Tumor-infiltrating CD8+ lymphocytes predict different clinical outcomes in organ and non–organ-confined urothelial carcinoma of the bladder following radical cystectomy

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Tumor-infiltrating lymphocytes (TILs) are associated with better clinical outcomes in many tumors. TILs represent a cell-mediated immune response against the carcinoma. CD8+ TILs are a crucial component of cell-mediated immunity. The significance of CD8+ TILs has not been reported respectively in organ and non–organ-confined urothelial carcinoma (UC) of the bladder. We explored the prognostic value of CD8+ TILs in the two groups. The presence of CD8+ TILs was assessed by immunohistochemical staining of whole tissue sections from 75 organ and 51 non–organ-confined disease patients with long-term follow-up, and its correlation with clinicopathological features and overall survival (OS) was determined. The CD8+ TIL immunohistochemical staining score was 0 (<1%), 1 (≥1%), 2 (≥5%), or 3 (≥10%) based on the percentage of positively stained cells out of total cells. A patient was considered CD8 negative if the score was 0. There were no associations between CD8+ TILs and age, sex, nuclear grade, and adjuvant or neoadjuvant chemotherapy in organ and non-organ-confined disease. The presence of CD8+ TILs was seen more frequently in pTa-$_1$ than pT$_2$ stage (p=0.033) in organ-confined disease. No associations between CD8+ TILs and pT stage, pN stage were found in non–organ-confined disease. CD8+ TILs were associated with better OS (log-rank test, P = 0.036) in non-organ-confined disease, but with poorer OS (log-rank test, P = 0.040) in organ-confined disease by the Kaplan–Meier method. In multivariate analysis, CD8+ TILs were an independent favorable prognostic factor in non-organ-confined disease, but were an independent unfavorable prognostic factor in organ-confined disease. These results suggest that CD8+ TILs have clinically significant anti-tumor activity in non-organ-confined disease, but may have pro-tumor activity in organ-confined disease. Therefore, we should be cautious if CD8+ TILs are aimed to be exploited in the treatment of bladder cancer.

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2 **non–organ-confined urothelial carcinoma of the bladder following radical cystectomy**

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13 **Abstract**

14 Tumor-infiltrating lymphocytes (TILs) are associated with better clinical outcomes in many

15 tumors. TILs represent a cell-mediated immune response against the carcinoma. CD8+ TILs are

16 a crucial component of cell-mediated immunity. The significance of CD8+ TILs has not been

17 reported respectively in organ and non–organ-confined urothelial carcinoma (UC) of the bladder.

18 We explored the prognostic value of CD8+ TILs in the two groups. The presence of CD8+ TILs

19 was assessed by immunohistochemical staining of whole tissue sections from 75 organ and 51

20 non–organ-confined disease patients with long-term follow-up, and its correlation with

21 clinicopathological features and overall survival (OS) was determined. The CD8+ TIL

22 immunohistochemical staining score was 0 (<1%), 1 (≥1%), 2 (≥5%), or 3 (≥10%) based on the

23 percentage of positively stained cells out of total cells. A patient was considered CD8 negative if

24 the score was 0. There were no associations between CD8+ TILs and age, sex, nuclear grade, and

25 adjuvant or neoadjuvant chemotherapy in organ and non-organ-confined disease. The presence

26 of CD8+ TILs was seen more frequently in pTa-$_1$ than pT$_2$ stage (p=0.033) in organ-confined

27 disease. No associations between CD8+ TILs and pT stage, pN stage were found in non–organ-

28 confined disease. CD8+ TILs were associated with better OS (log-rank test, P = 0.036) in non-

29 organ-confined disease, but with poorer OS (log-rank test, P = 0.040) in organ-confined disease

30 by the Kaplan–Meier method. In multivariate analysis, CD8+ TILs were an independent

31 favorable prognostic factor in non-organ-confined disease, but were an independent unfavorable

32 prognostic factor in organ-confined disease. These results suggest that CD8+ TILs have

33 clinically significant anti-tumor activity in non-organ-confined disease, but may have pro-tumor

34 activity in organ-confined disease. Therefore, we should be cautious if CD8+ TILs are aimed to

35 be exploited in the treatment of bladder cancer.

36

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Introduction

Bladder cancer is the ninth leading cause of cancer death in both sexes, with an estimated 429,800 new cases and 165,100 deaths in 2012 worldwide (Torre et al., 2015; Ferlay et al., 2015). In the US, bladder cancer was the second most common genitourinary cancer, with projected 79,030 new cases and 16,870 related deaths in 2017 (Siegel et al., 2017). In China, there were an estimated 80,500 new cases and 32,900 deaths from bladder cancer in 2015 (Chen et al., 2016).

Bladder cancer can be divided into organ-confined (pTa/pT1/carcinoma in situ [CIS]/pT2 NX/N0 M0) and non–organ-confined (pT3/T4N any M any or pT any N+M any or pT any N any M+) disease at cystectomy based on extravesical invasion (Boorjian et al., 2008). More than 90% of bladder cancers are urothelial carcinoma (UC) (Kaufman et al., 2009), of which one characteristic is the presence of high rates of somatic mutations (2014). These mutations may produce an increased number of cancer neoantigens and enhance the ability of the host immune system to recognize tumor cells (Chen & Mellman, 2013; Sanmamed & Chen, 2014; Chen, 2014).

The presence of tumor-infiltrating lymphocytes (TILs) has been associated with favorable prognosis in several neoplasms (Pages et al., 2005; Galon et al., 2006; Laghi et al., 2009; Zhang et al., 2003; Wang et al., 2016; Mahmoud et al., 2011). Among the TILs, CD8+ TILs may play a central role in anti-tumor immunity. Non–organ-confined UC of the bladder has worse prognosis than organ-confined disease. However, only two reported studies have assessed the effect of CD8+ TILs in UC of the bladder following radical cystectomy,which suggest the presence of CD8+ TILs is associated with favorable prognosis.. If CD8+ TILs have the similar role in the two group patients of organ and non-organ confined disease, there hasn't been reported.

In this study, we evaluated CD8+ TILs by immunohistochemistry (IHC) in organ and non–organ-confined UC of the bladder following radical cystectomy, and explored the association with overall survival (OS).

Materials and Methods

Patients and tumor specimens

Archival pathology tumor blocks were obtained from the Department of Pathology of Sun Yat-sen University Cancer Center, Guangzhou, China. We retrieved 126 consecutive formalin-fixed, paraffin-embedded radical cystectomy specimens of UC of the bladder obtained between January 2000 and September 2009, including 51 non-organ-confined and 75 organ-confined disease. Representative tumor specimens were selected upon review of hematoxylin and eosin slides. We reviewed all sections to confirm the original diagnosis and staged them according to the 2010 American Joint Committee on Cancer TNM (tumor-node-metastasis) classification. The clinicopathological features were retrospectively reviewed from the patients' medical records. The OS was determined from the date of surgery to the date of death or censored on the date of the last follow-up. Written informed consent was obtained from all patients prior to the study. The Ethical Committee of Sun Yat-sen University Cancer Center approved all experimental methods in the study (YB2016-006).

CD8 IHC

CD8 IHC was conducted according to a standard method. Briefly, tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. For antigen retrieval, tissue slides were boiled in 10 mM citrate buffer (pH 6.0) in antigen retriever (Ascend Biotechnology, Guangzhou, China) at 120°C for 5 min. Nonspecific binding was blocked with Serotec Block ACE (AbD Serotec, Oxford, UK) for 15 min. The slides were incubated with anti-CD8 antibody (Clone SP16, Thermo Scientific, Fremont, USA) overnight at 4°C. All incubations were performed in a moist chamber. Subsequently, the slides were incubated with a horseradish peroxidase–labeled secondary antibody for 60 min at 37°C, and then visualized using 3,3′-diaminobenzidine.

87 **_Scoring of CD8+ TILs_**

88   Two independent pathologists blinded to the clinicopathologic information performed the

89   scoring. The CD8+ TIL score was 0 (<1%), 1 (≥1%), 2 (≥5%), or 3 (≥10%) based on the

90   estimated percentage of positively stained cells out of total cells. A patient with a score of 0 was

91   considered CD8 negative (CD8−). If there was a discrepancy in scoring, both pathologists

92   reviewed the case using a double-headed microscope to achieve consensus. About 31% of the

93   total cases required a consensus discussion between the reviewing pathologists for scoring. At all

94   times, the pathologists were blinded to the clinical outcome.

95 **_Statistical methods_**

96   The Fisher exact test was used to evaluate CD8 status between different categories. The

97   Kaplan–Meier method and Cox proportional hazards model were used for OS analysis;

98   differences in the Kaplan–Meier survival analysis were assessed using the log-rank test.

99   Statistical analysis was performed using IBM SPSS 19.0 (SPSS, Inc., Chicago, Illinois) for

100   Windows; the significance level was set at 0.05 (2-sided).

101 **Results**

102 **_Clinicopathological analysis of organ and organ-confined disease and presence of CD8+ TILs_**

103   Table 1 summarizes the clinicopathologic characteristics. The median age of the 51 patients

104   at surgical resection was 63 years, and the median follow-up time from date of surgical resection

105   was 30.5 months (range: 0.2–129.6 months) in non-organ-confined disease. In organ-confined

106   disease, median age of the 75 patients at surgical resection was 60 years, and median follow-up

107   time from date of surgical resection was 51.8 months (range: 0.3–139.7 months).

108   CD8+ TILs were detected in both the tumoral and intratumoral areas, and were distributed

109   mainly at the interface of the tumor and tumor-adjacent stroma (Fig.1). We compared CD8+

110   TILs with the clinicopathological characteristics of organ and non–organ-confined disease (Table

111   1). There were no associations between CD8+ TILs and age, sex, nuclear grade, and adjuvant or

112   neoadjuvant chemotherapy in both organ and non–organ-confined disease. In organ-confined

113   disease, the presence of CD8 TILs was seen more frequently in pTa-$_1$ than pT$_2$ stage (p=0.033).

114   No associations between CD8+ TILs and pT stage, pN stage were found in non–organ-confined

115   disease.

116

117   ***OS and Cox proportional hazards analysis***

118   We examined the relationship between the presence of CD8+ TILs and OS from the date of

119   cystectomy. There was no statistically significant association between CD8+ TILs and OS in all

120   126 UC of the bladder patients (OS, P = 0.899, Fig. 2A) by the Kaplan–Meier method . The

121   presence of CD8+ TILs was correlated with improved OS in 51 patients of non–organ-confined

122   disease (P = 0.036, Fig. 2B). On the contrast, it was associated with poorer OS in 75 patients of

123   organ-confined disease (P = 0.040, Fig.2C). The multivariate analysis showed that the presence

124   of CD8+ TILs was an independent prognostic predictor in both organ and non-organ-confined

125   disease. In organ-confined disease,the patients with CD8 negative had a better prognosis than

126   the patients with CD8 positive (RR: 0.212, 95% CI: 0.048–0.932, P = 0.0040; Table 2). However,

127   in non-organ-confined disease,the patients with CD8 negative had a worse prognosis than the

128   patients with CD8 positive (RR: 2,397, 95% CI: 1.031–5.573, P = 0.042; Table 2).

129   In the present study, the CD8+ TIL score was 0 (<1%), 1 (≥1%), 2 (≥5%), or 3 (≥10%) based

130   on the estimated percentage of positively stained cells out of total cells. It was no statistically

131   significant association between CD8+ TILs and OS in all 126 UC of the bladder patients by the

132   Kaplan–Meier method regardless of CD8 score = 1 & 2 & 3 vs CD8 score = 0, CD8 score = 0 &

133   1 vs CD8 score = 2 & 3). There was no statistically significant association in non-organ-confined

134   disease or pT3+4 stage (data not shown) when it was CD8 score = 0 & 1 vs CD8 score = 2 & 3;

135   but when it was CD8 score = 1 & 2 & 3 vs CD8 score = 0, there was statistically significant

136   association in non-organ-confined disease or pT3+4 stage (data not shown).

137

Discussion

139 We show that CD8+ TILs are significantly associated with favorable clinical outcome in non–

140 organ-confined disease, but with unfavorable clinical outcome in organ-confined disease

141 in the Kaplan-Meier survival analysis, although no statistically significant association between

142 CD8+ TILs and OS was found in all 126 UC of bladder. In multivariate Cox's proportional

143 hazards regression analysis, the presence of CD8+ TILs was an independent factor of prognosis

144 in both of organ-confined and non-organ confined disease. It was a favorable factor of prognosis

145 in non-organ-confined disease, but was an unfavorable factor in organ-confined disease.

146 Previous studies have shown that TILs (especially CD8+ TILs), which have anti-tumor

147 activity, are associated with good prognosis in patients with tumors (Galon et al., 2006;

148 Mahmoud et al., 2011; Hwang et al., 2012; Pages et al., 2005). CD8+ TILs mediate most anti-

149 tumor immune responses (Wang et al., 2016), bladder cancer studies also suggest that the

150 presence of CD8+ TILs is associated with favorable prognosis (Sharma et al., 2007; Faraj et al.,

151 2015). High CD8 density was associated with improved OS (P = 0.02) in a subset of 50

152 cystectomies for invasive bladder UC (≥ pT1) (Faraj et al., 2015). The authors defined high CD8

153 density as the presence of ≥60 CD8+ T cells per high-power field in a given tissue microarray

154 spot. A tumor was considered high density if 50% of its spots were of high density. This is

155 different from our study. A patient with a score of 0 (<1% of positively stained cells out of total

156 cells) was considered CD8 negative in our study. CD8+ T cells were detected in both the

157 tumoral and intratumoral areas, and were distributed mainly at the interface of the tumor and

158 tumor-adjacent stroma (Tumeh et al., 2014), the score of CD8+ TILs was accessed in tumor

159 periphery (i.e. the interface of the tumor and tumor-adjacent stroma) in the present study.

160 Another study showed that, in similar-staged bladder UC (pT2, pT3, or pT4), patients with more

161 CD8+ TILs within the tumor had better OS (P < 0.018) than patients with fewer CD8+ TILs

162 (Sharma et al., 2007). Multivariate analysis also revealed a significant association between CD8+

163   TILs and OS. The previous two reports had the similar results, and it was consistent with the

164   findings of the present study in non-organ confined disease. However, the presence of CD8+

165   TILs is an significant unfavorable predictor in organ-confined disease, which was also found in

166   the present study. The immune cells of CD8+ TILs have anti-tumor activity in non-organ

167   confined disease, and may have pro-tumor activity in organ-confined disease. But if the CD8+

168   TILs themselves had pro-tumor activity or CD8+ TILs' anti-tumor activity inhibited by other

169   powerful pro-tumor factors in organ-confined disease, such as tumor cells PD-L1 expression

170   (Chen & Flies, 2013) , it needs further investigation. Many studies suggest CD8+ TILs could

171   potentially be exploited in the treatment of cancer. If CD8+ TILs are aimed to be exploited in the

172   treatment of bladder cancer, that different therapeutic strategies should be considered, which was

173   indicated by the present study.

174   The efficacy of immunotherapy in superficial bladder tumors was first established in 1976

175   with BCG (Morales et al., 1976), but no immunotherapy had been approved for the treatment of

176   advanced disease, until anti-PD-1/PD-L1 monoclonal antibody were approved for the treatment

177   of bladder cancer patients (Powles et al., 2017). All of the patients in our study didn't receive any

178   immunotherapy.

179   The study limitations are the small number of patients included , the heterogeneity of the

180   patient population and contrasting results between organ and non-organ-confined disease.

181   Therefore, further investigation is necessary to verify our findings , and explore why the

182   presence CD8+ TILs have different effects in the two cohorts.

183   In conclusion, our results provide evidence of the prognostic importance of CD8+ TILs in

184   organ and non–organ-confined UC of the bladder. The presence of CD8+ TILs in non–organ-

185   confined disease correlates with improved OS, but it was correlated with poorer OS in organ-

186   confined disease. On this account, if CD8+ TILs are aimed to be exploited in the treatment of

187   bladder cancer, different treatment strategies should be considered.

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191   First Affiliated Hospital of Sun Yat-sen University) for their assistance with the scoring of CD8+

192   TILs.

193

194   **Figure legends**

195

196   Figure 1. Representative examples of CD8+ TILs immunostaining. CD8+ TILs are distributed

197   mainly at the interface of the tumor and tumor-adjacent stroma. A, B, C and D ($\times$100

198   magnification) are representative images of scores of 0, 1, 2, and 3, respectively.

199

200   Figure 2. Kaplan–Meier survival curves with log-rank testing. A. All UC of the bladder (CD8+,

201   90; CD8−, 36). B. Non–organ-confined UC of the bladder (CD8+, 35; CD8−, 16). C. Organ-

202   confined UC of the bladder (CD8+, 55; CD8-, 20).

203
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279
280

Figure 1

Representative examples of CD8+ TILs immunostaining.

CD8+ TILs are distributed mainly at the interface of the tumor and tumor-adjacent stroma. A, B, C and D (×100 magnification) are representative images of scores of 0, 1, 2, and 3, respectively.

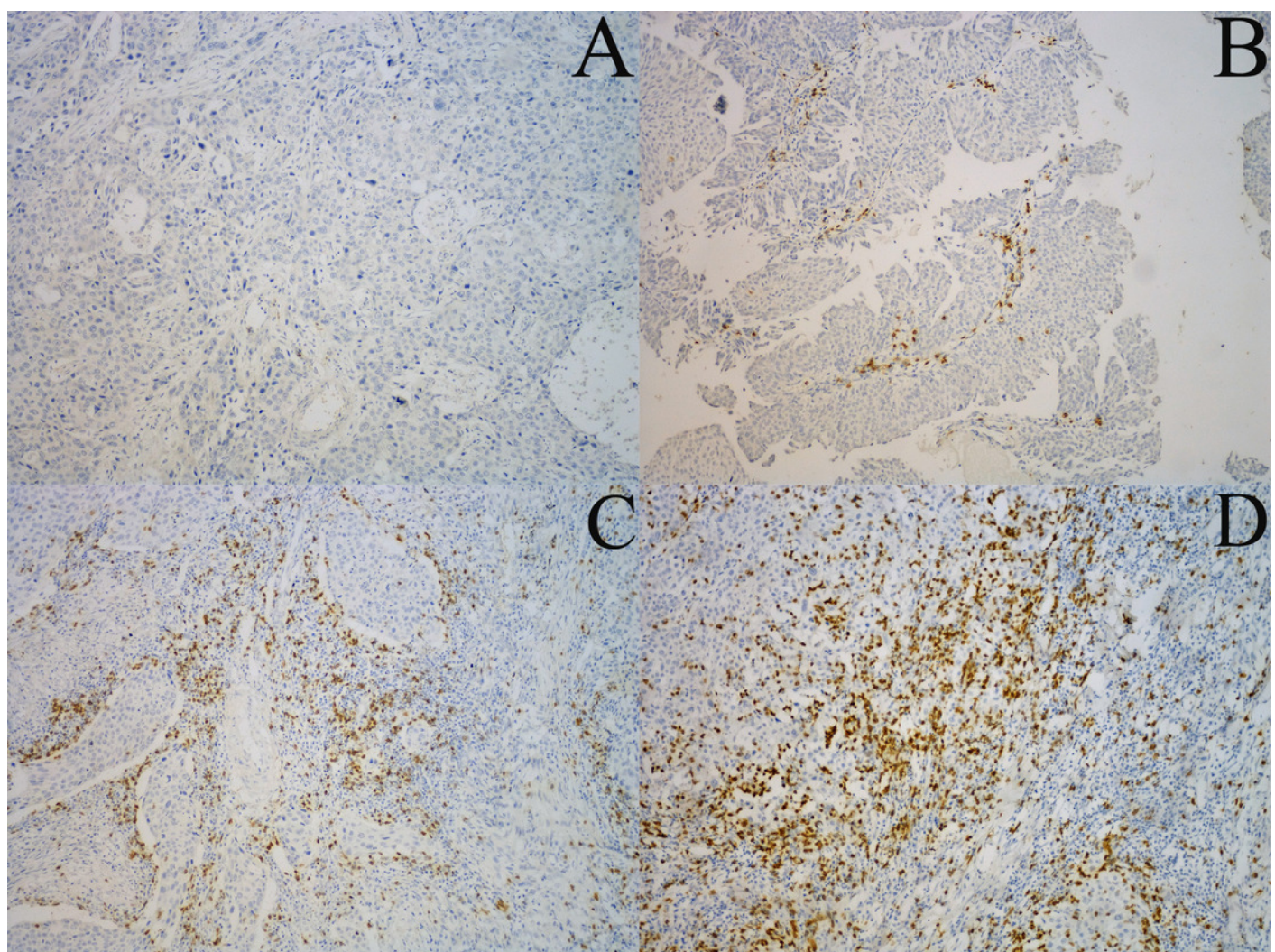
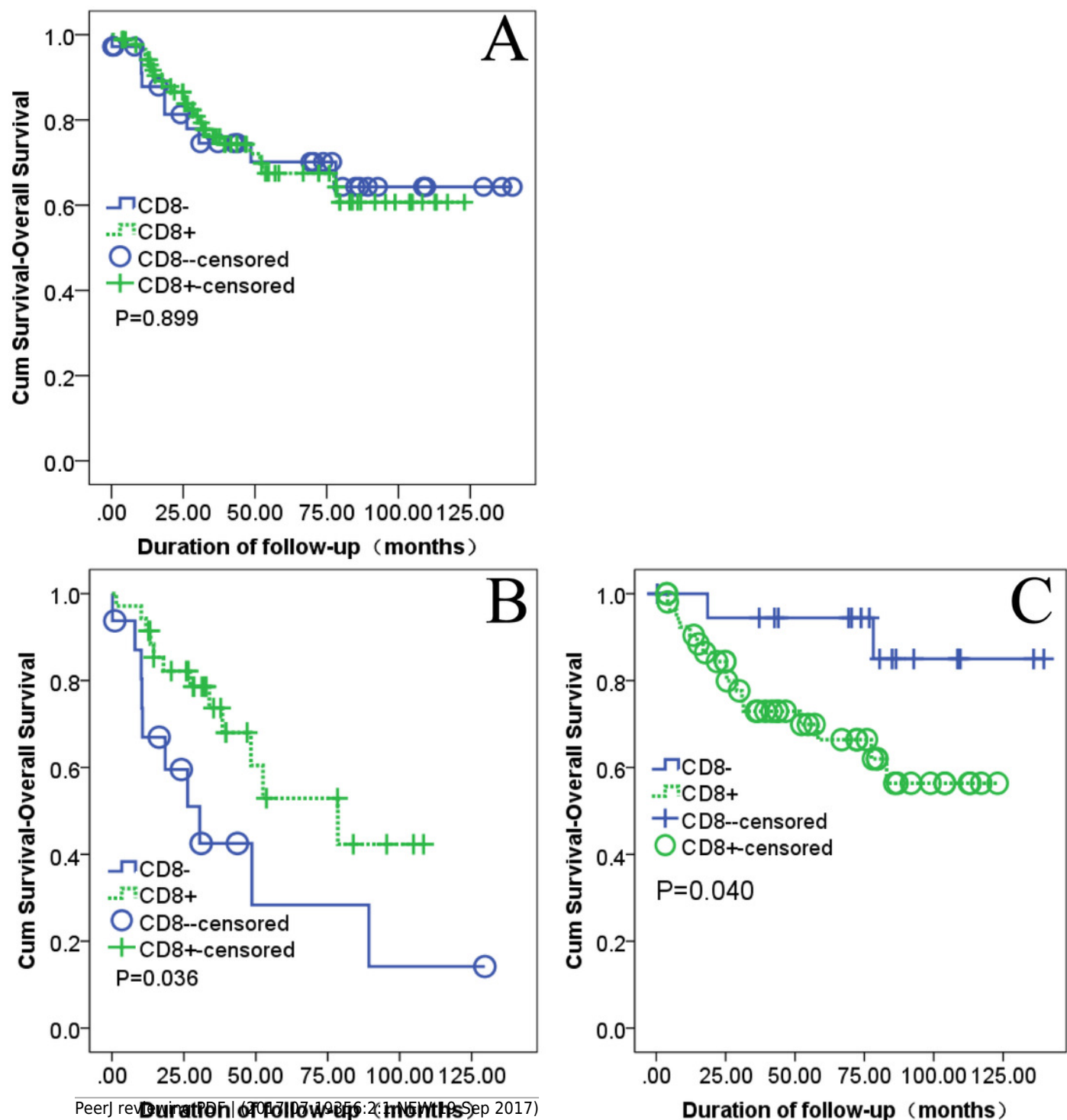



Figure 2

Kaplan–Meier survival curves with log-rank testing.

A. All UC of the bladder (CD8+, 90; CD8−, 36). B. Non–organ-confined UC of the bladder (CD8+, 35; CD8−, 16). C. Organ-confined UC of the bladder (CD8+, 55; CD8-, 20).

Correlation between clinicopathological features and CD8+ TILs

Characteristics	N	Organ-confined		P		Characteristics	N	Non-organ-confined		P	
		CD8+	CD8−	Fisher	exact test			CD8+	CD8−	Fisher	exact test
Age (years)				0.296						0.760	
<60	36	24	12				18	13	5		
≥60	39	31	8				33	22	11		
Sex				1.000						0.295	
Male	64	47	17				47	31	16		
Female	11	8	3				4	4	0		
pT stage				0.033		pT stage				0.345	
pTa-1	31	27	4			pT3	34	25	9		
pT2	44	28	16			pT4	17	10	7		
pN stage						pN stage				0.746	
						N-	35	23	12		
						N+	16	12	4		
Nuclear Grade				0.107							
Low	26	16	10								

High	49	39	10					
Adjuvant Chemotherapy, n (%)				0.114				0.527
Not Administered	70	53	17		34	22	12	
Administered	5	2	3		17	13	4	
Neo-adjuvant Chemotherapy, n (%)				1.000				0.643
Not Administered	65	48	17		5	3	2	
Administered	10	7	3		46	32	14	

Association between CD8 status and OS in the subset of 75 organ-confined and 51 non-organ-confined urothelia carcinoma of the bladder patients in the Cox proportional hazards model

	organ-confined			non-organ-confined		
	Risk ratio	95 % CI	P	Risk ratio	95 % CI	P
CD8−vs CD8+	0.212	0.048-0.932	0.040	2.397	1.031-5.573	0.042

1   Adjusted for age, sex, nuclear stage, T stage, N stage (only in non-organ-confined), neo-adjuvant

2   chemotherapy, and adjuvant chemotherapy.