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Intratumoral Leptin Expression and Tissue-Derived LEP rs2167270 Genotype Are Associated with Adverse Pathological Features and Substantial Prognosis in Sudanese Bladder Carcinoma

Bushra M. Bushra[1], Mohammed S. Abdelaziz[2], Alkhair A. Idris[3], Habsa A. A. Amshahar[4], Zeinab A. Mohamed[5], Ogail Y. Dawod[6], Mahmoud A. Aloriby[7,8], Yousef M. Hasen[9], Ahmed A. Alhaddad[8,10], Jummah O. Alsaity[8], Ahmed S. Mikael[11], Tarek S. Bader[12], Farag A. Bleiblo[13]*

[1]Department of Histopathology and Cytology, Faculty of Graduate Studies and Scientific Research, National University, Khartoum, Sudan
[2]Department of Histopathology and Cytology, College of Medical Laboratory Sciences, Sudan University of Science and Technology, Khartoum, Sudan
[3]Department of Histopathology and Cytology, College of Medical Laboratory Sciences, Ahfad University for Women, Khartoum, Sudan
[4]Pathology Department, Faculty of Medicine, Sirte University, Sirte, Libya
[5]Department of Histopathology and Cytology, College of Medical Laboratory Sciences, Shendi University, Khartoum, Sudan
[6]Department of Physical Therapy, College of Nursing and Health Sciences, Jazan University, Jazan, Saudi Arabia
[7]Department of Pathology, University Medical Center, Libyan International Medical University, Benghazi, Libya
[8]Department of Cytotechnology, Faculty of Biomedical Sciences, University of Benghazi, Benghazi, Libya
[9]Department of Pathology, Faculty of Dentistry, University of Zawia, Zawia, Libya
[10]Department of Cytogenetics, Faculty of Applied Medical Sciences, Libyan International Medical University, Benghazi, Libya
[11]Research Centre, University Medical Center, Libyan International University, Benghazi, Libya
[12]Department of Zoology, Faculty of Science, Sirte University, Sirte, Libya
[13]Department of Microbiology, Faculty of Science, University of Benghazi, Benghazi, Libya
Email: *farag.bleiblo@uob.edu.ly

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Abstract

Bladder carcinoma shows marked pathological and molecular heterogeneity, and tissue-based molecular markers may help identify tumors with aggressive pathological features. In African populations, including Sudan, molecular pathology data on bladder carcinoma remain limited. This study assessed whether intratumoral leptin expression and tissue-derived *LEP* rs2167270 genotype were associated with histological grade, pathological pT category, muscle invasion, and lymphovascular invasion in Sudanese bladder carcinoma. This retrospective, multicenter, tissue-based study included 153 formalin-fixed, paraffin-em-

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bedded bladder carcinoma specimens from four Sudanese institutions. Leptin expression was evaluated by immunohistochemistry and classified using the immunoreactive score. LEP rs2167270 was genotyped by PCR-RFLP using amplifiable FFPE tumor DNA. Assay reliability was evaluated by repeat genotyping and Sanger sequencing. The cohort was predominantly male (120/153, 78.4%), and urothelial carcinoma was the main tumor type (136/153, 88.9%). High-grade tumors were found in 49/136 urothelial carcinomas (36.0%). Muscle invasion was found in 54/149 tumors (36.2%), and lymphovascular invasion was present in 56/149 tumors (37.6%). Moderate-to-strong leptin immunoreactivity was more frequent in high-grade than low-grade tumors (61.2% vs 32.2%; $p = 0.004$), T2-or-higher than Ta tumors (64.8% vs 32.7%; $p = 0.003$), muscle-invasive than non-muscle-invasive tumors (64.8% vs 30.5%; $p < 0.001$), and lymphovascular invasion-positive tumors (64.3% vs 30.1%; $p < 0.001$). Valid tissue-derived LEP rs2167270 genotypes were obtained in 127 cases. The GG genotype was more frequent with stronger leptin staining ($p = 0.005$) and showed distributional differences by grade, pT category, muscle invasion, and lymphovascular invasion; however, the pT-category pattern did not show a clear stepwise increase in GG frequency with increasing pT category. PCR-RFLP was concordant with Sanger sequencing in 30 cases. Higher intratumoral leptin expression and tissue-derived LEP rs2167270 GG genotype were associated with adverse pathological features. Studies with matched normal DNA, larger African cohorts, clinical follow-up, and functional validation are needed to determine whether the tissue-derived genotype reflects germline variation, biological relevance, and prognostic value.

Keywords

Bladder Carcinoma, Leptin, LEP rs2167270, Immunohistochemistry, Genotype, Tumor Biomarker

1. Introduction

Urinary bladder carcinoma is a common urological cancer and represents a significant global disease burden [1] [2]. Over 600,000 new cases and over 220,000 deaths were reported globally in 2022, with a clear male predominance [1] [2]. Bladder cancer is biologically and clinicopathologically heterogeneous, ranging from non-muscle-invasive tumors with variable risks of recurrence and progression to muscle-invasive tumors with a higher risk of local and metastatic spread [3] [4]. Histological grade, pathological stage, muscle invasion status, and lymphovascular invasion remain central pathological markers of aggressive tumor behavior and are essential for treatment decisions and risk stratification [3] [4].

In routine practice, clinical diagnosis and risk assessment rely strongly on conventional histopathology; however, microscopic morphology does not capture the underlying molecular and phenotypic heterogeneity of bladder carcinoma [3] [5]. Tumors with similar microscopic features may exhibit different molecular signa-

tures that support invasion, progression, and dissemination [5] [6]. This limitation has promoted investigation into tissue-based biomarkers that may add biological information to routine histopathological evaluation, especially in high-risk tumors where conventional pathological features do not completely explain tumor behavior [5]-[7].

In addition to its role in energy homeostasis, leptin is a pleiotropic adipokine with diverse biological effects [8] [9]. Leptin and leptin receptor signaling have been linked to cell proliferation, inflammatory signaling, angiogenesis, invasion, migration, and tumor microenvironment remodeling across distinct malignancies [8] [9]. However, these effects are not uniform across tumor types. In pathology-based bladder cancer studies, leptin may be better regarded as a candidate tissue marker associated with an aggressive tumor phenotype rather than as an independent predictor of clinical outcome [8] [9].

Evidence in bladder cancer remains limited. Tissue-based studies suggest that malignant urothelium may show increased leptin expression and that higher expression may be associated with pathological features such as higher pT category, muscle invasion, lymphovascular invasion, and adverse tumor features [10] [11]. Genetic evidence is more limited. The regulatory LEP rs2167270 polymorphism has been implicated in bladder cancer susceptibility and selected tumor features in a case-control study [12]. However, current evidence remains largely derived from non-African populations, with limited tissue-based leptin studies, sparse data linking LEP rs2167270 with bladder cancer pathology, and few studies with paired tumor-normal DNA, survival or recurrence follow-up, or validation cohorts [10]-[12].

We conducted a retrospective multicenter, tissue-based analysis of archived FFPE bladder carcinoma specimens from Sudanese patients. Leptin immunohistochemical expression and tissue-derived LEP rs2167270 genotype were evaluated in relation to histological grade, pathological pT category, muscle invasion, and lymphovascular invasion. These variables were used as pathological features associated with tumor aggressiveness rather than survival outcomes. The study also aimed to provide molecular and pathological data from an African population underrepresented in cancer biomarker research.

2. Materials and Methods

2.1. Study Design and Setting

This retrospective, cross-sectional, multicenter study used archived FFPE urinary bladder carcinoma specimens from Sudan. Tumor leptin expression was assessed by immunohistochemistry, and tissue-derived LEP rs2167270 genotype was evaluated using DNA from the same FFPE tumor blocks. Four centers were included based on urologic oncology case volume and availability of suitable archival tissue: Ibn Sina Hospital, Omdurman Military Hospital, Atbara Medical Complex, and El-Mak Nimr University Hospital. The source archives included FFPE bladder carcinoma blocks from cases diagnosed and stored from 2015 onward. The origi-

nal tissue specimens had been collected for routine diagnostic care and were not collected prospectively for this study. Research access to archived FFPE blocks and pathology records was performed under Sudanese university/institutional authorization, while the subsequent coded collaborative analysis and manuscript reporting were approved by the Institutional Review Board/Research Ethics Committee of Libyan International Medical University, Benghazi, Libya (LIMU-IRB-BC-2024-002; 19 December 2024). Cases were identified from the pathology archives of each participating center. Because this was a retrospective study based on archived FFPE material, sampling was availability-based rather than population-based. Cases were not selected according to leptin staining, LEP rs2167270 genotype, tumor grade, pT category, muscle invasion, or lymphovascular invasion. Within each archive, retrievable bladder carcinoma cases were screened against the predefined eligibility criteria, and cases were included when a representative FFPE tumor block was available, and tissue quality was sufficient for histopathological review, immunohistochemistry, and molecular analysis.

2.2. Case Selection and Eligibility Criteria

Eligible cases had histologically confirmed urinary bladder carcinoma and a representative FFPE block suitable for histopathological, immunohistochemical, and molecular analyses. All histological types were eligible, with no restriction by age or sex. No cancer-free control tissue, matched blood, buccal sample, or adjacent non-tumor tissue was available. Cases were excluded if poor fixation, inadequate preservation, extensive necrosis, or insufficient tumor tissue could affect pathological or molecular assessment. The final cohort therefore represents an availability-based archival series of technically suitable FFPE bladder carcinoma specimens. This approach may introduce selection bias related to archive completeness, block retrieval, and tissue preservation; this limitation was considered when interpreting the findings.

2.3. Specimens Processing and Sectioning of Tissues

Six serial sections were prepared from each FFPE block: one 3-μm section for haematoxylin and eosin staining, two 4-μm sections on positively charged slides for leptin immunohistochemistry, and three 10-μm sections in sterile DNase- and RNase-free tubes for DNA extraction. Histopathology, leptin immunohistochemistry, and tissue-derived LEP rs2167270 genotyping used archival tissue from the same case.

2.4. Histopathological Review and Pathological Endpoint Definitions

Histopathological diagnosis, tumor grade, depth of invasion, muscularis propria involvement, and lymphovascular invasion were reviewed on H&E-stained sections by a pathologist experienced in genitourinary pathology. Histological grading followed the 2022 World Health Organization classification. Pathological pT

category was assigned from TURBT sections according to the T component of the AJCC TNM 8th edition. Tumors were classified as Tis, Ta, T1, or T2 or higher. Further T3/T4 subdivision was not performed because TURBT specimens alone cannot reliably assess this. The main pathological endpoints were histological grade, pathological pT category, muscle invasion, and lymphovascular invasion. Because survival and follow-up data were unavailable, these endpoints were analyzed as pathological features associated with tumor aggressiveness, not as prognostic outcomes.

2.5. Leptin Immunohistochemistry

Leptin immunohistochemistry was performed on 4-μm FFPE sections using the Leica BOND-MAX system and Leica BOND Polymer Refine Detection Kit. Sections underwent deparaffinization, heat-mediated antigen retrieval for 20 minutes at 97˚C using Bond Epitope Retrieval Solution 1 (ER1; citrate-based, pH 6.0), endogenous peroxidase blocking, and DAB detection. A rabbit polyclonal anti-leptin antibody (GeneTex, USA; GTX37638) was used at 1:600 for 30 minutes at room temperature. Placental tissue served as positive control, and negative controls replaced the primary antibody with antibody diluent.

2.6. Immunohistochemical Scoring, Blinding, and Reproducibility

Leptin expression was assessed as cytoplasmic immunoreactivity using the German Immunoreactive Score. One representative tumor-bearing section was selected per case. Five viable high-power fields were scored, excluding necrosis, crush artifact, hemorrhage, and edge artifact. At least 200 tumor cells were assessed per case. IRS was calculated by multiplying the percentage score by staining intensity. Percentage scores were 0, 1, 2, 3, and 4 for no positive cells, <10%, 10% - 50%, 51% - 80%, and >80%, respectively; intensity scores were 0, 1, 2, and 3 for negative, weak, moderate, and strong staining. IRS values were grouped as negative (0), weak (1 - 3), moderate (4 - 8), and strong (9 - 12). Slides were scored by an experienced pathologist blinded to molecular and clinicopathological data. Intraobserver reproducibility was assessed by repeat scoring of 23 randomly selected leptin-stained cases by the same observer after completion of the initial scoring round. The repeat assessment was performed blinded to the original IRS category, LEP rs2167270 genotype, and clinicopathological data. Agreement was assessed by exact percent agreement for the four IRS-based categories: negative, weak, moderate, and strong.

2.7. DNA Extraction from FFPE Tissue

Genomic DNA was extracted from three 10-μm unstained FFPE sections obtained from the representative tumor block selected for each case using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany), according to the manufacturer's instructions. The corresponding H&E-stained section was reviewed to confirm the presence of viable tumor and to avoid blocks dominated by necrosis, hemorrhage,

cautery artifact, or insufficient tumor tissue. Manual macrodissection was not performed; therefore, DNA was extracted from whole FFPE tumor sections. Cases were accepted for genotyping when the selected block contained adequate viable tumor for molecular analysis on histological review and produced amplifiable DNA. Cases with insufficient tumor tissue, poor preservation, extensive necrosis, or failed PCR amplification were excluded from genotype analysis. Because matched blood, buccal, or adjacent non-tumor tissue was not available, genotype results are reported as tissue-derived LEP rs2167270 genotypes rather than confirmed germline genotypes.

2.8. Tissue-Derived LEP rs2167270 Genotyping by PCR-RFLP

LEP rs2167270, also referred to as LEP G19A or −19G>A, was genotyped by PCR-RFLP. PCR was performed using DreamTaq Green PCR Master Mix in a 20-µL reaction containing 50 ng DNA. The primers were 5'-CCCGCGAGGTGCACACTG-3' and 5'-AGGAGGAAGGAGCGCGCC-3', generating a 353-bp amplicon. PCR products were digested with MspA1I for 4 h at 37˚C. The G allele yields 294-bp and 59-bp fragments, whereas the A allele remains undigested at 353 bp. Fragments were resolved on 2% agarose gels. The 59-bp fragment was interpreted cautiously because it may be faint or poorly resolved. Results are reported as tissue-derived LEP rs2167270 genotypes from FFPE tumor DNA, not confirmed germline genotypes. Fifteen randomly selected samples were re-genotyped by an independent blinded investigator.

2.9. Sanger Sequencing Confirmation

To verify tissue-derived LEP rs2167270 genotyping by PCR-RFLP, 30 randomly selected samples were analyzed by Sanger sequencing. PCR used DreamTaq Green PCR Master Mix with cycling conditions of 95˚C for 3 min; 35 cycles of 95˚C for 30 s, 58˚C for 30 s, and 72˚C for 1 min; followed by 72˚C for 7 min. Amplicons were purified using ExoSAP-IT and sequenced in the forward direction using BigDye Terminator v3.1 chemistry. The sequencing primer was 5'-CTGGAGGGACATCAAGGATTT-3'. Sequencing was performed on a SeqStudio Genetic Analyzer, and chromatograms were analyzed using BioEdit version 7.7.1.

2.10. Variables, Endpoints, and Analytical Subsets

Clinical and pathological data were extracted from hospital records and pathology reports into a structured database. Baseline variables included age, sex, and histological subtype. Main variables were leptin expression and tissue-derived LEP rs2167270 genotype. Leptin expression was analyzed as negative, weak, moderate, or strong; genotype was analyzed as AA, GA, or GG. The main pathological endpoints were histological grade, pathological pT category, muscle invasion, and lymphovascular invasion. Histological grade was evaluated in 136 urothelial carcinoma cases. Pathological pT category, muscle invasion, and lymphovascular invasion were evaluated in 149 cases; 4 cases were excluded because of poor or ab-

sent histopathological representation. Valid tissue-derived LEP rs2167270 genotyping was obtained in 127 cases; 26 cases were excluded because of insufficient or poor-quality DNA. Genotype-based clinicopathological analyses included 111 cases for histological grade and 123 cases for pathological pT category, muscle invasion, and lymphovascular invasion.

2.11. Statistical Analysis

Analyses were performed using IBM SPSS Statistics version 23. Categorical variables were summarized as frequencies and percentages, with 95% confidence intervals for selected baseline proportions. Associations were assessed using Pearson's chi-square test or Fisher's exact test, as appropriate. Fisher's exact test was used to assess leptin expression and pT category because of sparse Tis counts. For selected binary comparisons, odds ratios with 95% confidence intervals were calculated. Negative/weak leptin staining was grouped as lower expression and moderate/strong staining as higher expression. The association between tissue-derived LEP rs2167270 genotype and pT category was evaluated using a fixed-margin Monte Carlo exact test because of sparse cells. Cramér's V estimated association strength. All tests were two-sided, with $p < 0.05$ considered statistically significant. Because this was exploratory, p values were interpreted as evidence of association, not proof of causality or prognostic value. No formal correction for multiple testing was applied. Each analysis used the subset with complete data. To assess whether genotyping attrition could have biased genotype-pathology associations, key pathological features were compared between cases with successful LEP rs2167270 genotyping and cases excluded because of insufficient or poor-quality FFPE DNA. Comparisons included histological subtype, urothelial carcinoma grade, pathological pT category, muscle invasion, and lymphovascular invasion. Fisher's exact test was used for binary comparisons because the genotyping-failure group was small. Because the cohort included urothelial carcinoma, squamous cell carcinoma, and adenocarcinoma, sensitivity analyses were performed after restricting the cohort to urothelial carcinoma only. These analyses were used to assess whether the associations between leptin immunohistochemical expression, tissue-derived LEP rs2167270 genotype, and pathological features were influenced by pooling different histological subtypes. Histological grade analyses were already restricted to urothelial carcinoma because grading was applicable only to urothelial carcinoma in this cohort. For pT category, muscle invasion, lymphovascular invasion, and leptin-genotype associations, the primary analyses were repeated after excluding squamous cell carcinoma and adenocarcinoma.

2.12. Ethics Approval and Use of Archived Human Tissue

This retrospective multicenter study used archived FFPE urinary bladder carcinoma tissue blocks and coded clinicopathological data from Sudanese patients. Written institutional authorization for sample collection was issued by the Faculty of Medical Laboratory Sciences, National University-Sudan; the available letters

were dated 18 September 2022, 20 October 2022, and 26 November 2024. Ethical approval for the Libyan-Sudanese collaborative retrospective analysis and reporting was obtained from the Institutional Review Board/Research Ethics Committee of Libyan International Medical University, Benghazi, Libya (LIMU-IRB-BC-2024-002; 19 December 2024). The study involved no direct patient contact, intervention, or prospective collection of identifiable personal data. Based on the retrospective design and coded archived tissue, the Committee approved the study without requiring patient contact. Patient-identifying information was not included in the database, figures, tables, or manuscript. The study followed the Declaration of Helsinki and relevant institutional regulations.

3. Results

3.1. Study Cohort and Clinicopathological Characteristics

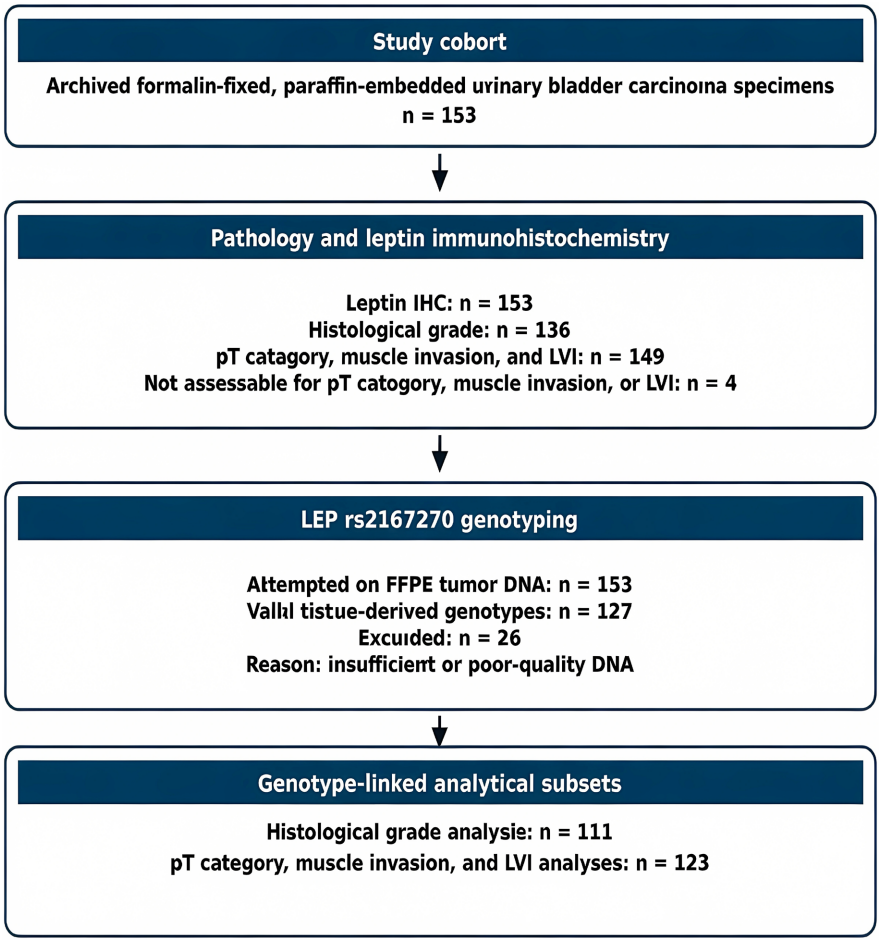


Figure 1. Study cohort and analytical subsets. Flow chart showing the archived FFPE urinary bladder carcinoma specimens included in the study and the subsets available for pathological review, leptin immunohistochemistry, and tissue-derived LEP rs2167270 genotyping. The chart shows the number of cases available for each endpoint and the number excluded from genotyping because of insufficient or poor-quality DNA. Abbreviations: FFPE, formalin-fixed, paraffin-embedded; IHC, immunohistochemistry; LEP, leptin gene; LVI, lymphovascular invasion; pT, pathological primary tumor category.

Table 1. Clinicopathological characteristics and analytical availability in the urinary bladder carcinoma cohort. Data are shown as n/N (%). Percentages were calculated using the denominator shown for each variable. The 95% CIs are binomial confidence intervals. Abbreviations: CI, confidence interval; FFPE, formalin-fixed, paraffin-embedded; LEP, leptin gene; pT, pathological tumor category. Tumor grade was assessed only in urothelial carcinoma cases. Pathological pT category, muscle invasion, and lymphovascular invasion were assessable in 149 cases. Valid tissue-derived LEP rs2167270 genotyping was obtained in 127/153 cases. For genotype-based clinicopathological analyses, 111 cases were evaluable for histological grade and 123 cases were evaluable for pathological pT category, muscle invasion, and lymphovascular invasion.

Domain	Category	n/N (%)	95% CI
Sex	Male	120/153 (78.4)	71.3 - 84.2
Sex	Female	33/153 (21.6)	15.8 - 28.7
Age group, years	18 - 39	8/153 (5.2)	2.7 - 10.0
Age group, years	40 - 59	68/153 (44.4)	36.8 - 52.4
Age group, years	>59	77/153 (50.3)	42.5 - 58.1
Histological subtype	Urothelial carcinoma	136/153 (88.9)	82.9 - 92.9
Histological subtype	Squamous cell carcinoma	9/153 (5.9)	3.1 - 10.8
Histological subtype	Adenocarcinoma	8/153 (5.2)	2.7 - 10.0
Tumor grade*	Low grade	87/136 (64.0)	55.6 - 71.6
Tumor grade*	High grade	49/136 (36.0)	28.4 - 44.4
Pathological pT category†	Ta	52/149 (34.9)	27.7 - 42.8
Pathological pT category†	Tis	5/149 (3.4)	1.4 - 7.6
Pathological pT category†	T1	38/149 (25.5)	19.2 - 33.1
Pathological pT category†	T2 or higher	54/149 (36.2)	29.0 - 44.2
Muscle invasion†	Absent	95/149 (63.8)	55.8 - 71.0
Muscle invasion†	Present	54/149 (36.2)	29.0 - 44.2
Lymphovascular invasion†	Absent	93/149 (62.4)	54.4 - 69.8
Lymphovascular invasion†	Present	56/149 (37.6)	30.2 - 45.6
Genotyping	Valid tissue-derived LEP rs2167270 genotyping	127/153 (83.0)	76.3 - 88.1

*Tumor grade was evaluated only in urothelial carcinoma; †Pathological pT category, muscle invasion, and lymphovascular invasion were assessable in 149 cases.

A total of 153 histologically confirmed urinary bladder carcinoma cases were included. The 153 cases were contributed by Ibn Sina Hospital (n = ___), Omdurman Military Hospital (n = ___), Atbara Medical Complex (n = ___), and El-Mak Nimr University Hospital (n = ___). Center-specific percentages were calculated using the full cohort denominator of 153 cases. The cohort structure and analytical subsets are shown in Figure 1, and baseline clinicopathological characteristics are summarized in Table 1. The cohort was predominantly male (120/153, 78.4%), with a male-to-female ratio of 3.6:1. Patient age ranged from 26 to 89 years. The largest age group was >59 years (77/153, 50.3%), followed by 40 - 59 years (68/153, 44.4%) and 18 - 39 years (8/153, 5.2%). Urothelial carcinoma was the most common histological subtype (136/153, 88.9%), followed by squamous cell carcinoma (9/153, 5.9%) and adenocarcinoma (8/153, 5.2%).

Histological grade was assessed only in urothelial carcinoma and was available for 136 cases; 87/136 tumors (64.0%) were low grade and 49/136 (36.0%) were high grade. Pathological pT category was available for 149/153 cases (97.4%): 52/149 (34.9%) were Ta, 5/149 (3.4%) were Tis, 38/149 (25.5%) were T1, and 54/149 (36.2%) were T2 or higher. Muscle invasion was present in 54/149 cases (36.2%), and lymphovascular invasion was detected in 56/149 cases (37.6%). Four cases were not assessable for these pathological variables because of inadequate muscularis propria and/or insufficient representative tumor tissue. Valid tissue-derived LEP rs2167270 genotyping was obtained in 127/153 cases (83.0%). For genotype-based clinicopathological analyses, 111 cases were evaluable for histological grade and 123 cases for pathological pT category, muscle invasion, and lymphovascular invasion.

3.2. Leptin Immunohistochemical Expression and Pathological Features

Leptin immunohistochemical expression differed across pathological subgroups of urinary bladder carcinoma (Table 2). Representative sections showed cytoplasmic leptin staining in tumor cells, with brown DAB reactivity and hematoxylin nuclear counterstain. Moderate-to-strong leptin expression was detected in 30/49 high-grade urothelial carcinomas (61.2%) and 28/87 low-grade urothelial carcinomas (32.2%). By pathological pT category, moderate-to-strong leptin expression was most frequent in T2-or-higher tumors (35/54, 64.8%). Ta and T1 tumors were more often negative or weakly positive (35/52, 67.3%; and 26/38, 68.4%, respectively). None of the Tis cases showed moderate or strong leptin staining.

Moderate-to-strong leptin expression was more frequent in muscle-invasive tumors than in non-muscle-invasive tumors (35/54, 64.8% vs 29/95, 30.5%). A similar pattern was observed for lymphovascular invasion. Moderate-to-strong staining was more frequent in lymphovascular invasion-positive tumors (36/56, 64.3%), whereas tumors without lymphovascular invasion were more often negative or weakly positive (65/93, 69.9%) (Table 2; Figure 2).

Leptin expression was significantly associated with histological grade (p =

0.004), pathological pT category (p = 0.003, Fisher's exact test), muscle invasion (p < 0.001), and lymphovascular invasion (p < 0.001). The association was small-to-moderate for pT category (Cramér's V = 0.232) and moderate for histological grade (V = 0.310), muscle invasion (V = 0.356), and lymphovascular invasion (V = 0.359). In unadjusted binary comparisons, moderate-to-strong leptin expression was associated with high-grade urothelial carcinoma (OR, 3.33; 95% CI, 1.61 - 6.89), muscle invasion (OR, 4.19; 95% CI, 2.06 - 8.51), and lymphovascular invasion (OR, 4.18; 95% CI, 2.05 - 8.52).

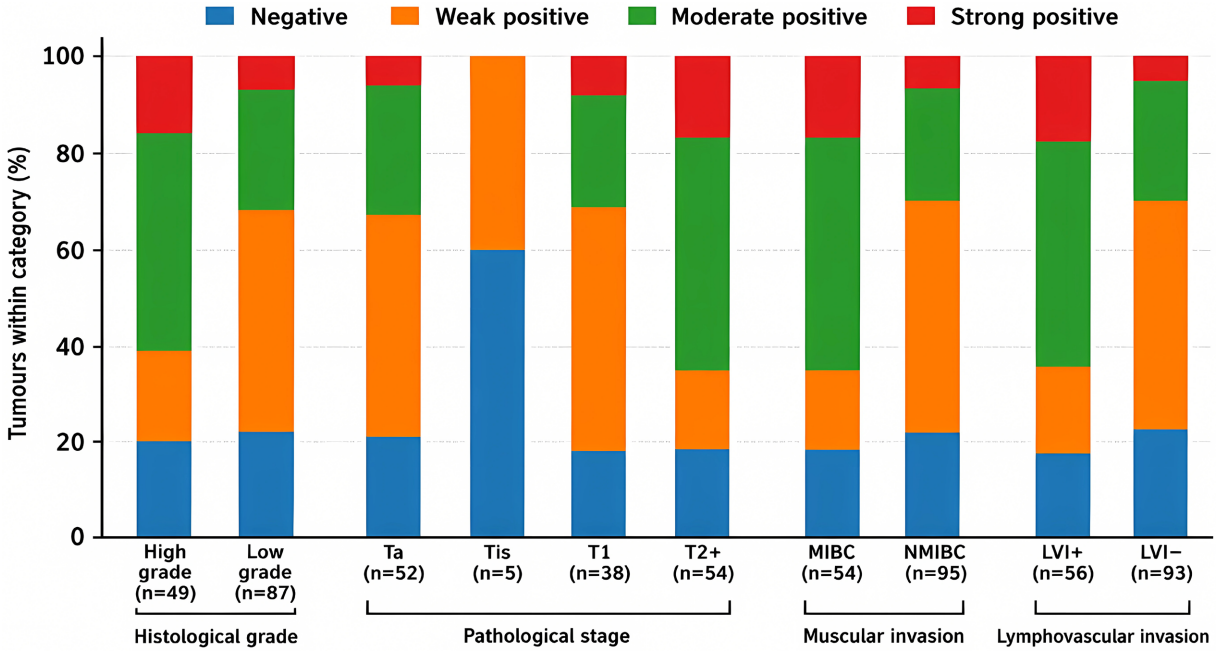


Figure 2. Leptin immunohistochemical expression across pathological subgroups. Stacked bar chart showing the distribution of negative, weak, moderate, and strong leptin staining across histological grade, pathological pT category, muscle invasion, and lymphovascular invasion status. Moderate-to-strong leptin expression was more frequent in high-grade tumors, T2-or-higher tumors, muscle-invasive tumors, and lymphovascular invasion-positive tumors. Abbreviations: LVI, lymphovascular invasion; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; pT, pathological tumor category.

Table 2. Leptin immunohistochemical expression according to pathological features associated with tumor aggressiveness. Data are shown as n (%). The moderate-to-strong category combines moderate and strong leptin staining to summarize higher leptin expression across pathological subgroups. Abbreviations: IHC, immunohistochemistry; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; pT, pathological tumor category. Pearson's chi-square test was used for histological grade, muscle invasion, and lymphovascular invasion. Fisher's exact test was used for pathological pT category because of sparse counts in the Tis subgroup. Cramér's V was used to estimate effect size. *p < 0.05.

Variable	Category	Negative n (%)	Weak positive n (%)	Moderate positive n (%)	Strong positive n (%)	Moderate-to-strong n (%)	Test	Cramér's V	p value
Histological grade	High grade (n = 49)	10 (20.4)	9 (18.4)	22 (44.9)	8 (16.3)	30 (61.2)	Pearson χ^2	0.310	**0.004***
	Low grade (n = 87)	19 (21.8)	40 (46.0)	22 (25.3)	6 (6.9)	28 (32.2)	—	—	—

Continued

Pathological pT category	Ta (n = 52)	11 (21.2)	24 (46.2)	14 (26.9)	3 (5.8)	17 (32.7)	Fisher's exact	0.232	**0.003***
	Tis (n = 5)	3 (60.0)	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	—	—	—
	T1 (n = 38)	7 (18.4)	19 (50.0)	9 (23.7)	3 (7.9)	12 (31.6)	—	—	—
	T2 or higher (n = 54)	10 (18.5)	9 (16.7)	26 (48.1)	9 (16.7)	35 (64.8)	—	—	—
Muscle invasion	MIBC (n = 54)	10 (18.5)	9 (16.7)	26 (48.1)	9 (16.7)	35 (64.8)	Pearson χ^2	0.356	**<0.001***
	NMIBC (n = 95)	21 (22.1)	45 (47.4)	23 (24.2)	6 (6.3)	29 (30.5)	—	—	—
Lymphovascular invasion	Present (n = 56)	10 (17.9)	10 (17.9)	26 (46.4)	10 (17.9)	36 (64.3)	Pearson χ^2	0.359	**<0.001***
	Absent (n = 93)	21 (22.6)	44 (47.3)	23 (24.7)	5 (5.4)	28 (30.1)	—		

3.3. Tissue-Derived LEP rs2167270 Genotype Distribution and Associations with Leptin Expression and Pathological Features

Valid tissue-derived LEP rs2167270 genotype data were obtained in 127 bladder carcinoma cases. The remaining 26 cases were excluded from molecular analysis because the extracted FFPE DNA was of insufficient quantity and/or quality. To evaluate possible bias from DNA attrition, key pathological features were compared between the 127 successfully genotyped cases and the 26 genotyping failures. Urothelial carcinoma accounted for 111/127 successfully genotyped cases (87.4%) and 25/26 genotyping failures (96.2%). Among urothelial carcinomas, high-grade tumors were present in 37/111 successfully genotyped cases (33.3%) and 12/25 genotyping failures (48.0%) (Fisher's exact test, p = 0.175). Pathological pT category was assessable in 123 successfully genotyped cases and all 26 genotyping failures. T2-or-higher tumors were present in 46/123 successfully genotyped cases (37.4%) and 8/26 genotyping failures (30.8%) (Fisher's exact test for T2-or-higher vs lower pT category, p = 0.655). Muscle invasion was present in 42/123 successfully genotyped cases (34.1%) and 12/26 genotyping failures (46.2%) (p = 0.268). Lymphovascular invasion was present in 44/123 successfully genotyped cases (35.8%) and 12/26 genotyping failures (46.2%) (p = 0.375). Overall, no statistically significant imbalance was detected between successfully genotyped cases and genotyping failures for the key pathological features examined, although the small number of failed cases limits the precision of this comparison. Genotype distribution across leptin immunohistochemical expression categories is shown in Table 3

and Figure 3. The GG genotype was least frequent in leptin-negative tumors (7/26, 26.9%) and most frequent in strongly positive tumors (8/12, 66.7%). The AA genotype was most frequent in leptin-negative tumors (11/26, 42.3%) and was not detected in strongly positive tumors. Genotype distribution differed significantly across leptin staining categories ($p = 0.005$; Cramér's $V = 0.295$).

Genotype distribution also differed across pathological subgroups. The GG genotype was more frequent in high-grade than low-grade urothelial carcinoma (22/37, 59.5% vs 31/74, 41.9%). The AA genotype was less frequent in high-grade than low-grade tumors (1/37, 2.7% vs 15/74, 20.3%). Across pathological pT categories, GG genotype frequencies were 19/38 (50.0%) in Ta tumors, 3/5 (60.0%) in Tis tumors, 22/34 (64.7%) in T1 tumors, and 29/46 (63.0%) in T2-or-higher tumors. GG was also more frequent in muscle-invasive than non-muscle-invasive tumors (25/42, 59.5% vs 34/81, 42.0%) and in lymphovascular invasion-positive than lymphovascular invasion-negative tumors (25/44, 56.8% vs 34/79, 43.0%).

Genotype distribution was significantly associated with leptin expression category ($p = 0.005$), histological grade ($p = 0.032$), pathological pT category (fixed-margin Monte Carlo exact test; $p = 0.022$), muscle invasion ($p = 0.012$), and lymphovascular invasion ($p = 0.039$). Effect sizes were small-to-moderate for leptin expression (Cramér's $V = 0.295$), histological grade ($V = 0.249$), pathological pT category ($V = 0.249$), muscle invasion ($V = 0.268$), and lymphovascular invasion ($V = 0.229$).

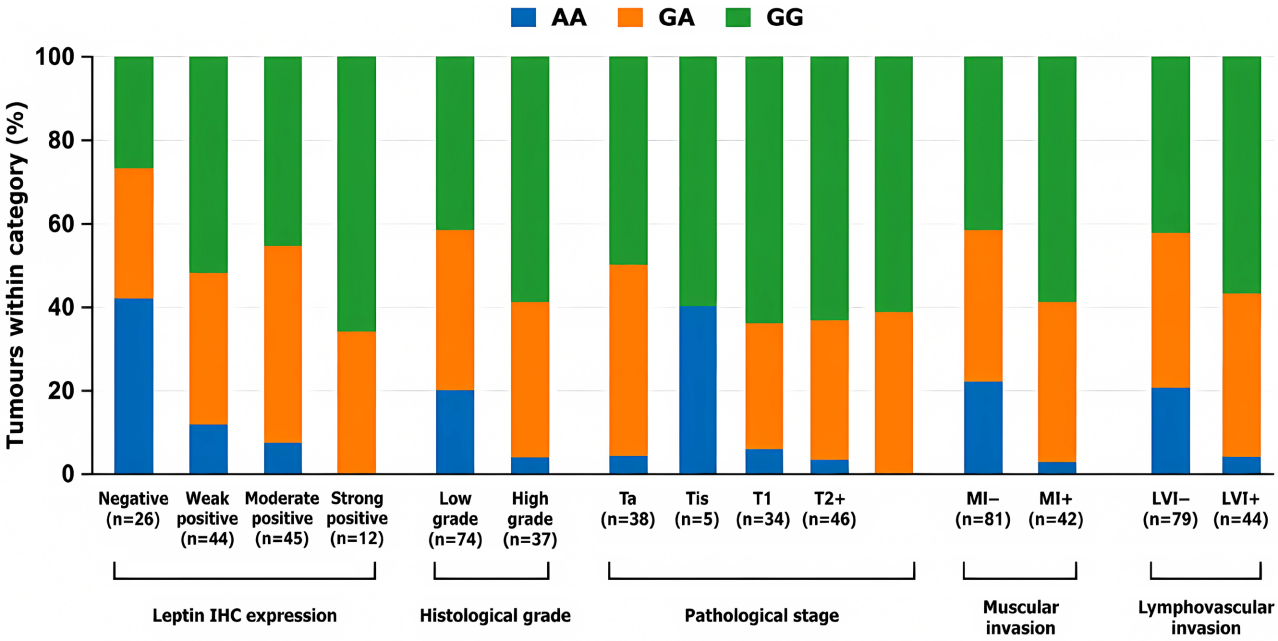


Figure 3. Tissue-derived LEP rs2167270 genotype distribution across leptin expression and pathological subgroups. Stacked bar chart showing the proportional distribution of AA, GA, and GG genotypes across leptin IHC staining categories, histological grade, pathological pT category, muscle invasion, and lymphovascular invasion status. The tissue-derived GG genotype was more frequent in strongly leptin-positive tumors and differed across several pathological subgroups. For pT category, the result represents a distributional difference rather than a clear stepwise increase with advancing pT category. Abbreviations: IHC, immunohistochemistry; LEP, leptin gene; LVI, lymphovascular invasion; pT, pathological tumor category.

Table 3. Tissue-derived LEP rs2167270 genotype distribution by leptin expression and clinicopathological variables. Data are shown as n (%). Genotype distributions are presented across leptin immunohistochemical staining categories, histological grade, pathological pT category, muscle invasion, and lymphovascular invasion status. Abbreviations: IHC, immunohistochemistry; LEP, leptin gene; LVI, lymphovascular invasion; pT, pathological tumor category. Pearson's chi-square test was used for histological grade, muscle invasion, and lymphovascular invasion. Fisher's exact test was used for leptin IHC expression. Fixed-margin Monte Carlo exact analysis was used for pathological pT category because of sparse cell counts. Cramér's V was used to estimate effect size. *p < 0.05.

Variable	Category	AA n (%)	GA n (%)	GG n (%)	Test	Cramér's V	*p* value
Leptin IHC expression	Negative (n = 26)	11 (42.3)	8 (30.8)	7 (26.9)	Fisher's exact test	0.295	**0.005***
	Weak positive (n = 44)	5 (11.4)	16 (36.4)	23 (52.3)	—	—	—
	Moderate positive (n = 45)	3 (6.7)	21 (46.7)	21 (46.7)	—	—	—
	Strong positive (n = 12)	0 (0.0)	4 (33.3)	8 (66.7)	—	—	—
Histological grade	Low grade (n = 74)	15 (20.3)	28 (37.8)	31 (41.9)	Pearson χ^2	0.249	**0.032***
	High grade (n = 37)	1 (2.7)	14 (37.8)	22 (59.5)	—	—	—
Pathological pT category	Ta (n = 38)	2 (5.3)	17 (44.7)	19 (50.0)	Fixed-margin Monte Carlo exact analysis	0.249	**0.022***
	Tis (n = 5)	2 (40.0)	0 (0.0)	3 (60.0)	—	—	—
	T1 (n = 34)	2 (5.9)	10 (29.4)	22 (64.7)	—	—	—
	T2 or higher (n = 46)	1 (2.2)	16 (34.8)	29 (63.0)	—	—	—
Muscle invasion	Absent (n = 81)	18 (22.2)	29 (35.8)	34 (42.0)	Pearson χ^2	0.268	**0.012***
	Present (n = 42)	1 (2.4)	16 (38.1)	25 (59.5)	—	—	—
Lymphovascular invasion	Absent (n = 79)	17 (21.5)	28 (35.4)	34 (43.0)	Pearson χ^2	0.229	**0.039***
	Present (n = 44)	2 (4.5)	17 (38.6)	25 (56.8)	—	—	—

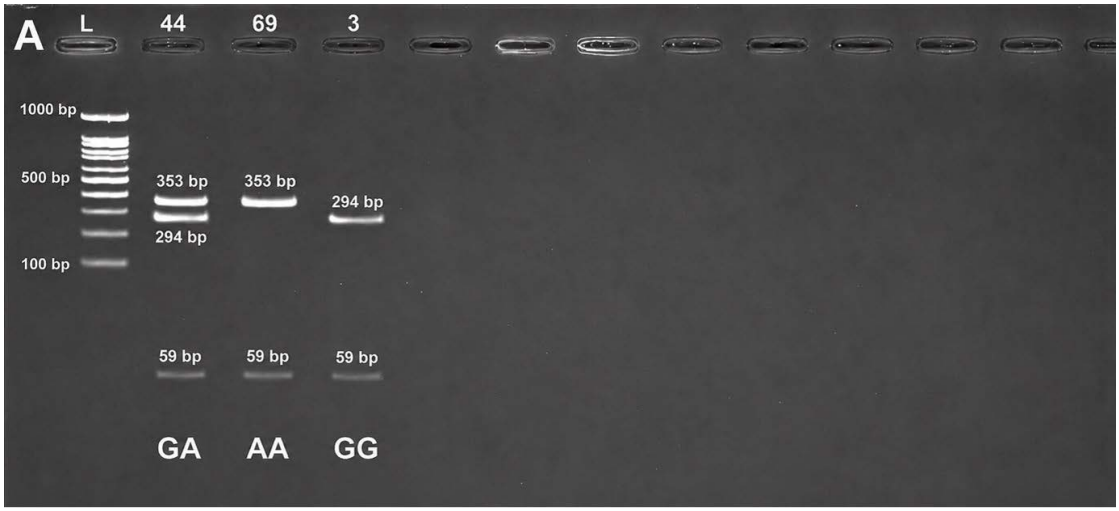
3.4. Analytical Validation of Tissue-Derived LEP rs2167270 Genotyping

Tissue-derived LEP rs2167270 genotyping was evaluable in 127 FFPE urinary bladder carcinoma cases. Only samples with successful PCR amplification were processed for restriction digestion and genotype analysis. As an internal reproducibility check, 15 randomly selected samples were re-analyzed by an independent blinded investigator. Repeat PCR-RFLP showed full concordance with the original genotype assignments (15/15, 100.0%).

Sanger sequencing was performed in 30 randomly selected cases to evaluate PCR-RFLP genotype agreement (Table 4). Representative PCR-RFLP profiles and sequencing chromatograms are provided in Figure 4. Sanger sequencing was concordant with PCR-RFLP genotype calls in all 30 validation samples (30/30, 100.0%; exact binomial 95% CI, 88.4% - 100.0%). No additional sequence variants were detected within the sequenced target region. Together, repeat genotyping and Sanger sequencing supported the reliability of tissue-derived LEP rs2167270 PCR-RFLP genotyping in this FFPE bladder carcinoma cohort.

Table 4. Analytical validation of tissue-derived LEP rs2167270 genotyping. Summary of PCR-RFLP genotyping performance, blinded repeat testing, Sanger sequencing validation, expected restriction profiles, and PCR-RFLP/Sanger sequencing concordance. Abbreviations: CI, confidence interval; FFPE, formalin-fixed, paraffin-embedded; LEP, leptin gene; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism. Concordance was calculated in the 30-case Sanger sequencing validation subset. The 95% CI is an exact binomial confidence interval. The 59-bp restriction fragment was faint or poorly resolved on agarose gel because of its small size.

Validation component	Finding
Cases with evaluable LEP rs2167270 genotype results	127
Samples excluded from genotyping analysis	26
Blinded repeat-genotyping subset	15
Concordance on repeat genotyping	15/15 (100.0%)
Validation subset selected for Sanger sequencing	30
Concordance between PCR-RFLP and Sanger sequencing	30/30 (100.0%)
Exact 95% CI for sequencing concordance	88.4% - 100.0%
Expected PCR amplicon size	353 bp
AA PCR-RFLP pattern	Single undigested 353-bp fragment
GA PCR-RFLP pattern	353-bp, 294-bp, and faint 59-bp fragments
GG PCR-RFLP pattern	294-bp fragment with faint 59-bp band
Additional sequence variants detected in the sequenced target region	None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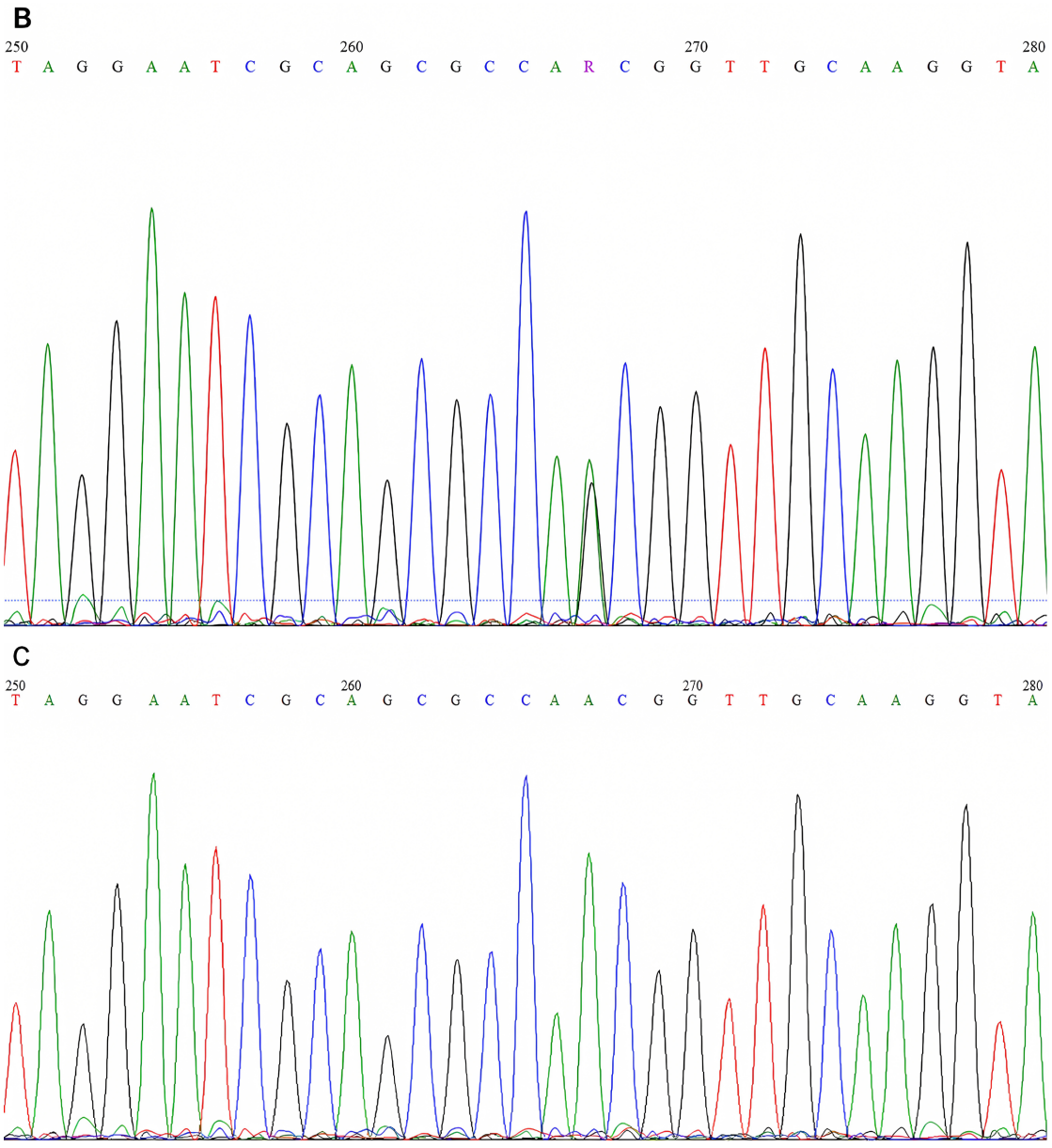
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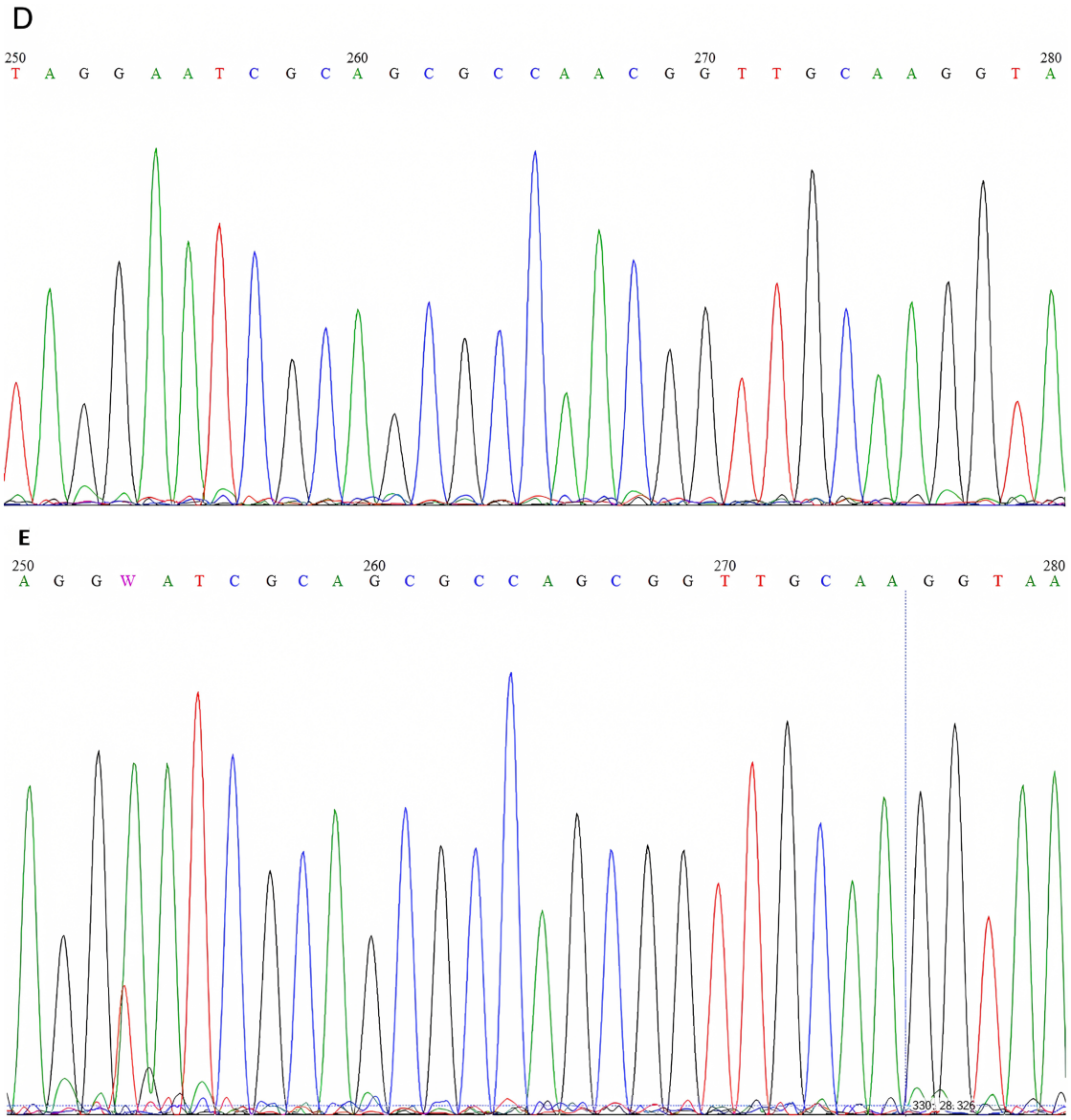

Figure 4. PCR-RFLP and Sanger sequencing validation of tissue-derived LEP rs2167270 genotypes. Representative analytical validation of tissue-derived LEP rs2167270 genotyping. (A) Agarose gel electrophoresis of PCR-RFLP products showing the expected restriction patterns for AA, GA, and GG genotypes. (B) Agarose gel electrophoresis of PCR products selected for Sanger sequencing, showing a single amplicon at approximately 353 bp. (C) Representative Sanger sequencing chromatogram of the GA genotype, showing overlapping guanine and adenine peaks at the rs2167270 site. (D) Representative chromatogram of the AA genotype, showing a single adenine peak. (E) Representative chromatogram of the GG genotype, showing a single guanine peak. Sequence-based genotype calls were concordant with PCR-RFLP assignments in the 30-case validation subset.

4. Discussion

This retrospective multicenter cross-sectional study included 153 histologically diagnosed bladder carcinoma cases from four institutions in Sudan. The study evaluated leptin immunohistochemical expression as a tissue-based marker associated with adverse pathological features. Moderate-to-strong leptin staining was more frequent in high-grade than low-grade urothelial carcinomas (61.2% vs 32.2%; p =

0.004), in muscle-invasive than non-muscle-invasive tumors (64.8% vs 30.5%; $p < 0.001$), in T2-or-higher tumors than Ta tumors (64.8% vs 32.7%; $p = 0.003$), and in lymphovascular invasion-positive than lymphovascular invasion-negative tumors (64.3% vs 30.1%; $p < 0.001$). The tissue-derived LEP rs2167270 GG genotype showed a related pattern for high grade, muscle invasion, and lymphovascular invasion. For pT category, genotype distribution differed statistically, but the pattern was not stepwise because GG was already frequent in Ta and Tis tumors and remained similarly frequent in T1 and T2-or-higher tumors. These findings support an association between leptin immunoreactivity, tissue-derived LEP rs2167270 genotype, and adverse pathological features in this Sudanese cohort. Because survival and follow-up data were unavailable, the findings should be interpreted as pathological associations, not as evidence of causality, confirmed germline effect, or prognostic value.

Bladder cancer is heterogeneous, and its behavior is not fully captured by histological grade or pathological pT category alone [1] [13]. Non-muscle-invasive and muscle-invasive tumors differ in molecular profile, immune context, and clinical behavior [14]. Tissue-based biomarkers may therefore provide additional biological information when interpreted alongside routine histopathology [15]. Adipokines, including leptin, may participate in inflammatory, angiogenic, and invasion-related pathways within the tumor microenvironment [16] [17]. This provides biological plausibility for the observed association, but these mechanisms were not directly tested here.

The Sudanese and sub-Saharan African context is relevant because bladder cancer patterns may differ from those in high-income settings [18]-[22]. Recent Sudanese data indicate that urothelial carcinoma is the main histological type, although squamous cell carcinoma remains relatively more frequent than in many Western cohorts, possibly reflecting the historical contribution of urogenital schistosomiasis [18] [19]. Schistosomiasis status was not assessed in this cohort. Limited access to early diagnosis may also contribute to late presentation and a higher burden of advanced disease across sub-Saharan Africa [20]-[22]. In this cohort, urothelial carcinoma predominated, and substantial proportions of tumors were high grade or muscle invasive. Assessing leptin expression in relation to these pathological features provides regionally relevant biomarker data from an underrepresented African population.

The immunohistochemical findings are consistent with previous bladder-specific reports on leptin expression. Earlier studies reported that stronger leptin staining was associated with adverse clinicopathological features in bladder carcinoma, and one study also evaluated survival outcomes [11] [23]. In this cohort, moderate-to-strong leptin expression was most frequent in T2-or-higher tumors and lymphovascular invasion-positive tumors. However, direct comparison should be cautious because the studies differed in cohort composition, pathological classification, analytical design, and outcome-data availability. Survival data were available in previous work but not here; therefore, our findings should not be interpreted as prog-

nostic evidence.

The tissue-derived LEP rs2167270 genotype findings were consistent with leptin immunohistochemistry. Among 127 genotyped tumors, GG was least frequent in leptin-negative tumors (7/26, 26.9%) and most frequent in strongly leptin-positive tumors (8/12, 66.7%). GG was also more frequent in high-grade, muscle-invasive, lymphovascular invasion-positive tumors, and tumors with higher pathological pT category. In contrast, previous case-control and meta-analytic studies suggested that the A allele may be associated with lower bladder cancer or urinary-system cancer risk [12] [24] [25]. This apparent difference may reflect the distinction between cancer susceptibility and pathological features among established tumors. The cohort included only confirmed bladder carcinoma cases and no cancer-free controls; therefore, it cannot assess cancer susceptibility. Earlier LEP rs2167270 studies mainly evaluated germline susceptibility and were conducted largely in Asian populations, whereas our analysis used tissue-derived FFPE tumor DNA from a Sudanese cohort. Because matched normal DNA was unavailable and genotype subgroups were small, these findings are preliminary and require validation.

Several leptin-related pathways provide biological plausibility for the association with adverse pathological features. After binding to LEPR, leptin can activate JAK2/STAT3, PI3K/AKT, and MAPK/ERK signaling in experimental cancer models [26]. Non-urothelial tumor models have linked these pathways to invasion, migration, epithelial-mesenchymal transition, angiogenic signaling, VEGF-related mechanisms, and vasculogenic mimicry [26]-[29]. However, most mechanistic data come from non-bladder cancer models and should be interpreted cautiously. The strongest associations of leptin expression were observed with muscle invasion and lymphovascular invasion. These cross-sectional tissue data do not show causality, but they support the possibility that higher leptin expression may mark a more invasive pathological phenotype.

Leptin staining in this cohort should be interpreted as intratumoral immunoreactivity, not as a proxy for systemic adiposity. Higher serum leptin levels have been reported in bladder cancer patients than cancer-free controls [30]. However, serum leptin, BMI, and other metabolic or anthropometric variables were unavailable. Therefore, tissue leptin expression cannot evaluate body weight, adiposity, or metabolic status.

This study has strengths, including a multicenter Sudanese cohort, analysis of leptin expression and tissue-derived LEP rs2167270 genotype in the same tumor specimens, clinically relevant pathological endpoints, blinded IHC scoring, repeat IHC assessment, blinded repeat PCR-RFLP genotyping, and Sanger sequencing validation. Limitations include the retrospective cross-sectional design, archived FFPE tumor tissue, absence of survival or recurrence follow-up, absence of cancer-free controls, valid genotyping in 127/153 cases (83.0%), small genotype subgroups, and absence of matched blood, buccal, or adjacent non-tumor DNA. Therefore, the LEP rs2167270 findings should be interpreted as tissue-derived genetic associations and require validation in larger studies using matched normal DNA

and clinical follow-up.

5. Conclusion

Elevated intratumoral leptin immunoreactivity and the tissue-derived LEP rs2167270 GG genotype were associated with adverse pathological features of bladder carcinoma, including high grade, advanced pT category, muscle invasion, and lymphovascular invasion. These findings support a tissue-based association between leptin-related markers and aggressive pathological phenotype in this Sudanese cohort. However, the study was based on archived tumor tissue and did not include survival follow-up or matched normal DNA. Therefore, the findings should be interpreted as pathological associations and not as evidence of causality, confirmed germline effect, or independent prognostic value.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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