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Urologic Oncology: Seminars and Original Investigations 37 (2019) 800–807

UROLOGIC ONCOLOGY

Laboratory-Bladder cancer

Significance of CLASP2 expression in prognosis for muscle-invasive bladder cancer patients: A propensity score-based analysis

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Received 24 January 2019; received in revised form 27 April 2019; accepted 4 May 2019

Abstract

Background: Cytoplasmic linker-associated protein 2 (CLASP2) belongs to a family of microtubule plus-end tracking proteins that localize to the distal ends of microtubules and is involved in various microtubule-dependent processes. We previously showed that CLASP2 is involved in the epithelial-to-mesenchymal transition of bladder urothelial cancer. This research aimed to explore the significance of CLASP2 expression as a prognostic marker for muscle-invasive bladder urothelial cancer (MIBC) patients after radical cystectomy-pelvic lymph node dissection (RC-PLND).

Methods: CLASP2 expression was analyzed in 76 benign bladder tissues and 160 MIBC tissues by tissue immunohistochemistry. Survival analysis and multiple regression analysis following propensity score matching were performed to investigate the correlation between high CLASP2 expression and MIBC patients' survival.

Results: CLASP2 expression was increased in MIBC patients, especially those with high-stage tumors or lymph node metastasis. In the follow-up of MIBC patients after propensity score matching, whether MIBC patients received adjuvant chemotherapy after RC-PLND, high CLASP2 expression was significantly associated with a poor prognosis. MIBC patients with low CLASP2 expression who received adjuvant chemotherapy tended to have an improved survival prognosis.

Conclusion: CLASP2 expression is correlated with malignant progression of MIBC. High CLASP2 expression predicted a poor prognosis for MIBC patients after RC-PLND.

Keywords: Cytoplasmic linker-associated protein 2; Muscle invasive bladder cancer; Adjuvant chemotherapy; Prognosis

Abbreviations: AC, adjuvant chemotherapy; BC, bladder urothelial cancer; CLASP2, cytoplasmic linker-associated protein 2; DFS, disease-free survival; EMT, epithelial-mesenchymal transition; ECOG, Eastern Cooperative Oncology Group; GC, gemcitabine and cisplatin; IHC, immunohistochemistry; MIBC, muscle-invasive bladder urothelial cancer; OS, overall survival; PS, performance status; RC-PLND, radical cystectomy-pelvic lymph node dissection; TNM, tumor-node-metastasis; UICC, Union for International Cancer Control

1. Introduction

Bladder cancer (BC) is the second most common genitourinary malignancy and the fourth most common cancer in the United States, representing approximately 5% of all new cancers with an estimated 17,240 deaths expected in the United States alone in 2018 [1,2]. BC is staged based on the tumor-node-metastasis (TNM) system, which describes the extent of invasion [3]. According to the 8th edition of the TNM classification published by the Union for International Cancer Control (UICC), BC can be divided into nonmuscle-invasive BC and muscle-invasive BC (MIBC) [4]. Approximately 30% of patients are diagnosed with MIBC at the time of their initial presentation, and they have a less favorable prognosis with a 5-year survival rate of less than 50% [5]. The prognosis and treatment of BC are usually determined based on traditional clinicopathological parameters including

Financial support for this study: This work was supported by the following grants: the National Natural Science Foundation of China (No. 81001137 and No. 81874094), Innovation and Entrepreneurship in South Central University project (No. 2017gczd032), and the project from Health and Family Planning Commission of Hunan Province (No. C20180105).

Conflict of interest: The authors declare that they have no competing interests.

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https://doi.org/10.1016/j.urolonc.2019.05.003
1078-1439/

tumor stage and grade, which cannot accurately reflect disease behavior. Despite improvements in surgical techniques for radical cystectomy-pelvic lymph node dissection (RC-PLND), only half of patients with extravesical extension and 15% to 35% of patients with nodal metastasis are long-term survivors [6–9].

Perioperative systemic treatment has been a positive addition to the treatment of BC and has achieved better disease control and improved survival for BC patients. Although a great deal of evidence supports the use of neoadjuvant chemotherapy for MIBC patients [10,11], adjuvant chemotherapy (AC) is administered more frequently in daily clinical practice [12]. Evidence also advocates that AC might be beneficial for MIBC patients [13,14]. The first-line chemotherapeutic agent of choice is cisplatin, which is usually administered in combination with gemcitabine [15]. However, chemoresistance seems to exist, depending on the molecular subtype of BC [16]. This resistance can lead to possible adverse effects for nonresponders with unnecessary toxicity that causes deterioration in patients' physical condition and a delay in initiating the alternatively effective therapy. Therefore, to further understand the prognosis for MIBC patients and to improve the therapeutic index of AC, a precise evaluation of prognosis following radical surgery is necessary.

Epithelial-to-mesenchymal transition (EMT) is a biological process in which epithelial cells lose their epithelial characteristics and acquire a migratory and mesenchymal phenotype [17,18]. This process has been documented in BC [19–21]. We previously discovered that cytoplasmic linker-associated protein 2 (CLASP2) was involved in the EMT of BC, and that CLASP2 could promote proliferation, migration, and invasion in BC cell lines [22]. In this study, to investigate the association between CLASP2 and BC progression status as well as survival prognosis, CLASP2 expression in the bladder tissues of MIBC patients was detected and a propensity score-based analysis was conducted.

2. Materials and methods

2.1. Bladder specimens and follow-up of MIBC patients

One hundred sixty MIBC patients, and 76 patients with benign bladder biopsies were included in this study from January 2010 to January 2015 in the Department of Urology, Xiangya Hospital of Central South University (Changsha, PR China). The MIBC samples were collected from RC-PLND operations. Benign samples were collected from bladder biopsies, and pathologists ruled out any malignancy.

Tumor staging was performed according to the standard TNM staging guidelines of the UICC and tumor grading was performed according to the 2004 World Health Organization (WHO) grading system [4,23]. No patient underwent neoadjuvant chemotherapy. Patients with BC $\geq pT_2$ (N0-3) were recommended for treatment with AC after RC-PLND [24]. The chemotherapy regimen of gemcitabine plus cisplatin (GC) was as follows: gemcitabine 1000 mg/m^2, 1 day-1, 8 day-1; cisplatin 70 mg/m^2, 2 day-1. This regimen was administered in a 21-day cycle over 2 cycles. Patients with pure urothelial carcinoma or mixed histology with squamous and/or glandular differentiation were included in the analysis. Patients with all other histology variants and cT_{4b} disease were excluded from the analysis. The median follow-up time of these patients was 30 months (ranged 12–36 months). Disease-free survival (DFS) was defined as the period after RC-PLND when no local BC recurrence or metastases could be detected. The study protocol was reviewed and approved by the Xiangya Hospital Ethics Committee. All patients were provided with written consent for the tissue samples.

2.2. Tissue immunohistochemistry (IHC)

IHC staining was performed on the samples from the primary BC tissues and benign bladder tissues. The samples were fixed in 4% neutral buffered para-formaldehyde, embedded in paraffin, and cut into 5 μm slices. After deparaffinization, hydration, and antigen retrieval, these sections were incubated with corresponding primary antibody, followed by incubation with biotinylated secondary antibody (Vector, Burlingame, CA) and then visualized by the VECTASTAIN ABC peroxidase system and 3, 3′-diaminobenzidine (DAB) kit (Vector, Burlingame, CA). The primary antibody was a rabbit anti-CLASP2 antibody (Abcam, Cambridge, United Kingdom, dilution 1:100). CLASP2 immunoreactivity was scored according to the intensity and extent of staining by two independent pathologists who lacked prior knowledge of the patients' clinicopathological characteristics. Briefly, the scoring classification used for either the tumor or the benign epithelium was as follows: low: no staining, weak staining or strong staining in less than 50% of tumor cells; or high: strong staining in more than 50% of tumor cells. In cases of discrepant results, the values were discussed until an agreement was reached.

2.3. Statistical analysis

The propensity score matching was performed between MIBC patients with low and high CLASP2 expression. To conduct propensity score matching, the records of MIBC patients with low CLASP2 expression ($n = 68$) and high CLASP2 expression ($n = 92$) were obtained. Multiple clinical and pathologic features including patient data (age, sex, Eastern Cooperative Oncology Group Performance Status [ECOG PS]), tumor data (pathological tumor stage, pathological lymph node status), and treatment of AC were collected. Because of inherent differences among patient groups in terms of baseline patient and disease characteristics, nearest neighbor (1:1) propensity score matching was used to adjust for those differences.

Comparisons were performed using an unpaired t test or a chi-squared test, as appropriate. DFS and overall survival (OS) were examined by comparing the low- CLASP2-expression group and the high-CLASP2-expression group using the Kaplan-Meier and log-rank tests. Cox proportional

hazards regression was performed to evaluate the association of prognostic outcomes with CLASP2 expression, patient characteristics, tumor characteristics, and AC. The influence of AC on the prognosis for patients with low CLASP2 expression and those with high CLASP2 expression was evaluated separately. All statistical analyses were performed by SPSS Statistics 24. *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. Higher CLASP2 expression was found in patients with a higher stage tumor and lymph node metastasis

Of the 160 MIBC patients, 89 were male with an average age of 64 years (ranged 55−75 years), and 71 were female with an average age of 66 years (ranged 55−76 years). All patients underwent RC-PLND with 83 patients diagnosed as stage T_2 and 77 patients diagnosed as $T_{3\text{-}4a}$. Five patients were found to have carcinoma in situ, and 23 patients had lymph node metastasis after RC-PLND. IHC revealed that CLASP2 was mainly expressed in the cytoplasm (Fig. 1). After comparing CLASP2 expression between the benign and cancerous tissues, CLASP2 expression in benign bladder tissues was found to be low, but it was significantly higher in BC tissues, especially in BC tissues from patients with high-stage BC or lymph node metastasis (Fig. 1). Among the benign bladder tissues, only 2 (2.6%) samples showed high CLASP2 expression. However, 37 of 75 (49.3%) T_2N_0 BC samples and 35 of 62 (56.5%) $T_{3\text{-}4a}N_0$ BC samples exhibited high CLASP2 expression (Table 1). Moreover, the highest level of CLASP2 expression was found to be in BC tissues from patients with lymph node metastasis, with 20 of 23 (87.0%) samples showing high CLASP2 expression (Table 1).

These data indicated that increased CLASP2 expression was involved in BC development and progression. CLASP2 expression could be a representative marker of the status of BC progression.

3.2. High CLASP2 expression was significantly associated with a poor prognosis for MIBC patients after RC-PLND

A summary of the characteristics of MIBC patients before and after propensity score matching is shown in Table 2. Before matching, 68 patients (42.5%) had low

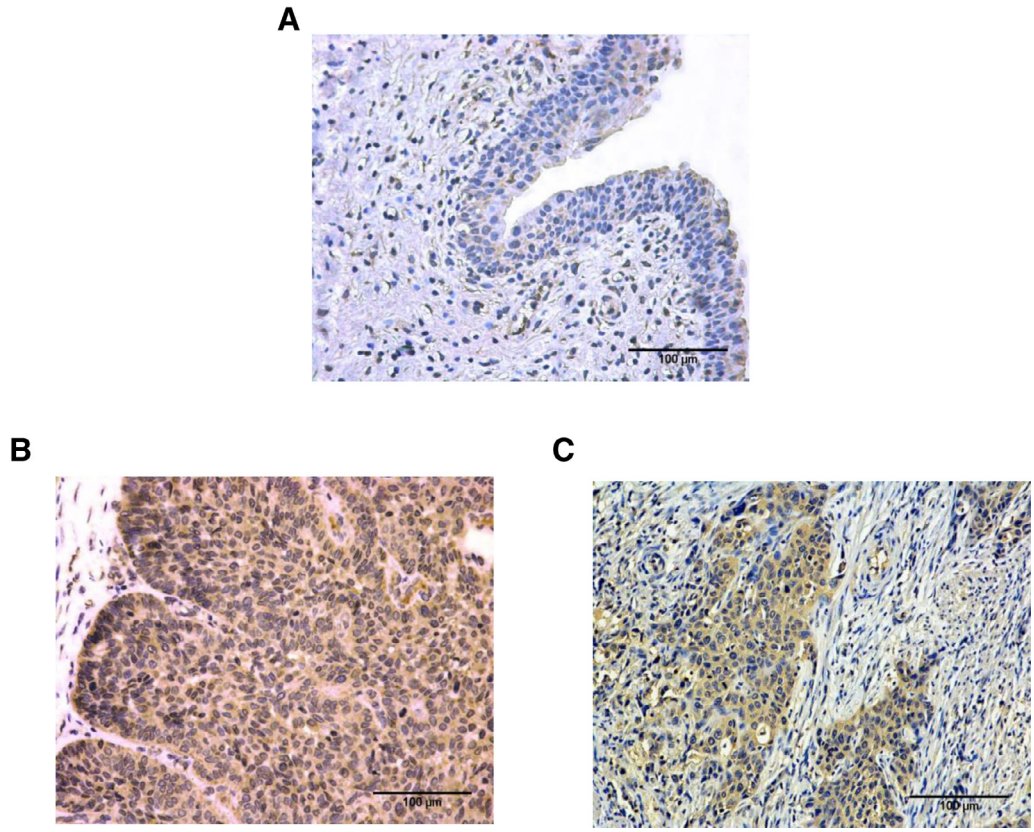

Fig. 1. CLASP2 immunohistochemistry. (A) Low CLASP2 expression in benign bladder tissue (magnification × 200). (B) High CLASP2 expression in T_2 BC (magnification × 200). (C) High CLASP2 expression in T_3 BC with lymph node metastasis (magnification × 200). BC = bladder urothelial cancer; CLASP2 = cytoplasmic linker-associated protein 2.

Table 1
Comparison of CLASP2 expression between benign bladder tissues and MIBC tissues of different stages. (L: low expression; H: high expression; a, b, c, d: group symbols).

	Total N = 236	CLASP2-L cases (%)	CLASP2-H cases (%)	χ^2	*P*
Benign bladder tissues[a]	76	74(97.4)	2(2.6)		
T_2N_0[b]	75	38 (50.7)	37 (49.3)	47.69	<0.01[ab]
$T_{3\text{-}4a}N_0$[c]	62	27 (43.5)	35 (56.5)	0.69	0.49[bc]
Lymph node metastatic BC[d]	23	3 (13.0)	20 (87.0)	6.84	0.01[cd]

BC = bladder urothelial cancer; CLASP2 = cytoplasmic linker-associated protein 2; MIBC = muscle-invasive BC.

Table 2
Clinical information of MIBC patients with low or high CLASP2 expression after RC-PLND. (L: low expression; H: high expression).

Variables	Before match			After propensity score match (1:1)		
	CLASP2-L (*n* = 68)	CLASP2-H (*n* = 92)	*P*	CLASP2-L (*n* = 68)	CLASP2-H (*n* = 68)	*P*
Age (years old)	55~76	55~75	0.06	55~76	55~75	0.15
Sex						
Female	26	45		26	36	
Male	42	47	0.20	42	32	0.12
ECOG PS≥1						
No	50	59		50	46	
Yes	18	33	0.23	18	22	0.57
pT						
T2	38	45		38	36	
≥T3	30	47	0.43	30	32	0.86
pN positive						
No	65	72		65	63	
Yes	3	20	**<0.01**	3	5	0.72
(N1/N2/N3)	(3/0/0)	(10/8/2)	(0.44)	(3/0/0)	(3/1/1)	(1.00)
AC						
No	30	43		30	36	
Yes	38	49	0.75	38	32	0.39

AC = adjuvant chemotherapy; CLASP2 = cytoplasmic linker-associated protein 2; ECOG PS = Eastern Cooperative Oncology Group Performance Status; MIBC = muscle-invasive BC; RC-PLND = radical cystectomy-pelvic lymph node dissection,

CLASP2 expression, and 92 patients (57.5%) had high CLASP2 expression. There was a significant difference in the distribution of pathological lymph node metastasis between the two groups. After matching, the characteristics of patients between the 2 groups were evenly distributed.

Kaplan-Meier analysis revealed that high CLASP2 expression was associated not only with poor DFS but also with poor OS (Fig. 2). The 3-year DFS rate was 83.8% in the low-CLASP2-expression group vs. 55.9% in the high-CLASP2-expression group (log rank: $P < 0.01$) (Fig. 2A). And the 3-year OS rate was 86.8% vs. 60.3%. (log rank: $P < 0.01$) (Fig. 2B).

Propensity score-adjusted univariate and multivariate analyses with the Cox proportional hazards regression model were undertaken to identify the prognostic variables (Tables 3 and 4). Only the significant factors of the univariate analysis were included in the multivariate analysis. The multivariate analysis showed that high CLASP2 expression (hazard ratio [HR] 5.47, 95% confidence interval [CI] 2.61−11.47; $P < 0.01$), pT ≥ T_3 (HR 13.19, 95% CI 5.42−32.13; $P < 0.01$) and pN positivity (HR 8.67, 95% CI 3.44−21.89; $P < 0.01$) were all independent predictors of worse DFS, while AC (HR 0.17, 95% CI 0.08−0.34; $P < 0.01$) was associated with better DFS (Table 3). Furthermore, high CLASP2 expression (HR 4.34, 95% CI 1.98−9.52; $P < 0.01$), pT ≥ T_3 (HR 9.52, 95% CI 3.92−23.12; $P < 0.01$), pN positivity (HR 8.11, 95% CI 3.01−21.84; $P < 0.01$), and ECOG PS ≥ 1 (HR 2.13, 95% CI 1.07−4.24; $P = 0.03$, $P < 0.05$) were all independent predictors of worse OS (Table 4).

Meanwhile, patient survival in subgroups of No AC and AC was analyzed separately. In the patients who did not receive AC after RC-PLND, the patients with low CLASP2 expression had a better prognosis than those with high CLASP2 expression not only in DFS (log rank: $P = 0.031$, $P < 0.05$) but also in OS (log rank: $P = 0.040$, $P < 0.05$) (Fig. 3A). Similarly, in the patients who received AC after RC-PLND, patients with low CLASP2 expression also had

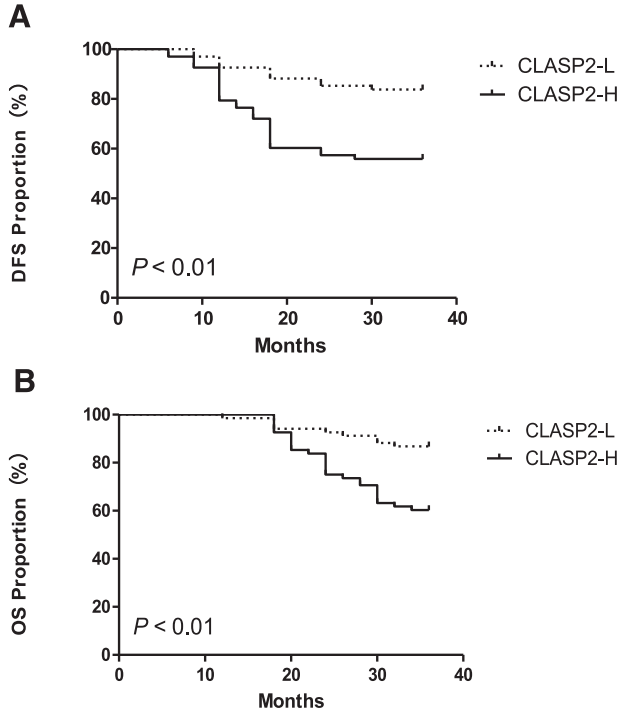

Fig. 2. **(A)** Kaplan-Meier estimated DFS curves for patients with low CLASP2 expression compared with the DFS curves for patients with high CLASP2 expression. (L: low expression; H: high expression). **(B)** Kaplan-Meier estimated OS curves for patients with low CLASP2 expression compared with the OS curves for patients with high CLASP2 expression. (L: low expression; H: high expression). CLASP2 = cytoplasmic linker-associated protein 2; DFS = disease-free survival; OS = overall survival.

a better prognosis than those with high CLASP2 expression in DFS (log rank: $P < 0.01$) and OS (log rank: $P < 0.01$) (Fig. 3B). Therefore, whether the MIBC patients received AC after RC-PLND, high CLASP2 expression was significantly associated with a poor prognosis. High CLASP2 expression was an adverse prognostic biological marker for MIBC patients.

Further survival analysis in the low- and high-CLASP2-expression groups revealed that, in the patients with low CLASP2 expression, the 3-year DFS rate was 65.6% in those who received AC vs. 47.2% in patients who did not receive AC (log rank: $P = 0.034$, $P < 0.05$) and the 3-year OS rate was 71.9% vs. 50% (log rank: $P = 0.025$, $P < 0.05$) (Fig. 4A). However, in the patients with high CLASP2 expression, there was no prominent improvement in DFS (log rank: $P = 0.12$, $P > 0.05$) or OS (log rank: $P = 0.06$, $P > 0.05$) (Fig. 4B). Based on this outcome, the MIBC patients with low CLASP2 expression who received AC tended to have an improved survival prognosis.

4. Discussion

Postoperative cisplatin-based chemotherapy has been widely used for muscle- invasive or metastatic BC [25]. Unfortunately, because of the chemo-resistance of cancer cells, the effective response is not sustained in more than 50% of cases, resulting in a 5-year survival rate of 15% [26,27]. It is important to better understand the factors that determine the chemotherapy response and affect prognosis for BC patients. Emerging evidence suggests that the EMT process in BC promotes the acquisition of invasive and chemo-resistance properties [28,29]. We previously found that CLASP2 could promote EMT and BC progression [22]. To further explore the clinical value of CLASP2 as a predictive biomarker in MIBC patients, we investigated the correlation between CLASP2 and BC progression as well as patient prognosis.

Our findings showed that increased CLASP2 expression was a general feature of BC progression and was associated with lymph node metastasis. CLASP2 may be a biological marker of aggressiveness for BC and a predictive factor for patient survival. We further conducted propensity score-based analysis to explore the significance of CLASP2 in the survival prognosis for MIBC patients. Propensity score matching eliminated the bias of pathological lymph node metastasis in MIBC patients with low and high CLASP2 expression, thus balancing the cohort groups. Deep analysis revealed that high CLASP2 expression was significantly correlated with poor DFS and OS in MIBC patients after

Table 3
Propensity score-adjusted univariate and multivariate Cox regression analysis for factors affecting DFS. (H: high expression).

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age (≥ 65)	0.44	1.02	0.97–1.09			
Sex (male)	0.38	0.76	0.41–1.40			
ECOG PS ≥ 1	0.12	1.65	0.88–3.09			
pT ≥ T_3	< 0.01	7.76	3.43–17.55	<0.01	13.19	5.42–32.13
pN positivity	< 0.01	10.33	4.54–23.53	<0.01	8.67	3.44–21.89
AC	0.01	0.43	0.23–0.82	<0.01	0.17	0.08–0.34
CLASP2-H	< 0.01	3.22	1.61–6.44	<0.01	5.47	2.61–11.47

AC = adjuvant chemotherapy; CI = confidence interval; CLASP2 = cytoplasmic linker-associated protein 2; DFS = disease-free survival; ECOG PS = Eastern Cooperative Oncology Group Performance Status; HR = hazard ratio; OS = overall survival.

Table 4
Propensity score-adjusted univariate and multivariate Cox regression analysis for factors affecting OS. (H: high expression).

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age (≥ 65)	0.18	1.04	0.98−1.11			
Sex (male)	0.36	0.74	0.38−1.41			
ECOG PS ≥ 1	0.01	2.36	1.22−4.54	0.03	2.13	1.07−4.24
pT ≥ T_3	<0.01	6.35	2.78−14.52	<0.01	9.52	3.92−23.12
pN positivity	<0.01	10.21	4.45−23.43	<0.01	8.11	3.01−21.84
AC	<0.01	0.36	0.18−0.72	<0.01	0.12	0.05−0.28
CLASP2-H	<0.01	3.41	1.60−7.27	<0.01	4.34	1.98−9.52

AC = adjuvant chemotherapy; CI = confidence interval; CLASP2 = cytoplasmic linker-associated protein 2; ECOG PS = Eastern Cooperative Oncology Group Performance Status; HR = hazard ratio; OS = overall survival.

RC-PLND, which was consistent with the HR of high CLASP2 expression in the propensity score-adjusted multivariate Cox regression analysis. These data verified the tight association between CLASP2 expression and prognosis for MIBC patients. Meanwhile, Whether the MIBC patients received AC after RC-PLND, better DFS and OS were found in the patients with low CLASP2 expression than in those with high CLASP2 expression further demonstrating the association. Moreover, MIBC patients with low CLASP2 expression who received AC after RC-PLND tended to have improved DFS and OS, while those with high CLASP2 expression did not, implying an underlying correlation might exist between CLASP2 and chemoresistance. Based on these findings, CLASP2 could be used as a risk stratification standard for MIBC patients. Low CLASP2 expression might be a favorable factor not only

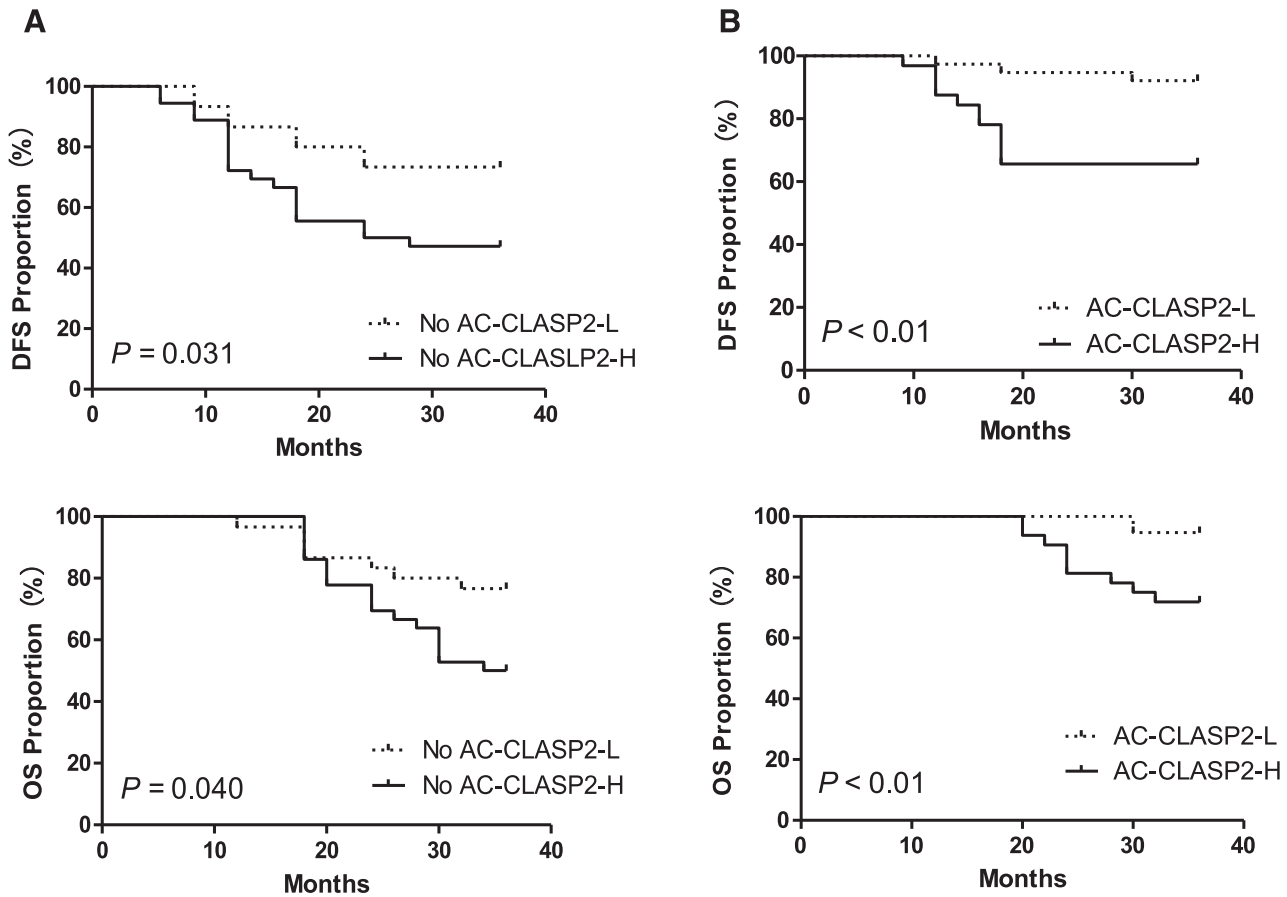

Fig 3. **(A)** Kaplan-Meier estimated DFS and OS curves for patients not receiving AC with low CLASP2 expression compared with the DFS and OS curves for those with high CLASP2 expression. (L: low expression; H: high expression) **(B)** Kaplan-Meier estimated DFS and OS curves for patients receiving AC with low CLASP2 expression compared with the DFS and OS curves for those with high CLASP2 expression. (L: low expression; H: high expression). AC = adjuvant chemotherapy; CLASP2 = cytoplasmic linker-associated protein 2; DFS = disease-free survival; OS = overall survival.

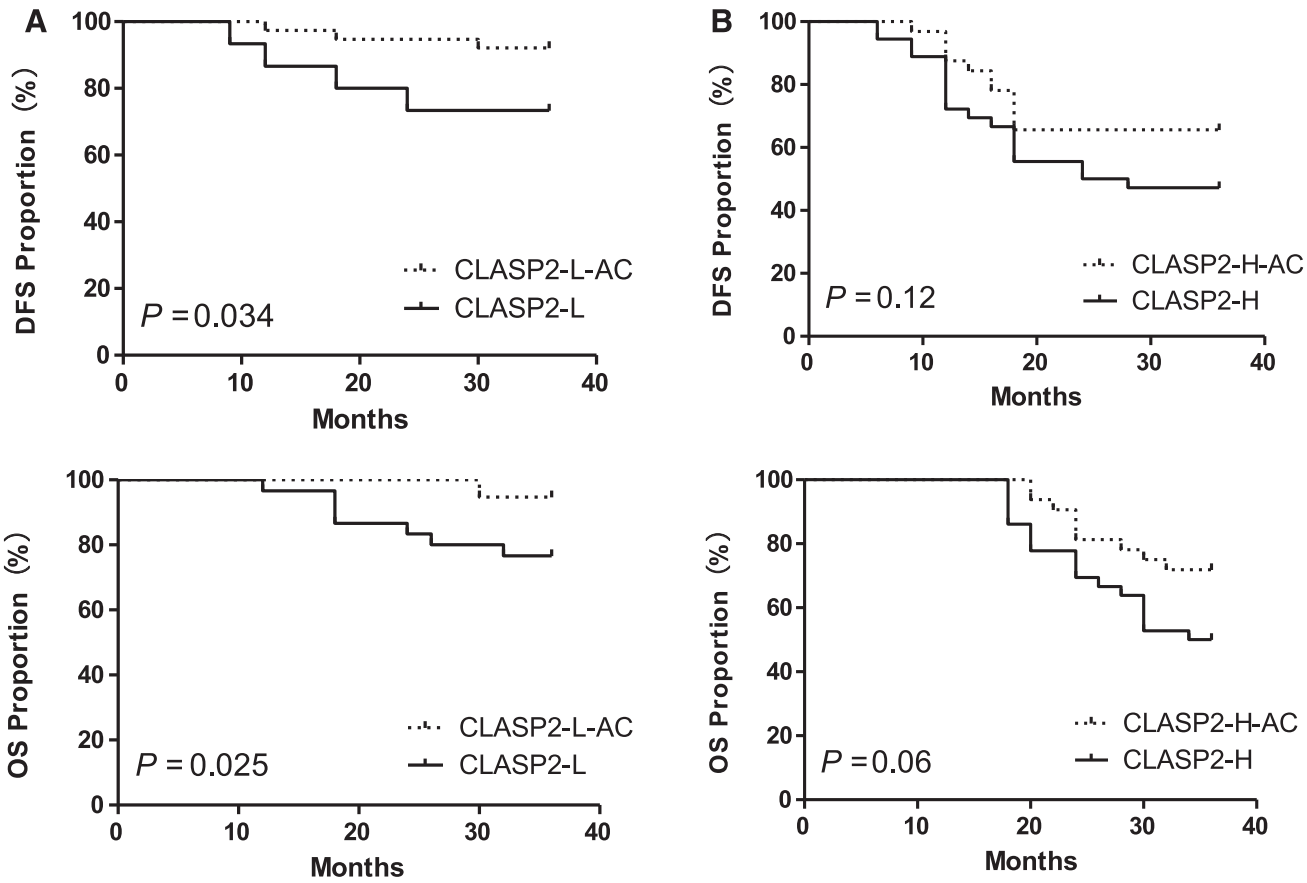

Fig. 4. **(A)** Kaplan-Meier estimated DFS and OS curves for patients with low CLASP2 expression who received AC compared with those who did not receive AC. (L: low expression) **(B)** Kaplan-Meier estimated DFS and OS curves for patients with high CLASP2 expression who received AC compared with those who did not receive AC. (H: high expression). AC = adjuvant chemotherapy; CLASP2 = cytoplasmic linker-associated protein 2; DFS = disease-free survival; OS = overall survival.

for patient survival but also for the therapeutic index of AC. In contrast, high CLASP2 expression could be an adverse factor in the clinical assessment of MIBC patients. Very few studies related to CLASP2 in other cancers have been reported. Our study suggests that there may be a great potential for CLASP2 in acquiring information concerning cancer therapy or prognosis in the future.

5. Conclusion

In summary, high CLASP2 expression in BC patients is correlated with a higher BC stage and with an increased risk of lymph node metastasis compared with low CLASP2 expression. High CLASP2 expression is significantly associated with poor prognosis for MIBC patients after RC-PLND.

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