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ORIGINAL ARTICLE

# Clinicopathological significance of PLAC1 expression in patients with bladder urothelial carcinoma

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**ABSTRACT**

Ectopic expression of Placenta-specific 1 (PLAC1), a Cancer/Testis Antigen (CTA), was reported in various tumors, but its clinical relevance and prognostic value in bladder urothelial carcinoma (UC) remain unclear. This study evaluated PLAC1 expression in the membrane, cytoplasm, and nucleus of tumor cells and its clinicopathological significance in bladder UC patients. We analyzed 177 bladder UC samples to assess nuclear, cytoplasmic, and membranous PLAC1 expression by immunohistochemistry (IHC) on tissue microarrays (TMAs) and examined its associations with clinicopathological characteristics and prognosis. Higher cytoplasmic expression of PLAC1 was associated with increased histological grade ($P< 0.001$), advanced pathological primary tumor (pT) staging ($P= 0.038$), lamina propria involvement ($P= 0.013$), and lamina propria/muscularis (L/M) involvement ($P= 0.038$). A significant association was identified between the increased nuclear expression of PLAC1 and tumor size ($P= 0.036$) and higher histological grades (intensity of staining, $P= 0.045$). PLAC1 expression was not significantly associated with disease-specific survival (DSS) or progression-free survival (PFS) in bladder UC patients.

Cytoplasmic PLAC1 expression significantly correlates with higher tumor grade and advanced stage in bladder cancer, suggesting its potential as an exploratory biomarker. The association between PLAC1 expression and tumor invasiveness warrants further functional and prospective studies to clarify its biological significance.

***Keywords:*** PLAC1 (Placenta-specific1), Bladder urothelial carcinoma (UC), Immunohistochemistry, Tissue microarray (TMA), Histological grade, pT Stage, Bladder cancer

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## Introduction

Bladder cancer represents the most frequently diagnosed malignancy of the urinary tract and ranks as the ninth most prevalent cancer worldwide, accounting for approximately 549,000 new cases annually. The incidence is four times higher in men than in women (1-5). Urothelial carcinoma (UC) is the most prevalent histological type of bladder cancer, and it accounts for 95% of all bladder cancer cases (6, 7). Squamous cell carcinoma (SCC), adenocarcinoma, and small cell carcinoma (SCLC) comprise the remaining 5% (8, 9). Although bladder cancer patients receive common treatments such as surgery, radiation therapy, chemotherapy, and cystectomy (10), more than half of them experience tumor recurrence or progression (7), necessitating the development of new approaches like targeted immunotherapy (10). A prerequisite for the development of targeted cancer therapies is the identification of tumor-specific antigens with therapeutic function (11).

The PLAC1 gene, a member of the cancer-testis antigens family, is located on the X chromosome and encodes a protein that consists of 212 amino acids (12, 13). PLAC1 is expressed in the placenta and plays a role in trophoblast function. Much lower levels of expression were reported in the testis (13) (14) and cerebellum (15), and no detectable expression was found in other normal tissues (16). Ectopic expression of PLAC1 has been demonstrated in several types of cancer, including breast, melanoma, colon, prostate, lung, liver, pancreas, stomach, ovary, uterus, and cervix (17-20), and numerous studies have shown its involvement in various tumor cell processes, including proliferation, motility, migration, and invasion (17, 21, 22). From a clinical point of view, increased expression of PLAC1 was identified as an indicator of worse prognosis among several cancers, such as cervical cancer (17, 21).

Cancer immunotherapy has revolutionized cancer treatment over the past decade. Selecting an appropriate target is one of the most challenging steps in the targeted therapy approach. One of the key parameters in selecting a marker for cancer immunotherapy is the lack of expression in normal tissues. The characteristics of PLAC1 support the idea that it could be a promising target for cancer immunotherapy. Here, we present the first investigation into the PLAC1 expression in bladder UC tissues to correlate its expression with clinicopathological parameters.

## Methods

### Patients and tissue samples

This retrospective study was conducted on 177 archived formalin-fixed, paraffin-embedded (FFPE) bladder urothelial carcinoma tissues and 10 non-malignant tissues from the Hasheminejad Hospital, a center of urology and nephrology, in Tehran, Iran, obtained between 2008 and 2011. Cases were selected based on the availability of adequate tissue for tissue microarray construction and the availability of clinicopathological and follow-up data. None of the patients had a history of chemo or radiotherapy before surgery. Patients' clinicopathological parameters, such as age, sex, tumor size (The largest tumor dimension), pT stage, histologic grade, muscularis propria invasion, lamina propria involvement, lamina propria /muscularis (L/M) involvement, distant metastases, and recurrence of tumors, were collected from patient's documents and analyzing the hematoxylin and eosin (H&E) stained sections. The pathological tumor staging adhered to the pTNM classification system for bladder UC (23). The American joint committee on cancer/international union against cancer (AJCC/UICC) and the pTNM systems were used for defining the pT stage of malignancies, respectively (24-26). Furthermore, we calculated the disease-specific survival (DSS), defined as the interval from surgery to death, and PFS calculated from the initial surgery date and the last check-up if the patient showed no symptoms of illness, relapse of malignancy, or distant metastasis. This work has been endorsed by Human Research Ethics Committee of Iran University of Medical Sciences (Ref No: IR.IUMS.REC.1398.102).

### Tissue microarray (TMA) construction

A precision arraying device (Tissue Arrayer Minicore; ALPHELYS, Plaisir, France) was used to prepare bladder UC TMAs, as previously reported (27). In brief, H&E-stained slides were evaluated by pathologists to identify three representative tumor tissue areas in each block. Selected areas were then punched out (0.6 mm diameter) from each block and inserted to the recipient paraffin blocks. A well-established and widely accepted method in tissue

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microarray (TMA) construction, as supported by numerous scientific publications, involves the use of a 0.6-mm-diameter punch for inserting tissue into recipient blocks (28, 29). The 0.6-mm punch is favored in most studies due to its advantages, such as minimizing damage to the donor block, preserving more tissue, and allowing a higher number of cores to be incorporated into a single recipient block (29). Research on TMA methodology indicates that utilizing two or more cores enhances accuracy to 95–99%, addressing potential issues related to heterogeneous antigen expression (30-32). After constructing the TMA blocks, 4 µm sections were cut and placed onto adhesive slides. To improve the accuracy and validity of the results, at least three cores were stained for each sample, and then each core was individually scored. For each tissue sample, the final score was calculated by averaging the values obtained from the three cores.

**Immunohistochemistry (IHC) staining**

After dewaxing by xylene, the TMA slides were rehydrated using graded ethanol (100%, 90%, 70% and 50%). Then, antigen retrieval was performed using Tris-EDTA buffer (pH = 9) at 95 ◦C for 30 minutes in a water bath. After washing three times with Tris-buffered saline (TBS), pH 7.4, endogenous peroxidase activity was quenched by addition of 3% $H_2O_2$ for 15 minutes at room temperature. The slides were washed and then non-specific bindings were blocked using 5% sheep serum diluted in protein block (Dako, Denmark) for 30 minutes.The tissue slides were then treated with the anti-PLAC1 antibody (rabbit polyclonal antibody against a synthetic peptide adjacent to the N-terminus of human PLAC1 (14) at a 1/1000 dilution for 1 hour at room temperature. After washing four times with TBS, Mouse/Rabbit PolyVue HRP/DAB Detection Kit (Envision-HRP Kit, Bio pharmadx) was added as the secondary antibody. After washing as above, 3, 30-diaminobenzidine (DAB; Dako) chromogen substrate was used to visualize the PLAC1 expression pattern, and then TMA slides were counterstained by hematoxylin and dehydrated with graded alcohol (50%, 70%, 90% and 100%), cleaned in xylenes (Dako, Denmark), and mounted.

It should be noted that, to validate specificity of antibody, we employed three complementary methods: ELISA using recombinant PLAC1 (33) coated plate, Western blot analysis of placenta lysate (14), and immunohistochemical staining of placenta sections, which served as a positive control. In the negative reagent control, non-immune rabbit serum (1/1000) was used instead of the primary antibody.

**Immunostaining evaluation and scoring**

PLAC1 immunostaining was assessed by two pathologists, unaware of the clinicopathological data, employing a semi-quantitative scoring system. Staining intensity was evaluated using a semi-quantitative scale ranging from 0 to 3, where 0 denoted no staining, 1 indicated weak staining, 2 represented moderate staining, and 3 corresponded to strong staining. Additionally, the proportion of tumor cells exhibiting positive staining was recorded as a percentage between 0% and 100%. These two values were subsequently multiplied to generate a histochemical score (H-score), with possible scores ranging from 0 to 300. Finally, the H-scores were classified as positive or negative for nuclear and membranous expression, but for cytoplasmic expression, a cutoff of 200 was considered, and a median H-score was used to classify the specimens as high or low.

**Statistical analysis**

Statistical analysis was performed with the use of SPSS software (Version 22.0; IBM Corp., Armonk, NY, USA). The categorical data were presented as N (%), whereas the quantitative data were provided as mean, standard deviation (SD), and median (Q1–Q3). Furthermore, Pearson's $\chi^2$ and Spearman's correlation analyses were used to examine the significance of the association and correlation between PLAC1 expression and clinicopathological characteristics. The estimation of OS (overall survival), DSS, and PFS curves was conducted using the Kaplan-Meier analysis. To compare the survival curves between groups, we employed the log-rank test, presenting the results with 95% confidence intervals (CIs). A p-value of 0.05 was judged statistically significant.

The cutoffs for PLAC1 expression were determined after evaluating the distribution of H-scores using the Shapiro–Wilk test. As cytoplasmic H-scores were not normally distributed, the median H-score was used as the cutoff to classify cases into low and high expression groups. This median-based approach is commonly applied in retrospective immunohistochemical biomarker studies when biologically validated thresholds are not available (34,

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35). For nuclear and membranous PLAC1 expression, the median H-score was zero because the majority of cases showed no detectable staining. Therefore, cases were categorized as positive (any detectable expression) or negative (no detectable expression). This approach was selected to reflect the underlying distribution of the data and to allow meaningful statistical comparisons between groups. Given the exploratory nature of this biomarker study, these cutoff definitions were considered the most appropriate for evaluating associations between PLAC1 expression and clinicopathological characteristics.

## Results

### Characteristics of bladder cancer patients

This investigation assessed a total of 177 cases of bladder UC. The median patients' age was determined to be 67 (SD = 12.928) years, (ranging from 25 to 95) years; 87 (49.2%) were under the age of 67, while 90 (50.8%) were above the age of 67. The studied population included 134 (75.7%) male and 43 (24.3%) female patients, yielding a sex ratio of approximately 3.11. Tumor sizes ranged from 1 to 13 cm, and tumors were divided into two groups based on mean tumor size (2.5 cm). While 108 (61.0%) of the cases had tumors that were larger than the mean (size ≥ 2.50), 69 (39.0%) of the cases had tumors that were smaller than the mean (size < 2.50). Table 1 summarizes the clinicopathological features of the patient.

### Expression of PLAC1 in bladder UC tissues

We assessed the protein expression level of PLAC1 by immunohistochemistry (IHC) on tissue microarray (TMA) sections in terms of intensity, positive tumor cell percentage, and H-score. In this study, rabbit anti-human PLAC1 antibody (Avicenna Research Institute) was used as the primary antibody. The reactivity of antibody was validated by indirect ELISA using recombinant full length extracellular domain of human PLAC1 (33) coated plate. As demonstrated in Figure S1, the antibody recognized recombinant human PLAC1. Furthermore, the antibody's reactivity to detect human PLAC1 was previously confirmed by Western blot analysis using placenta lysate, where it specifically identified the 24 kDa PLAC1 molecule, as reported in earlier study (14). Additionally, immunostaining of human placenta (Figure 1), serving as a positive control, revealed a specific staining pattern, further confirming the antibody's specificity.

**Table 1. Patients and clinicopathological parameters of bladder UC tissues**

| Patients and tumor characteristics | | Total tumor tissue samples N (%) |
|---|---|---|
| **Number of tumor samples** | | **177** |
| **Age** | Median age<br>Range (years)<br>67 ≤<br>67 > | 67<br>25- 95<br>90 (50.8)<br>87 (49.2) |
| **Gender** | Male<br>Female<br>(Male/Female) | 134 (75.7)<br>43 (24.3)<br>3.11 |
| **Mean tumor size (cm)** | 2.5 ≤<br>2.5 > | 108 (61.0)<br>69 (39.0) |
| **Histological grade** | Low<br>High | 91 (51.4)<br>86 (48.6) |
| **pT stage** | pTa<br>pT1<br>pT2<br>pT3<br>pT4 | 90 (50.9)<br>70 (39.5)<br>17 (9.6)<br>0 (0.0)<br>0 (0.0) |
| **Lamina propria involvement** | Involved<br>Not Involved | 90 (50.8)<br>87 (49.2) |
| **Muscularis invasion** | Involved<br>Not Involved | 17 (9.6)<br>160 (90.4) |

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| Patients and tumor characteristics | | Total tumor tissue samples N (%) |
|---|---|---|
| **Lamina propria / Muscularis (L/M) involvement** | L-/M-<br>L+/M+ or L-/M+<br>L+/M- | 90 (50.8)<br>17 (9.6)<br>70 (39.5) |
| **Tumor recurrence** | Present<br>Absent | 41 (23.2)<br>136 (76.8) |
| **Distant metastasis** | Present<br>Absent | 25 (14.1)<br>152 (85.9) |
| N (%) exhibits number of cases and their percentage in brackets. | | |

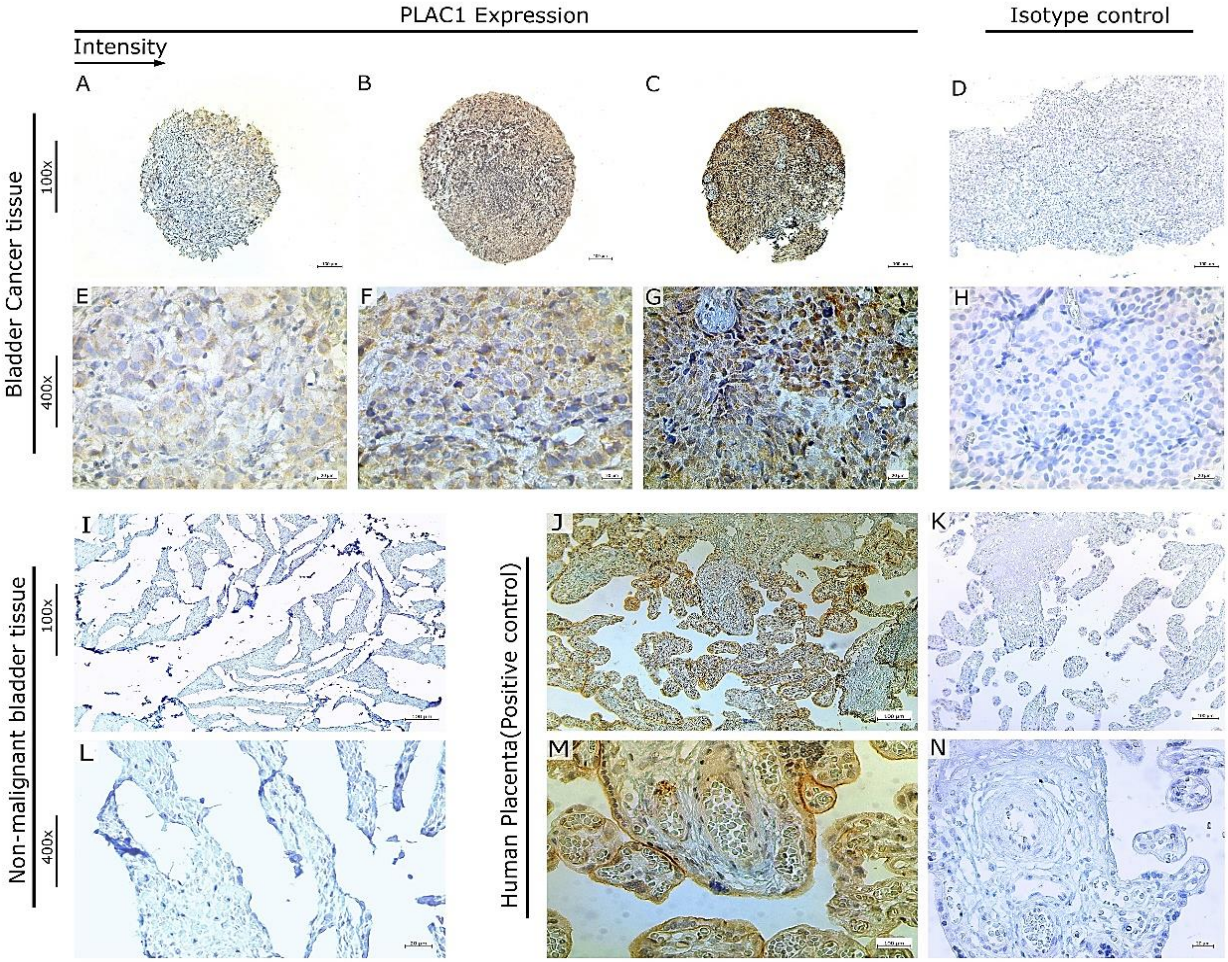


**Figure 1. Immunohistochemical staining of PLAC1 in bladder UC tissues, non-malignant bladder tissue, and positive (placenta tissues) and negative (isotype) controls. (A, E) Weak, (B, F) moderate, and (C, G) strong PLAC1 expression in bladder UC. (D, H) Isotype control (whole bladder UC tissue). (I, L) PLAC1 expression in non-malignant bladder tissue (whole tissue), No PLAC1 expression was detected in non-malignant bladder tissues. (J, M) PLAC1 expression in human term placenta tissue as a positive control (whole tissue), (K, N) Isotype control (whole placenta tissue). Figures are shown with a magnification of 100X and 400X**.

The presence of PLAC1 was detected in bladder tumor tissues at varying ranges in the cell membrane, cytoplasm, and nucleus (Figure 1). PLAC1 expression was found in 25 (14.1%), 173 (97.7%), and 10 (5.6%)



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bladder cancer cases in the nucleus, cytoplasm, and membrane, respectively (Table 2). Using the median H-score (=200) as a cut-off, cytoplasmic expression of PLAC1 was found to be low in 98 (55.4%) samples and high in 79 (44.6%) samples. For membranous and nuclear PLAC1 expression, patients are divided into positive and negative groups. Positive and negative membranous expression was found in 10 (5.6%) and 167 (94.4%) cases, respectively. Nuclear expression was positive in 25 cases (14.1%) and negative in 152 (85.9%) (Table 2). PLAC1 expression was assessed in whole tissues from nine non-malignant samples. No expression was observed in non-malignant tissues. As expected, no staining was seen in negative reagent controls (Figure 1).

**Table 2. PLAC1 expression (Intensity of staining, percentage of positive tumor cells, and H-score) in bladder UC tissues samples**

| Scoring system | | Nuclear expression N (%) | Cytoplasmic expression N (%) | Membranous expression N (%) |
|---|---|---|---|---|
| **Intensity of staining** | Negative (0)<br>Weak (+1)<br>Moderate (+2)<br>Strong (+3) | 152 (85.9)<br>8 (4.5)<br>4 (2.3)<br>13 (7.3) | 4 (2.3)<br>45 (25.4)<br>50 (28.2)<br>78 (44.1) | 167 (94.4)<br>2 (1.1)<br>3 (1.7)<br>5 (2.8) |
| **Percentage of positive tumor cells** | <25%<br>25–50%<br>51 -75%<br>> 75% | 177 (100)<br>0 (0.0)<br>0 (0.0)<br>0 (0.0) | 6 (3.4)<br>2 (1.1)<br>10 (5.6)<br>159 (89.8) | 176 (99.4)<br>0 (0.0)<br>1 (0.6)<br>0 (0.0) |
| **H-score (cut off)** | Positive<br>Negative | 152 (85.9)<br>25 (14.1) | Median (200.0)<br>Low ≤ - 98 (55.4)<br>High > -79 (44.6) | 167 (94.4)<br>10 (5.6) |
| **Total** | | 177 | 177 | 177 |
| H-score indicates histological score. | | | | |

**Associations between cytoplasmic PLAC1 expression and clinicopathological characteristics of bladder UC**

Pearson's $\chi^2$ test demonstrated a statistically significant association between cytoplasmic PLAC1 expression and higher histological grade (H-score *P < 0.001*, intensity *P < 0.001*), advanced pT stage (H-score *P = 0.038*, intensity *P = 0.003*), lamina propria involvement (H-score *P = 0.013*, intensity *P < 0.001*), as well as L/M involvement (H-score *P = 0.038*, intensity *P = 0.003*) (Table 3). Further, Spearman's correlation test was carried out to show the correlation between PLAC1 protein expression and clinicopathological features. Our analysis showed a significant correlation between a high level of cytoplasmic expression of PLAC1 and degree of grade (P <0.001), lamina propria involvement (P = 0.013), L/M involvement (P = 0.01), and stage (P = 0.027).

**Associations between nuclear and membranous PLAC1 expression and clinicopathological characteristics of bladder UC**

Pearson's $\chi 2$ test exhibited a significant association between increased nuclear expression of PLAC1 protein and tumor size (H-score *P = **0.036***) as well as higher histological grade (intensity of staining *P = 0.045*) (Table 4).

Furthermore, no significant association was found between membranous PLAC1 expression and clinicopathological characteristics (Table 5).

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**Table 3. The association between cytoplasmic PLAC1 expression and clinicopathological characteristic in bladder UC tissues (Intensity of staining and H-score)**

| Patients and tumor characteristics | | Total No.cases (% of total) | Intensity of staining N (%) | | | | *P- value* | H-score (cut off = 200) N (%) | | *P- value* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 (Negative) | 1+ (Weak) | 2+ (Moderate) | 3+ (Strong) | | Low (≤200) | High (>200) | |
| **Bladder carcinoma tissues** | | **177** | **4(2.3)** | **45(25.4)** | **50(28.2)** | **78(44.1)** | | **98(55.4)** | **79(44.6)** | |
| **Median age (years)** | 67 ≤<br>67 > | 90(50.8)<br>87(49.2) | 0(0.0)<br>4(100.0) | 27(60.0)<br>18(40.0) | 23(46.0)<br>27(54.0) | 40(51.0)<br>38(48.7) | 0.106 | 49(50.0)<br>49(50.0) | 41(51.9)<br>38(48.1) | 0.802 |
| **Gender** | Male<br>Female | 134(75.7)<br>43(24.3) | 3(75.0)<br>1(25.0) | 30(66.7)<br>15(33.0) | 38(76.0)<br>12(24.0) | 63(80.0)<br>15(19.2) | 0.378 | 71(72.4)<br>27(27.6) | 63(79.7)<br>16(20.3) | 0.260 |
| **Mean tumor size (cm)** | 2.5 ≤<br>2.5 > | 108(61.0)<br>69(39.0) | 3(75.0)<br>1(25.0) | 32(71.0)<br>13(28.9) | 27 (54.0)<br>23 (46.0) | 46(59.0)<br>32(41.0) | 0.330 | 63(64.3)<br>35(35.7) | 45(57.0)<br>34(43.0) | 0.321 |
| **Histological grade** | Low<br>High | 91(51.4)<br>86(48.6) | 1(25.0)<br>3(75.0) | 35(77.8)<br>10(22.2) | 27 (54.0)<br>23 (46.0) | 28(35.9)<br>50(64.1) | <0.001 | 63(64.3)<br>35(35.7) | 28(35.4)<br>51(64.6) | <0.001 |
| **pT stage** | pTa<br>pT1<br>pT2<br>pT3<br>pT4 | 90(50.9)<br>70(39.5)<br>17(9.6)<br>0(0.0)<br>0(0.0) | 0(0.0)<br>3(75.0)<br>1(25.0)<br>0(0.0)<br>0(0.0) | 34(75.6)<br>10(22.2)<br>1(2.2)<br>0(0.0)<br>0(0.0) | 24(48.0)<br>19(38.0)<br>7(14.0)<br>0(0.0)<br>0(0.0) | 32(41.0)<br>38(48.7)<br>8(10.3)<br>0(0.0)<br>0(0.0) | 0.003 | 58(59.2)<br>31(31.6)<br>9(9.2) | 32(40.5)<br>39(49.4)<br>8(10.1) | 0.038 |
| **Lamina propria involvement** | Involved<br>Not Involved | 90(50.8)<br>87(49.2) | 0 (0.0)<br>4(100.0) | 34(75.6)<br>11(24.4) | 24(48.0)<br>26(52.0) | 32(41.0)<br>46(59.0) | <0.001 | 58(59.2)<br>40(40.8) | 32(40.5)<br>47(59.5) | 0.013 |
| **Muscularis invasion** | Involved<br>Not Involved | 17(9.6)<br>160(90.4) | 1(25.0)<br>3(75.0) | 1(2.2)<br>44(97.8) | 7(14.0)<br>43(86.0) | 8(10.3)<br>70(89.7) | 0.167 | 9(9.2)<br>89(90.8) | 8(10.1)<br>71(89.9) | 0.832 |
| **Lamina propria / Muscularis (L/M) involvement** | L-/M-<br>L+/M+ or L-/M+<br>L+/M- | 90(50.8)<br>17(9.6)<br>70(39.5) | 0 (0.0)<br>1(25.0)<br>3(75.0) | 34(75.6)<br>1(2.2)<br>10(22.2 | 24(48.0)<br>7(14.0)<br>19(38.0) | 32(41.0)<br>8(10.3)<br>38(48.7) | 0.003 | 58(59.2)<br>9(9.2)<br>31(31.6) | 32(40.5)<br>8(10.1)<br>39(49.4) | 0.038 |
| **Tumor recurrence** | Present<br>Absent | 41(23.2)<br>136(76.8) | 1(25.0)<br>3(75.0) | 10(22.2)<br>35(77.8) | 13(26.0)<br>37(74.0) | 17(21.8)<br>61(78.2) | 0.953 | 25(25.5)<br>73(74.5) | 16(20.3)<br>63(79.7) | 0.410 |
| **Distant metastasis** | Present<br>Absent | 25(14.1)<br>152(85.9) | 1(25.0)<br>3(75.0) | 6(13.3)<br>39(86.7) | 8 (16.0)<br>42 (84.0) | 10(12.8)<br>68(87.2) | 0.881 | 16(16.3)<br>82(83.7) | 9(11.4)<br>70(88.6) | 0.349 |

H-score indicates histological score.*P value*; Pearson's $\chi 2$ test.Values in bold and italic are statistically significant.

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**Table 4. The association between nuclear PLAC1 expression and clinicopathological characteristics in bladder UC tissues (Intensity of staining and H-score)**

| Patients and tumor characteristics | | Total No. cases | Intensity of staining N (%) | | | | P- value | H-score (Cut-off = Positive or negative) N (%) | | P- value |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 (Negative) | 1+ (Weak) | 2+ (Moderate) | 3+ (Strong) | | Negative | Positive | |
| **Bladder carcinoma tissues** | | 177 | 152(85.9) | 8 (4.5) | 4 (2.3) | 13 (7.3) | | 152(85.9) | 25(14.1) | |
| **Median age (years)** | 67 ≤<br>67 > | 90 (50.8)<br>87 (49.2) | 75(49.3)<br>77(50.7) | 5 (62.5)<br>3 (37.5) | 1(25.0)<br>3(75.0) | 9(69.2)<br>4(30.8) | 0.334 | 75(49.3)<br>77(50.7) | 15(60.0)<br>10(40.0) | 0.323 |
| **Gender** | Male<br>Female | 134 (75.7)<br>43 (24.3) | 116(76.3)<br>36(23.7) | 4 (50.0)<br>4 (50.0) | 4(100.0)<br>0 (0.0) | 10(76.9)<br>3(23.1) | 0.241 | 116 (76.3)<br>36 (23.7) | 18(72.0)<br>7(28.0) | 0.641 |
| **Mean tumor size (cm)** | 2.5 ≤<br>2.5 > | 108 (61.0)<br>69 (39.0) | 88(57.9)<br>64(42.1) | 6 (75.0)<br>2 (25.0) | 3 (75.0)<br>1 (25.0) | 11 (84.6)<br>2 (15.4) | 0.199 | 88 (57.9)<br>64 (42.1) | 20(80.0)<br>5(20.0) | 0.036 |
| **Histological grade** | Low<br>High | 91(51.4)<br>86(48.6) | 74(48.7)<br>78(51.3) | 8(100.0)<br>0 (0.0) | 2(50.0)<br>2(50.0) | 7(53.8)<br>6(46.2) | 0.045 | 74(48.7)<br>78(51.3) | 17(68.0)<br>8 (32.0) | 0.073 |
| **pT stage** | pTa<br>pT1<br>pT2<br>pT3<br>pT4 | 90(50.9)<br>70(39.5)<br>17(9.6)<br>0(0.0)<br>0(0.0) | 73(48.0)<br>62(40.8)<br>17(11.2)<br>0(0.0)<br>0(0.0) | 7(87. 5)<br>1(12.5)<br>0(0.0)<br>0(0.0)<br>0(0.0) | 2(50.0)<br>2(50.0)<br>0(0.0)<br>0(0.0)<br>0(0.0) | 8(61. 5)<br>5(38.5)<br>0(0.0)<br>0(0.0)<br>0(0.0) | 0.312 | 73(48.0)<br>62(40.8)<br>17(11.2)<br>0(0.0)<br>0(0.0) | 17(68.0)<br>8(32.0)<br>0(0.0) | 0.086 |
| **Lamina propria involvement** | Involved<br>Not Involved | 90(50.8)<br>87(49.2) | 73(48.0)<br>79(52.0) | 7(87.5)<br>1(12.5) | 2(50.0)<br>2(50.0) | 8(61.5)<br>5(38.5) | 0.146 | 73(48.0)<br>79(52.0) | 17(68.0)<br>8(32.0) | 0.064 |
| **Muscularis invasion** | Involved<br>Not Involved | 17(9.6)<br>160(90.4) | 17(11.2)<br>135(88.8) | 0(0.0)<br>8(100.0) | 0(0.0)<br>4(100.0) | 0(0.0)<br>13(100.0) | 0.377 | 17(11.2)<br>135 (88.8) | 0(0.0)<br>25(100.0) | 0.079 |
| **Lamina propria / Muscularis (L/M) involvement** | L-/M-<br>L+/M+ or L-/M+<br>L+/M- | 90(50.8)<br>17(9.6)<br>70(39.5) | 73(48.0)<br>17(11.2)<br>62(40.8) | 7(87.5)<br>0(0.0)<br>1(12.5) | 2(50.0)<br>0(0.0)<br>2(50.0) | 8(61.5)<br>0(0.0)<br>5(38.5) | 0.312 | 73(48.0)<br>17(11.2)<br>62(40.8) | 17(68.0)<br>0(0.0)<br>8(32.0) | 0.086 |
| **Tumor recurrence** | Present<br>Absent | 41(23.2)<br>136(76.8) | 36(23.7)<br>116(76.3) | 2(25.0)<br>6(75.0) | 1(25.0)<br>3(75.0) | 2(15.4)<br>11(84.6) | 0.922 | 36(23.7)<br>116(76.3) | 5(20.0)<br>20(80.0) | 0.686 |
| **Distant metastasis** | Present<br>Absent | 25(14.1)<br>152(85.9) | 24(15.8)<br>128(84.2) | 1(12.5)<br>7(87.5) | 0(0.0)<br>4(100.0) | 0(0.0)<br>13(100.0) | 0.367 | 24(15.8)<br>128(84.2) | 1(4.0)<br>24(96.0) | 0.117 |

H-score indicates the histological score. P value; Pearson's $\chi 2$ test. Values in bold and italic are statistically significant.

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**Table 5. The association between membranous PLAC1 expression and clinicopathological characteristics in bladder UC tissues (Intensity of staining and H-score)**

| Patients and tumor characteristics | | Total no. cases | Intensity of staining N (%) | | | | *P- value* | H-score (Cut off = Positive or negative) N (%) | | *P- value* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 (Negative) | 1+ (Weak) | 2+ (Moderate) | 3+ (Strong) | | Negative | Positive | |
| **Bladder carcinoma tissues** | | 177 | 167(94.4) | 2(1.1) | 3(1.7) | 5(2.8) | | 167(94.4) | 10(5.6) | |
| **Median age (years)** | 67 ≤<br>67 > | 90 (50.8)<br>87 (49.2) | 88 (52.7)<br>79(47.3) | 0 (0.0)<br>2(100.0) | 1(33.3)<br>2(66.7) | 1(20.0)<br>4(80.0) | 0.206 | 88(52.7)<br>79(47.3) | 2(20.0)<br>8(80.0) | *0.045* |
| **Gender** | Male<br>Female | 134 (75.7)<br>43 (24.3) | 126 (75.4)<br>41 (24.6) | 2(100.0)<br>0 (0.0) | 2 (66.7)<br>1 (33.3) | 4 (80.0)<br>1 (20.0) | 0.842 | 126(75.4)<br>41 (24.6) | 8 (80.0)<br>2 (20.0) | 0.744 |
| **Mean tumor size (cm)** | 2.5 ≤<br>2.5 > | 108 (61.0)<br>69 (39.0) | 103 (61.7)<br>64 (38.3) | 1 (50.0)<br>1 (50.0) | 2 (66.7)<br>1 (33.3) | 2 (40.0)<br>3 (60.0) | 0.777 | 103(61.7)<br>64 (38.3) | 5 (50.0)<br>5 (50.0) | 0.462 |
| **Histological grade** | Low<br>High | 91 (51.4)<br>86 (48.6) | 85 (50.9)<br>82 (49.1) | 2(100.0)<br>0 (0.0) | 1 (33.3)<br>2 (66.7) | 3 (60.0)<br>2 (40.0) | 0.485 | 85 (50.9)<br>82 (49.1) | 6 (60.0)<br>4 (40.0) | 0.576 |
| **pT stage** | pTa<br>pT1<br>pT2<br>pT3<br>pT4 | 90 (50.9)<br>70 (39.5)<br>17 (9.6)<br>0 (0.0)<br>0 (0.0) | 84 (50.3)<br>67 (40.1)<br>16 (9.6)<br>0 (0.0)<br>0 (0.0) | 2(100.0)<br>0 (0.0)<br>0 (0.0)<br>0 (0.0)<br>0 (0.0) | 1 (33.3)<br>2 (66.7)<br>0 (0.0)<br>0 (0.0)<br>0 (0.0) | 3 (60.0)<br>1 (20.0)<br>1 (20.0)<br>0 (0.0)<br>0 (0.0) | 0.662 | 84 (50.3)<br>67 (40.1)<br>16 (9.6) | 6 (60.0)<br>3 (30.0)<br>1 (10.0) | 0.810 |
| **Lamina propria involvement** | Involved<br>Not Involved | 90 (50.8)<br>87 (49.2) | 84 (50.3)<br>83 (49.7) | 2(100.0)<br>0 (0.0) | 1 (33.3)<br>2 (66.7) | 3 (60.0)<br>2 (40.0) | 0.477 | 84 (50.3)<br>83 (49.7) | 6 (60.0)<br>4 (40.0) | 0.551 |
| **Muscularis invasion** | Involved<br>Not Involved | 17 (9.6)<br>160 (90.4) | 16 (9.6)<br>151 (90.4) | 0 (0.0)<br>2(100.0) | 0 (0.0)<br>3 (100.0) | 1 (20.0)<br>4 (80.0) | *0.764* | 16 (9.6)<br>151(90.4) | 1 (10.0)<br>9 (90.0) | 0.965 |
| **Lamina propria / Muscularis (L/M) involvement** | L-/M-<br>L+/M+ or L-/M+<br>L+/M- | 90 (50.8)<br>17 (9.6)<br>70 (39.5) | 84 (50.3)<br>16 (9.6)<br>67 (40.1) | 2(100.0)<br>0 (0.0)<br>0 (0.0) | 1 (33.3)<br>0 (0.0)<br>2 (66.7) | 3 (60.0)<br>1 (20.0)<br>1 (20.0) | 0.662 | 84(50.3)<br>16 (9.6)<br>67 (40.1) | 6 (60.0)<br>1 1(0.0)<br>3 (30.0) | 0.810 |
| **Tumor recurrence** | Present<br>Absent | 41 (23.2)<br>136 (76.8) | 37 (22.2)<br>130 (77.8) | 1 (50.0)<br>1 (50.0) | 1 (33.3)<br>2 (66.7) | 2 (40.0)<br>3 (60.0) | 0.599 | 37 (22.2)<br>130(77.8) | 4 (40.0)<br>6 (60.0) | 0.194 |
| **Distant metastasis** | Present<br>Absent | 25 (14.1)<br>152 (85.9) | 22 (13.2)<br>145 (86.8) | 1 (50.0)<br>1 (50.0) | 0 (0.0)<br>3 (100.0) | 2 (40.0)<br>3 (60.0) | 0.139 | 22 (13.2)<br>145(86.8) | 3 (30.0)<br>7 (70.0) | 0.138 |
| | H-score indicates histological score. *P value*; Pearson's $\chi 2$ test. Values in bold and italic are statistically significant. | | | | | | | | | |

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**Association of PLAC1 expression and clinical outcomes**

In the present study, a total of 136 (76.8%) patients were found to have no history of metastasis or recurrence. However, 41 (23.2%) patients had a history of either metastasis or recurrence. Metastasis was seen in 25 (14.1%) cases, whereas recurrence occurred in 41 (23.2%) patients. During the follow-up period, 33 (18.6%) patients died from cancer-related causes. The mean duration of follow-up for DSS and PFS was 76.04 (SD, 23.7) and 72.18 (27.4) months, respectively. The median follow-up was 82.00 (77, 92) months for DSS and 80.00 (70, 91) months for PFS, with a range of 1–98 months. No significant correlation was observed between cytoplasmic expression status and patient outcomes (DSS and PFS) based on the Kaplan-Meier survival analysis. (DSS; $P$ = 0.249, PFS; $P$ = 0.342) as well as positive or negative membranous and nuclear expression of PLAC1 (DSS; P = 0.079, PFS; P = 0.212, DSS; P = 0.128, PFS; P = 0.414, respectively). Because no significant associations were observed between PLAC1 expression patterns and DSS or PFS in Kaplan–Meier analyses, multivariable Cox regression analyses were not performed. Given the exploratory nature of this biomarker study, the results should be interpreted accordingly.

## Discussion

Bladder cancer remains a significant clinical challenge because of its high rates of recurrence, even after early-stage tumor removal. For this reason, continuous and extensive follow-up is necessary (36). Novel treatment approaches such as immunotherapy may reduce recurrence rates and improve patient survival. Some lines of evidence suggests that a combination of surgical intervention and targeted immunotherapy is the most optimal approach to improve treatment efficacy (37).

After its discovery as an essential molecule in placentation, PLAC1 is now being investigated as a putative biomarker and a molecule of potential relevance in cancer immunotherapy research. A lot of data indicate a significant restriction in PLAC1 expression in normal tissues, although it is expressed in various cancers (20). Based on these characteristics (lack of expression in normal tissues and ectopic expression), PLAC1 has attracted interest as a molecule in cancer therapeutic research and therefore warrants further investigation in the context of cancer biology.

Although PLAC1 is investigated in a wide variety of malignancies (38), there are currently no studies that specifically examine its expression and function in bladder cancer. Therefore, this study is the first report to evaluate the protein expression pattern and clinicopathological significance of PLAC1 in a panel of bladder UC tissues by immunohistochemistry on TMA slides. IHC offers several advantages, including high specificity and sensitivity in detecting target antigens within tissue samples. It allows for the visualization of protein expression and localization at the cellular level, aiding in accurate disease diagnosis. IHC is also valuable for prognostic and predictive biomarker analysis, guiding personalized treatment strategies.

According to published data, PLAC1 is a molecule that has varying subcellular localization expression in malignant tissues (20). In the present investigation, PLAC1 expression in cancer cells was classified as membranous, cytoplasmic and nuclear. The staining patterns of each sample exhibited varying percentages of stained tumor cells, along with differential expression of PLAC1 protein in the membrane, cytoplasm, and nucleus, with intensities ranging from weak to strong. It is critical to investigate the subcellular localization of molecules in malignant tissues (20). A molecule may play different roles at different sublocalizations especially in cancer cells (39), indicating the importance of identifying the subcellular localization of the molecule of interest. The different sublocalizations of PLAC1 expression in cancer cells may reveal its various functions. Importantly, our data demonstrated that PLAC1 cytoplasmic overexpression in the bladder UC tissues is associated with the most critical clinicopathological aspects, such as advanced pT stage, histological grade, lamina propria involvement and L/M involvement. Nuclear expression is only associated with histological grade, but membranous expressions do not correlate with clinicopathological characteristics in bladder UC patients. However, given the very small number of membranous-positive cases, the study was likely underpowered to detect such associations, and these findings should therefore be interpreted with caution. In addition, nuclear PLAC1 expression was associated with larger tumor size and higher histological grade.

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However, given the limited number of cases showing nuclear positivity, these findings should be considered exploratory and require confirmation in larger cohorts. Our findings suggest that there is a potential association between higher PLAC1 expression and more aggressive behavior in bladder UC tissue as judged by advanced pT stage, histological grade, lamina propria involvement and L/M involvement. Several lines of evidence indicate the correlation between PLAC1 expression and tumor progression and invasion across various cancer types (20). In confirmation of this, one research study on breast cancer cells revealed that knocking down of PLAC1 using siRNA led to reduced cell migration and invasion as well as their proliferation by inducing cell cycle arrest (17).

In another study, it has been shown that knocking down of PLAC1 in Bel-7402 and HepG2 cells resulted in the inhibition of epithelial-mesenchymal transition (EMT), leading to reduced cellular migration and invasion (38). This effect was accompanied by an increase in the expression of E-cadherin and a reduction in the expression of vimentin, twist, and snail, which are known markers of EMT. Reducing tumor cell proliferation and increasing apoptosis after knock down of PLAC1 with small interfering RNA (siRNA) in Bel-7402 and HepG2 cells supported the oncogenic potential of PLAC1 (38). Additional research has shown that a reduction in PLAC1 expression leads to a decrease in the phosphorylation of Akt, the consequence of which is the reduction of EMT (38, 40). As mentioned above and considering the role of PLAC1 in cancer cell phenotype (21), it may hold some promise as a molecule of interest in cancer therapeutic research. However, functional studies are needed to fully understand its role and validate its clinical applicability.

In the current study, a significant correlation between cytoplasmic and nuclear expression of PLAC1 and histological grades was shown. Histological grade in malignancies reflects the level of differentiation of cancer cells that ranges from well-differentiated (low grade) to poorly differentiated (high grade), and represents the tumor cell behavior (41, 42). In line with our findings, Yin, Y., et al. showed a significant correlation between PLAC1 expression and tumor differentiation and grade in pancreatic ductal adenocarcinoma (43).

Another study has shown that PLAC1 protein levels were elevated in moderately and poorly differentiated tumor tissues compared to highly differentiated tumor tissues in non-small cell lung cancer (44). Although this study showed an association between PLAC1 expression and tumor aggressiveness, no significant association with survival was observed. This apparent discrepancy could reflect the multifactorial nature of survival outcomes, which are influenced not only by tumor biology but also by treatment strategies, comorbidities, and individual patient responses (45, 46). Furthermore, other factors could have contributed to this outcome, including sample size, follow-up time, and potential confounding factors, which was not considered in our analysis. Future studies with larger, uniformly treated patient cohorts and extended follow-up are warranted to clarify the true prognostic role of PLAC1. Taken together, the association of PLAC1 expression with advanced pT stage and higher histological grades suggests that PLAC1 could be explored as a potential biomarker in bladder UC, particularly in advanced disease. However, further research is needed to validate its clinical utility and biological significance.

In conclusion, our study highlights the potential value of PLAC1 as a biomarker in bladder cancer. The following features make it a candidate biomarker for further investigation in bladder cancer: lack of expression in most normal tissues, and a high number of patients expressing this molecule in bladder UC. Furthermore, the correlation between PLAC1 expression and advanced disease stages suggests its potential involvement in disease progression. Further research is needed to clarify the functional role of PLAC1 in tumorigenesis and explore its possible utility as a molecule of interest in cancer therapeutic research.

**Limitations**

Although this study offers important findings, some limitations must be noted. First, the small sample size may reduce the extent to which these findings can be generalized and also diminish statistical power. In addition, PLAC1 expression was not detected in non-malignant bladder tissues. However, the limited number of non-malignant samples should be taken into consideration when interpreting this observation. Second, although cytoplasmic PLAC1 expression was significantly associated with several adverse clinicopathological features, no significant association

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with DSS or PFS was observed, limiting conclusions regarding its prognostic utility. Third, the lack of animal studies has limited the possibility of investigating molecular mechanisms behind these findings. Fourth, the molecular mechanisms underlying the observed association was not investigated, leaving a gap in the comprehension of the functional pathways involved. Finally, the study did not examine the dynamic expression of PLAC1 during tumorigenesis, which could provide critical insights into its role in cancer progression. Addressing these limitations in future research will be essential to strengthen the conclusions and enhance the translational potential of this work.

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