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ORIGINAL ARTICLE

Expression of APOA4 gene in urothelial bladder carcinoma: a potential diagnostic immunohistochemical biomarker

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ABSTRACT

Objective: To investigate the immunohistochemical expression of Apolipoprotein A-IV (APOA4) as a potential diagnostic and prognostic biomarker in urothelial bladder carcinoma (UBC).

Methods: This cross-sectional study was conducted from January 2020 to December 2022, at the Institute of Pathology and Diagnostic Medicine, Khyber Medical University, Peshawar, Pakistan. Formalin-fixed paraffin-embedded tumor tissues were stained with hematoxylin and eosin for histopathological classification and with APOA4 antibody for immunohistochemistry (IHC). Stained sections were evaluated microscopically using a blinded method, and APOA4 expression was quantified with a semi-quantitative Histoscore (0–300). Statistical analysis was performed using the Mann-Whitney U test, with diagnostic validity assessed by Receiver operating characteristic (ROC) curve analysis.

Results: A total of 121 participants were included (67 UBC patients, 54 controls). Among UBC cases, 31 (46%) were non-invasive low-grade papillary, 24 (36%) were non-invasive high-grade papillary, and 11 (16%) were invasive urothelial carcinoma. The mean proportion of APOA4-positive cells was 44% in cases versus 9% in controls, with mean Histoscores of 76 and 10, respectively ($p<0.0001$). ROC analysis showed an AUC of 0.79, with sensitivity and specificity varying according to cutoff thresholds.

Conclusion: APOA4 expression was significantly higher in UBC tissues compared to controls, with increasing expression correlating with tumor grade and stage. These findings suggest that APOA4 has potential diagnostic and prognostic value in UBC, warranting further validation in larger, prospective studies.

Keywords: Urinary Bladder Neoplasms (MeSH); Urothelial bladder carcinoma (Non-MeSH); Carcinoma, Transitional Cell (MeSH); Apolipoproteins A (MeSH); APOA4 (Non-MeSH); ApoA-IV (Non-MeSH); Biomarkers (MeSH); Diagnosis (MeSH); Point-of-Care Testing (MeSH); Neoplasam (MeSH).

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INTRODUCTION

Bladder cancer (BC) is the tenth most common malignancy worldwide and occurs more frequently in men than in women, accounting for nearly 170,000 deaths annually.[1] In Pakistan, it is the fourth most prevalent cancer.[2] Established risk factors include advanced age, male sex, cigarette smoking, obesity, and alcohol consumption.[3,4] Additional risks are linked to exposure to aromatic and polycyclic amine hydrocarbons, long-term use of analgesics, cyclophosphamide therapy, infection with Schistosoma haematobium, and prior pelvic irradiation. Most BC cases are urothelial carcinomas, which most commonly present with hematuria and urinary urgency.[5]

Urothelial carcinoma is subdivided by morphology and pathogenesis into papillary lesions (papilloma, low malignant potential, and papillary carcinoma) and flat lesions (urothelial carcinoma in situ and invasive).[6] For the initial diagnosis of bladder cancer, cystoscopic examination remains the standard, while histologic staging is a critical component of the diagnostic process.[7] Most patients (75-85%) present with non-muscle-invasive bladder cancer (NMIBC), primarily stage pTa (70%), pT1 (20%), and carcinoma in situ (CIS) (10%). Approximately 30% of NMIBC cases progress to muscle-invasive bladder cancer (MIBC), and up to 80% experience at least one recurrence.[8] The choice of intravesical therapy is influenced by tumor stage, grade, multifocality, and patient tolerability.[9] Bacillus Calmette-Guérin (BCG) instillation is the established gold standard adjuvant treatment for NMIBC.[10] In contrast, MIBC not amenable to endoscopic resection requires management with radical cystectomy.[11]

Cancer cells reprogram lipid metabolism to support membrane synthesis, generate lipid-derived second messengers, and provide energy. In addition, they secrete lipid metabolites that modulate immune cell function and contribute to a pro-tumorigenic microenvironment.[12] Apolipoproteins (APOs), the major protein components of lipoproteins, are central to lipid transport and metabolism.[13] By regulating processes such as apoptosis resistance, inflammation, angiogenesis, metastasis, and sustained cell proliferation, they play a pivotal role in cancer development and progression.[14]

Previous studies suggest that APOA4 may serve as a potential biomarker in ovarian cancer, hepatocellular carcinoma,[15] and oral cancers.[16] Several immunohistochemical (IHC) markers have been employed to aid in the diagnosis and prognostication of bladder cancer, including CK7, CK20, GATA3, p63, HMWCK, Uroplakin II, and thrombomodulin.[17] IHC biomarkers are particularly valuable in the differential diagnosis of glandular and spindle cell lesions, variants of urothelial carcinoma, and flat lesions.[18]

However, their sensitivity is limited in low-grade bladder cancer. For example, positive GATA3 staining of prostatic basal cells may create diagnostic confusion, [17] while CK20 and p63 show reduced sensitivity in high-grade urothelial carcinoma. Similarly, Uroplakin III and thrombomodulin exhibit poor sensitivity in high-grade tumors, limiting their clinical utility. [19] Given these limitations and the emerging evidence of APOA4 involvement in other malignancies, investigating its role in urothelial carcinoma may provide a more reliable diagnostic and prognostic marker. In this context, the present study aimed to establish immunohistochemical expression thresholds for APOA4 in cases of urothelial carcinoma.

METHODS

Ethical clearance was obtained from the Institutional Ethical Committee of Khyber Medical University, Peshawar (Ref: DIR/KMU-EB/DP/000619, dated April 30, 2019), and study approval was granted by the Advanced Studies and Research Board of Khyber Medical University (Ref: DIR/KMU-AS&RB/DP/000908, dated December 3, 2024). Following informed consent, 121 patients were enrolled between January 2020 and December 2022 at the Institute of Kidney Diseases, Hayatabad, Peshawar. Data were collected using a non-probability convenience sampling technique.

Biopsy samples were collected and transported according to standard protocols to the Histopathology Laboratory of the Institute of Pathology and Diagnostic Medicine, Khyber Medical University, for processing. Hematoxylin and eosin-stained sections of formalin-fixed, paraffin-embedded tumor specimens were examined independently by a consultant histopathologist and the primary author. Diagnoses were established in accordance with the 2016 WHO classification of urinary system tumors.[20]

IHC for APOA4 was performed on biopsy tissue sections using Abclonal's A9792 antibody, following the manufacturer's protocol. The procedure included tissue preparation and deparaffinization, antigen retrieval with specific buffer, blocking of endogenous peroxidase activity, incubation with the primary antibody, sequential washing, application of the secondary antibody, visualization with chromogen, counterstaining, dehydration, mounting, and microscopic examination. Digital image acquisition and analysis were subsequently carried out to evaluate APOA4 expression.[21]

The plasma-thrombin technique was utilized. Urine-containing tubes were centrifuged at 3000 rpm for ten minutes. Following centrifugation, blood plasma and thrombin were added to the sediment to form a cohesive pellet, and the supernatant was discarded. The pellet was subsequently fixed in 10% formalin and underwent processing akin to tissue samples. Paraffin-embedded Cell Block sections were stained with hematoxylin and eosin.

The immunohistochemically stained section slides were analyzed microscopically using a blinded method by a consultant histopathologist and the primary author. All findings were recorded in an Excel spreadsheet. A semi-quantitative Histoscore was calculated based on the percentage of cells showing negative, weakly stained, moderately stained, and strongly stained characteristics (scored as 0 ×% negative cells, 1×% weakly stained cells, 2×% moderately stained cells, and 3×% strongly stained cells, respectively). This Histoscore ranged from 0 to 300 and was subsequently subjected to statistical analysis.

Power analysis was performed using OpenEpi to calculate the minimum required sample size, with parameters set at 80% power ($\beta=0.20$), a 5% significance level ($\alpha=0.05$), and an expected effect size based on clinical relevance. This ensured adequate power to minimize Type II errors. Mean APOA4 expression levels in urothelial carcinoma and non-tumor tissues were compared using the Mann-Whitney U test. Sensitivity and specificity were calculated from Histoscores using standard formulas: specificity=true negatives / (true negatives+false positives), and sensitivity=true positives / (true positives+false negatives). Receiver operating characteristic (ROC) curves were generated in SPSS (version 24), plotting sensitivity against 1-specificity at varying thresholds, with the area under the curve (AUC) used to assess diagnostic accuracy. A p-value <0.05 was considered statistically significant.

RESULTS

A total of 121 participants were included, comprising 67 urothelial carcinoma cases and 54 controls. The mean age of the control group was 63.15±1.54 years (range: 28–98), while that of the patient group was 60.75±1.80 years. Among the 54 controls, 6 (11%) were female and 48 (89%) were male, whereas the patient group (n=67) included 14 females (21%) and 53 males (79%).

In the control group, cystitis was the most frequent finding (28/54; 51.9%), followed by urothelial hyperplasia (18/54; 33.3%). In the patient group, non-invasive low-grade papillary urothelial carcinoma was the predominant diagnosis (31/67; 46.3%), followed by non-invasive high-grade papillary urothelial carcinoma (24/67; 35.8%). Additional histopathological details are provided in Table I.

Tumor cells of urothelial carcinoma showing cytoplasmic immunohistochemical staining for APOA4 were considered positive (Table II). The mean percentage of APOA4-positive cells among cases was 44%, compared to 9% in controls. Likewise, the mean Histoscore for APOA4 expression was 76 in cases and 10 in controls.

Tumor cells of urothelial carcinoma displaying immunohistochemical staining of APOA4 in their cytoplasm were considered positive cells (Table III). The mean positivity was 44±39.2 in cases versus 9±28.9 in controls ($p<0.0001$). Similarly, the mean Histoscore was 76±78.5 in cases compared to 10±42.8 in controls ($p<0.0001$), indicating markedly elevated APOA4 expression in tumor tissues.

The sensitivity and specificity of APOA4 were evaluated using three positivity cutoffs: 5%, 10%, and 20% of cells showing any degree of staining (Figure 1). These thresholds were derived from (ROC) curve analysis. Sensitivity was

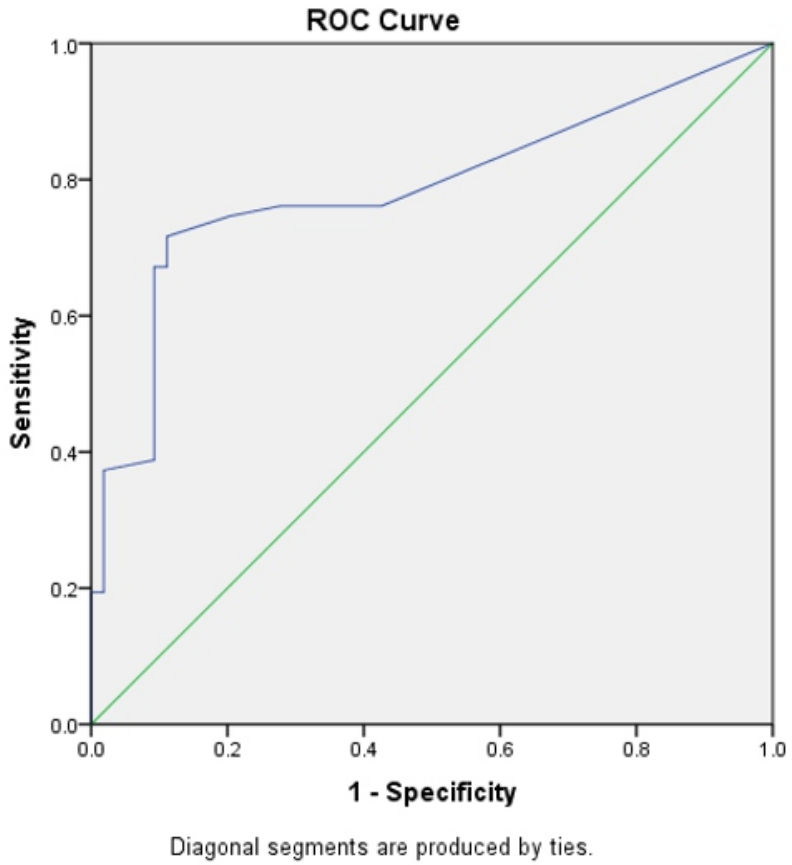

Figure 1: ROC curves based on Histoscore in case and control tissue immunohistochemical staining for APOA4.

plotted against 1- specificity to generate the ROC curve and corresponding coordinates (Figure 2). A part showing mild intensity of staining at 20X magnification, B part showing mild intensity of staining at 40X magnification, C part showing moderate intensity of staining at 20X magnification, D part showing moderate intensity of staining at 40X magnification, E part showing strong intensity of staining at 20X magnification. F part showing strong intensity of staining at 40X magnification, G part showing high-grade UC H&E 10x and H showing the APOA4 IHC Expression in the cytoplasm of tumor cells in urine specimen of high-grade U.C. 40X.

Each part highlights different levels of staining intensity, observed at specific magnifications, indicating the varied expression of APOA4 in the tumor cytoplasm.

The area under the curve was calculated to be 0.79 (95% CI: 0.75-0.83).

The diagnostic validity of a biomarker lies in its ability to accurately differentiate disease from non-disease states. Our results suggest that APOA4 holds promise; however, its clinical utility depends on establishing precise and validated cutoff values (Figure 3). This necessitates further validation in larger and more diverse cohorts to ensure consistent performance across populations and settings. Importantly, the choice of cutoff should reflect clinical context: a lower threshold with higher sensitivity may be preferable for screening, while higher specificity would be essential in confirmatory diagnostics to minimize false positives and unnecessary interventions.

APOA4 demonstrated a sensitivity of 76% and a specificity of 72% in detecting urothelial carcinoma, with a negative predictive value of 86% and a positive predictive value of 77%. The area under the ROC curve (AUC) was 0.79, indicating good diagnostic accuracy. However, the sensitivity of APOA4 varied across cutoff values. Lower thresholds improved sensitivity by capturing more true positives but reduced specificity, while higher thresholds increased specificity and reduced false positives at the expense of sensitivity. This trade-off, common in biomarker research, emphasizes the need to establish an optimal balance between sensitivity and specificity to minimize both false negatives and false positives for APOA4 to achieve clinical utility.

The sensitivities and specificities of APOA4 were calculated using three cutoffs (Tables III–VI). With increasing cutoff thresholds, specificity improved while sensitivity declined.

The positive likelihood ratio reflects the test's ability to confirm disease, whereas the negative likelihood ratio indicates its capacity to rule out disease. Positive and negative predictive values (PPV and NPV) provide the probabilities of true positive and true negative results, respectively, given the test outcome. Accuracy denotes the overall proportion of correctly classified cases (Table III).

Among 71 urine samples analyzed, APOA4 negativity was noted in 27 cases, of which 26 (93.3%) were urine cytology negative and 1 (3.7%) was cytology positive. APOA4 positivity was observed in 43 cases, comprising 23 (53.5%) cytology negative and 20 (46.5%) cytology positive samples (Table IV).

In comparison of APOA4 status between urine cell blocks and biopsy samples (n=71), 27 cases were negative (8 in urine cell blocks and 19 in biopsies), while 43 cases were positive (12 in urine cell blocks and 31 in biopsies) in urothelial bladder cancer patients (Table V).

Compared with established biomarkers for urothelial carcinoma, APOA4 must demonstrate either superior or at least comparable diagnostic performance. In the comparison of urine cell block and biopsy samples, APOA4 negativity was observed in 27 cases, including 8 (29.6%) detected in urine cell blocks and 19 (70.4%) in biopsy samples. APOA4 positivity was found in 43 cases, with 12 (53.5%) identified in urine cell blocks and 31 (46.5%) in biopsy

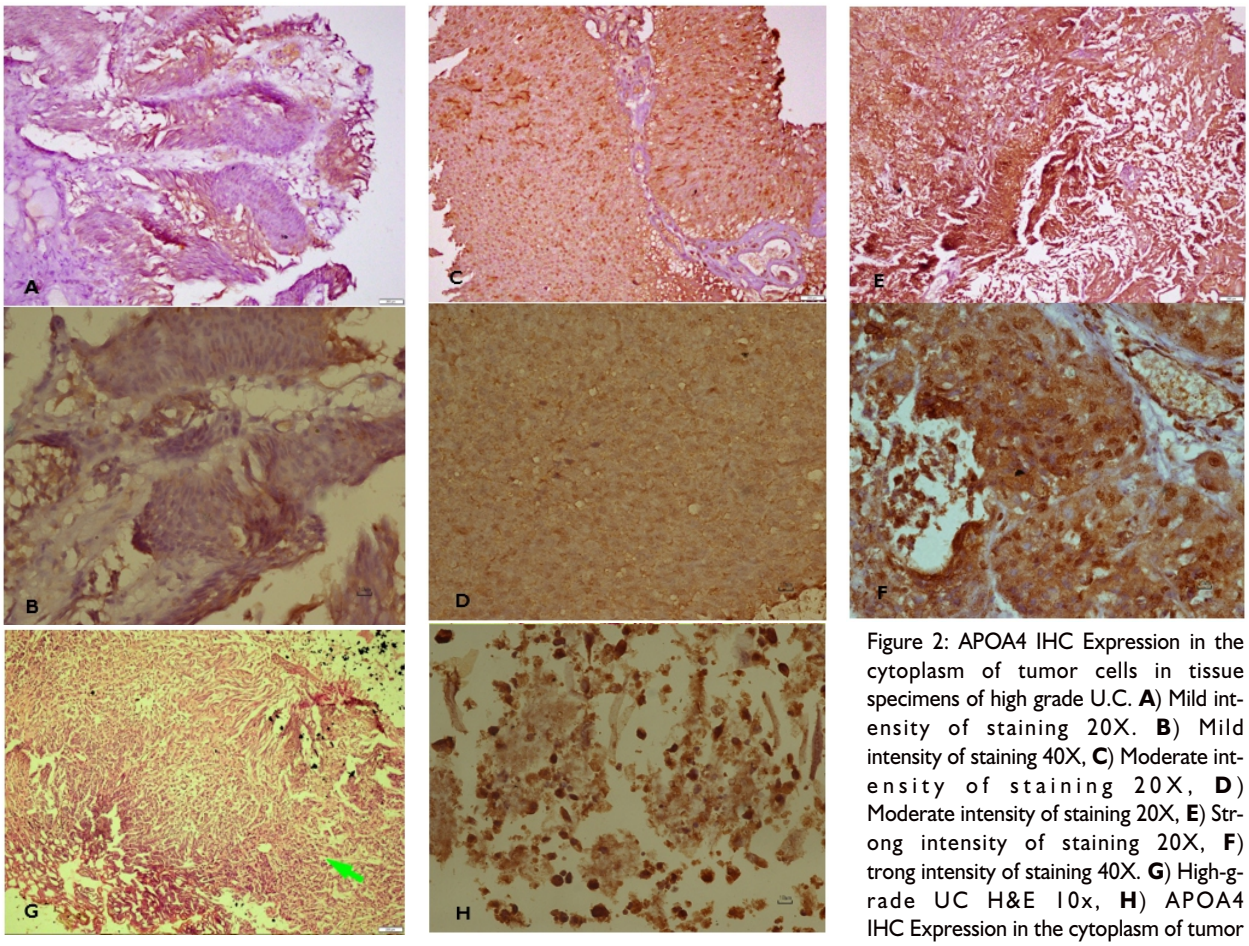

Figure 2: APOA4 IHC Expression in the cytoplasm of tumor cells in tissue specimens of high grade U.C. **A**) Mild intensity of staining 20X. **B**) Mild intensity of staining 40X, **C**) Moderate intensity of staining 20X, **D**) Moderate intensity of staining 20X, **E**) Strong intensity of staining 20X, **F**) trong intensity of staining 40X. **G**) High-grade UC H&E 10x, **H**) APOA4 IHC Expression in the cytoplasm of tumor cells in urine specimen of high-grade U.C. 40X.

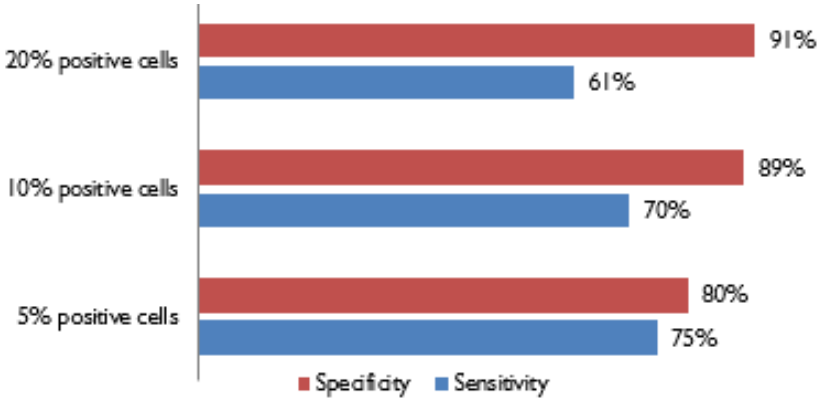

Figure 3: Sensitivity and specificity analysis at different cutoffs of positive tumor cells for immunostaining of APOA4

samples (Table VI). Standard markers such as cytokeratins and other molecular markers already play well-defined roles in diagnosis, so any new biomarker must provide added value, whether through greater accuracy, ease of application, cost-effectiveness, or improved detection of specific bladder carcinoma subtypes.

DISCUSSION

This study provides important insights into the potential of APOA4 as a diagnostic biomarker for urothelial bladder carcinoma. Our findings demonstrate that the cutoff values used to define positivity significantly influence the sensitivity and specificity of APOA4, emphasizing the need to optimize these thresholds to achieve maximum diagnostic accuracy. Comparable results have been reported in other populations; for instance, a study from Tehran involving 106 urothelial carcinoma cases reported a mean age of 62.98 years (range: 38-89 years),[22] while a study from China found a median age of 65 years (range: 24-92 years).[23] These findings are consistent with our study, where the mean age was 61.1 years (range: 28-98 years). We observed a marked male predominance, with males nearly five times more affected than females (101 vs. 20). Similar trends were reported in studies from Tehran, which documented 85 male and 21 female patients, and from China, where the male-to-female ratio was 2:1.[22, 23] In this study, 82% of cases were diagnosed as non-invasive urothelial carcinomas, while 16.5% were invasive at presentation. In comparison, a study from Tehran reported 59.4% non-

Table I: Histopathological distribution of urothelial findings in control and patient groups

Group (n)	Diagnosis	Frequency (%)
Control (n=54)	Cystitis	28 (51.9)
	Urothelial hyperplasia	18 (33.3)
	Normal urothelium	8 (14.8)
Patients (n=67)	Non-invasive low-grade papillary urothelial carcinoma	31 (46.3)
	Non-invasive high-grade papillary urothelial carcinoma	24 (35.8)
	Invasive urothelial carcinoma	11 (16.4)
	Papillary urothelial neoplasm of low malignant potential	1 (1.5)

N: Frequency

Table II: Immunohistochemical staining of APOA4 in urothelial carcinoma

APOA4	Statistics	Cases	Control	p-value*
Positivity	Mean±SD	44±39.2	9±28.9	<0.0001
	Median	43 (0-48.75)	0 (0-1)	
Histoscore	Mean	76±78.5	10±42.8	<0.0001
	Median	35 (0-60)	0 (0-.75)	

*Mann Whitney U Test; SD=Standard Deviation

Table III: Sensitivity, specificity, negative predictive value, positive predictive value, likelihood ratio of positive and negative test results of APOA4 Immunohistochemical staining of cases and control subjects

Cut Offs	Sensitivity	Specificity	PPV	NPV	+likelihood ratio	likelihood ratio
5%+cells	75%	80%	82%	72%	3.66	0.32
10%+cells	70%	89%	89%	71%	6.31	0.34
20%+cells	61%	91%	92%	65%	6.61	0.43

NPV: Negative predictive value, PPV: positive predictive value

Table IV: Status of APOA4 immunocytochemistry in urine samples in urothelial bladder cancer patients

Urine Cell Block	Urine Cytology Negative	Urine Cytology Positive	Total
APOA4 Negative	26 (93.3%)	1 (3.7%)	27
APOA4 Positive	23 (53.5%)	20 (46.5%)	43
APOA4 inconclusive	1 (100%)	0 (0%)	1
Total	50 (70.4%)	21 (29.6%)	71

invasive and 40.5% invasive cases, whereas another study documented 56.2% non-invasive tumors, 30% invasive tumors, and 13.8% with unknown pathological stage at the time of biopsy.[22,23] Our findings are consistent with recent studies evaluating the diagnostic role of APOA4 in urothelial bladder carcinoma. Tumor cells demonstrated cytoplasmic immunohistochemical staining with intensities ranging from weak to strong, a pattern also reported by KumarP, et al., (2015).[24] Soukup V, et al., (2019) reported comparable diagnostic performance of APOA4 as a biomarker for urothelial bladder carcinoma. Using the ELISA technique on urine samples, they demonstrated a sensitivity of 55.6% and a specificity of 83.3% in detecting urothelial carcinoma.[25] Furthermore, the positive and negative predictive values observed in our study were comparable to those reported by Soukup V, et al., (2019), who demonstrated a PPV of 55.6% and an NPV of 83.3% for APOA4 in detecting urothelial bladder carcinoma.[25] In contrast, Kumar P, et al., (2015) reported higher diagnostic performance, with sensitivity and specificity exceeding 90%.[24] This discrepancy may be attributed to their use of a combination biomarker model rather than APOA4 alone, employing ELISA and western blot techniques for validation.

The positive and negative likelihood ratios observed in our study further support the diagnostic potential of APOA4. These results are in line with Soukup V, et al. (2019), who reported likelihood ratios of 3.33 (positive) and 0.53 (negative) for APOA4 in evaluating its diagnostic validity.[25] Beyond bladder cancer, APOA4 has also been explored in other malignancies. In ovarian cancer, it demonstrated low sensitivity (40.8%), leading investigators to conclude that it was not a sufficiently reliable diagnostic marker.[15] In hepatocellular carcinoma, an Indian study found APOA4 expression positively correlated with viral load, ALT, AST, INR, albumin, and bilirubin compared with controls.[16] Additionally, a study from Taiwan suggested APOA4 as a promising diagnostic biomarker for oral cancer, reporting high sensitivity (83.3%) and specificity (89.7%).[23]

The IHC biomarkers GATA3, Uroplakin, and p63 are widely used to further characterize bladder cancer, while CK7, CK20, and PSA help distinguish prostatic adenocarcinoma from bladder cancer.[26] However, limitations exist due to variability in sensitivity and specificity, particularly in high-grade urothelial carcinoma, where the accuracy of these markers in differentiating urothelial carcinoma from primary prostatic adenocarcinoma has been questioned.[27] Reported sensitivity and specificity for GATA3 range from 70% to 83.3% and 62% to 80.9%, respectively.[28,29] For p63, different studies have reported sensitivity between 83.3% and 85%

Table V: Comparison of APOA4 status in a urine cell block with biopsy sample in urothelial bladder cancer patients

Variables	Urine Cell Block	Biopsy Sample	Total
APOA4 Negative	8 (29.6%)	19 (70.4%)	27
APOA4 Positive	12 (53.5%)	31 (46.5%)	43
APOA4 inconclusive	1 (100%)	0 (0%)	1
Total	21 (29.6%)	50 (70.4%)	71

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APOA4 inconclusive	1 (100%)	0 (0%)	1
Total	21 (29.6%)	50 (70.4%)	71

and specificity between 66.6% and 100%.[29,30] In this context, the sensitivity and specificity values observed for APOA4 in our study are comparable to those of these established IHC biomarkers, supporting its potential diagnostic and prognostic utility.

Limitations of the study

This study has certain limitations. Participant recruitment was constrained by financial challenges and COVID-related restrictions, which affected sample size, though it remained adequate for preliminary analysis, its broader applicability requires confirmation in larger cohorts. Selection bias may also have influenced results, as only patients presenting to the outpatient department with hematuria were included. Although measures were taken to control confounding variables, complete elimination of bias is difficult. Future studies with larger and more diverse populations are needed to validate these findings.

CONCLUSION

Our study highlights the potential of APOA4 as a diagnostic biomarker for urothelial bladder carcinoma. The observed variability in sensitivity and specificity at different cutoff values emphasizes the need for validation and optimization. With large-scale prospective studies to establish optimal thresholds, APOA4 could serve as a valuable diagnostic tool, contributing to earlier detection and improved patient outcomes. Combining APOA4 with other biomarkers in a multi-marker panel may enhance diagnostic accuracy, as multimodal approaches often outperform single markers. Further exploration of the biological role of APOA4 in urothelial bladder carcinoma could provide deeper insights into its function, refine its diagnostic value, and uncover potential therapeutic targets through a better understanding of the molecular mechanisms underlying its expression and association with disease progression.

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AUTHORS' CONTRIBUTION

The Following authors have made substantial contributions to the manuscript as under:

NS: Conception, acquisition, analysis and interpretation of data, drafting the manuscript, approval of the final version to be published

WS, AA: Study design, analysis and interpretation of data, drafting the manuscript, approval of the final version to be published

TAK: Conception and study design, critical review, approval of the final version to be published

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

Authors declared no conflict of interest, whether financial or otherwise, that could influence the integrity, objectivity, or validity of their research work.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

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