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Elevated Sall4 expression correlates with prostate cancer Gleason score and metastasis using immunohistochemistry and RNAscope®

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ABSTRACT

Background: Prostate cancer lacks reliable biomarkers to distinguish between indolent and aggressive forms, posing diagnostic and prognostic challenges. Sall4, primarily found in embryonic stem cells, is reactivated in various cancers, but its role in prostate cancer remains unclear.

Objectives: This study aims to evaluate Sall4 protein and mRNA levels in malignant and normal prostate tissues and explore their association with clinical data.

Materials and methods: This study was conducted from December 2022 to April 2024 at Al-Hussein Teaching Hospital, Thi-Qar, Iraq. Sall4 protein and mRNA expression levels were assessed in 40 normal tissues and 194 malignant prostate tissues using immunohistochemistry and RNAscope® methods. The data were analyzed using unpaired t-tests.

Results: The study identified a significant increase in nuclear Sall4 protein expression, assessed by immunohistochemistry, in prostate cancer tissues compared to normal tissues (p=0.001). Similarly, Sall4 mRNA levels, measured using RNAscope®, were significantly higher in malignant tissues (p<0.001). Increased Sall4 expression at both protein and mRNA levels was significantly associated with higher Gleason scores (protein: p=0.003; mRNA: p=0.009), lymph node involvement (protein: p=0.002; mRNA: p=0.006), and metastasis (protein: p=0.001; mRNA: p=0.017). However, no significant correlation was found between Sall4 expression and tumor size.

Conclusion: Elevated Sall4 expression may be associated with prostate tumorigenesis and aggressiveness. Further studies are needed to clarify its role and evaluate its potential as a prognostic biomarker for prostate cancer.

Introduction

Prostate cancer (PCa) is a significant global health concern, characterized by a high fatality rate and widespread impact on men's health.[1,2] It accounts for approximately 300,000 new cases and 41,000 deaths annually in the United States[3] and ranks as the tenth most predominant malignancy in Iraq, accounting for 1,224 newly diagnosed cases in 2019.[4] The vast majority of PCa cases, approximately 95%, are known to be acinar adenocarcinomas, which develop in glandular areas of the prostate.[5,6] In contrast, only around 5% of these cases have ductal adenocarcinomas, which grow from the glandular

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epithelial cells and are often identified through histopathological analysis.[6]

Two key systems assess PCa progression: the Gleason grade and Tumor-Node-Metastasis (TNM) systems.[7] The Gleason grading system classifies PCa based on microscopic tissue architecture, ranging from 1 to 5, indicating how closely the cancer resembles normal tissue.[8] Less aggressive cancers resemble healthy tissue, while more aggressive ones show abnormal features and higher metastatic potential.[9] PCa is heterogeneous, with varying patterns in the same tumor, so the Gleason score combines the two most common grades in biopsy or prostatectomy samples, with the highest score being 10. A Gleason score of 7 (e.g., 3+4 or 4+3) reflects different proportions of grade 3 and 4 components.[9,10] The TNM system stages cancer based on tumor size (T), lymph node involvement (N), and metastasis (M), providing key prognostic information and guiding treatment.[11] However, neither system can reliably differentiate between aggressive and non-aggressive tumors or predict outcomes after initial therapy, such as relapse or sustained remission.[8]

Spalt-like transcription factor 4 (Sall4), a zinc finger transcription factor, is crucial in maintaining pluripotency and promoting oncogenesis, with its expression linked to aggressive cancer behaviors. Sall4 is predominantly found in embryonic stem cells and during embryonic development, with its expression markedly absent or reduced in the majority of healthy adult human tissues. However, it has been reactivated in various cancers, including approximately 30% of solid tumors, such as PCa.[12-14]

Its role in regulating key pathways associated with cell survival and proliferation makes it a compelling candidate for further investigation in PCa. Sall4 may contribute to the progression of PCa primarily by acting as a transcriptional activator of the Bmi-1 gene, which is well-known for its function in controlling stem cell self-renewal and differentiation as well as preventing cells from dying.[15-17] Another study on transgenic mice with the Sall4 B mutation showed lower levels of PTEN expression than wild-type controls, indicating that Sall4 may inhibit PTEN transcription. Elevated Sall4 levels could, therefore, contribute to PCa development by inhibiting PTEN and fostering tumorigenic processes, emphasizing the need to explore further Sall4's regulatory impact on PTEN in PCa.[18] Yang and colleagues have demonstrated that Sall4 upregulates several anti-apoptotic genes, including Bmi-1, Bcl2, DAD1, TEGT, BIRC7, and BIRC4, while negatively regulating pro-apoptotic genes such as TNF, TP53, and PTEN.[18]

These findings encourage us to assess the expression level of Sall4 in normal and malignant prostate tissues and to investigate its correlation with PCa clinical data using immunohistochemistry (IHC) and RNAscope® methods.

Materials and methods

Patients and control samples

This retrospective study was approved by the Ethics Board of Al Hussein Teaching Hospital, Thi-Qar, Iraq (Approval No. 2021159, dated 7/12/2022). A total of 234 prostate samples were analyzed, including 194 formalin-fixed, paraffin-embedded malignant specimens and 40 normal prostate (NP) tissues as controls. Gleason scores and pathological stages were assessed by histopathologists. Patients who received chemotherapy or hormonal therapy were excluded. Normal testis tissue served as a positive control in both IHC and RNAscope® assays, while negative controls omitted the primary antibody. Clinical data related to prostate tissues are summarized in Table 1.

Table 1. The distribution of normal and malignant prostate cases according to clinical data.

Prostate clinical data		Number	%	*p* value
Number of samples	Normal	40	17.1	<0.0001
	Malignant	194	82.9	
Age range	Normal	21-50		<0.0001
	Malignant	20-85		
Gleason score	Low Gleason score	30	15.5	
	High Gleason score	160	82.5	
	N/A	4	2	
T category	T1	18	9.3	
	T2	103	53.1	
	T3	48	24.7	
	T4	17	8.8	
	N/A	8	4.1	

Table 1. The distribution of normal and malignant prostate cases according to clinical data (continued).

Prostate clinical data		Number	%	*p* value
N category	N0	154	81	
	N1	31	8.8	
	N/A	9	4.6	
M category	M0	148	76.3	
	M1	33	17	
	N/A	13	6.7	

Immunohistochemistry

IHC was performed on prostate tissue sections using two anti-Sall4 antibodies: a rabbit polyclonal (1:100, Abcam, Ab29112, UK) and a mouse monoclonal (1:100, Novus Bio, H00057167-M03, UK). Detection used the DAKO Envision™+HRP (DAB) kit (K4010, DAKO, UK). This method was carried out according to the previously protocol described by Alalwany, *et al.*[19] Sections were deparaffinized with Histoclear (H5-200, National Diagnostics, UK), rehydrated through graded ethanol (100%, 95%, 70%) (20821 330, VWR, UK), and permeabilized with 0.5% Triton X-100 (T8787-100ML, Sigma, UK) in PBS (BR00140, OXOID, UK).

Heat-induced epitope retrieval was done at 90 °C for 30 minutes in Tris/EDTA buffer (pH 9) (T/p90/630, Fisher Scientific, UK), followed by cooling. Endogenous peroxidase activity was blocked with %3 hydrogen peroxide, and non-specific binding was reduced using %10 normal goat serum (G9023, Sigma, UK) and %0.05 BSA (A4503, Sigma, UK) in PBS.

Primary antibodies were applied overnight at 4 °C. After PBS washes, sections were incubated with secondary antibody for 30 minutes, developed with DAB for 5 minutes, counterstained with hematoxylin (H-3401, Vector, UK), and mounted using DPX (06522, Sigma-Aldrich, UK). Images were captured using a Nikon Eclipse E800 microscope.

IHC quantification

The H-score approach was used to evaluate the Sall4 immunostaining in prostate tissue samples by histopathologists. This method quantifies protein expression in IHC by evaluating the percentage and intensity of stained cells. The H-score range is 0-300.[25] Nuclear staining was shown to have four intensity levels: 0, indicating no staining, 1, indicating mild staining, 2, indicating moderate staining, and 3, indicating severe staining. The formula used to determine the H-score was as follows:

"H-score = (3 × % strongly positive nuclei) + (2 × % moderately positive nuclei) + (1 × % weakly positive nuclei)". The total score ranges from 0 to 300.

RNAscope® protocol

RNAscope® was used to detect mRNA molecules in tissue samples via light microscopy, following the manufacturer's instructions over two days using RNAscope® 2.5. HD detection reagent –Brown (Cat. No. 322310, ACD, Italy). First, tissue sections were incubated in a dry oven at 60°C for an hour to ensure adherence, followed by deparaffinization and rehydration as described in the IHC protocol. Endogenous peroxidase activity was blocked by applying H_20_2 for 10 minutes, followed by washing with deionized water (ddH_2O). Target retrieval (cat. no 322000, ACD, Italy) was performed by heating the slides at 98 °C in 1x target retrieval solution (cat. no 310091, ACD, Italy) for 15 minutes, then rinsing with ddH_2O and 100% ethanol.

On the second day, RNAscope® amplifier reagents were brought to room temperature, and probes were pre-warmed to 40 °C in a HybEZ II oven to dissolve any precipitated salts. Tissue sections were treated with protease pulse (Cat. No. 322330, ACD, Italy), incubated for 30 minutes at 40 °C in a humidified tray, and washed with ddH_2O to remove RNA-binding proteins, permeabilize tissue, and enhance probe penetration.

Next, mRNA probes were added, and the sections were incubated for two hours at 40 °C. After a two-minute wash with 1x wash buffer, six amplification stages (AMP 6-1) were carried out, with incubation times of 30, 15, 30, 30, and 15 minutes, respectively, at 40 °C. Each amplification step was followed by two washes in buffer for three minutes. DAB was applied to the sections for 10 minutes at room temperature, followed by ddH_2O washing. Nuclear staining was done with 50% hematoxylin for two minutes, rinsed under tap water, and briefly washed with 0.02% ammonia water. Finally, dehydration, clearing, mounting, and photography were performed as per the IHC protocol.

RNAscope® quantification

Using the methods advised by the manufacturer, the RNAscope® score was employed to assess the expression of mRNA Sall4 in tissue samples. Briefly, the number of brown spots inside the prostate cells were counted and scored after five random fields were photographed at a 20x magnification. The positive cells were scored using the following scoring system: 0 represented no staining, one represented 1-3 dots/cell, two represented 4-9 dots/cell, three represented 10-15 dots/ cell, and <10% dots are in clusters, and four represented >15 dots/cell and >10% dots are in clusters.

Statistical analysis

The data of this study was analyzed by GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Data were first assessed for normality using the Shapiro–Wilk test. Variables that followed a normal distribution ($p>0.05$) were analyzed using unpaired t-tests to compare means between groups. For non-normally distributed data, non-parametric tests were considered. For statistical significance, a significance level of $p<0.05$ was used.

Results

Clinical data of study population

The current study used 194 PCa (82.9%) and 40 NP tissue samples (17.1%). In PCa, the age range was between 20 and 80, while in NP, it was between 21 and 50. In addition, the majority of PCa cases were classified as high Gleason scores (N=160, 82.9%), whereas only 30 PCa cases (15.5%) were classified as low Gleason scores. Of the 121 PCa patients (62.4%) with T1-2 malignancies, 65 cases (33.5%) had T3-4 malignancies. The majority of PCa cases (N=154, 81%) had no lymph node invasion, whereas the remaining cases (N=31, 8.8%) had lymph node invasion. A total of 33 cases (17%) of PCa were identified as metastatic, while the majority of cases (N=148, 76.3%) were non-metastatic, as detailed in Table 1.

Sall4 expression in prostate samples using IHC

IHC was performed on normal and malignant prostate tissues to assess the immunostaining of anti-Sall4. The results indicated that normal and malignant prostate tissue showed nuclear Sall4 immunostaining, with signal intensities varying from strong to moderate. Variable intensities of nuclear Sall4 were observed in NP tissue. Staining intensity ranged from strong (Figure 1B, arrow) to negative (Figure 1A, arrow). PCa tissues also exhibited nuclear Sall4 staining, with signal strength varying significantly from strong (Figure 1C, arrow) to moderate (Figure 1D, arrow), weak (Figure 1E, arrow), and negative (Figure 1F, arrow). Sall4 has been previously reported in spermatogonia cells of the testis.[27] In this study, testis was used as a positive control for Sall4 expression, and IHC analysis revealed strong nuclear staining in the spermatogonia cells (Figure 1G, arrow). Negative control, no primary antibody added, showed free of Sall4 staining (Figure 1H, arrow).

Sall4 immunostaining validation using testis and postate tissues

To validate Sall4 immunostaining in both prostate and positive control, Testis tissues, IHC was conducted on sections to ensure that the two independent Sall4 antibodies (rabbit polyclonal and mouse monoclonal) exhibited a consistent staining pattern. The rabbit polyclonal Sall4 antibody result showed that strong nuclear Sall4 was seen in both the Testis (Figure 2A, arrow) and Prostate (Figure 2C, arrow). Sall4 mouse monoclonal also showed a similar expression pattern in the Testis (Figure 2B, arrow) and Prostate (Figure 2D, arrow) tissues.

Sall4 expression in prostate samples using RNA-scope®

RNAscope® analysis was performed on normal and malignant prostate tissues. The findings demonstrated that Sall4 mRNA stained both groups. The glandular epithelial cells of NP had Sall4 mRNA staining (Figure A, arrow). Malignant tissues were found to have varied degrees of Sall4 mRNA staining; these included widespread (Figure 3B, arrows), scattered (Figure 3C, arrow), and negative (Figure 3D, arrow) staining. Furthermore, it was shown that the spermatogonia cells in the testis tissue expressed Sall4 mRNA (Figure 2E, arrow). The negative control's prostate sections had no staining (Figure 3F, arrow).

Quantification of Sall4 staining in prostate tissue samples

The measurement of Sall4 expression in prostate samples was investigated using both methods. Quantitative analysis of IHC data demonstrated a significantly higher nuclear Sall4 immunostaining in PCa compared to NP tissues ($p=0.001$) (Table 2 and Figure 4A). This elevation of Sall4 expression exhibited a positive correlation with a high Gleason score ($p=0.003$) (Table 2 and Figure 4B). Furthermore, it was demonstrated that Sall4 nuclear expression was markedly elevated in N1 patients compared to those with (N0) ($p=0.002$) (Table 2 and Figure 4C). Nuclear Sall4 staining and metastasis showed a strong correlation ($p=0.001$) (Table 2 and Figure 4D). In contrast, Sall4 expression did not show a significant association with tumor size ($p=0.728$) (Table 2).

The quantification of Sall4 mRNA revealed higher mRNA levels of Sall4 in PCa than in normal individuals ($p<0.001$) (Table 2 and Figure 5A). Increased Sall4 at the mRNA level was seen in the high Gleason score compared to the low Gleason score ($p=0.009$) (Table 2 and Figure 5B). A significant association was seen between high mRNA levels of Sall4 and the presence of lymph node involvement (N1) ($p=0.006$) (Table 2 and Figure 5C) as well as metastatic PCa patients ($p=0.017$) (Table 2 and Figure 5D). However, this study did not show a significant association between Sall4 mRNA levels and tumor size ($p=0.852$) (Table 2).

Table 2. Sall4 quantification results in Prostate cancer clinical data.

Comparison	The main findings	Nuclear Sall4 result		Sall4 mRNA result	
		Mean±SD	*p* value	Mean±SD	*p* value
NP vs PCa	Increased in PCa	0.650±1.988	0.001	0.003±0.025	<0.001
		12.24±19.93		0.488±0.826	
Gleason scores (LGS vs HGS)	Increased in high Gleason score	8.743±16.31	0.003	0.960±0.838	0.009
		22.88±26.17		1.543±1.275	
Stage T (T1-2 vs T3-4)	No difference	11.26±18.57	0.728	0.498±0.88	0852
		12.39±20.57		0.489±0.746	
Stage N (NO vs N1)	Increased in N1	0.464±18.30	0.002	0.516±0.853	0.006
		21.73±20.36		1.061±1.254	
Stage M (M0 vs M1)	Increased in M1	9.345±17.71	0.001	0.484±0.813	0.017
		22.02±22.13		0.931±1.254	

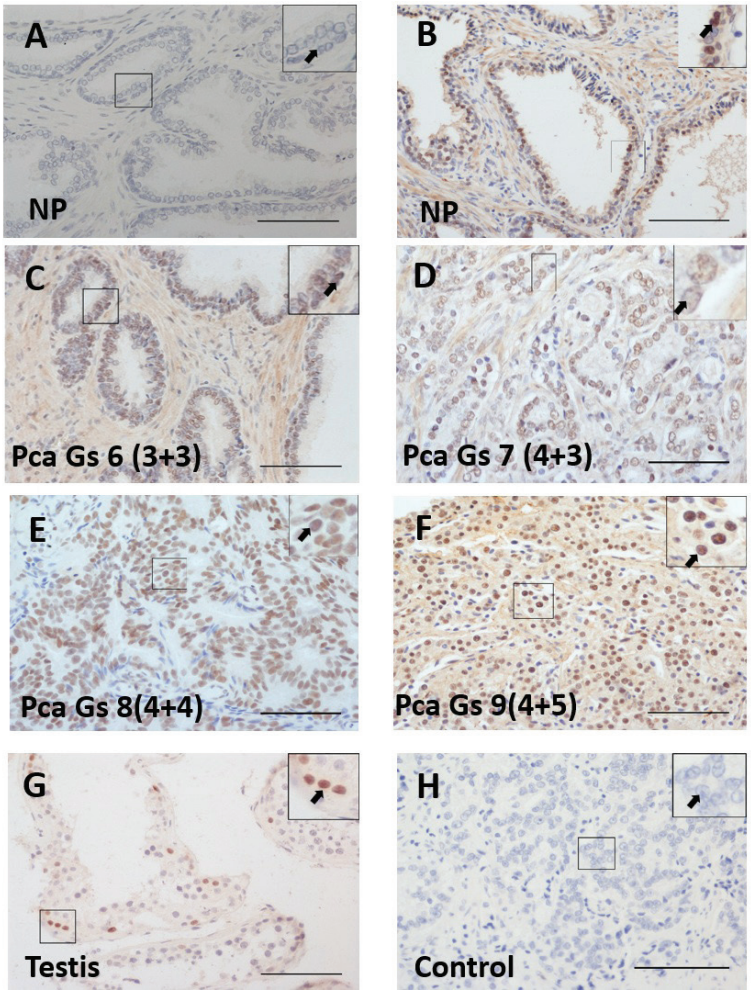

Figure 1. *The expression of Sall4 in normal and malignant tissues. A: no Sall4 expression was seen in NP (arrow), B: strong nuclear Sall4 staining was found in NP (arrow). C: PCa Gleascon score (Gs) 6 showed weak nuclear Sall4 staining (arrow). D: PCa Gs 8 tissue has weak nuclear Sall4 staining (arrow). E: PCa Gs 8 tissues showed moderate nuclear Sall4 staining (arrow). F: strong nuclear Sall4 staining was seen in PCa high Gs 8 (arrow), G: positive control, testis tissue, had strong nuclear Sall4 (arrow), H: no background staining is observed in prostate tissue, confirming the specificity of the assay. Scale bars = 100 µm; insets show 2x magnification.*

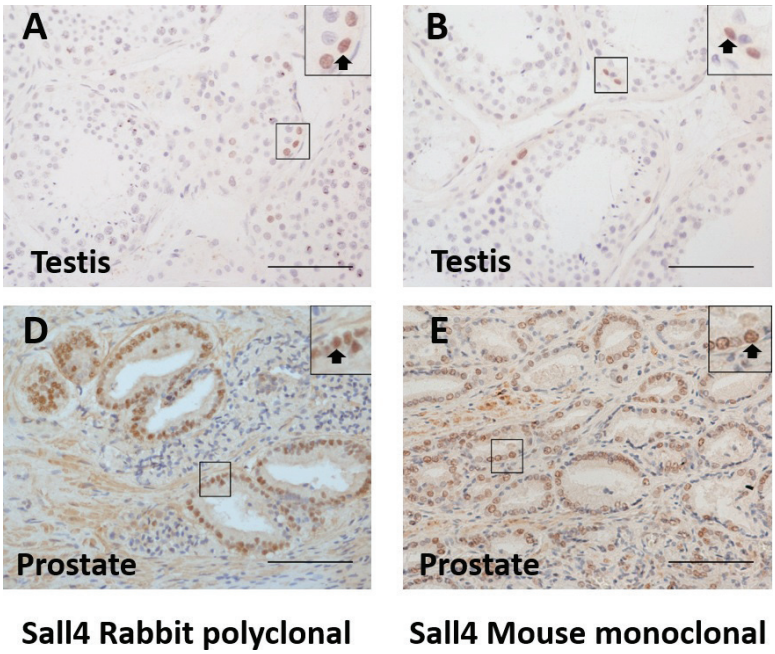

Figure 2. *Validation of Sall4 staining using two antibodies. A and C: nuclear Sall4 staining (rabbit polyclonal) was observed in both tissues of the Testis and Prostate (arrows), B and D: intense nuclear Sall4 staining (mouse monoclonal) detected in tissues of the testis and prostate (arrows).*

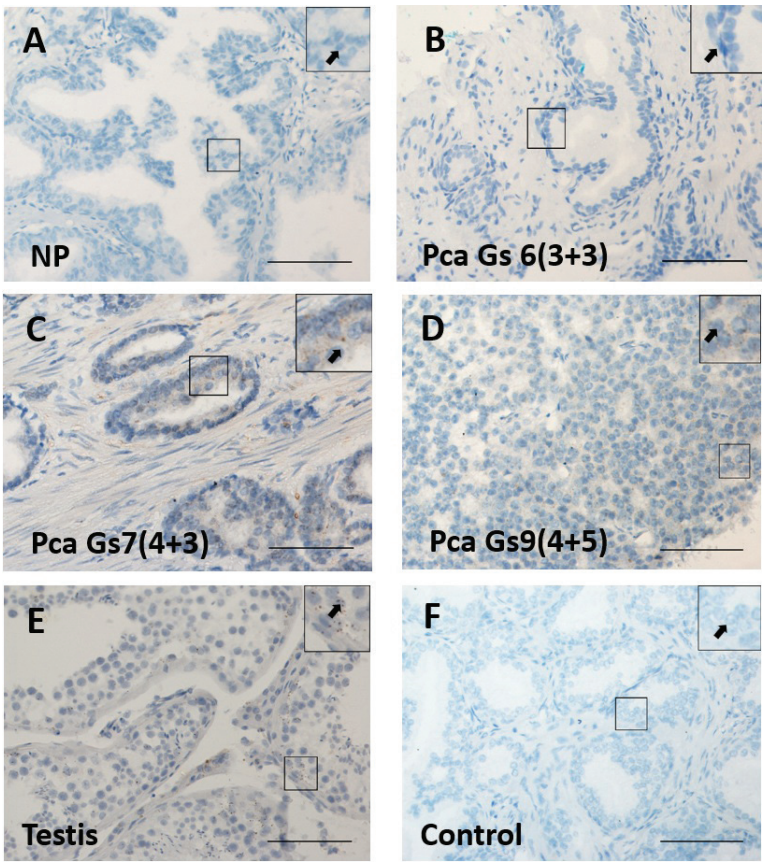

Figure 3. *Sall4 mRNA in prostate samples. A: mRNA staining (arrow) of Sall4 was detected in a few epithelial cells of NP, B: no Sall4 mRNA dots were observed in PCa Gs 6 (arrow), C: mRNA dots of Sall4 were scattered in PCa Gs 7 (arrow), D: Sall4 mRNA dots were found in PCa Gs 9 (arrow), E: Sall4 mRNA dots were observed in the spermatogonia cells of the testis (arrow), F: negative control had no Sall4 mRNA dots (arrow). Scale bars = 100 µm; insets show 2x magnification.*

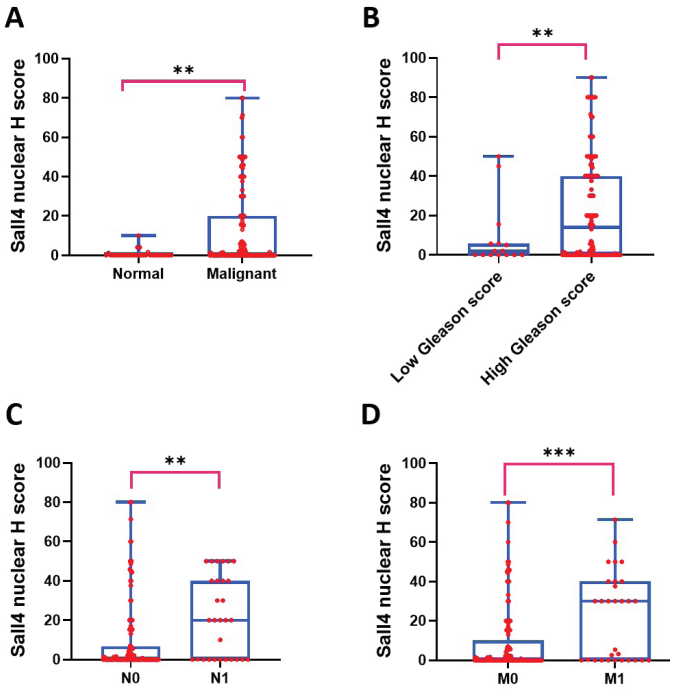

Figure 4. *Sall4 expression quantification in prostate samples using IHC. A: PCa had more nuclear expression of Sall4 than NP (P 0.001), B: there was a noticeable increase in nuclear Sall4 expression from high Gleason scores to low scores (p=0.003), C: Sall4 expression was increased significantly in N1 compared to N0 (p=0.002), D: increased Sall4 expression was associated with metastasis (p=0.001). Each case's five randomly selected photographs make up the data. Unpaired t-tests were used in this study. NP (N=40), PCa (N=194), Low Gleason score (N=30), High Gleason score (N=160), T1-2 (N=121), T3-4 (N=65), N0 (N=154), N1(N=31), M0(N=148), M1(N=33).*

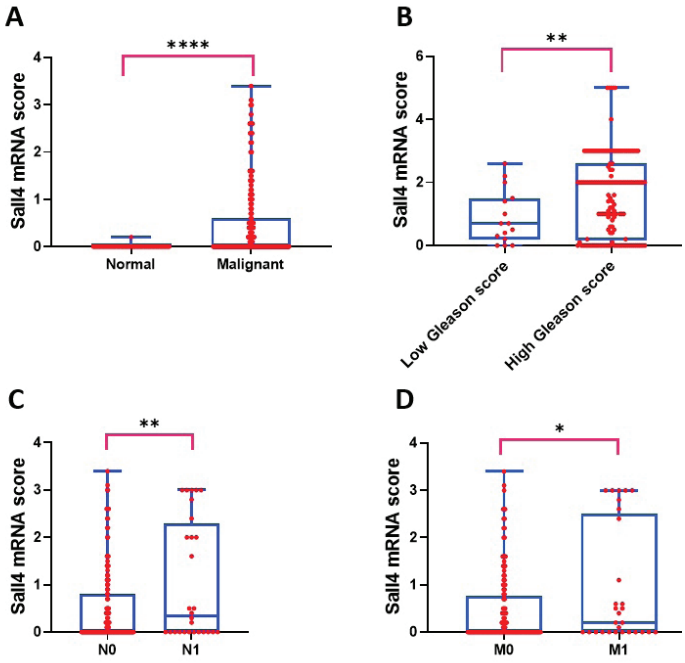

Figure 5. *Sall4 mRNA quantification in Prostate samples using RNAscope®. A: PCa had more Sall4 mRNA than NP (p<0.0001), B: higher Sall4 mRNA was significantly related to Gleason Score (p=0.009), C: increased Sall4 mRNA level in N1 compared to N0 (p=0.006), D: a significant association was detected between Sall4 mRNA level and metastasis (p=0.002). Each case's five randomly selected photographs make up the data. Unpaired t-tests were used in this study. NP (N=40), PCa (N=194), Low Gleason score (N=30), High Gleason score (N=160), T1-2 (N=121), T3-4 (N=65), N0(N=154), N1(N=31), M0(N=148), M1(N=33).*

Discussion

Sall4, a key regulator of pluripotency and differentiation, is linked to oncogenesis and tumor progression.[12] While its role in various cancers is documented, its involvement in PCa remainsunder-explored. This study used RNAscope® and IHC assays to investigate Sall4 levels in prostate samples. Sall4 is significantly up-regulated at protein and mRNAlevels in PCa compared to NP, correlating positively with Gleason score. Its expression is significantly associated with PCa pathological stage, including N and M, but not T, suggesting Sall4's role in tumor grade and stage.

Since there are few studies on Sall4 in PCa, this study may represent a pioneering effort to thoroughly investigate Sall4 expression in PCa. It evaluates both protein and mRNA levels and explores their associations with the Gleason score and pathological stage. SALL4 is primarily known as an embryonic stem cell marker and is typically low or absent in most adult tissues; occasional low-level expression has been reported in regenerative or proliferative epithelial compartments.[12] However, the SALL4 staining observed in a few NP tissues, such as in Figure 1B, may be attributed to non-specific background staining or focal expression in certain basal or progenitor-like epithelial cells. Nonetheless, this finding was not consistent across all NP samples examined, and overall, SALL4 expression was significantly higher in Pca tissues compared to NP. A Chinese study found that increased Sall4 staining in cancer patients with a high Gleason score (≥) compared to those with a low Gleason score (<7), as well as in advanced vs early stages.20 This study agreed with the current data. In addition to this Chinese study, previous studies identified Sall4 levels in various cancer types, including PCa samples, but they did not particularly investigate the relationship between PCa clinical characteristics and Sall4 expression.

For example, modest Sall4 expression was seen in NP tissues (57%) in research examining non-small cell lung carcinomas using a tissue microarray with 112 benign tissue controls, including 7 NP samples.[29] This observation aligns with our research results.[21] Conversely, another investigation revealed that none of the 62 PCa samples in a large cohort had nuclear Sall4 staining.[22] This discrepancy may be due to differences in sample size, the type of antibody, or the scoring system used. To the best of our knowledge, no previous study has utilized RNAscope® technology to evaluate Sall4 mRNA levels in prostate tissue, whether malignant or non-malignant, or to explore the correlation between Sall4 expression and clinical data related to malignancy. In this study, RNAscope in situ hybridization was used rather than quantitative RT-PCR and RNA-sequencing to assess mRNA expression, due to the former's superior ability to preserve tissue architecture and provide spatial resolution at the single-cell level. RNAscope® allows for direct visualization of transcript localization within intact tissue, making it especially valuable in heterogeneous tissues such as prostate cancer. This technique also minimizes issues related to RNA degradation commonly encountered in FFPE samples and allows for precise localization of transcripts, offering both morphological and molecular insights.[23]

This research addresses this significant gap by utilizing advanced RNAscope® assays to elucidate the differential expression patterns of Sall4 and their potential correlation with PCa clinical parameters, thereby contributing novel insights into its role and relevance in the pathology of PCa.

To provide context for our findings, we compared Sall4 expression in PCa with other cancer types. This analysis enhances our understanding of Sall4's role across different malignancies, aligning with research on Sall4 in colon and lung carcinomas, further supporting its involvement in various cancers.[21,24] The anti-Sall4 and mRNA data from this investigation, however, differ from those of Hao *et al.*, who found that colorectal carcinoma (CRC) had less Sall4 staining than either atypical hyperplasia or normal colon tissues.[25] Discrepancies in Sall4 expression observed across different studies may be attributed to variations in sample types, sample sizes, or the antibodies used. The IHC data for Sall4 expression in malignant versus normal tissues across several cancer types are complicated. Further research, including large cohorts of various cancer kinds and monitoring both Sall4 expression, is required, and somewhat inconsistent, suggesting that tumor type may affect these results.

In addition, our data about the correlation between Sall4 level and cancer grade partially agreed with the previous study, which found a low level of Sall4 mRNA in moderately differentiated CRC tissues compared to well-differentiated and poorly differentiated tissues, as measured by RT-PCR.24 These observations suggest that Sall4 could be used as a candidate biomarker for PCa prognosis, offering insights into its role in disease progression and differentiation. This current study, however, disagreed with another CRC study.[25] This discrepancy could be due to differences in cancer type and diagnostic methodologies, as RT-PCR was used in that study to quantify Sall4 mRNA. Furthermore, other research has reported no statistical association between Sall4 level and cancer grades in various malignancies.[21,26,27] These inconsistencies suggest that Sall4 may have varied roles in different cancers, potentially influenced by the specific cancer type and molecular environment.

The current results show a positive association between Sall4 expression and pathological stages, such as lymph node status and metastasis, suggesting its link to PCa progression. This finding suggests that SALL4 may influence tumor aggressiveness and metastatic potential more than primary tumor volume. SALL4 may play a greater role in promoting processes such as invasion, migration, and angiogenesis, which are more reflective of metastatic behavior than of

localized tumor growth. This finding is supported by other study which found that SALL4 knockdown impaired cell proliferation and migration but had limited effect on overall cell mass or apoptosis.[20] Thus, SALL4 may contribute to disease progression through mechanisms that are not directly dependent on tumor size, highlighting the importance of evaluating multiple tumor characteristics when assessing oncogenic drivers. This aligns with a previous CRC study, which found a correlation between Sall4 mRNA and lymph node metastases, but not clinical stage T.[24] Our findings were inconsistent with previous lung cancer[21,27],esophageal cancer[22] and hepatocellular carcinoma studies.[28] The absence of a correlation between SALL4 expression and tumor size suggests that Sall4 may be more relevant to tumor aggressiveness and metastatic potential rather than proliferative volume, aligning with findings in other cancers where it influences invasiveness independently of size.

Clinical approaches for diagnosing and prognosing PCa include IHC-based tumor biomarkers, clinical staging, Gleason scores, and PSA levels. IHC is widely used to identify key proteins in cells and tissues.[23,29] However,somevariables,suchastheselectionofantibodies, antibody concentrations, and antigen retrieval techniques, might affect IHC findings and cause variations in staining results.[30] The lack of a standardized IHC protocol further complicates its use, and non-specific staining can result in false-positive findings.30 Despite its limitations, IHC remains a clinically relevant and widely accessible method for protein localization. Despite its variability, IHC remains IHC remains a clinically relevant and widely accessible method for protein localization. We used RNAscope® to complement IHC and overcome its limitations.

Sall4 may play a role in PCa development and progression, as shown by consistent results from two antibodies and a Sall4 probe using high-specificity approaches. Combining RNAscope® and IHC provides more accurate expression data than IHC alone.

Conclusion

This study is the first to show a positive correlation between elevated Sall4 levels and Gleason grades and metastasis in PCa, suggesting Sall4's role in disease initiation and progression. Sall4 emerges as a potential biomarker for PCa, with diagnostic and prognostic value. Further research using additional prostate cancer cell lines, larger cohorts including more normal prostate tissue samples, and complementary molecular techniques such as RNA-sequencing and multivariate analyses will be essential to validate and extend our findings.

Ethical approval

The approval for conducting this retrospective study was obtained from the Ethics Board of Al Hussein Teaching Hospital in Thi-Qar, Iraq (approval number: 2021159, date: 7/12/2022). The study was conducted in accordance with applicable ethical guidelines, and patient confidentiality was strictly maintained throughout the research process.

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Conflict of interest

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Both Authors designed the study, collected data, conducted experiments and statistical analysis, and drafted the report, reviewed and edited the final text.

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