

# Prognostic Role of Epithelial-Mesenchymal Transition Markers “E-Cadherin, $\beta$ -Catenin, ZEB1, ZEB2 and p63” in Bladder Carcinoma

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## Abstract

**Background:** This study aimed to investigate the expression of epithelial-mesenchymal markers' E-cadherin,  $\beta$ -catenin, zinc-finger E-box-binding homeobox 1 (ZEB1), zinc-finger E-box-binding homeobox 2 (ZEB2) and p63 in transitional cell carcinoma (TCC) and squamous cell carcinoma (SCC) variants of bladder carcinoma (BC) and their correlation with clinicopathological parameters of prognostic importance.

**Methods:** In this retrospective study, 91 patients were enrolled (66 with TCC and 25 with SCC). All patients had full clinical and follow-up data and available paraffin blocks. Immunohistochemical analysis was performed and correlated with clinicopathological factors.

**Results:** In TCC cases, reduced E-cadherin,  $\beta$ -catenin positivity and p63 expression rate were evident in the setting of increased expression of ZEB1 and ZEB2. Patients with ZEB2 positive tumors were more likely to die compared to those with negative ZEB2 ( $P = 0.024$ ). Moreover, in patients with muscle-invasive BCs, an intense p63 expression was associated with poor overall survival (OS) ( $P < 0.001$ ). For patients with SCC, there was a reduction in E-cadherin and  $\beta$ -catenin positivity with elevated p63 expression and concomitant increased ZEB1 and ZEB2 expression. Poor prognosis was evident in association with reduced E-cadherin, positive nuclear  $\beta$ -catenin/reduced membranous  $\beta$ -catenin, ZEB1 and ZEB2 positive cases as well patients with elevated p63 expression ( $P < 0.001$ ). TCC and SCC cases showed similar poor prognosis in association with elevated p63 expression ( $P < 0.001$ ).

**Conclusions:** In both TCC and SCC variants, epithelial-mesenchymal transition (EMT) process is evident; however, its molecular mechanism shows some variations, specifically this notably different p63 expression pattern among two carcinoma variants with the simi-

lar impact of elevated p63 expression pattern on prognosis.

**Keywords:** E-cadherin;  $\beta$ -catenin; ZEB1; ZEB2; p63; Bladder carcinoma

## Introduction

Bladder cancer is the most common malignancy of the urinary tract. It accounts for about 3.2% of all cancers worldwide and ranks the ninth highest cancer incidence, which is estimated to be 380,000 annually. It is the 13th mortality cause among all cancers with approximately 150,000 yearly deaths worldwide [1]. In Egypt, urinary bladder tumors constitute 30% of all cancer cases with an incidence of 13.5/100,000 patients. It is the third most prevalent cancer and accounts for 12.7% of male cancers with the majority of cases presented with an invasive form. Transitional cell carcinoma (TCC) represents about 90% of bladder cancer. The remaining 10% include squamous cell carcinoma (SCC), adenocarcinoma and other rare types [2]. Bladder carcinoma (BC) has high recurrence and mortality rates. BCs are categorized as non-muscle-invasive (NMIBCs) which occur in 70% to 80% of the cases, whereas the remaining 20% to 30% usually present with the invasive form (MIBCs). Most of the patients with NMIBCs are treated by endoscopic resection; however, the majority of patients have cancer recurrences after resection in 50-70% of the cases. Almost half of the patients with MIBCs usually present with distant metastases at the time of diagnosis [3].

Based on embryological studies, tumor progression and metastasis could be attributed to transformation in epithelial to mesenchymal cells, epithelial-mesenchymal transition (EMT) [4, 5]. During this process, cell-to-cell adhesion molecules are down-regulated and cell polarity may be lost. These changes increase cell migration and invasion of surroundings [6-8].

Loss of epithelial cell-to-cell interactions alters cell morphology and motility [9]. This interaction is mediated by cadherins, which include E-, P-, and N-isoforms [10]. E-cadherin, an epithelial-specific cadherin, plays a key role in selective cell adhesion within epithelial tissues and is necessary for normal cell integrity [11]. This function takes place at the plasma membrane, where  $\beta$ -catenin combines with the cytoplasmic domain of E-cadherin, in conjunction with  $\alpha$ -catenin, and binds to the

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microfilament network of the cytoskeleton [9]. This process is adversely affected during EMT when E-cadherin is down-regulated [12]. Reduction of E-cadherin is associated with translocation of  $\beta$ -catenin from cell membrane to nucleus. The newly located  $\beta$ -catenin activates WNT signaling pathway, resulting in EMT and metastasis formation [13]. Thus, E-cadherin is considered as a suppressor for malignant cell invasion and metastasis, and subsequently, its reduced expression is expected to increase tumor undifferentiation and invasiveness [14].

The EMT is controlled by several transcription factors within the cells, including Slug, Snail, Twist, zinc-finger E-box-binding homeobox 1 (ZEB1) and zinc-finger E-box-binding homeobox 2 (ZEB2) [15]. ZEB1 is the vertebrate homologue of the ZFH gene family of zinc finger/homeodomain proteins. It is encoded by the TCF8 gene [16]. It is considered a key inducer of malignant tumor progression [17, 18]. It acts as a transcriptional repressor of E-cadherin through binding to its gene promoter [19, 20]. Smad-interacting protein 1 (SIP1)/ZEB2 is a member of the  $\delta$ EF-1 family, which is a two-handed zinc finger nuclear factor. Its expression is associated with EMT during development. ZEB2 represses the transcription of junctional protein gene coding contributing to the dedifferentiated state. This function is mediated at SIP1-binding sites. Real-time polymerase chain reaction (RT-PCR) has shown evidence of ZEB2 upregulation in several cancer types [21, 22]. Thus, ZEB2 is believed to act as a biomarker of biological aggressiveness and metastasis [16, 23].

Another important transcription factor is p63, which is a member of the p53 family. It has two main isoforms (TAp63 and  $\Delta$ Np63), each with a distinct function [24, 25]. Normally, TAp63 isoform is essential for the initiation of epithelial stratification and maintenance of basal cells proliferative capacity. It may also be responsible for cell cycle arrest, by transactivating p53 target genes that promote apoptosis.  $\Delta$ Np63 improves cells proliferation by enhancing cell response to maturation signals [26]. However, during this process, it exerts dominant-negative activities against TAp63 and p53, suggesting its involvement in EMT during tumor progression [27], and thus, it is considered as an oncoprotein [28].

This study aimed at exploring the molecular mechanisms involved in EMT seen in BC. Further, we investigated whether EMT biomarkers could identify aggressive MIBC behavior in Egyptian patients with TCC and SCC.

## Materials and Methods

This study is a retrospective study that took place at Minia University Hospital and Minia Oncology Center, Minia, Egypt, from 2005 to 2012.

### Patients

Ninety-one patients with BC were enrolled in this study, 66 patients with TCC and 25 with SCC. All patients had full clinical and follow-up data and available paraffin blocks. Those patients were treated with curative intent by senior urologists as

follows: radical cystectomy was performed for both invasive disease or high-risk superficial TCC for which conservative measures failed and SCC cases. Patients' data were obtained including age, sex, bilharzial status, and follow-up data.

### Histopathological evaluation

The original hematoxylin and eosin (H&E) stained sections were retrieved and re-assessed to confirm the original diagnosis, histopathological type, tumor grade and stage. TCC and SCC were staged according to the American Joint Committee on Cancer guidelines [29] and graded according to the World Health Organization (WHO) classification system for the urinary tract tumors [30].

### Immunohistochemistry

Five- $\mu$ m sections from pre-diagnosed cases were transferred to adhesive slides from representative formalin-fixed, paraffin-embedded blocks. Then they were processed by deparaffinizing in xylene before they were dehydrated in a series of graded ethanol. Then they were prepared for immunohistochemistry staining using the streptavidin-biotin immunoperoxidase complex method. Briefly, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide, for 30 min, at room temperature. Antigen was then retrieved by microwave treatment (600W) for 10 min using 0.01M sodium citrate buffer (pH 6.0). The slides were then left to cool at room temperature before incubation with mouse monoclonal antibody E-cadherin (clone 4A2C7, 1:100 dilution, Thermo Fisher Scientific) for 30 min, pre-diluted rabbit monoclonal antibody  $\beta$ -catenin (clone E247, Thermo Fisher Scientific) for 60 min, mouse monoclonal antibody ZEB1 (clone 2A8A6, 1:200 dilution, Abcam) for 30 min, rabbit polyclonal antibody ZEB2 (1:50 dilution, Abcam) for 30 min, mouse monoclonal antibody p63 (clone 4A4, 1:50 dilution, Abcam) followed by biotinylated secondary antibody for 30 min at room temperature. The reaction was visualized with an avidin-biotin complex immunoperoxidase system using 3,3'-diaminobenzidine (DAB) as a chromogen (Sigma). Sections were then counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted with distyrene, plasticizer, and xylene (DPX). Negative control sections were treated with phosphate-buffered saline (PBS) instead of the primary antibody.

### Immunohistochemical analysis

All cases were interpreted blindly regardless of the clinicopathological data of the patients. For E-cadherin, membranous expression was graded based on the proportion of positive cells and classified as normal (> 90%) and reduced expression group (0-90%) [31]. For  $\beta$ -catenin, the expression of cancer cells was classified as: > 70% of cell membranes stained as normal, otherwise as reduced, and > 10% of nuclei or cytoplasm stained as positive [32]. For ZEB1 (nucleus) and ZEB2

**Table 1.** Clinicopathological Characteristics of Patients With TCC and SCC

Variables	TCC patients (%) (n = 66)	SCC patients (%) (n = 25)
Age (years)	≤ 59.5: 35 (53%); > 59.5: 31 (47%)	≤ 51.5: 14 (56%); > 51.5: 11 (44%)
Gender		
Men	57 (86.4%)	21 (84%)
Women	9 (13.6%)	4 (16%)
Bilharzial status		
No	36 (54.5%)	9 (36%)
Yes	30 (45.5%)	16 (64%)
Grade	Low grade: 16 (24.2%); high grade: 50 (75.8%)	G1: 1 (4%); G2: 18 (72%); G3: 6 (24%)
Stage	NMIBC: 25 (37.9%); MIBC: 41 (62.1%)	T1: 3 (12%); T2: 18 (72%); T3:4 (16%)
Lymph node metastases		
No	43 (65.2%)	19 (76%)
Yes	23 (34.8%)	6 (24%)

TCC: transitional cell carcinoma; SCC: squamous cell carcinoma; NMIBC: non-muscle-invasive bladder carcinoma; MIBC: muscle-invasive bladder carcinoma.

(plasma membrane or cytoplasm), reactions were categorized as positive (staining of ≥ 5% of cells) or negative (no staining or tumor cells < 5%) [33]. For p63, positive nuclear immunohistochemical reactions were categorized as negative (stained cells < 10%), weak (≥ 10% and < 80% of cells are stained) and high (intense) (stained cells 80-100%) [26].

### Statistical analysis

Data were analyzed using the SPSS statistical package version 20. Firstly, a descriptive analysis of clinicopathological features was performed. Associations between immunohistochemical markers expression and various clinicopathological features were assessed using Chi-square test. Spearman's correlation analysis was used to analyze the correlation between the studied markers. The Kaplan-Meier method was used for survival analysis. P values less than 0.05 were considered to be statistically significant.

This study was approved by the Research Ethics Committee of Faculty of Medicine of Minia University vides approval letter No. 165:2/2019. The Institutional Review Board of Faculty of Medicine of Minia University also approved this study.

## Results

### Patient demographics and clinicopathologic findings

For the TCC patient group (n = 66), the mean age was 59.5 years (range: 44 - 78). For patients with SCC (n = 25), the mean age was 51.5 ± 4.3 years. Patients with SCC were significantly younger than those with TCC (P = 0.03). In the TCC group, 86.4% (n = 57) were men and 13.6% (n = 9) were women; similarly, in the SCC group, a male predominance was also noticed with a man/woman ratio of 5.3:1 (Table 1).

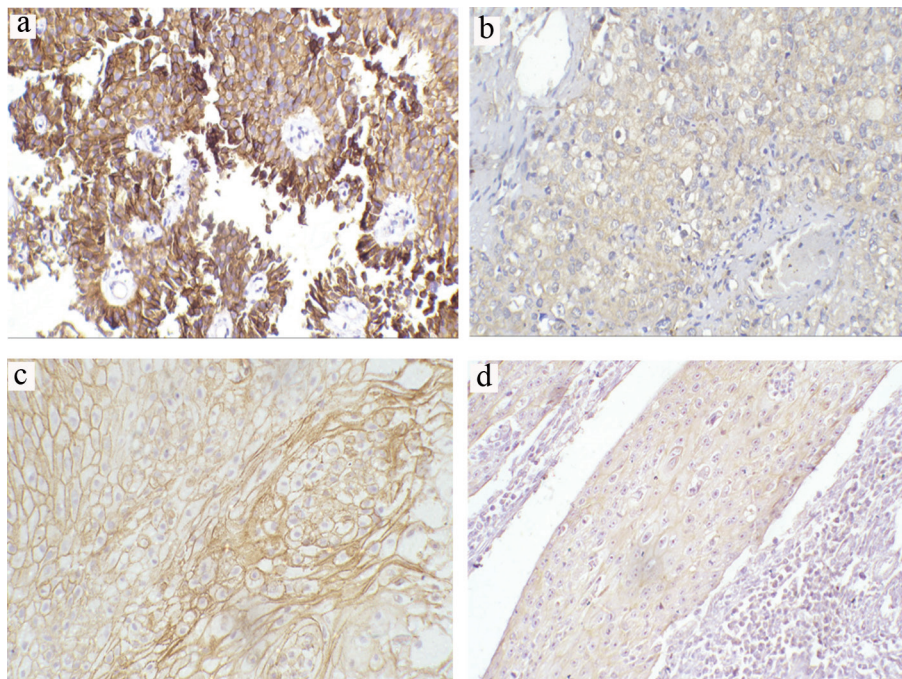
In TCC patients' group, carcinoma was low grade in 24.2% (n = 16) and high grade in 75.8% (n = 50). Urinary carcinoma (UC) with superficial invasion (NMI-UC) was diagnosed in 37.9% (n = 25), while UC with muscle invasion (MI-UC) was found in 62.1% (n = 41) cases. For SCC, carcinoma was graded I in one patient (4%), II in 72% (n = 18), and III in 24% (n = 6). Muscle invasion (T2-3) was evident in 88% (n = 22) of the cases. Lymph node (LN) metastasis was diagnosed in 34.8% (n = 23) of the TCC cases and in 24% of SCC. Bilharzias was evident in 45.5% of TCC and in 68% (n = 17) of SCC patients (Table 1).

### Association between clinicopathological data and E-cadherin expression

For patients with TCC, 32 tumors (48.5%) showed a normal positive staining pattern and 34 (51.5%) showed reduced E-cadherin expression (Fig. 1a, b). Correlation between E-cadherin expression and histopathological features showed a positive association between reduced expression and tumor grading (P = 0.015) (60% in high grade as compared to only 25% of low-grade types). E-cadherin reduced expression was also seen in 65.9% of MIBCs compared to 28% of NMIBCs (P = 0.003). Regarding LN metastases, 73.9% of node-positive tumors showed reduced expression (P = 0.008, Table 2).

For patients with SCC, 44% (n = 11) exhibited abnormal reduced E-cadherin expression. Among these, 33.3% (n = 6) were observed in 18 patients with moderate differentiation and 83.3% (n = 5) were found in five patients with poor differentiation. No reduced expression was detected in well-differentiated SCC patients (n = 1). There was a significant association between E-cadherin expression and the degree of differentiation (P = 0.04; Table 3; Fig. 1c, d).

Abnormal E-cadherin expression was also observed in 44.4% (n = 8) of the T2 subtype patients (n = 18) as well as in



**Figure 1.** Representative immunohistochemical staining of E-cadherin in TCCs and SCCs (a-d) (magnification  $\times 200$ ). (a) Preserved normal positive membranous expression in low-grade non-muscle-invasive TCC. (b) Reduced membranous expression in high-grade invasive TCC. (c) Preserved normal positive membranous expression in moderately differentiated SCC. (d) Reduced membranous expression in poorly differentiated SCC. TCC: transitional cell carcinoma; SCC: squamous cell carcinoma.

75% ( $n = 3$ ) of the four patients with T3 subtype. No reduced expression was reported in T1 non-invasive subtype ( $n = 1$ ). However, this association between reduced expression and tumor stage did not reach a significant level ( $P = 0.07$ , Table 3). On the other hand, a significant association was detected between reduced E-cadherin expression and node-positive tumors ( $P = 0.026$ ).

#### Association between clinicopathological data and membranous as well as nucleo-cytoplasmic $\beta$ -catenin expressions

For TCC patients, 75.8% ( $n = 50$ ) of patients showed reduced  $\beta$ -catenin membranous expression while 51.5% ( $n = 34$ ) demonstrated ectopic nucleocytoplasmic expression (Fig. 2a-c). There was a significant association between reduced membranous  $\beta$ -catenin and tumor grade ( $P < 0.001$ ) and stage ( $P \leq 0.001$ ). There was also a significant difference in the expression pattern of reduced membranous  $\beta$ -catenin between node-negative and node-positive tumors ( $P = 0.006$ ). Nucleocytoplasmic localization of  $\beta$ -catenin was significantly and strongly associated with muscle invasion ( $\geq T2$ ) in 65.9% ( $n = 27$ ) compared with non-muscle-invasive tumors (28%;  $n = 7$ ) ( $P = 0.003$ ). Further, there was a significant correlation with tumor high grade ( $P < 0.001$ ) (Table 2).

For SCC patients, reduced membranous expression was detected in 56% ( $n = 14$ ) whereas 36% showed ectopic nucleocytoplasmic expression (Fig. 2d, e). There was a significant correlation between reduced membranous expression and nucleocytoplasmic localization with tumor high grade ( $P = 0.008$

and  $P = 0.017$ , respectively). However, only reduced membranous expression showed a significant correlation with high tumor stage ( $P = 0.008$ ). On the other hand, nucleocytoplasmic  $\beta$ -catenin exhibited a significant correlation with node-positive tumors ( $P = 0.012$ ) (Table 3).

#### Association between clinicopathological data and ZEB1 and ZEB2 expression

For TCC, positive nuclear ZEB1 expression was shown in 36.4% ( $n = 24$ ) (Fig. 3a) with 44% recorded in high-grade tumors and 12% in low-grade tumors. Expression was significantly different between the two tumor grades ( $P = 0.023$ ); however, distribution was almost equally observed in invasive (36%) and non-invasive subtypes (36.6%). There was no significant association between ZEB1 expression and tumor stage ( $P = 0.962$ ) nor with LN metastasis ( $P = 0.46$ ). Cytoplasmic immunoreactivity for ZEB2-positive samples was detected in 57.6% ( $n = 34$ ) (Fig. 3b). There was a significant correlation between ZEB2-positive expression and tumor stage ( $P = 0.006$ ) and LN metastasis (87% of node-positive tumor exhibited positive ZEB2 expression) ( $P < 0.001$ ). On the other hand, there was no significant difference in the expression pattern of ZEB2 between low- and high-grade tumors ( $P = 0.647$ , Table 2).

For SCC, positive ZEB1 expression was noticed in 28% ( $n = 7$ ) (Fig. 3c). There was a significant correlation between positive expression and tumor grade ( $P = 0.04$ ), stage ( $P = 0.04$ ) and node-positive tumors ( $P = 0.001$ ). Cytoplasmic immuno-

**Table 2.** Expression of E-Cadherin, β-Catenin, ZEB1, ZEB2 and p63 in TCC and Their Association With the Clinicopathological Characters

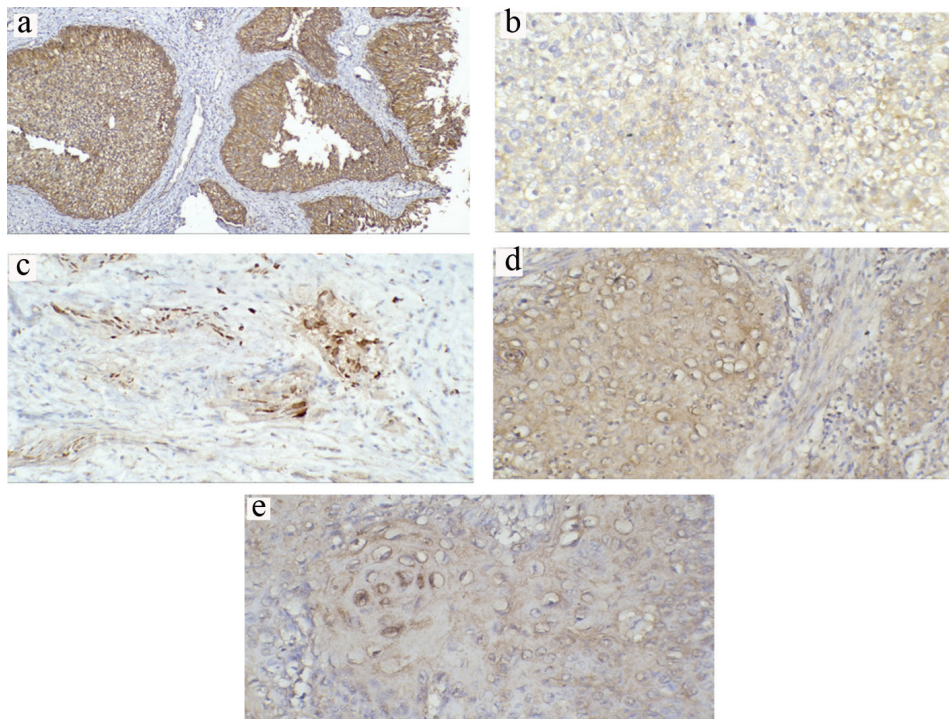
Variable	No. of cases	E-cadherin		Membranous β-catenin		Nucleo-cytoplasmic β-catenin		ZEB1		ZEB2		p63	
		Normal	Reduced	Normal	Reduced	-ve	+ve	-ve	+ve	-ve	+ve	Weak	Intense
Age													
≤ 59.5 years	35	17 (48.6%)	18 (51.5%)	8 (22.9%)	27 (77.1%)	17 (48.6%)	18 (51.4%)	27 (77.1%)	8 (22.9%)	16 (45.7%)	19 (54.3%)	7 (20%)	17 (48.6%)
> 59.5 years	31	15 (48.4%)	16 (51.6%)	8 (25.8%)	23 (74.2%)	15 (48.4%)	16 (51.6%)	16 (51.6%)	15 (48.4%)	12 (38.7%)	19 (61.3%)	3 (9.7%)	17 (54.8%)
P value		0.988		0.780		0.988		0.015*		0.50		0.506	
Sex													
Men	57	28 (49.1%)	29 (50.9%)	13 (22.8%)	44 (77.2%)	27 (47.4%)	30 (52.6%)	37 (64.9%)	20 (35.1%)	25 (43.9%)	32 (56.1%)	8 (14%)	31 (54.4%)
Women	9	4 (44.4%)	5 (55.6%)	3 (33.3%)	6 (66.7%)	5 (55.6%)	4 (44.4%)	5 (55.6%)	4 (44.4%)	3 (33.3%)	6 (66.7%)	2 (22.2%)	3 (33.3%)
P value		0.794		0.493		0.648		0.588		0.553		0.497	
Bilharzial status													
No	36	16 (44.4%)	20 (55.6%)	8 (22.2%)	28 (77.8%)	16 (44.6%)	20 (55.6%)	22 (61.1%)	14 (38.9%)	12 (33.3%)	24 (66.7%)	6 (16.7%)	19 (52.8%)
Yes	30	16 (53.3%)	14 (46.7%)	8 (26.7%)	22 (73.3%)	16 (53.3%)	14 (46.7%)	20 (66.7%)	10 (33.3%)	16 (53.3%)	14 (46.7%)	4 (13.3%)	15 (50%)
P value		0.470		0.675		0.470		0.640		0.102		0.849	
Grade													
Low grade	16	12 (75%)	4 (25%)	12 (75%)	4 (25%)	14 (87.5%)	2 (12.5%)	14 (87.5%)	2 (12.5%)	6 (37.5%)	10 (62.5%)	2 (12.5%)	4 (25%)
High grade	50	20 (40%)	30 (60%)	4 (8%)	46 (92%)	18 (36%)	32 (64%)	28 (56%)	22 (44%)	22 (44%)	28 (56%)	8 (16%)	30 (60%)
P value		0.015*		< 0.001*		< 0.001*		0.023*		0.640		0.015*	
Stage													
NMIBC	25	18 (72%)	7 (28%)	12 (48%)	13 (52%)	18 (72%)	7 (28%)	16 (64%)	9 (36%)	16 (64%)	9 (36%)	2 (8%)	9 (36%)
MIBC	41	14 (34.1%)	27 (65.9%)	4 (9.8%)	37 (90.2%)	14 (34.1%)	27 (65.9%)	26 (63.4%)	15 (36.6%)	12 (29.3%)	29 (70.7%)	8 (10.5%)	25 (61%)
P value		0.003*		< 0.001*		0.003*		0.96		0.006*		0.009*	
Lymph node metastasis													
No	43	26 (60.5%)	17 (39.5%)	15 (34.9%)	28 (65.1%)	23 (53.5%)	20 (46.5%)	26 (60.5%)	17 (39.5%)	25 (58.1%)	18 (41.9%)	9 (20.9%)	16 (37.2%)
Yes	23	6 (26.1%)	17 (73.9%)	1 (4.3%)	22 (95.7%)	9 (39.1%)	14 (60.9%)	16 (69.6%)	7 (30.4%)	3 (13%)	20 (87%)	1 (4.3%)	18 (78.3%)
P value		0.008*		0.006*		0.260		0.460		< 0.001*		0.006*	

NMIBC: non-muscle-invasive bladder carcinoma; MIBC: muscle-invasive bladder carcinoma; ZEB: zinc-finger E-box-binding homeobox; TCC: transitional cell carcinoma. \*Significant association.

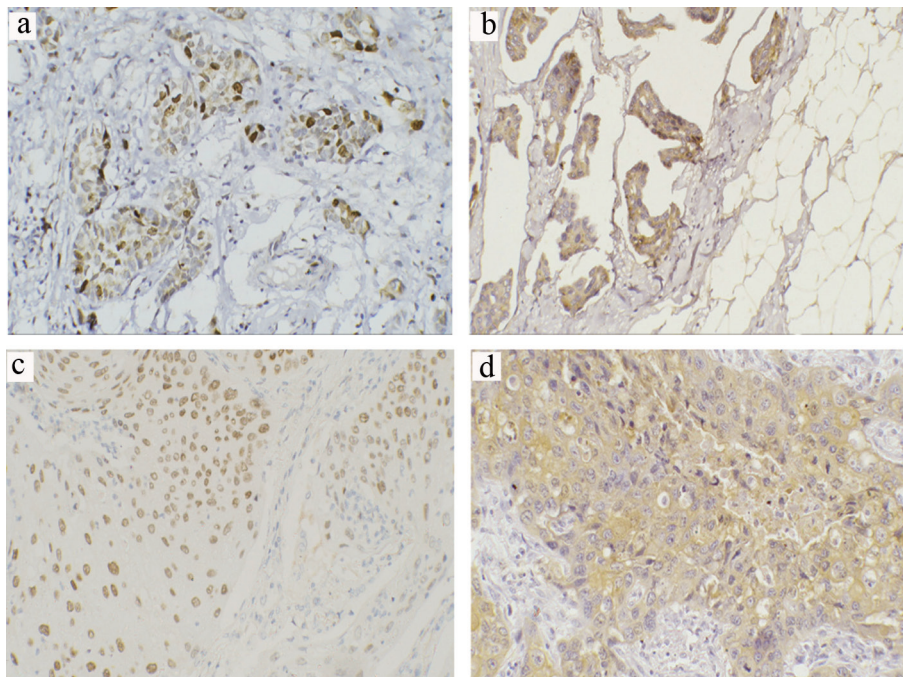
**Table 3.** Expression of E-Cadherin, β-Catenin, ZEB1, ZEB2 and p63 in SCC and Their Association With the Clinicopathological Characters

Variable	No. of cases	E-cadherin		Membranous β-catenin		Nucleo-cytoplasmic β-catenin		ZEB1		ZEB2		p63		
		Normal 14 (56%)	Reduced 11 (44%)	Normal 11 (44%)	Reduced 14 (56%)	-ve 16 (64%)	+ve 9 (36%)	-ve 18 (72%)	+ve 7 (28%)	-ve 16 (64%)	+ve 9 (36%)	-ve 2 (8%)	Weak 18 (72%)	Intense 5 (20%)
Age														
≤ 51.5 years	14	9 (64.3%)	5 (35.7%)	6 (42.9%)	8 (57.1%)	10 (71.4%)	4 (28.6%)	10 (71.4%)	4 (28.6%)	10 (71.4%)	4 (28.6%)	1 (7.1%)	9 (64.3%)	4 (28.6%)
> 51.5 years	11	5 (45.5%)	6 (54.5%)	5 (45.5%)	6 (54.5%)	8 (72.7%)	3 (27.3%)	8 (72.7%)	3 (27.3%)	6 (54.5%)	5 (45.5%)	1 (9.1%)	9 (81.8%)	1 (9.1%)
P value		0.346		0.897	0.383	0.943	0.383	0.943	0.383	0.383	0.482			
Sex														
Men	21	8 (38.1%)	13 (61.9%)	11 (52.4%)	10 (47.6%)	15 (71.4%)	6 (28.6%)	16 (76.2%)	5 (23.8%)	15 (71.4%)	6 (28.6%)	1 (4.8%)	16 (76.2%)	4 (19%)
Women	4	3 (75%)	1 (25%)	3 (75%)	1 (25%)	2 (50%)	3 (75%)	2 (50%)	2 (50%)	3 (75%)	3 (75%)	1 (25%)	2 (50%)	1 (25%)
P value		0.288		0.604	0.116	0.548	0.116	0.548	0.116	0.116	0.438			
Bilharzial status														
No	8	4 (50%)	4 (50%)	3 (37.5%)	5 (62.5%)	5 (62.5%)	3 (37.5%)	6 (75%)	2 (25%)	5 (62.5%)	3 (37.5%)	1 (12.5)	5 (62.5%)	2 (25%)
Yes	17	10 (58.8%)	7 (41.2%)	8 (47.1%)	9 (52.9%)	11 (64.7%)	6 (35.3%)	12 (70.6%)	5 (29.4%)	11 (64.7%)	6 (35.3%)	1 (5.9%)	13 (76.5%)	3 (17.6%)
P value		1.000		1.000	0.900	1.000	0.900	1.000	0.915	0.915	0.752			
Grade														
G1	1	1 (100%)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)
G2	18	12 (66.7%)	6 (33.3%)	10 (55.6%)	8 (44.4%)	14 (77.8%)	4 (22.2%)	15 (83.3%)	3 (16.7%)	14 (77.8%)	4 (22.2%)	1 (5.6%)	15 (83.3%)	2 (11.1%)
G3	6	1 (16.7%)	5 (83.3%)	0 (0%)	6 (100%)	1 (16.7%)	5 (83.3%)	2 (33.3%)	4 (66.7%)	1 (16.7%)	5 (83.3%)	0 (0%)	3 (50%)	3 (50%)
P value		0.040*		0.008*	0.017*	0.040*	0.017*	0.040*	0.017*	0.017*	0.046*			
Stage														
T1	3	3 (100%)	0 (0%)	3 (100%)	0 (0%)	3 (100%)	0 (0%)	3 (100%)	0 (0%)	3 (100%)	0 (0%)	1 (33%)	2 (66.7%)	0 (0%)
T2	18	10 (55.6%)	8 (44.4%)	8 (44.4%)	10 (55.6%)	12 (66.7%)	6 (33.3%)	14 (77.8%)	4 (22.2%)	13 (72.2%)	5 (27.8%)	1 (5.6%)	15 (83.3%)	2 (11.1%)
T3	4	1 (25%)	3 (75%)	0 (0%)	4 (100%)	1 (25%)	3 (75%)	1 (25%)	3 (75%)	0 (0%)	4 (100%)	0 (0%)	1 (25%)	3 (75%)
P value		0.079		0.008*	0.072	0.048*	0.003*	0.048*	0.003*	0.003*	0.046*			
Lymph node metastasis														
No	19	13 (68.4%)	6 (31.6%)	9 (47.4%)	10 (52.6%)	15 (78.9%)	4 (21.1%)	17 (89.5%)	2 (10.5%)	15 (78.9%)	4 (21.1%)	2 (10.5)	16 (84.2%)	1 (5.3%)
Yes	6	1 (16.7%)	5 (83.3%)	5 (83.3%)	1 (16.7%)	1 (16.7%)	5 (83.3%)	1 (16.7%)	5 (83.3%)	1 (16.7%)	5 (83.3%)	0 (0%)	2 (33.3%)	4 (66.7%)
P value		0.026*		0.180	0.012*	0.001*	0.012*	0.001*	0.012*	0.012*	0.004*			

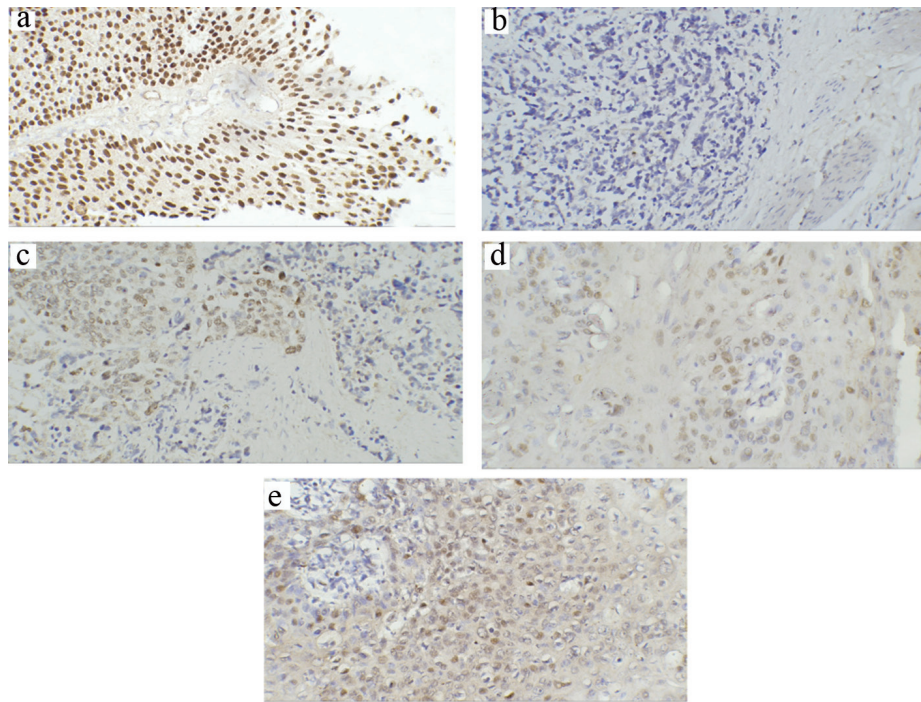
ZEB: zinc-finger E-box-binding homeobox; SCC: squamous cell carcinoma. \*Significant association.



**Figure 2.** Representative immunohistochemical staining of  $\beta$ -catenin in TCCs and SCCs (a-e) (magnification  $\times 200$ ). (a) Normal positive membranous expression in low-grade non-muscle-invasive TCC. (b) Reduced membranous expression in high-grade invasive TCC. (c) Predominant nuclear expression in invasive TCC. (d) Reduced membranous expression with prominent cytoplasmic expression in invasive SCC. (e) Nuclear expression in invasive SCC. TCC: transitional cell carcinoma; SCC: squamous cell carcinoma.



**Figure 3.** Representative immunohistochemical staining of ZEB1 and ZEB2 in TCCs and SCCs (a-d) (magnification  $\times 200$ ). (a) Positive ZEB1 nuclear expression in invasive TCC. (b) Positive ZEB2 cytoplasmic expression in invasive TCC. (c) Positive ZEB1 nuclear expression in invasive SCC. (d) Positive ZEB2 cytoplasmic expression in high-grade invasive SCC. TCC: transitional cell carcinoma; SCC: squamous cell carcinoma; ZEB: zinc-finger E-box-binding homeobox.



**Figure 4.** Representative immunohistochemical staining of p63 in TCCs and SCCs (a-e) (magnification × 200). (a) Strong staining of all cell layers in low-grade superficial TCC. (b) Loss of p63 expression in invasive TCC. (c) Some preserved positive p63 expression in invasive TCC. (d) Positive p63 in moderately differentiated SCC (G2) showing positivity in the cells of the basal layer. (e) Diffuse and high intense positivity for p63 in poorly differentiated SCC (G3). TCC: transitional cell carcinoma; SCC: squamous cell carcinoma.

reactivity for ZEB2-positive samples was detected in 36% (n = 9) (Fig. 3d). There was a significant correlation between

**Table 4.** Correlation of E-Cadherin Expression With p63, ZEB1, ZEB2 and Nucleo-Cytoplasmic β-Catenin Expression in TCC Cases

	E-cadherin		Spearman	P value
	Normal	Reduced		
p63			0.512	0.000
-ve	6 (60%)	4 (40%)		
Weak	4 (11.8%)	30 (88.2%)		
Intense	22 (100%)	0 (0%)		
ZEB 1			-0.481	0.000
-ve	28 (66.7%)	14 (33.3%)		
+ve	4 (16.7%)	20 (83.3%)		
ZEB2			-0.517	0.000
-ve	22 (78.6%)	6 (21.4%)		
+ve	10 (26.3%)	28 (73.7%)		
Nucleo-cytoplasmic β-catenin			-0.757	0.000
-ve	28 (87.5%)	4 (12.5%)		
+ve	4 (11.8%)	30 (88.2%)		

ZEB: zinc-finger E-box-binding homeobox; TCC: transitional cell carcinoma.

ZEB2-positive expression and tumor grade (P = 0.017), stage (P = 0.003) and LN metastasis (P = 0.012) (Table 3).

**Table 5.** Correlation of E-cadherin Expression With p63, ZEB1, ZEB2 and Nucleo-Cytoplasmic β-Catenin Expression in SCC Cases

	E-cadherin		Spearman	P value
	Normal	Reduced		
p63			-0.263	0.242
-ve	2 (100%)	0 (0%)		
Weak	10 (55.6%)	8 (44.4%)		
Intense	2 (40%)	3 (60%)		
ZEB1			-0.704	0.000
-ve	14 (77.8%)	4 (22.2%)		
+ve	0 (0%)	7 (100%)		
ZEB2			-0.678	0.000
-ve	13 (81.3%)	3 (18.8%)		
+ve	1 (11.1%)	8 (88.9%)		
Nucleo-cytoplasmic β-catenin			-0.846	0.000
-ve	14 (87.5%)	2 (12.5%)		
+ve	0 (0%)	9 (100%)		

ZEB: zinc-finger E-box-binding homeobox; SCC: squamous cell carcinoma.



### Association between clinicopathological data and p63 expression

For TCC, 62% of positive expression was recorded in low-grade and 24% in high-grade tumors. There was a significant difference in expression between the two grades ( $P = 0.015$ ). Regarding tumor stage, pTa and pT1 tumors were seen in 37.8% ( $n = 25$ ); only 8% ( $n = 2$ ) showed lost p63 expression, while 36% ( $n = 9$ ) displayed weak staining and 56% ( $n = 14$ ) with high (intense) homogenous staining (Fig. 4a). For  $\geq T2$  cases ( $n = 41$ ), negative immunostaining was observed in 19.5% ( $n = 8$ ), while weak staining was seen in 61% ( $n = 25$ ) and few subsets of invasive TCC did retain significant intense p63 expression in 19.5% ( $n = 8$ ) (Fig. 4b, c). A significant difference was noticed between the different tumor stages (pTa, pT1 and pT2 or more) ( $P = 0.009$ , Table 2).

For SCC, 8% ( $n = 2$ ) exhibited negative p63 expression, while 72% ( $n = 18$ ) displayed weak staining and 20% ( $n = 5$ ) showed strong homogenous staining. There were significant correlations between p63 expression and histological differentiation ( $P = 0.04$ ), invasion depth ( $P = 0.04$ ) and LN metastasis ( $P = 0.003$ ) (Table 3). Negative p63 expression (1/1) was evident in grade I SCC case, while half of the patients with high grade (3/6) showed high (intense) positive expression (Fig. 4d, e). For tumor stage, none of the patients with non-invasive T1 stage exhibited strong staining, while 75% ( $n = 3$ ) of invasive T3 stage showed high staining pattern. Further, there was a significant difference in p63 expression pattern between node-negative and node-positive tumors ( $P = 0.004$ ).

### A correlation analysis for different markers expression among TCC and SCC cases

In TCC cases, E-cadherin showed a significant moderate positive association with p63 expression ( $r = 0.512$ ,  $P < 0.001$ ), whereas it was inversely associated with ZEB1 ( $r = -0.481$ ,  $P < 0.001$ ), ZEB2 ( $r = -0.517$ ,  $P < 0.001$ ), and nuclear  $\beta$ -catenin ( $r = -0.757$ ,  $P < 0.001$ ) (Table 4). For SCC, E-cadherin expression was moderately to strongly inversely correlated with expression of ZEB1 ( $r = -0.7$ ,  $P < 0.001$ ), ZEB2 ( $r = -0.6$ ,  $P < 0.001$ ), and nuclear  $\beta$ -catenin ( $r = -0.8$ ,  $P < 0.001$ ). However, the association with p63 was weak and insignificant ( $r = -0.2$ ,  $P = 0.2$ ) (Table 5).

### Five-year overall survival (OS) rate

For TCC patients, the median follow-up was 55 months (range: 10 - 60). OS was 48.5%. Reduced E-cadherin expression was not significantly associated with the OS ( $P = 0.103$ ). The lack of association was also true for high (intense) p63 expression ( $P = 0.09$ ), nucleocytoplasmic/reduced membranous  $\beta$ -catenin expression ( $P = 0.8$  and  $P = 0.284$ , respectively) as well as positive ZEB1 staining ( $P = 0.08$ ). However, patients with ZEB2 positive tumors were more likely to die compared to those with negative ZEB2 ( $P = 0.024$ ). The 5-year OS was 49.4 for patients with ZEB2-negative tumors compared to

34.9 in ZEB2-positive tumors (Fig. 5a-f). Interestingly, when confined to patients with MIBCs, an association was noticed between p63 high expression and poor OS; specifically, high (intense) p63 expression was associated with a significantly worse median OS of 19 months, whereas patients with lower levels of p63 had an improved median OS of 24 months ( $P < 0.001$ ). Further, other markers did not show an association with OS ( $P > 0.05$ ) (Fig. 5g).

For SCC, the median follow-up was 51 months (range: 24 - 60). OS was 44% ( $n = 11$ ). E-cadherin aberrant or absent immunoreactivity correlated with OS ( $P = 0.001$ , Fig. 6a). The OS was significantly poorer in patients with positive nucleocytoplasmic  $\beta$ -catenin/reduced membranous  $\beta$ -catenin, ZEB1 and ZEB2 positive cases as well patients with high (intense) p63 expression ( $P < 0.001$ , Fig. 6b-f).

### Discussion

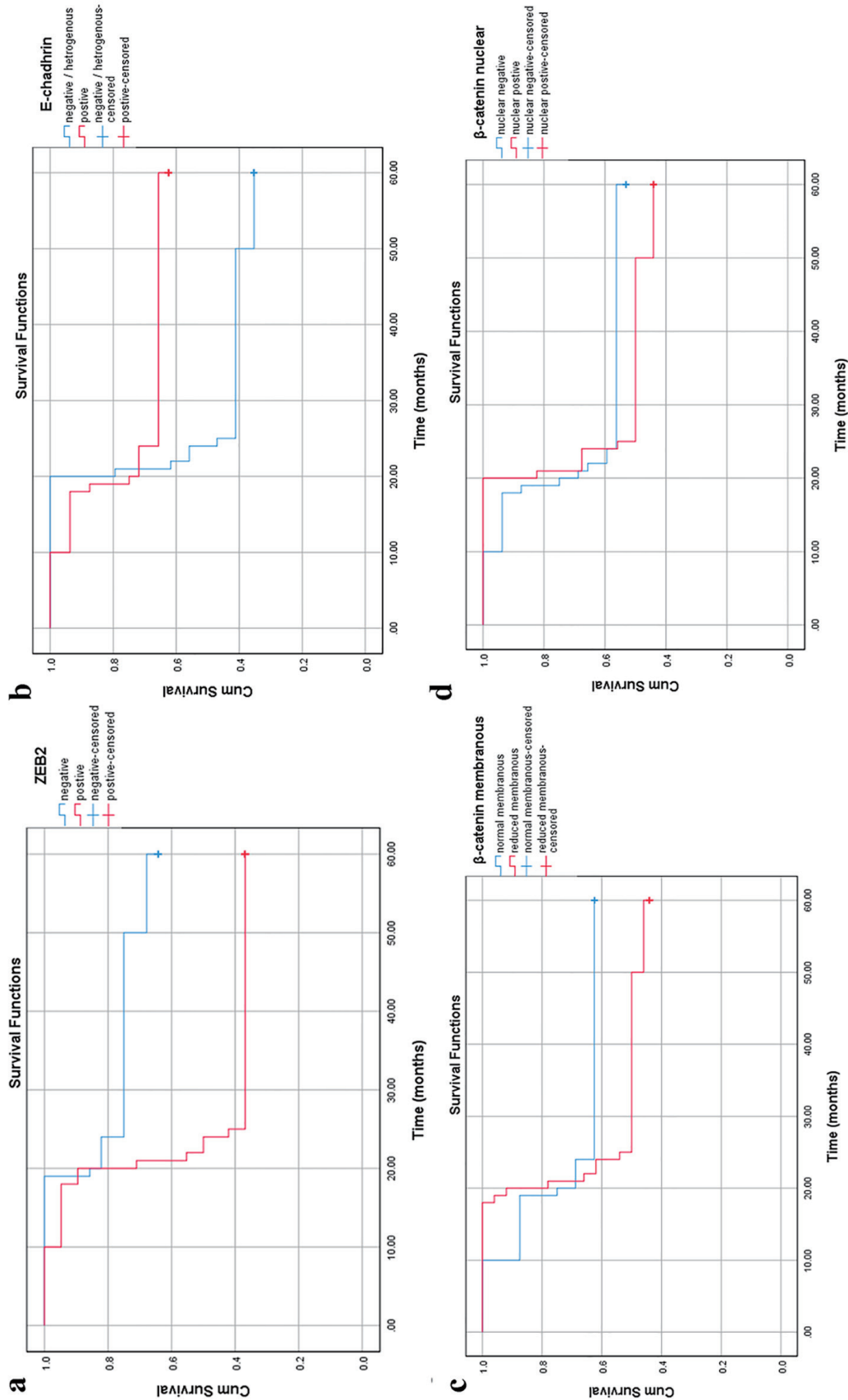
The spectrum of bladder tumors is broad and includes TCC, adenocarcinoma and SCC and other rare types. TCC is the most prevalent tumor representing 90% of bladder cancer cases and therefore has been studied most extensively [34]. SCC constitutes only a small percentage of all bladder tumors and therefore it has received less attention.

This study enrolled 66 patients (72.5%) with TCC and 25 patients (27.5%) with SCC. The distribution of carcinoma type in the studied sample is comparable to that reported earlier in Egypt [35]. The lower prevalence of SCC compared to TCC may be attributed to a reduction in schistosomal infection, increased cigarette smoking and occupation-related chemical exposure. In this study, the mean age at diagnosis for SCC cases was younger than that of TCC, which is an agreement with other work [36]. Male predominance was almost similar in both types of tumors to what has been previously reported [37, 38]. Also, the prevalence of bilharzial infection was significantly lower in TCC compared to SCC cases as previously reported in the literature [39]. The invasiveness of tumors was greater in SCC compared to TCC implying a more aggressive nature of SCC.

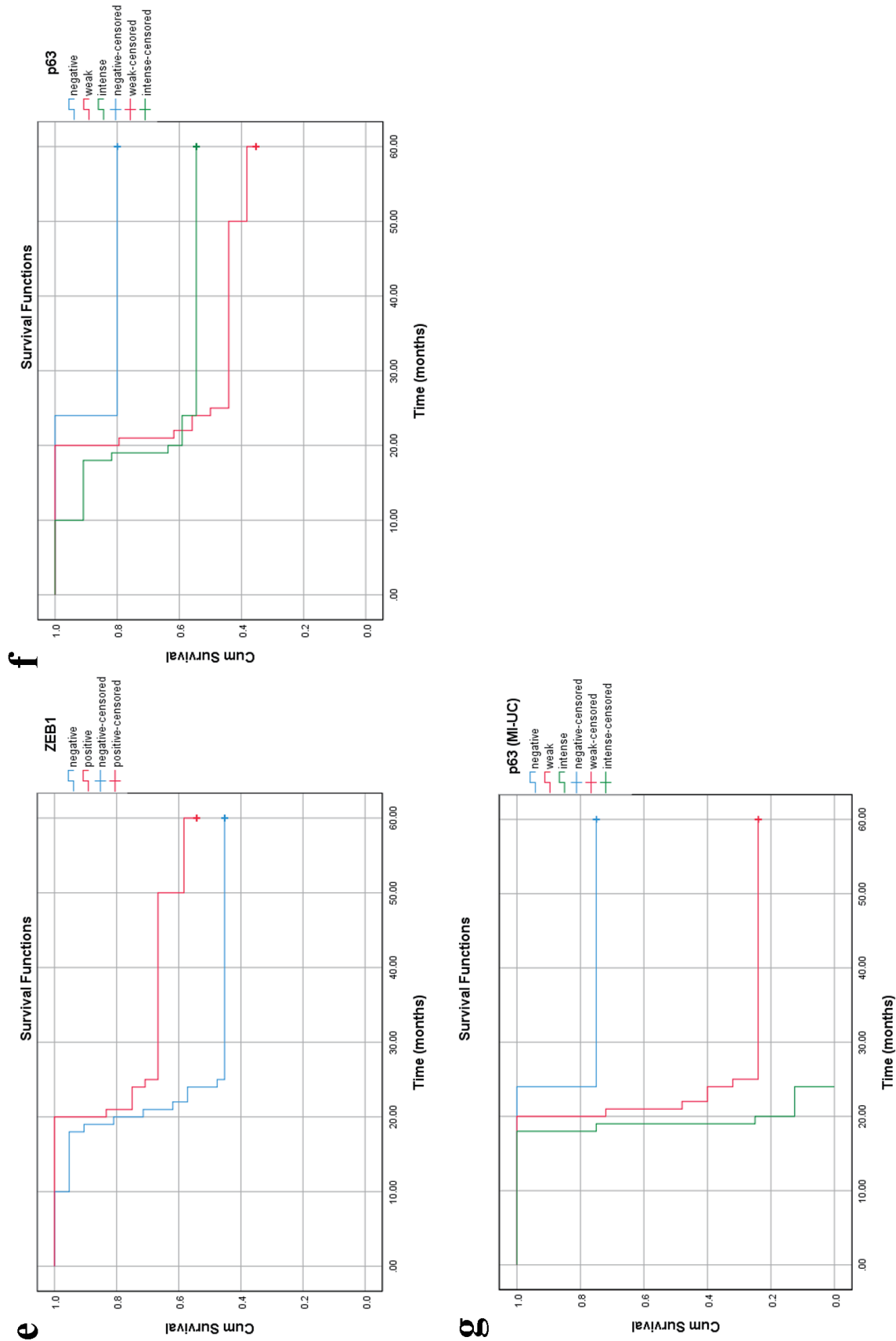
To the authors' knowledge, this study is the first to investigate EMT markers (E-cadherin,  $\beta$ -catenin, p63, ZEB1 and ZEB2) expression in urothelial tumors of TCC and SCC variants. In this study, patients with TCC and SCC showed reduced E-cadherin expression in association with increased tumor grading, depth of invasion and LN metastasis. The TCC results are consistent with previous reports [31, 40, 41]. Similarly, current results are in line with that reported for SCC of non-bladder origin [42-46].

In TCC cases, E-cadherin was not associated with OS; meanwhile, conflicting results have been recorded [40, 41] regarding the relation of reduced E-cadherin expression and poor outcome. However, altered E-cadherin expression in SCC variant was associated with poor survival which is in an agreement with previous reports on SCC of non-bladder origin [42, 47].

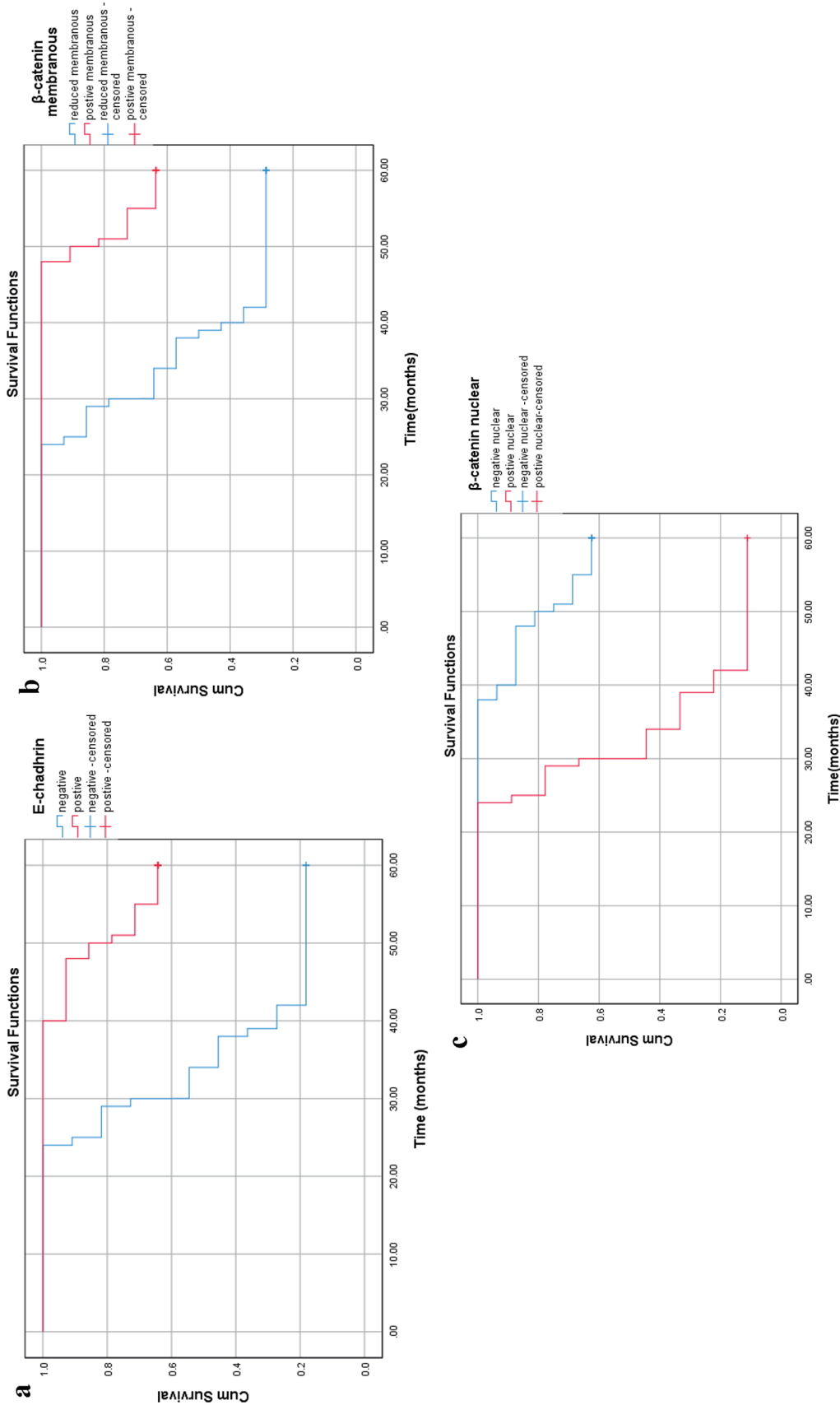
$\beta$ -Catenin plays a role in cell-to-cell adhesion and regulates urothelial homeostasis and carcinogenesis [48]. Our



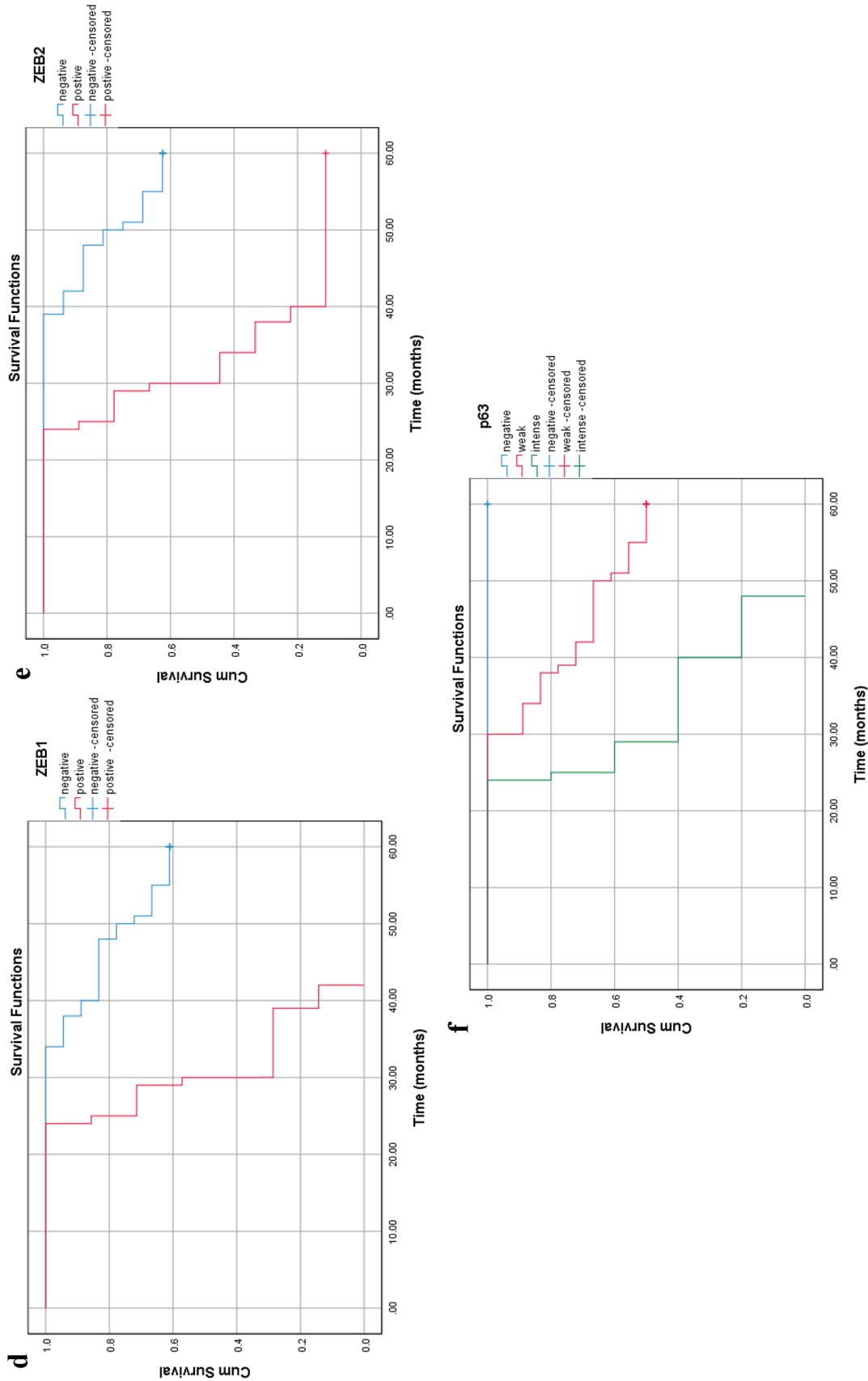
**Figure 5.** Survival analysis in the TCC group according to cumulative Kaplan-Meier methods (a-g). (a) ZEB2 cytoplasmic expression: negative group (blue) is associated with longer survival than positive group (red) ( $P = 0.024$ ). (b-f) Reduced membranous E-cadherin, reduced membranous β-catenin, nuclear β-catenin, positive ZEB1, intense p63 expressions without significant correlations ( $P > 0.05$ ). (g) Intense p63 expression is associated with poor survival in MI-UC ( $P < 0.001$ ). MI-UC: urinary carcinoma with muscle invasion; TCC: transitional cell carcinoma; ZEB: zinc-finger E-box-binding homeobox. - (continued on the next page)



**Figure 5. (continued)** Survival analysis in the TCC group according to cumulative Kaplan-Meier methods (a-g). (a) ZEB2 cytoplasmic expression: negative group (blue) is associated with longer survival than positive group (red) ( $P = 0.024$ ). (b-f) Reduced membranous E-cadherin, reduced membranous  $\beta$ -catenin, nuclear  $\beta$ -catenin, positive ZEB1, intense p63 expressions without significant correlations ( $P > 0.05$ ). (g) Intense p63 expression is associated with poor survival in MI-UC ( $P < 0.001$ ). MI-UC: urinary carcinoma with muscle invasion; TCC: transitional cell carcinoma; ZEB: zinc-finger E-box-binding homeobox.



**Figure 6.** Survival analysis in the SCC group according to cumulative Kaplan-Meier methods (a-f). (a) Reduced membranous E-cadherin, (b) reduced membranous β-catenin, (c) nuclear β-catenin, (d) positive ZEB1 and (e) positive ZEB2 and (f) intense p63 expressions with significant correlations with shorter survival (P < 0.001). SCC: squamous cell carcinoma; ZEB: zinc-finger E-box-binding homeobox. - (continued on the next page)



**Figure 6.** (continued) Survival analysis in the SCC group according to cumulative Kaplan-Meier methods (a-f). (a) Reduced membranous E-cadherin, (b) reduced membranous  $\beta$ -catenin, (c) nuclear  $\beta$ -catenin, (d) positive ZEB1 and (e) positive ZEB2 and (f) intense p63 expressions with significant correlations with shorter survival ( $P < 0.001$ ). SCC: squamous cell carcinoma; ZEB: zinc-finger E-box-binding homeobox.

study showed that  $\beta$ -catenin reduced expression in patients with TCC. This reduction was significantly associated with muscle-invasive tumors and high tumor grade. This association agrees with that reported by previous studies [32, 49-53]. On the other hand, the evidence is lacking to support a difference in nuclear and membranous  $\beta$ -catenin expression pattern in node-negative and node-positive tumors, which could be attributed to the relatively small sample size enrolled in this study, which may have led to type II statistical error.

In SCC cases, our study demonstrated that both reduced  $\beta$ -catenin membranous expression and the presence of nuclear/cytoplasmic  $\beta$ -cat expression correlated with higher tumor grade; meanwhile reduced membranous expression was strongly associated with tumor stage. These findings are similar to those previously reported [45, 54, 55]. The loss of membranous expression and cytoplasmic and nuclear accumulation of  $\beta$ -catenin have been reported in different SCC tumors [56-58]. Further, this study showed a significant correlation between the presence of nuclear/cytoplasmic  $\beta$ -catenin expression and both node-positive tumors and OS, which is consistent with previous reports [2, 55, 59]. However, it should be noted that the correlation of these biomarkers with nodal metastasis and their diagnostic usefulness is still controversial [2, 55, 59-62].

For ZEB1 and ZEB2, which control EMT [16], nuclear ZEB1 expressions correlated only with tumor grade in TCC, which may imply its potential role in tumor differentiation. No evidence existed to suggest a correlation between this transcriptional repressor and tumor stage, LN metastasis and OS. Lee et al (2014) reported ZEB-1 immunoreactivity in association with high-grade urothelial carcinoma [33]. Although Kenney et al (2011) reported no association between ZEB1 positive expression and tumor grade, which contradicts with our results, yet the lack of association with nodal involvement, or OS is similar to the current results [63], so ZEB1 may participate only in tumor differentiation of TCC variant. On the contrary, for patients with SCC, ZEB1 was up-regulated in association with the differentiation status, tumor stage, LN metastasis as well as OS. Similar results have been reported in SCC of non-bladder origin [64, 65]. This was also true for undifferentiated endometrial carcinomas and high Gleason score prostate carcinomas [66, 67] as well as in metastatic tumors including LN metastasis and more invasive tumors [68, 69]. The association reported in SCC patients may suggest the association between nuclear ZEB1 expression and tumor phenotype aggressiveness and increased cell motility, and hence, their invasiveness. Such an association could predict a poorer prognosis of bladder SCC.

In contrast, this study showed ZEB2 positive expression in TCC that correlated with tumor stage, positive LN metastases and OS. These findings may imply a potential role for ZEB2 in TCC progression and invasion, and hence, its potential diagnostic value as a biomarker of biological aggressiveness. Sayan et al showed a ZEB2 positive expression in TCC in association with decreased OS, despite lacking for ZEB1-positive expression [70]. Meanwhile in SCC, ZEB2 increased expression correlated with all clinicopathological factors including histological differentiation, pathological stage, LN metastasis and OS, a relationship that was previously reported in gastric cancer, renal cell carcinoma, breast cancer, and oral SCC [22,

71-76].

p63 is selectively expressed in the basal cell or progenitor cell compartment of stratified epithelia including urothelium in human tissues [25, 77, 78]. Several studies have shown that p63 may play a key role in the differentiation of transitional epithelium [24, 78]. Two main isoforms (TAp63 and  $\Delta$ Np63) with different functions are known. In normal bladder,  $\Delta$ Np63 is absent or weakly expressed in these cells, whereas, in BCs, elevated expression of  $\Delta$ Np63 was identified. It seems that  $\Delta$ Np63 allows cells to respond to signals required for maturation [77, 79]. This may suggest the contribution of  $\Delta$ Np63 to BC progression. A few authors proposed the use of p63 as a prognostic marker; however, it should be emphasized that variation in p63 expression and its role in bladder cancer development are still controversial [24, 51, 79, 80].

In this study, we reported diminished p63 expression in TCC as tumor grade increases. Further, weaker p63 expression was associated with increased tumor stage from superficial to invasive subtypes. These findings agreed with previous studies [24, 26, 51, 80, 81]. This suggests that, in the normal urothelium, p63 may play a growth-suppressive and differentiation-associated role, in which diminished p63 expression in TCCs may represent the loss of differentiation-associated, and therefore, growth-inhibitory p63 isoforms.

Considering the muscle-invasive subtypes, this study reports a high p63 expression in association with poor OS in patients with TCC. This is consistent with the findings of Choi et al who reported retention of this marker in primary bladder muscle-invasive tumors in association with poor outcome [81]. However, these results contradict with previous studies that associated the loss of p63 with shorter survival in patients with bladder cancer [51, 79]. However, the latter studies included patients with superficial cancers who usually have good survival, whereas the current study investigated more specific subgroups of patients with the muscle-invasive disease.

In patients with SCC, p63 overexpression was related to the degree of differentiation, stage and LN metastasis, all of which are signs of tumor aggressiveness. This could be attributed to the maintenance of the Np63 isoforms in squamous cancers leading to a greater immature cellular phenotype, thereby promoting tumor growth [82, 83]. Reports on SCC variant of bladder origin are lacking. Yet, the current findings are in agreements with the reported association of p63 expression and SCC differentiation in experimental animal models, head and neck squamous cell cancer, oral SCC, nasopharyngeal cancer, lung cancer, and epidermal tumor [83-89]. Our results are partly in agreement with Saghraevanian et al who showed significantly high p63 expression in association with tumor stage and LN metastases in oral SCCs [90, 91]. In the current study, we demonstrated that a higher p63 (intense) expression was significantly associated with poor OS; it should be emphasized that the prognostic value of p63 expression is still controversial [90, 92-94]. This lack of consensus may be attributed to testing different p63 isoforms with different biologic properties and the small number of cases that were included in these studies.

Interestingly, our results demonstrate distinctive p63 expression patterns according to phenotypic variants of BCs. Decreased expression of  $\Delta$ Np63 is a common feature in high-

grade invasive urothelial carcinomas, indicating that urothelial carcinogenesis is distinct from the SCC transformation strongly associated with overexpression of DNp63 [51, 95]. Furthermore, it is interesting to emphasize that, as shown by our results, although this different pattern of p63 expression exists among two different variants of BC, it was clearly noticed that, the tumor cases with the highest p63 expression had the worst prognosis in both tumor groups. These results can highlight the important role of high p63 expression among different phenotypes of BC regardless of the different mechanism of action that differs from each tumor type. Also, these results can possibly advance our understanding of the initiating mechanisms, pathogenesis and prognosis of SCC variant versus TCC variant and also result in novel therapeutic target in cancer treatment.

Lastly, it has been shown that the clinical behavior of BC is difficult to predict based on classic histopathologic parameters alone [96]. So, to enhance the detection of tumor aggressiveness, use of biomarkers in conjunction with classic pathological prognostic factors such as stage, grade, LN involvement and lymphovascular invasion may improve prognosis and guide multimodal treatment approaches in the era of personalized medicine. More recently, another view of EMT became an important issue. The latest studies have demonstrated that numerous master regulators of EMT, including ZEB proteins, could have a crucial role in control of key cellular features, such as cell cycle, susceptibility to apoptosis and senescence. At early stages, ZEB proteins may potentially contribute to tumorigenesis via cooperation with activated oncogenes by overriding oncogene-induced senescence and apoptosis. They may control tumor cell dissemination via reversible EMT and help the formation of dormant metastases by causing reversible cell cycle arrest. At later stages, ZEB proteins have an effect on cancer cell properties by regulating expression of p53 family members such as p63, inducing resistance against anti-proliferative therapies [97-99].

In the light of previous findings, in our study we reported that positive ZEB2 expression which represents a poor prognostic feature in both TCC and SCC patients has shown a well-established anti-apoptotic activity independent of its effects on cell adhesion, thus suggesting that ZEB2 indicates a poor prognosis, especially in patients who will be treated with adjuvant postoperative radiotherapy/and or chemotherapy. Parallely, as seen in our study, patients with ZEB1-positive bladder SCC exhibited worse survival outcomes. This can be explained on the basis of that ZEB1 expression protects cells from apoptosis and contributes to drug resistance in cancer cells, in part through regulation of p53/p63/ p73 family members. So, detection of ZEB1 and ZEB2 expression level in bladder tumors could facilitate prospective determining of their resistance (or sensitivity) to specific radiotherapy/chemotherapy treatments. Additionally, the inhibition of ZEB1, ZEB2 or its cofactors could be used to reverse drug resistance in cancer patients. Altogether, ZEB1 and ZEB2 may be used as an important prognostic and/or therapeutic cancer targets in the near future.

As regards to probable role of p63 in the new therapeutic options, pre-surgical (neoadjuvant) cisplatin-based neoadjuvant chemotherapy (NAC) is considered as the current standard-of-care for high-risk MIBC [100]. Although about 40% of patients with high-risk muscle-invasive bladder cancers can be

cured with surgery and cisplatin-based chemotherapy, progression in patients with cisplatin-resistant disease is highly rapid and fatal [101]. Therefore, there is a great ought to discover new therapies that focus on the cisplatin-resistant subset of muscle-invasive tumors.

Previous studies have reported that p63 stimulates the expression of miR205 which has a well-established role in blocking EMT that has been implicated in drug resistance [6, 102]. So it seems that the effects of p63 in promoting sensitivity to antimetabolic chemotherapy are also linked to its effects on EMT [103]. Interestingly, our results as well as these previous findings could indicate that for patients with the lethal, p63-positive muscle-invasive tumors who exhibited worse survival outcome, they could get most benefit from treatment with antimetabolic chemotherapy.

To this end, we can emphasize that use of a panel of five markers, including E-cadherin,  $\beta$ -catenin, p63, ZEB1, and ZEB2 was suggestive to best represent variations in specific biomarker expression that may be associated with differences in patient outcomes or patient responsiveness to therapies. So these biomarkers may be used to guide the optimal treatment approach after radical cystectomy.

## Conclusions

Patients with TCC of non-muscle-invasive subtypes showed increased expression of the epithelial markers E-cadherin,  $\beta$ -catenin and p63, while invasive tumors showed higher expression of mesenchymal markers including ZEB1 and ZEB2. Yet, a worse prognosis was seen in association with elevated p63 expression denoting an “epithelial” phenotype of invasive tumors. For patients with SCC, there was a reduction in E-cadherin and  $\beta$ -catenin positivity, elevated p63 expression and concomitant increased expression of ZEB1 and ZEB2. Poor prognosis was evident in association with elevated p63 expression. Taken together, in both TCC and SCC variants, EMT process is evident; however its molecular mechanism shows some variations, specifically this notably different p63 expression pattern among two carcinoma variants with the similar impact on prognosis. Thus, with available number investigated in this study, E-cadherin,  $\beta$ -catenin and p63, as well as ZEB1 and ZEB2 biomarkers levels may have significant prognostic value, and hence, may impact developing targeted therapy specifically for SCC variant. Future, prospective cohort studies with a larger sample size are required to confirm this conclusion.

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## Conflict of Interest

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

## Informed Consent

Considering the characteristic of this study as a retrospective study performed on paraffin blocks, the written informed consents were waived in this study.

## Author Contributions

RAM and EZIK contributed to collection of the samples, data statistics, manuscript writing and composition. AIA was responsible for data collection from medical records, clinical aspect of the project and the supervision and management of the project. All authors have contributed to and approved the final manuscript.

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