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*Original Article*

# Clinical significance of MACC1, Twist1, and KAI1 expressions in infiltrating urothelial carcinoma of the bladder

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**Abstract:** Background: Metastasis-associated in colon cancer 1 (MACC1), a candidate oncogene, promotes tumor cell invasion and metastasis in various cancers. Twist1, a key transcriptional gene of the epithelial-mesenchymal transition (EMT), is involved in EMT and metastasis in many cancers. KAI1, also known as CD82, was originally considered as a suppressor gene of tumor metastasis. In this study, we investigated the expressions and significance of MACC1, Twist1, and KAI1 in infiltrating urothelial carcinoma of bladder (IUCB). Methods: The expressions of MACC1, Twist1, and KAI1 in 195 IUCB specimens and their corresponding control specimens were investigated by immunohistochemistry. The patients' clinical, demographic, and follow-up data were collected. Results: The rates of the positive expressions of MACC1 and Twist1 were significantly higher in IUCB tissues than in normal bladder mucosa tissues, and their expressions were positively correlated with tumor stages, grades of differentiation, lymph node metastasis (LNM), and tumor-node-metastasis (TNM) stages. The rate of positive expression of KAI1 was significantly lower in IUCB than in the control tissues, and its expression was inversely associated with tumor stages, grades of differentiation, LNM, and TNM stages. Patients who expressed MACC1 or Twist1 had an unfavorable overall survival (OS) time when compared with patients who did not express these proteins. However, patients who expressed KAI1 had a favorable OS when compared with patients who did not express this protein. A multivariate analysis demonstrated that the expressions of MACC1, Twist1, and KAI1, tumor stages, grades of differentiation, LNM, as well as TNM stages were independent prognostic indicators for IUCB patients. Conclusion: Therefore, MACC1, Twist1, and KAI1 should be considered potentially promising biomarkers of IUCB prognosis.

**Keywords:** IUCB, MACC1, Twist1, KAI1, prognosis

## Introduction

In 2018, it was estimated that there were 549,000 new cases of bladder cancer and 200,000 associated deaths worldwide [1]. Bladder urothelial carcinoma is the most common type of bladder cancer. Many IUCB patients are diagnosed at the advanced stages in China because the disease does not show any apparent symptoms during the early stage. Along with the development of medical technology, the outcome of bladder urothelial carcinoma has been clearly improved. However, the overall survival (OS) time of patients with bladder urothelial carcinoma has not been significantly prolonged. This may be related to a candidate oncogene named metastasis-associated in colon cancer 1 (MACC1). MACC1, originally found in colon cancer cell lines in 2009, is a crucial regulator of the hepatocyte growth factor (HGF)/mesenchymal-epithelial transition (MET) signaling pathway [2]. The MACC1 gene is located on human chromosome 7p21.1 and encodes 7 exons and 6 introns [3]. MACC1 can regulate the HGF/MET signaling pathway by binding to the promoter of the MET to regulate its transcriptional activity [2, 4]. It has been demonstrated that MACC1 not only promotes tumor cell mobility and migration in vitro but also promotes tumor cell invasion and metastasis in vivo [2, 4, 5]. Accumulating evidence has demonstrated that MACC1 is likely involved in invasion and metastasis and is considered a prognostic biomarker in various human cancers [2-10].

**Table 1.** Patient characteristics

| Patient characteristics | Frequency (n) | Percentage (%) |
|---|---|---|
| Age (years) | | |
| ≤ 60 | 127 | 65.1 |
| > 60 | 68 | 34.9 |
| Gender | | |
| Male | 129 | 66.2 |
| Female | 66 | 33.8 |
| Location | | |
| Side | 92 | 47.2 |
| Posterior | 67 | 34.4 |
| Top | 22 | 11.3 |
| Triangle | 14 | 7.2 |
| Alcohol | | |
| No | 88 | 45.1 |
| Yes | 107 | 54.9 |
| Size (cm) | | |
| ≤ 2.0 | 116 | 59.5 |
| > 2.0 | 79 | 40.5 |
| Smoking | | |
| No | 92 | 47.2 |
| Yes | 103 | 52.8 |
| Depth | | |
| Mucosa | 101 | 51.8 |
| Submucosa | 94 | 48.2 |
| Grades | | |
| Low | 102 | 52.3 |
| High | 93 | 47.7 |
| Lymph node metastasis | | |
| No | 183 | 93.8 |
| Yes | 12 | 6.2 |
| TNM stages | | |
| I+II | 173 | 88.7 |
| III+IVA | 22 | 11.3 |

Twist1, a highly conservatively basic helix-loop-helix (bHLH) transcriptional factor, is a crucial regulator of the epithelial-mesenchymal transition (EMT) [11]. It is well known that EMT is able to promote carcinoma cell invasion and metastasis by losing epithelial features and gaining mesenchymal features [12, 13]. Twist1, located on chromosome 7p21.2, encodes 2 exons and 1 intron [14]. Previous studies have demonstrated that Twist1 regulates EMT by directly inhibiting E-cadherin expression and enhancing N-cadherin synthesis [11, 15]. The overexpression of Twist1 can induce significant cancer cell morphological changes and reduce adhesion between cell and cell or cell and extracellular matrix, thus strengthening tumor cells mobility and migration ability [16, 17].

The inactivation of the suppressor of tumor metastasis is an early event in tumor invasion and metastasis. KAI1, also known CD82, was originally identified in prostate cancer cell lines [18]. The KAI1 gene that is located on human chromosome 11p11.2 belongs to the transmembrane 4 protein superfamily (TM4SF). The KAI1 gene encodes 10 exons and 9 introns. The KAI1 gene can inhibit tumor cell mobility and migration through enhancing cell-cell adhesion [19]. Previous studies have indicated that a low expression of KAI1 could promote malignant transformation and progression, further causing invasion, metastasis, and poor prognosis [7, 11, 19, 20].

Overall, studies on MACC1, Twist1, and KAI1 have indicated that they are associated with cancer invasion and metastasis. However, the associations between these proteins and IUCB have been not widely reported. The objective of this study is to assess the hypothesis that these proteins are associated with IUCB progression and prognosis.

## Methods

### *Patients and specimens*

We recruited 195 patients who were diagnosed with IUCB from January 2010 to December 2012 by the Department of Pathology of our hospital and collected specimens of carcinoma tissues and corresponding normal urothelial epithelial tissue from all patients. The corresponding adjacent normal bladder mucosa tissues were collected 3 cm away from tumor's edge. The clinicopathological characteristics, demography and follow-up data were also collected. Patients who received any anti-cancer therapy were excluded from the study. Overall survival (OS) time was assessed from the surgical removal date to December or his or her death date. The tumor-node-metastasis stages were assessed according to the 8$^{th}$ edition of the guidelines issued by American Joint Committee on Cancer (AJCC). The grades of differentiation were assessed according to the guidelines issued by the World Health Organization (WHO). The other clinicopathological characteristics are shown in **Table 1**.

*Reagent and immunohistochemistry*

Rabbit anti-human polyclonal antibody against MACC1 and mouse anti-human monoclonal antibody against Twist1 and KAI1 were purchased from Abcam, Co., (USA). Other reagents were purchased from Fuzhou Maxin Biotechnology Development Co., (China). Both the experimental and control tissues were fixed in a 10% buffer formalin solution and were embedded in paraffin. Then continuous 4-μm-thick sections were cut. Subsequently, all slices were deparaffinized and dehydrated in xylene and gradient alcohol. The immunohistochemistry was carried out according to the Elivision™ Plus method, and the procedure was carried out according to the kit's instructions. A citrate buffer solution (pH 6.0) was used for antigen repair. Endogenous peroxidase activity was blocked by a 3% $H_2O_2$ solution. After the were washed with PBS several times, all the slices were blocked with goat serum. Then the primary antibodies of MACC1, Twist1, and KAI1 were added and incubated at 4°C overnight. Subsequently, reagents A and B were added. All the slices were developed in a diaminobenzidine (DAB) substrate solution, and then they were re-dyed with hematoxylin.

*Assessment of immunostaining*

To preserve the intratumoral cell heterogeneity of the biomarker expressions, ten high-power-fields (HPF) were selected randomly. Two pathologists who were blinded to the patients' data assessed the immunostaining results through semi-quantitative points. The immunostaining results were calculated according to staining intensity and extent [21]. The intensity scores were graded as follows: no staining was 0; light-yellow staining was 1; yellow staining was 2; brown-yellow was 3. The extent scores were graded as follows: percentage of positive cells < 11% was 1; 10% < percentage of positive cells < 51% was 2; 50% < percentage of positive cells < 76% was 3; percentage of positive cells > 75% was 4. The final scores (range 0-12) were calculated by multiplying the intensity score by the extent score. A result > 2 was considered positive. An average of the final result of each specimen was taken. If there was a difference between the final results from the two independent pathologists, the results were re-assessed.

*Statistical analysis*

We used a Chi-square test to assess the expressions of MACC1, Twist1, and KAI1 in IUCB and in the control tissues as well as the associations between their expressions and the IUCB clinicopathological characteristics. The correlation analysis was performed using the Spearman coefficient test. A univariate OS analysis was carried out using the Kaplan-Meier method with a log-rank test. The multivariate OS analysis was carried out using the Cox regression model test. $P < 0.05$ was considered statistically significant. The statistical analysis was carried out using SPSS 19.0 software for Windows (Chicago, IL, USA).

## Results

*Expressions of MACC1, Twist1, and KAI1 in IUCB, and their association with the clinicopathological characteristics*

The positive staining of MACC1 was mainly confined to the cytoplasms, the positive staining of Twist1 was mainly confined to the cytoplasms and nuclei, and the positive staining of KAI1 was mainly confined to the membrane and cytoplasms. The positive expression of MACC1 in the IUCB group (66.7%, 130/195) was significantly higher when compared with its expression in the control group (7.7%, 15/195; P < 0.001; **Figure 1A** and **1B**). MACC1 expression was positively associated with grades of differentiation, lymph node metastasis (LNM), depth of invasion, and TNM stages, but not with the patients' ages, gender, tumor location, alcohol status, smoking status, or tumor size (**Table 2**).

There was a significant difference (P < 0.001) in Twist1 expression between the IUCB (64.1%, 125/195) and the control cells (5.1%, 10/195; P < 0.001; **Figure 1C** and **1D**). The expression of Twist1 was positively associated with grades of differentiation, LNM, depth of invasion, and TNM stages, but not with the patients' ages, gender, tumor location, alcohol status, smoking status, or tumor size (**Table 2**). The positive expression of KAI1 in IUCB group (40.0%, 78/195) was significantly lower than that in the control group (92.3%, 180/195; P<0.001; **Figure 1E** and **1F**). Positive expression of KAI1 was inversely correlated with grades of differentiation, LNM, depth of invasion and TNM stages (**Table 2**).

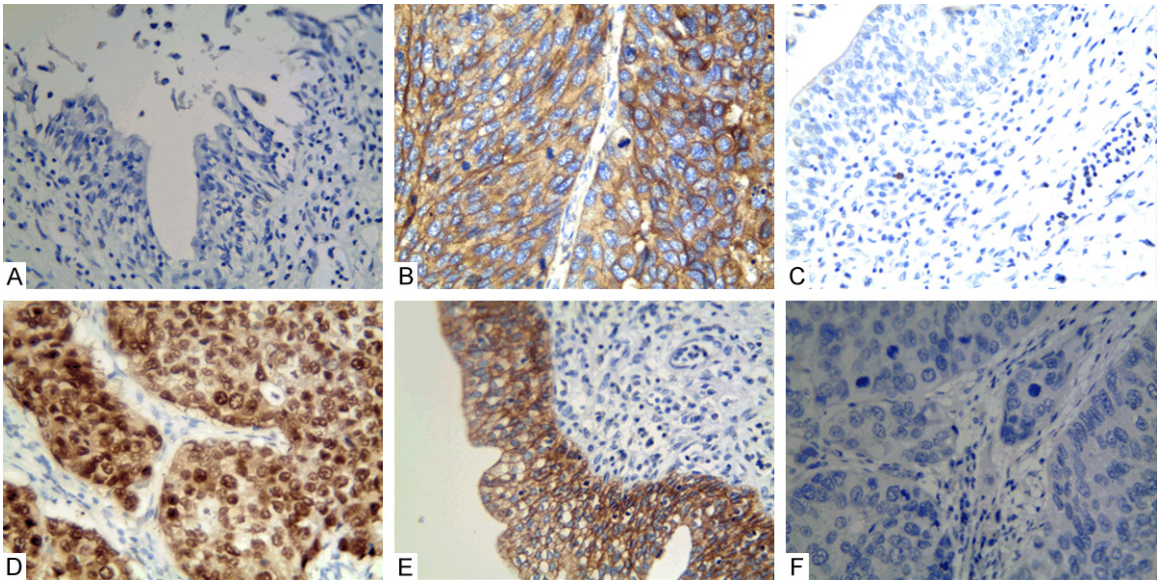


**Figure 1.** The immunostaining of MACC1, Twist1, and KAI1 in IUCB and the control tissues. A. Negative staining of MACC1 in the control tissues (100 magnification). B. Positive staining of MACC1 in the cytoplasms of the IUCB cells (400 magnification). C. Negative staining of Twist1 in the control tissues (100 magnification). D. Positive staining of Twist1 in the cytoplasms and nuclei of the IUCB cells (400 magnification). E. Positive staining of KAI1 in the membranes and cytoplasms of the control tissues (100 magnification). F. Negative staining of KAI1 in the IUCB cells (400 magnification).

*Spearman coefficient test*

The spearman correlation coefficient analysis indicated a negative association between KAI1 expression and MACC1 ($r = -0.244$, $P = 0.001$) and between KAI1 expression and Twist1 ($r = -0.327$, $P < 0.001$). And there was a positive association between MACC1 expression and Twist1 expression ($r = 0.537$, $P < 0.001$; **Table 3**).

*Univariate and multivariate analyzes*

As shown in **Figure 2A**, the univariate OS analysis demonstrated that the OS time of patients who expressed MACC1 was significantly lower than that of the patients who did not express the proteins (log-rank = 45.338, $P < 0.001$). As shown in **Figure 2B**, the OS time for the Twist1-positive patients was significantly lower than the OS time of the Twist1-negative patients (log-rank = 45.129, $P < 0.001$). As shown in **Figure 2C**, the OS time for the KAI1-positive patients was significantly longer than it was for those who did not express the protein (log-rank = 42.962, $P < 0.001$). In other univariate analyzes, the OS time significantly correlated with the following characteristics: grades of differentiation (log-rank = 20.813, $P < 0.001$; **Figure 2D**), depth of invasion (log-rank = 73.905, $P < 0.001$; **Figure 2E**), LNM (log-rank = 121.521, $P < 0.001$; **Figure 2F**), and TNM stages (log-rank = 93.567, $P < 0.001$; **Figure 2G**).

The multivariate analysis demonstrated that the expressions of MACC1, Twist1, and KAI1, depth of invasion, grades of differentiation, LNM, and TNM stages were independent factors for IUCB (**Table 4**).

## Discussion

Recurrence and metastasis are the main reasons for cancer treatment failure. The activation of a tumor metastasis gene should be closely related to these processes. The metastasis-associated in colon cancer 1 (MACC1) gene, which is considered an oncogene, was first identified in colon cancer cell lines [3]. MACC1 regulates the HGF/MET signaling pathway through the activation of MET transcriptional activity. The overexpression of MACC1 should affect tumorigenicity, EMT, mobility, migration, invasion, and metastasis [2, 4, 22]. In this study, we found that IUCB tissue expressed a higher level of MACC1 than the control tissue. MACC1 expression was positively associated with grades of differentiation, depth

**Table 2.** The associations between the expressions of MACC1, Twist1, and KAI1 and the clinicopathological characteristics of invasive bladder carcinoma (IUCB)

| Variables | MACC1 | | P | Twist1 | | P | KAI1 | | P |
|---|---|---|---|---|---|---|---|---|---|
| | - | + | | - | + | | - | + | |
| Age (years) | | | 0.595 | | | 0.285 | | | 0.111 |
| < 60 | 44 | 83 | | 49 | 78 | | 71 | 56 | |
| ≥ 60 | 21 | 47 | | 21 | 47 | | 46 | 22 | |
| Gender | | | 0.521 | | | 0.827 | | | 0.902 |
| Male | 45 | 84 | | 47 | 82 | | 77 | 52 | |
| Female | 20 | 46 | | 23 | 43 | | 40 | 26 | |
| Location | | | 0.174 | | | 0.089 | | | 0.161 |
| Side | 32 | 60 | | 27 | 65 | | 60 | 32 | |
| Posterior | 26 | 41 | | 32 | 35 | | 33 | 34 | |
| Top | 3 | 19 | | 6 | 16 | | 14 | 8 | |
| Triangle | 4 | 10 | | 5 | 9 | | 10 | 4 | |
| Alcohol | | | 0.263 | | | 0.186 | | | 0.217 |
| No | 33 | 55 | | 36 | 52 | | 57 | 31 | |
| Yes | 32 | 75 | | 34 | 73 | | 60 | 47 | |
| Size (cm) | | | 0.149 | | | 0.845 | | | 0.311 |
| ≤ 2.0 | 34 | 82 | | 41 | 75 | | 73 | 43 | |
| > 2.0 | 31 | 48 | | 29 | 50 | | 44 | 35 | |
| Smoking | | | 0.085 | | | 0.366 | | | 0.412 |
| No | 25 | 67 | | 30 | 62 | | 58 | 34 | |
| Yes | 40 | 63 | | 40 | 63 | | 59 | 44 | |
| Depth | | | < 0.001 | | | < 0.001 | | | < 0.001 |
| Mucosa | 47 | 54 | | 48 | 53 | | 45 | 56 | |
| Submucosa | 18 | 76 | | 22 | 72 | | 72 | 22 | |
| Grades | | | 0.001 | | | 0.027 | | | 0.007 |
| Low | 45 | 57 | | 44 | 58 | | 52 | 50 | |
| High | 20 | 73 | | 26 | 67 | | 65 | 28 | |
| LNM | | | 0.027 | | | 0.018 | | | 0.045 |
| No | 65 | 118 | | 70 | 113 | | 106 | 77 | |
| Yes | 0 | 12 | | 0 | 12 | | 11 | 1 | |
| TNM stages | | | 0.005 | | | < 0.001 | | | 0.014 |
| I+II | 64 | 109 | | 70 | 103 | | 98 | 75 | |
| III+IVA | 1 | 21 | | 0 | 22 | | 19 | 3 | |

**Table 3.** Correlations among the expressions of MACC1, Twist1, and KAI1 in IUCB

| Variable | MACC1 | | r | P | Twist1 | | r | P |
|---|---|---|---|---|---|---|---|---|
| | - | + | | | - | + | | |
| MACC1 | | | | | | | 0.537 | < 0.001* |
| - | | | | | 47 | 18 | | |
| + | | | | | 23 | 107 | | |
| KAI1 | | | -0.244 | 0.001@ | | | -0.327 | < 0.001@ |
| - | 28 | 89 | | | 27 | 90 | | |
| + | 37 | 41 | | | 43 | 35 | | |

*: positive association; @: negative association.

of invasion, LNM, and TNM stages. The OS analysis showed that IUCB patients expressing MACC1 survived for less time than patients who did not express MACC1. These findings are similar to those of other studies [2-11, 23], suggesting that MACC1 should be considered a useful and valuable biomarker for the prediction of IUCB invasion and prognosis.

Accumulating evidence has demonstrated that EMT can make tumor cells become more aggressive. Twist1, a key regulator of EMT through the promotion of N-cadherin synthesis and the inhibition of E-cadherin expression, is a transcriptional factor [11, 15]. Twist1 activates the EMT process and promotes cell invasion, metastasis, tumorigenicity, and cancer stem cell phenotypes [24]. In this study, we found that IUCB tissue expressed a higher level of Twist1 the control tissue did. Twist1 overexpression was positively correlated with grades of differentiation, depth of invasion, LNM, and TNM stages. Similar to MACC1, the OS analysis suggested that patients who expressed Twist1 survived for less time than those who did not express the protein. These findings suggest that Twist1 is involved in the invasion and metastasis process and should be considered a potential promising predictor for IUCB patients.

The inactivation of the suppressor gene for tumor metastasis also promotes tumor cell invasion and metastasis. KAI1 is a tumor metastasis suppressor in various human cancers. A positive expression

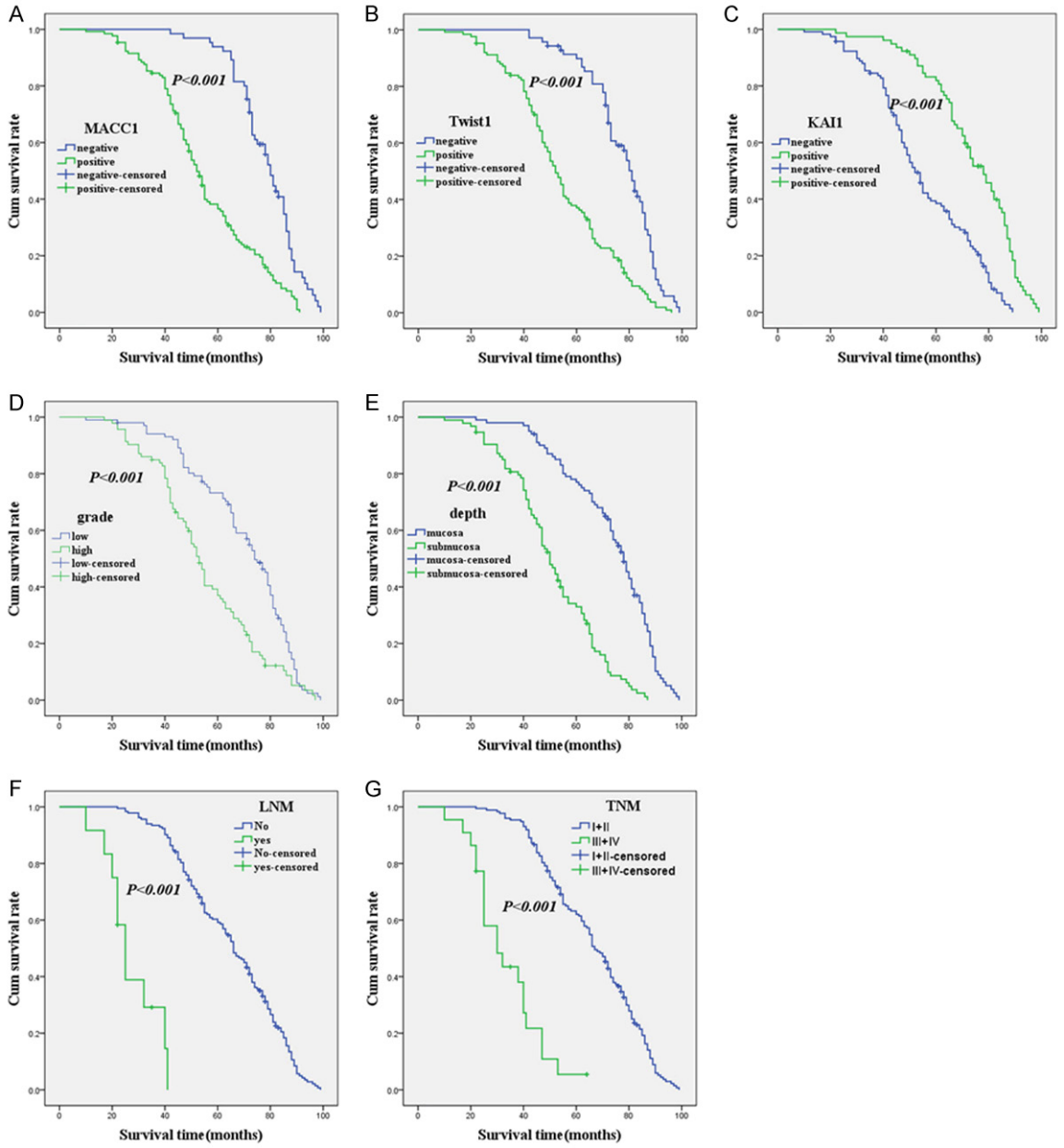


**Figure 2.** A Kaplan-Meier analysis of the survival rate of patients with IUCB. The y-axis represents the percentage of patients; the x-axis represents their survival in months. (A) Overall survival of all patients in relation to MACC1 (log-rank = 45.338, P < 0.001). (B) Overall survival of all patients in relation to MACC1 (log-rank = 45.129, P < 0.001). (C) Overall survival of all patients in relation to KAI1 expression (log-rank = 42.962, P < 0.001); In the (A-C) analyses, the green line represents patients with positive expressions of biomarkers, and the blue line represents the negative expressions of the biomarkers. (D) Overall survival of all patients in relation to the grades of differentiation (log-rank = 20.813, P < 0.001; the blue line represents patients with low grades, and the green line represents patients with high grades). (E) Overall survival of all patients in relation to depth of invasion (log-rank = 73.905, P < 0.001; the blue line represents patients in the mucosa group, the green line represents patients in the submucosa group). (F) Overall survival of all patients in relation to LNM (log-rank = 121.521, P < 0.001; the blue line represents patients with no LNM group, the green line represents patients with the LNM group). (G) Overall survival of all patients in relation to TNM stages (log-rank = 93.567, P < 0.001); the blue line represents the patients in stages I-II; the green line represents the patients in stages III-IV.

of KAI1 is able to suppress secondary metastasis without influencing primary tumor proliferation [25]. In our study, a positive expression of KAI1 was significantly lower in IUCB tissues

**Table 4.** Results of the multivariate analyses of the overall survival (OS) times

| Covariate | B | SE | P | HR | 95% CI |
|---|---|---|---|---|---|
| MACC1 | 0.702 | 0.211 | 0.001 | 2.018 | 1.336-3.049 |
| Twist1 | 0.762 | 0.202 | < 0.001 | 2.142 | 1.441-3.185 |
| KAI1 | -0.679 | 0.193 | < 0.001 | 0.507 | 0.347-0.741 |
| Depth | 1.222 | 0.197 | < 0.001 | 3.395 | 2.306-4.997 |
| Grades | 0.789 | 0.175 | < 0.001 | 2.201 | 1.563-3.099 |
| LNM | 1.217 | 0.507 | 0.016 | 3.376 | 1.251-9.112 |
| TNM stages | 0.977 | 0.380 | 0.010 | 2.656 | 1.261-5.594 |

than it was in control tissues. In addition, a positive expression of KAI1 was inversely associated with grades of differentiation, depth of invasion, LNM, and TNM stages. The OS analysis indicated that, unlike MACC1 and Twist1, patients who expressed KAI1 survived for a longer time than those who did not express the protein. Our findings indicate that a low expression of KAI1 should promote IUCB progression and metastasis, which is consistent with previous studies [7, 11, 19, 20, 25].

In this study, a Spearman correlation analysis demonstrated that the expression of KAI1 was negatively correlated with the expression of MACC1 or Twist1. And there was a positive association between the expressions of MACC1 and Twist1. A multivariate analysis showed that positive expressions of MACC1, Twist1, and KAI1, grades of differentiation, depth of invasion, LNM, and TNM stages were independent, prognostic indicators for IUCB patients. As a tumor metastasis suppressor, KAI1 can inhibit HGF activation by linking the MET gene to form a complex [26, 27]. This may suppress the activation of MACC1 to reduce the mobility and migration of tumor cells. KAI1 enhances cell-cell adhesion and the cell-extracellular matrix (ECM) by strengthening the stabilization of the E-cadherin/β-catenin complex to reduce metastasis [19]. A Low expression of KAI1 may lose its ability to stabilize the E-cadherin/β-catenin complex. At the same time, the overexpression of Twist1 should promote EMT by inhibiting E-cadherin expression and inducing N-cadherin synthesis. And the overexpression of MACC1 can promote EMT via the HGF/MET signaling pathway [6, 28]. So, the overexpression of MACC1 and Twist1, and the low expression of KAI1 should promote IUCB progression and also indicates a worse prognosis for patients.

## Conclusion

Our study found that the expressions of MACC1, Twist1, and KAI1 are associated with the duration of OS for IUCB patients. Therefore, MACC1, Twist1, and KAI1 can serve as useful and valuable biomarkers for IUCB and can provide some potential promising targets for treating IUCB patients.

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Tissue samples for diagnostic and research purposes were obtained with each patient's consent, and the research was approved by the ethical committee of Bengbu Medical University and performed in accordance with the guidelines of the Declaration of Helsinki.

## Disclosure of conflict of interest

None.

## Abbreviations

IUCB, infiltrating urothelial carcinoma of bladder; MACC1, metastasis-associated in colon cancer 1; EMT, epithelial-mesenchymal transition; TNM, tumor-node-metastasis; LNM, lymph node metastasis; OS, overall survival; HGF/MET, hepatocyte growth factor/mesenchymal-epithelial transition; bHLH, basic helix-loop-helix; TM4SF, transmembrane 4 protein superfamily; WHO, World Health Organization; AJCC, American Joint Committee on Cancer; HPF, high-power-field; DAB, diaminobenzidine; ECM, cell-extracellular matrix.

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