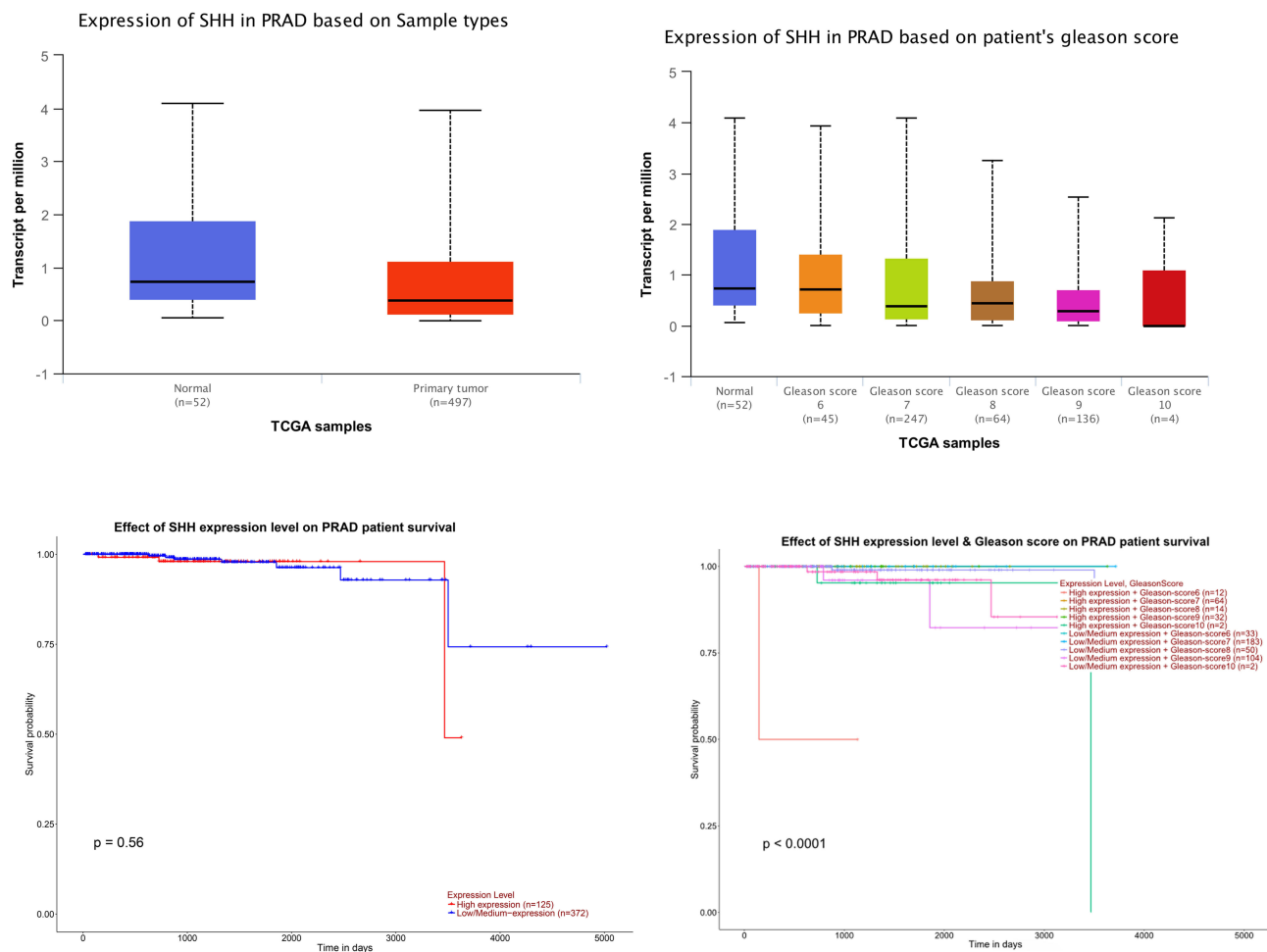


Figure 6 TCGA data comparing the relative expression of Shh in normal tissues versus tumor tissues, and survival analysis showed the association of gene expression levels with patients' overall survival.

of Shh as a potential prognostic marker and therapeutic target.

Abbreviations

Shh, sonic hedgehog; GRP78, 78 kDa glucose-regulated protein; AR, androgen receptor; ER, endoplasmic reticulum; IHC, immunohistochemistry; PRAD, prostate adenocarcinoma.

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Disclosure

The authors report no conflicts of interest in this work.

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