RESEARCH ARTICLE

Editorial Process: Submission:05/13/2025 Acceptance:01/29/2026 Published:02/05/2026

Crosstalk between *ZEB1* expression and *CD163*+ Tumor-Associated Macrophages in Muscle-Invasive Urothelial Carcinoma

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Abstract

Objective: There is an urgent need to investigate the molecular mechanisms underlying the invasion and metastasis of bladder cancer to develop more effective therapeutic strategies and thereby reduce tumor-related morbidity and mortality. This study aimed to evaluate the prognostic significance of *ZEB1* expression in bladder carcinoma (BC) and its association with tumor-associated macrophages (TAMs) within the tumor microenvironment (TME). **Methods:** A retrospective analysis was conducted on 48 patients with muscle-invasive bladder carcinoma (MIBC) who underwent radical cystectomy. Immunohistochemical staining for *ZEB1* and *CD163* was performed, followed by statistical analysis to assess their association with various clinicopathological parameters, including survival outcomes. **Results:** High *ZEB1* expression was significantly correlated with lymphovascular invasion, tumor necrosis, advanced disease stage, and nodal metastasis. Furthermore, elevated *ZEB1* expression was associated with significantly worse 3-year overall survival (OS) and disease-free survival (DFS). Similarly, a high density of *CD163*+TAMs within TME was associated with adverse clinicopathological parameters and poor survival outcomes. Notably, a strong positive correlation was observed between *ZEB1* expression and the density of *CD163*+ TAMs within the TME of BC. Multivariate analysis identified *ZEB1* expression as an independent predictor of recurrence and nodal metastasis. **Conclusion:** Elevated *ZEB1* expression is strongly associated with poor prognosis in BC and closely correlated with an increased density of *CD163*+TAMs, further contributing to adverse outcomes. These findings highlight the potential of *ZEB1* as a prognostic biomarker and underscore the therapeutic relevance of targeting TAMs in the management of BC.

Keywords: Bladder cancer- ZEB1- Microenvironment- Macrophages- CD163

Asian Pac J Cancer Prev, **27 (2)**, 499-507

Introduction

Bladder cancer is the tenth most common malignancy worldwide and the 13th leading cause of cancer-related mortality, with its incidence progressively increasing. It remains the most prevalent malignancy of the urinary system [1]. According to the Global Cancer Observatory (GLOBOCAN), approximately 573,000 new bladder cancer cases were diagnosed in 2020, reflecting a 3% increase in incidence. Bladder cancer is four times more common in men than women and typically occurs in individuals above 65 years of age [2].

Urothelial carcinoma is the predominant pathological subtype of bladder cancer. Approximately 75 % of bladder cancer cases are non–muscle-invasive bladder cancer (NMIBC), which is typically localized and manageable through transurethral resection of the bladder (TURB). However, the remaining 25 % of cases progress to muscle-invasive bladder cancer (MIBC), requiring more aggressive treatment strategies such as radical cystectomy, chemotherapy, immunotherapy, or radiotherapy [3].

The prognosis for bladder cancer worsens in cases of metastatic disease, with a five-year survival rate of less than 10%. Common metastatic sites include the liver, lungs, bones, and adrenal glands [4]. Despite advances in treatment, bladder cancer has a high recurrence rate of 60-70%, with many cases eventually progressing to muscle invasion or metastatic disease. Consequently, there is an urgent need to investigate the molecular mechanisms underlying bladder cancer invasion and metastasis to develop more effective therapeutic and reduce tumor-related morbidity and mortality [5].

Epithelial-mesenchymal transition (EMT) is a critical biological process in tumor progression, during which epithelial cells lose their phenotype and acquire mesenchymal characteristics. A hallmark of EMT is the

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loss of E-cadherin expression, which facilitates tumor invasion and metastasis [6]. Zinc finger E-box binding homeobox 1 (*ZEB1*), a transcription factor, plays a central role in EMT by regulating cellular plasticity and promoting tumor progression. Beyond its role in tissue development, *ZEB1* is implicated in several human malignancies, including breast, pancreatic, lung, liver, and colon cancers, where its dysregulated expression plays a critical role in disease progression [1]. Additionally, ZEB-1 expression is a significant barrier to chemotherapy and radiotherapy, acting as a key determinant of cancer prognosis [7].

Tumor-associated macrophages (TAMs) are essential components of the tumor microenvironment (TME) and are predominantly polarized into the M2 phenotype, which is associated with immunosuppression and tumor progression. The cluster of differentiation 163 (CD163) antibody is a well-established marker of M2 polarized macrophages, and its presence in various cancers is linked to poor clinical outcomes [8].

Given that EMT is a key driver of tumor cell invasion, it remains unclear whether invading tumor cells undergoing EMT are associated with M2 polarization and the density of TAMs within the TME. Therefore, this study aimed to evaluate the expression of *ZEB1* and *CD163*+ TAMs within the TME of muscle-invasive urothelial carcinoma and to assess their association with clinicopathological parameters, including survival outcomes.

Materials and Methods

Patient material and characterization

Patient data was guaranteed to remain confidential, with data sheets coded numerically to maintain anonymity, in accordance with the ethical guidelines of the Helsinki Declaration. The study was approved by the Ethics Committie of the South Egypt Cancer Institute, Assiut, Egypt (IRB NO: IORG0006563, Approval NO :757).

Forty-eight formalin-fixed, paraffin-embedded tumor tissue samples from patients with at least three years of follow-up data and who underwent radical cystectomy were retrieved from the pathology laboratory archives of the South Egypt Cancer Institute, Assiut, Egypt. The primary outcomewas to assess the association between *ZEB1* expression and tumor-associated macrophages (TAMs) within the tumor microenvironment. The minimum required sample size was calculated as 46 cases Psych Stat's two-sample proportion calculator (https://webpower.psychstat.org/models/prop02/), based on a correlation test to detect the relation between *ZEB1* and *CD163* expression in muscle-invasive bladder carcinoma. As no prior studies investigated this specific outcome, we hypothesized a moderate correlation (r=0.4) based on expert opinion. The parameters used for the calculation were alpha= 0.05 and power= 0.80.

Patient data were collected from 2019 to 2021. The following information was reviewed: sex, age at diagnosis, clinical stage, surgery details, postoperative residual disease, systemic therapy, local recurrence, and survival outcomes.

Immunohistochemistry procedures

Immunohistochemistry (IHC) was performed on the 48 tissue samples following the protocol described by Abdel-Hakeem et al. [9]. Formalin-fixed, paraffin-embedded tissue sections were cut to a thickness of 4-5 μm and mounted on coated, positively charged glass slides. The slides were heated in a water bath at 95°C for two hours, deparaffinized in xylene (two 10-minute cycles), and rehydrated in a descending series of alcohols (100%, 90%, 80%, and 70%;10 seconds each), followed by rinsing in distilled water. Antigen retrieval was performed by submerging the slides in Coplin jars containing Dako EnVisionTM FLEX Target Retrieval Solution (Code DM829), Citrate buffer (50x, pH 6.1), and heating in a microwave oven for 10-12 minutes (three times 5 minute cycles). The slides were allowed to cool at room temperature and washed three times with Dako EnVsionTM FLEX Wash Buffer (20x) (Code DM831) diluted in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked using Dako EnVisionTM FLEX peroxidase blocking reagent (Code SM801) for 5-10 minutes at room temperature. The sections were incubated for 24 hours at 4°C with primary antibody for *ZEB1* (Rabbit polyclonal antibody, Cat.NO: A16981) and *CD163* (Rabbit Monoclonal Antibody, Clone: EP324 ready to use, BIO SB) at a 1:100 dilution, following the manufacturer's protocol. The slides were then washed three times with PBS (3 minutes/each). After washing, the secondary antibody was applied for 20 minutes at room temperature. Diaminobenzidine (DAB) solution (Dako EnVisionTM FLEX DAB,Code DM827) was applied for 5-10 minutes, and sections were counterstained with Herris hematoxylin. Finally, sections were mounted using Dibutyl Phthalate Xylene (DPX) and examined under an OPTICA microscope equipped with a digital colored video camera (OPTICA 4083.B9 digital camera, Italy) [10].

Scoring of immunostaining

ZEB1 staining was observed in both nucleus and cytoplasm, with no exclusive nuclear staining detected. The intensity of *ZEB1* expression was scored on a scale from 0 to 3 (0 = negative, 1 = weak, 2 = medium, 3 = strong) and the staining extent was scored as 0 (0-5%), 1 (6-25%), 2 (26-75%) or 3 (75-100%). The intensity and extent scores were summed to obtain a final score. Final scores ≤ 3 were classified as low expression, and scores > 3 as high expression. All samples were evaluated independently by three observers [11].

For *CD163*, the percentage of stained cells was assessed relative to the total number of stromal cells within hot spots areas (1.0 mm2 within tumor centre). A score ranging from 0 to 100% was assigned, and cases were categorized into four grades: grade 0 (0-5 %), grade 1 (6-30 %), grade 2 (31 to 50 %), and grade 3 (> 50 %). Cases were then classified as low TAMs (grades 0 and 1) or high TAMs (grades 2 and 3) [12].

Statistical analysis

Data were analyzed using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 20. Continuous data were expressed as mean

± standard deviation (SD), or median and range for non-normally distributed data. Categorical data were presented as frequencies (number of cases) and relative frequencies (percentages). Quantitative variables were compared using the Student's t-test, and categorical data was analysing using the chi square ($\chi 2$) test [13]. Fisher's exact test was used when the expected frequency was less than 5. Survival analysis, including progression-free survival and overall survival, was performed using Kaplan-Meier's method with log rank test. A P-value < 0.05 was considered statistically significant..

Results

Clinicopathological characteristics

Forty-eight cases of muscle invasive urothelial carcinoma were retrospectively enrolled in this study. The mean age was 59 years. The tumor median size was 4.0 cm (range of 2.0 - 9.0 cm). Six patients had distant organ metastasis, whereas 14 (29.2%) of the selected cases showed local recurrence. The majority of cases (70.8 %) underwent adjuvant chemotherapy. Fourteen other patients (29.1%) were dead at the end of our follow up period.

Immunohistochemical profile of ZEB1 and CD163 in patients with muscle-invasive urothelial bladder carcinoma

The immunohistochemical scoring of *ZEB1* revealed that 32 cases (66.6%) of urothelial carcinoma exhibited high *ZEB1* expression, observed in nuclear and/or cytoplasmic (Figure 1). However, high immunostaining density of *CD163*+TAMs was observed in 54.2% of the examined urothelial carcinoma cases (Figure 1).

Association between ZEB1 expression, CD163+TAMs density, and different clinicopathological characteristics

Table 1 and 2 summarize the clinical and pathological characteristics associated with *ZEB1* expression and *CD163*+TAMs density. Results showed that patients with high *ZEB1* expression and *CD163*+TAMs high density exhibited worse clinicopathological features, including tumor necrosis, lymphovascular invasion, greater extent of invasion, advanced disease stage, positive nodal metastasis, and recurrence (Figure 2).

Survival analysis

Table 3 presents the clinicopathological parameters associated with poor prognosis during a 3-year (36-month) follow-up for overall survival (OS) and disease-free survival (DFS), as determined by Kaplan-Meier's survival

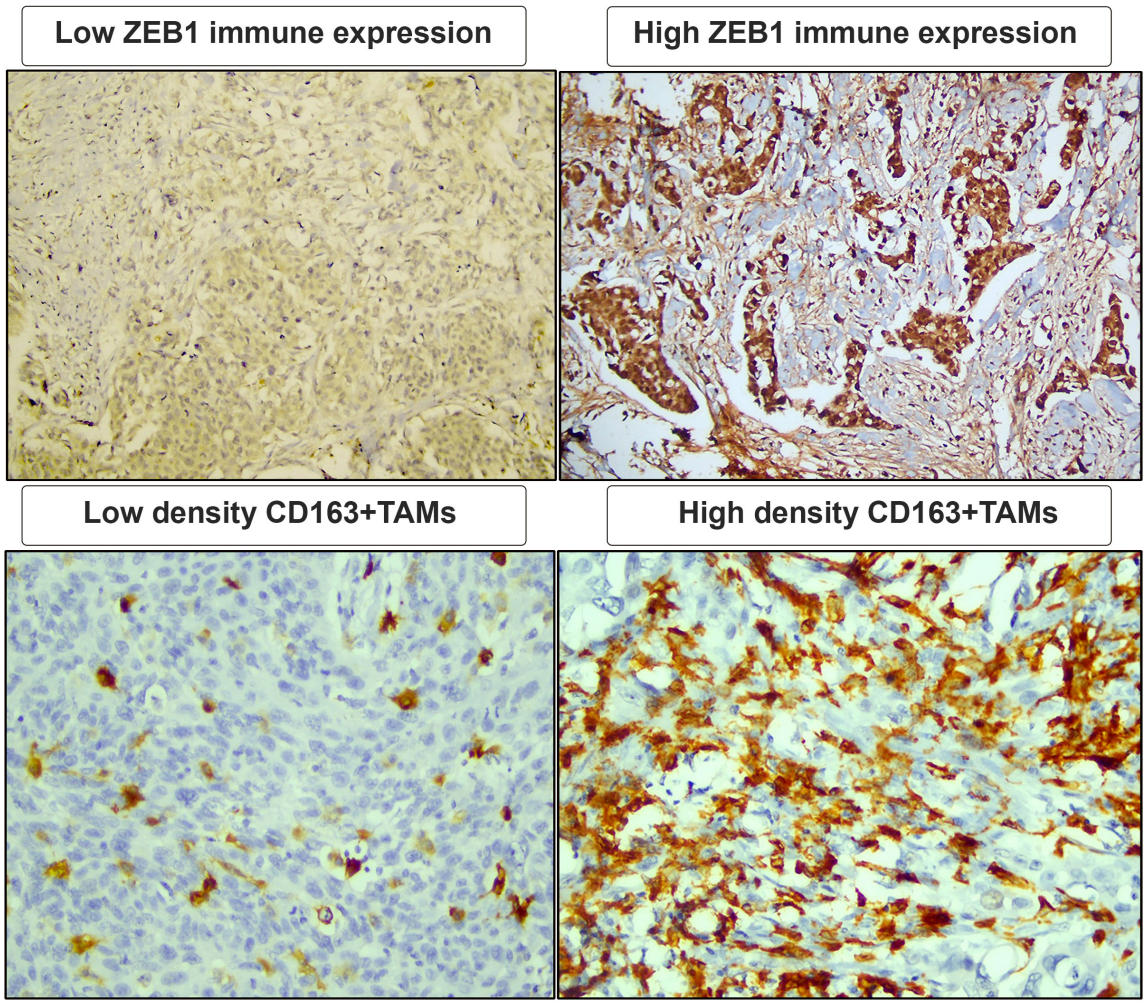

Figure 1. Immunohistochemical Staining and Scoring of *ZEB1* and *CD163*+TAMs.

Table 1. Correlation between *ZEB1* Expression and Demographic and Clinico-Pathological Parameters of Studied Participants

Variables		Low *ZEB 1* expression (n=16)		High *ZEB 1* expression (n=32)		P value
Age (years)	Mean ± SD	61.88 ± 5.20		58.00 ± 7.79		0.079 T
	Median (range)	63.5 (53 – 69)		61 (38 – 65)		
Gender	Male	16	-100.00%	28	-87.50%	0.286 F
	Female	0	0.00%	4	-12.50%	
Divergent differentiation of urothelial carcinoma	No	8	-50.00%	16	-50.00%	1.0 χ^2
	Squamous	8	-50.00%	16	-50.00%	
Bilharziasis	Absent	8	-50.00%	20	-62.50%	0.408 χ^2
	Present	8	-50.00%	12	-37.50%	
Necrosis	Absent	14	-87.50%	8	-25.00%	<0.001 χ^2
	Present	2	-12.50%	24	-75.00%	
Perineural invasion	Absent	4	-25.00%	6	-18.80%	0.712 F
	Present	12	-75.00%	26	-81.30%	
Lymphovascular tumor emboli	Absent	10	-62.50%	2	-6.30%	<0.001 F
	Present	6	-37.50%	30	-93.80%	
Pathological tumor stage (T)	T2	14	-87.50%	2	-6.30%	<0.001 F
	T3	2	-12.50%	24	-75.00%	
	T4	0	0.00%	6	-18.80%	
Pathological nodal stage (N)	N0	16	-100.00%	8	-25.00%	
	N1	0	0.00%	6	-18.80%	<0.001 F
	N2	0	0.00%	18	-56.30%	
Recurrence/ Metastasis	No	16	-100.00%	12	-37.50%	<0.001 χ^2
	Yes	0	0.00%	20	-62.50%	
Adjuvant therapy	No	2	-12.50%	6	-18.80%	0.331 F
	Yes (chemotherapy)	14	-87.50%	20	-62.50%	
	Yes (chemo and radiotherapy)	0	0.00%	4	-12.50%	
	Yes (radiotherapy)	0	0.00%	2	-6.30%	
CD163.categories	Low *CD163* expression	16	-100.00%	6	-18.80%	<0.001 χ^2
	High *CD163* expression	0	0.00%	26	-81.30%	

Data are presented as mean ± SD and median (range), or number (percentage). Significance defined by P-value ≤ 0.05. Student t test was used for comparing continuous data. Chi square (χ^2) test and Fisher Exact test were used for comparing categorical data.

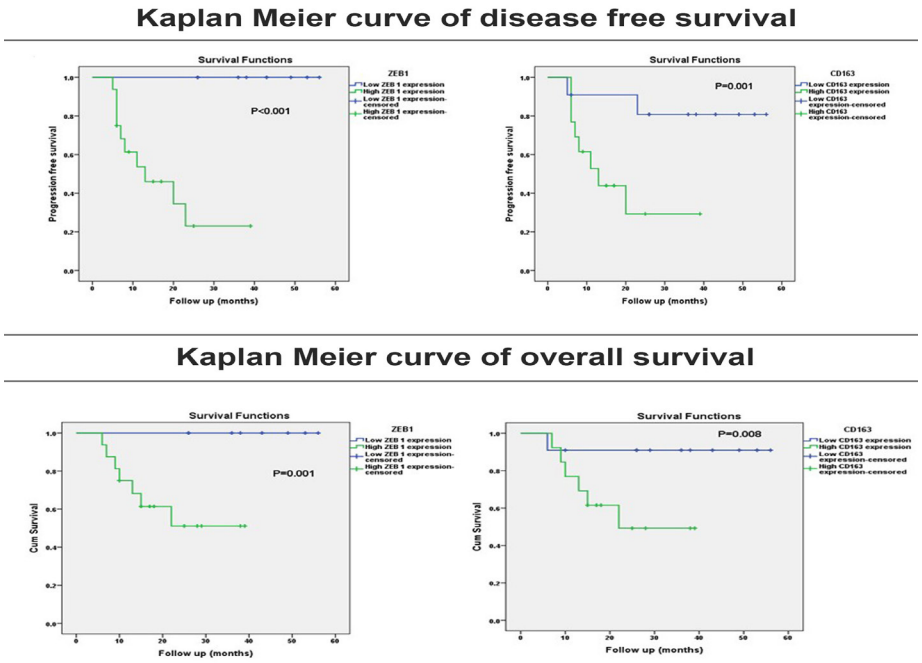

Figure 2. Kaplan Meier Curve of Disease-Free Survival and Overall Survival among the Studied Cases with Bladder Cancer According to *ZEB1* and *CD163* Expression

Table 2. Correlation between *CD163* Expression and Demographic and Clinico-Pathological Parameters of Studied Participants

Variables		Low *CD163* expression (n=22)		High *CD163* expression (n=26)		P value
Age (years)	Mean ± SD	59.82 ± 8.35		58.85 ± 6.25		0.647 T
	Median (range)	63 (38 – 69)		61 (43 – 65)		
Gender	Male	22	-100.00%	22	-84.60%	0.114 F
	Female	0	0.00%	4	-15.40%	
Divergent differentiation of urothelial carcinoma	No	10	-45.50%	14	-53.80%	0.562 χ^2
	Squamous	12	-54.50%	12	-46.20%	
Bilharziasis	Absent	12	-54.50%	16	-61.50%	0.624 χ^2
	Present	10	-45.50%	10	-38.50%	
Necrosis	Absent	16	-72.70%	6	-23.10%	0.001 χ^2
	Present	6	-27.30%	20	-76.90%	
Perineural invasion	Absent	4	-18.20%	6	-23.10%	0.735 F
	Present	18	-81.80%	20	-76.90%	
Lymphovascular tumor emboli	Absent	10	-45.50%	2	-7.70%	0.003 χ^2
	Present	12	-54.50%	24	-92.30%	
Pathological tumor stage (T)	T2	14	-63.60%	2	-7.70%	<0.001 F
	T3	6	-27.30%	20	-76.90%	
	T4	2	-9.10%	4	-15.40%	
Pathological nodal stage (N)	N0	16	-72.70%	8	-30.80%	0.010 F
	N1	2	-9.10%	4	-15.40%	
	N2	4	-18.20%	14	-53.80%	
Recurrence/ Metastasis	No	18	-81.80%	10	-38.50%	0.002 χ^2
	Yes	4	-18.20%	16	-61.50%	
Adjuvant therapy	No	4	-18.20%	4	-15.40%	0.141 F
	Yes (Chemotherapy)	18	-81.80%	16	-61.50%	
	Yes (Chemo and radiotherapy)	0	0.00%	4	-15.40%	
	Yes (Radiotherapy)	0	0.00%	2	-7.70%	

Data are presented as mean ± SD and median (range), or number (percentage). Significance defined by P-value ≤ 0.05. Student t test was used for comparing continuous data. Chi square (χ^2) test and Fisher Exact test were used for comparing categorical data.

analysis (Figure 2).

Cox regression survival analysis

Significant factors in the Kaplan-Meier analysis were further evaluated using univariate and multivariate Cox regression analyses to account for confounders affecting 3-year OS and DFS (Table 4 and 5). The results identified high *ZEB1* expression as an independent prognostic factor for nodal metastasis and recurrence.

Discussion

There is an urgent need to investigate the molecular mechanisms underlying bladder cancer invasion and metastasis to develop more effective therapeutic approaches and reduce tumor-associated morbidity and mortality. Our findings revealed that high *ZEB1* expression in patients undergoing radical cystectomy for muscle-invasive urothelial carcinoma is significantly associated with adverse clinicopathological characteristics, including lymphovascular invasion, tumor necrosis, advanced stage, nodal metastasis, and poor survival outcomes. These results suggests that *ZEB1* plays a crucial role in bladder cancer progression, consistent with previous studies by Zhu J et al. [5]. Ting et al. [14] and Lin J et al. [15], and Mahdavinezhad et al. [16], which linked overexpressed *ZEB1* to increased invasiveness in bladder cancer patients. Additionally, Li et al. [11] reported that *ZEB1* expression significantly contributes to bladder cancer progression.

ZEB1 is a transcription factor and key regulator of epithelial-to-mesenchymal transition (EMT), primarily suppresses of E-Cadherin expression, leading to loss of cell-cell adhesion and increased tumor migration. *ZEB1*-mediated EMT also influences critical tumor cell signaling pathways, such as the MAPK pathway in KRAS-mutant tumors [17]. Thus, *ZEB1* expression promotes tumor invasion, metastasis, and chemotherapy resistance across various cancer types [18-23]

Within the tumor microenvironment (TME), M2-like macrophages, marked by *CD163* expression, are a major component, and their accumulation is associated with poor prognosis in several cancer types. In this study, increased infiltration of *CD163*+ tumor-associated macrophages correlated with adverse prognostic parameters in bladder

Table 3. Disease Free and Overall Survival According to the Clinic-Pathological Details of the Studied Cases with Bladder Cancer (n=48)

	DFS (3 years)		OS (3 years)	
	Estimate ± SE	P value	Estimate ± SE	P value
Age		0.12		0.08
Mean ± SE	59.3 ± 7.3		59.3 ± 7.3	
Median (range)	61 (39-69)		61 (39-69)	
Gender		0.45		0.35
Male	55.0 ± 8.5%		70.0 ± 7.5%	
Female	65.0 ± 12.0 %		80.0 ± 10.0%	
Divergent differentiation of urothelial carcinoma		0.559		0.448
No	62.9 ± 10.6%		66.7 ± 9.6%	
Squamous	50.0 ± 10.2%		72.2 ± 9.8%	
Bilharziasis		0.154		0.024
Absent	44.9 ± 10.1%		57.1 ± 9.4%	
Present	70.0 ± 10.2%		88.9 ± 7.4%	
Necrosis		0.358		0.007
Absent	63.6 ± 10.3%		90.9 ± 6.1%	
Present	40.0 ± 11.9%		51.3 ± 10.3%	
Perineural invasion		0.372		0.445
Absent	40.0 ± 15.5%		80.0 ± 12.6%	
Present	58.6 ± 8.8%		66.6 ± 8.0%	
Lymphovascular tumor emboli		0.028		0.011
Absent	83.3 ± 10.8%		100.0 ± 0.0%	
Present	42.0 ± 9.4%		57.6 ± 8.9%	
T staging		0.001		<0.001
T2	87.5 ± 8.3%		100.0 ± 0.0%	
T3	35.9 ± 11.0%		57.7 ± 10.6%	
T4	0.0 ± 0.0%*		0.0 ± 0.0%*	
Positive L.N		<0.001		<0.001
Negative	82.5 ± 8.0%		90.9 ± 6.1%	
Positive	17.1 ± 10.1%*		47.6 ± 10.6%	
Distant metastasis		0.013		0.065
M0	64.4 ± 7.8%		75.9 ± 6.7%	
M1	0.0 ± 0.0%*		0.0 ± 0.0%*	
ZEB1 categories		<0.001		0.001
Low expression	100.0 ± 0.0%		100.0 ± 0.0%	
High expression	23.0 ± 9.4%		51.1 ± 9.9%	
CD163.categories		0.001		0.008
Low expression	80.8 ± 8.7%		90.9 ± 6.1%	
High expression	29.3 ± 10.8%		49.2 ± 10.9%	

* Follow up ended before 36 months of follow up.

carcinoma patients, including overall survival and disease-free survival. These findings align with previous studies [9, 12, 24, 25]. Notably, Chenard et al. [24] reported that a higher density of *CD163*+ macrophages is associated with shorter recurrence-free survival in both male and female patients. Furthermore, Wang et al. [12] reported that increased ratio of *CD163*+ TAMs in bladder cancer is associated with reduced patients survival time.

TAMs contribute to tumor progression through several mechanisms, including the secretion of pro-inflammatory cytokines such as IL-6, IL-10, and TGF-β, which suppress anticancer immune response and promote tumor progression [26]. These findings suggest that *CD163*+ TAMs may serve as a prognostic marker for bladder cancer survival, highlighting their potential pro-tumorigenic role in malignant progression.

Our study demonstrates a strong positive correlation between *ZEB1* expression and *CD163*+ TAM density in the TME of bladder cancer, suggesting a potential interplay between *ZEB1*-driven processes and macrophage

Table 4. COX Regression Analysis for Prediction of Recurrence

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Positive L.N						
Negative	Ref*					
Positive	8.34	2.703 – 25.728	<0.001			
ZEB1 categories						
Low expression	Ref*			Ref*		
High expression	70.658	1.779 – 2806.983	0.023	70.658	1.779 – 2806.983	0.023
CD163.categories						
Low expression	Ref*					
High expression	5.51	1.768 – 17.174	0.003			

BMI, body mass index; CI, Confidence interval; HR, Hazard ratio. * P value is significant ≤0.05

Table 5. COX Regression Analysis for Prediction of Death

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Positive L.N						
Negative	Ref*			Ref*		
Positive	9.944	2.180 – 45.355	0.003	9.944	2.180 – 45.355	0.003
ZEB1 categories						
Low expression	Ref*					
High expression	50.369	0.597 – 4247.785	0.083			
CD163.categories						
Low expression	Ref*					
High expression	5.963	1.323 – 26.874	0.02			

BMI, body mass index; CI, Confidence interval; HR, Hazard ratio. * P value is significant ≤0.05

infiltration. While the exact mechanisms remain to be elucidated, this association may involve *ZEB1*-mediated EMT influencing the secretion of macrophage-attracting factors, contributing to poor prognosis in bladder cancer patients. Further functional studies are needed to determine whether *ZEB1* directly or indirectly modulates TAM recruitment in bladder cancer.

The mechanisms by which *ZEB1* influences TAMs within the TME are still under investigation. Beyond its role in EMT, *ZEB1*modulates the EMT by upregulating inflammatory mediators such as IL8 and VEGF, which promote tumor growth, invasion, extracellular matrix remodeling, immune cell infiltration, and angiogenesis [17, 27].

Jiang et al. [26] hypothesized that M2 like TAMs predominantly accumulate in hypoxic zones within TME, where they play a significant role in tumorigenesis, growth, and progression. Hypoxia, is a key regulator of immunosuppressive mechanisms, is induced by *ZEB1* through extracellular lactate secretion, which, in turn, activates the transcription factor CCL8, attracting macrophages via the CCR2-NF-κB Pathway. Moreover, *ZEB1* induces the CCR2-MMP9-CCL2 loop between tumor cells and TAMs, sustaining the pro-tumor phenotype of TAMs and promoting tumor growth. Studies have also shown that M2-like TAMs frequently surround *ZEB1*-positive cells in ovarian and cervical cancers [28, 29]. Thus, bidirectional regulation between EMT and the tumor immune microenvironment may be mediated by *ZEB1* [27].

In conclusion, the results of this study provide insights for optimizing predictive models for bladder cancer prognosis. They also suggest a potential mechanism by which high *ZEB1* expression contributes to increased infiltration of M2-type tumor-associated macrophages (TAMs). However, this study has limitations, including a small sample size and restriction to patients who underwent cystectomy. However, including of patients who underwent transurethral resection of bladder tumor could enable a more comprehensive analysis. Additionally, this does not establish a direct mechanistic role *ZEB1* in macrophages recruitment. The observed association may reflect indirect effect mediated by epithelial-to-mesenchymal transition (EMT)related cytokines or other components of the microenvironment. Further functional studies are needed to validate whether *ZEB1* directly influences macrophage recruitment. Lastly, adjacent normal mucosa may exhibit molecular characteristics similar to tumor cells, potentially acting as a confounding variable. Therefore, future studies should include two control groups, normal tissue and healthy adjacent tissue to improve the robustness of the analysis.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

None.

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