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Depth of invasion to the bladder wall as a prognostic factor and its association with circulating cell-free DNA levels in patients with muscle-invasive bladder cancer

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Abstract

Background: Radical cystectomy (RC) is the standard surgical treatment for patients with muscle-invasive bladder cancer, but the prognosis is not favorable, and new prognostic factors need to be discovered. We investigated the potential of depth of invasion (DOI) as a prognostic factor in patients with muscle-invasive bladder cancer who underwent RC. Furthermore, we examined the association between preoperative levels of circulating cell-free DNA and DOI.

Materials and methods: We retrospectively reviewed patients who underwent RC between January 2007 and December 2017; those who received neoadjuvant chemotherapy were excluded. Depth of invasion was measured using hematoxylin-eosin–stained RC specimens.

Results: Of the 121 patients selected, 41 (33.9%) were eligible for analysis. The median follow-up period was 14 months and mean DOI was 17 mm (range, 2–75 mm). Long DOI (>17 mm) was significantly associated with shorter progression-free survival (hazard ratio, 14.5; 95% confidence interval, 3.9–53.97, $p < 0.0001$) and cancer-specific survival (hazard ratio, 18.97; 95% confidence interval, 4.04–88.99, $p = 0.0002$) compared with short DOI. Multivariate analysis revealed that DOI was an independent risk factor for cancer-specific survival. The levels of circulating cell-free DNA were significantly higher in patients with a longer DOI than in those with short DOI (65 vs. 20 ng/mL, respectively; $p = 0.028$).

Conclusions: Depth of invasion predicted with levels of circulating cell-free DNA and thus could be a useful prognostic factor.

Key words: Bladder cancer; Depth of invasion; Cell-free DNA

1. Introduction

Bladder cancer (BC) is the 10th most common form of cancer globally, with 549,393 new cases and 199,922 BC-related deaths reported in 2018.[1] It has traditionally been classified as nonmuscle-invasive BC (NMIBC) or muscle-invasive BC (MIBC), according to the invasion of the muscularis propria. Radical cystectomy (RC) is the standard surgical treatment for patients with MIBC.[2] Although the 5-year overall survival rate after RC can be as high as 80% in lymph node-negative and organ-confined disease (pT2N0M0), the rate decreases to 40% in patients with extravesical extension of the bladder wall (pT3) and to 15%–35% in those with lymph node involvement.[3,4] The primary reason for poor survival rates in patients with locally advanced MIBC is the presence of micrometastases at the time of RC, which cannot be detected using preoperative imaging.[5] These findings suggest that the extended use of neoadjuvant or adjuvant systemic chemotherapy should be considered in patients with advanced MIBC to eradicate micrometastases.

Currently, the TNM staging system, which is based on tumor invasion, lymph node involvement, and distant metastasis, is commonly used to predict cancer-specific survival (CSS) in patients with BC.[6,7] However, the predictive value of the T category is inconsistent because BC often shows heterogeneous behavior. Therefore, other clinically available markers have been explored to better predict postoperative outcomes. Recently, it was reported that depth of invasion (DOI) in oral cancer is a useful predictor of CSS by improving the T staging system.[8] No studies have reported the clinical significance of DOI in BC.[7,9]

Circulating cell-free DNA (cfDNA) in serum, which was discovered as early as in 1948,[10] is the fragmented DNA originating from cancer cells through the processes of necrosis and apoptosis.[11] In 1977, Leon et al.[12] observed that compared with healthy individuals, patients with cancer had increased levels of circulating cfDNA in the serum, indicating the potential of cfDNA as a diagnostic and monitoring marker during cancer treatment. Wang et al.[13] reported that circulating cfDNA levels in blood and urine have the potential to be useful for the early diagnosis of BC. Currently, no study has investigated the association between DOI in RC specimens and circulating cfDNA levels.

In this study, we investigated the potential of DOI to the bladder wall as a prognostic factor in patients with MIBC who underwent

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RC. Moreover, we examined the association between preoperative circulating cfDNA levels in serum and DOI.

2. Materials and methods

2.1. Patient selection

We retrospectively reviewed the medical charts of 121 patients who underwent RC between January 2007 and December 2017 at Nara Medical University. Among them, 65 patients who received neoadjuvant chemotherapy were excluded. Of the remaining 56 patients, 8 with pathological T0 and 7 with pathological T1 were also excluded because their DOI could not be analyzed. Ultimately, 41 patients were included in the analysis (Fig. 1). This study was approved by the Nara Medical University Ethics Committee (reference ID: 1256). Written informed consent was obtained from all the participants.

2.2. Quantification of the DOI in surgical specimens

Two investigators (S.F. and T.F.) independently reviewed the pathological specimens obtained by RC. The distance from the bladder mucosa to the invasion front of cancer cells was examined in RC specimens and defined as DOI (Fig. 2). Two values from the independent observations were averaged and considered as the DOI.

2.3. Quantification of the levels of circulating cfDNA

Before transurethral resection of the bladder tumor, blood was collected in serum-separating tubes and centrifuged at 3000*g* for 10 minutes. Serum samples were stored at −80°C until analysis. For use, initially, the serum samples were thawed gradually on ice and, then, 0.5 mL of serum per patient was subjected to cfDNA extraction using XCF™ COMPLETE Exosome and cell-free DNA Isolation Kit (System Biosciences Co, Palo Alto, CA). Circulating cfDNA levels were quantified using AccuBlue High Sensitivity dsDNA Quantitation Kit (Biotium Co software, Fremont, CA). Circulating cfDNA levels were expressed in nanograms of DNA per 1 mL of serum (in nanograms per milliliter). Serum samples obtained from patients with Ta NMIBC were used as noninvasive controls (DOI = 0).

2.4. Follow-up after RC

The follow-up schedule in this study was in accordance with the European Association of Urology Guidelines. Briefly, a computed tomography scan was performed every 3–6 months for initial 3 years, and annually thereafter. The period from RC to progression and death from BC was calculated as the time to progression-free survival (PFS) and CSS. Progression was defined as extraurinary recurrences, such as local recurrence and distant metastasis.

2.5. Statistical analysis

The Mann-Whitney and Fischer exact tests were used to examine the relationship between DOI and circulating cfDNA levels. The Kaplan-Meier analysis followed by the log-rank test was performed to examine whether DOI was associated with PFS and CSS. A subanalysis was similarly performed for the pT2-T3 and pT3-T4 groups. Multivariate logistic regression models were used to identify the independent risk factors. Statistical analyses were performed using SPSS II for Windows (version 26 J; SPSS, Inc, Chicago, IL). Figures were generated using GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, CA). A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics and the relationship with DOI

Patient characteristics are shown in Table 1. The median patient age was 72 years. The median follow-up duration was 14 months, during which progression was observed in 21 patients (51.2%), and 11 patients (26.8%) died of cancer. As the median DOI was 17 mm, we compared the clinicopathological factors between the long DOI group (>17 mm) and the short DOI group (≤17 mm), demonstrating that the former was associated with a higher pT stage, lower rate of concomitant carcinoma in situ, and positive surgical margin. Subgroup analyses were performed for pT2-T3 (24 patients) and pT3-T4 (31 patients). The median DOI was 11 and 18 mm for the pT2-T3 and pT3-T4 groups, respectively.

3.2. Association of DOI with oncological outcomes

We examined whether DOI was associated with PFS and CSS in the overall population of patients with MIBC using Kaplan-Meier survival analysis. The long DOI was significantly associated with a shorter PFS (Fig. 3A; hazard ratio [HR], 14.5; 95% confidence interval [CI], 3.9–53.97, $p < 0.0001$) and CSS (Fig. 3B; HR, 18.97; 95% CI, 4.04–88.99, $p = 0.0002$) compared with the short DOI group. Furthermore, the subgroup analysis of organ-confined MIBC (pT2 and pT3) demonstrated that the long DOI group had significantly shorter PFS (Fig. 3C; HR, 2.13; 95% CI, 0.52–8.69, $p = 0.049$), but not CSS (Fig. 3D; HR, 1.41; 95% CI = 0.19–10.29, $p = 0.74$).

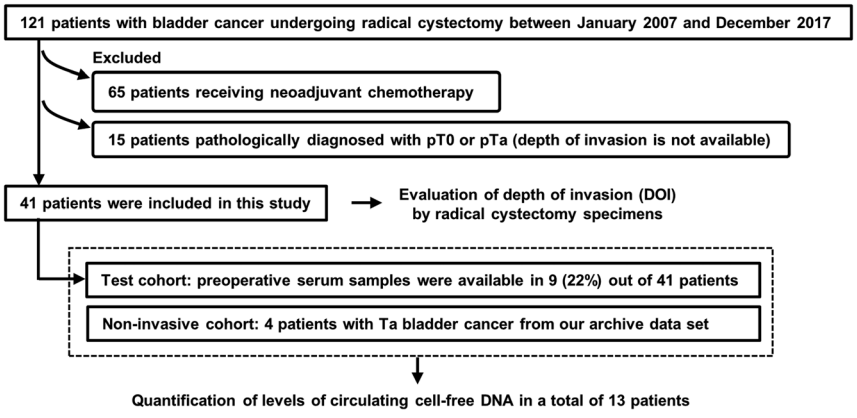

Figure 1. Flowchart of the selection process of the study population. Out of a total of 121 patients who underwent radical cystectomy between January 2007 and December 2017 at the Nara Medical University, 65 patients who received neoadjuvant chemotherapy were excluded. Of the remaining patients, 8 with pathological T0 and 7 with pathological T1 were also excluded as the depth of invasion could not be analyzed. Ultimately, a total of 41 patients were included in the analysis. Of the 41 patients, 9 patients had their levels of circulating cell-free DNA measured, and they were examined together with four pathological Ta patients as a combined control group.

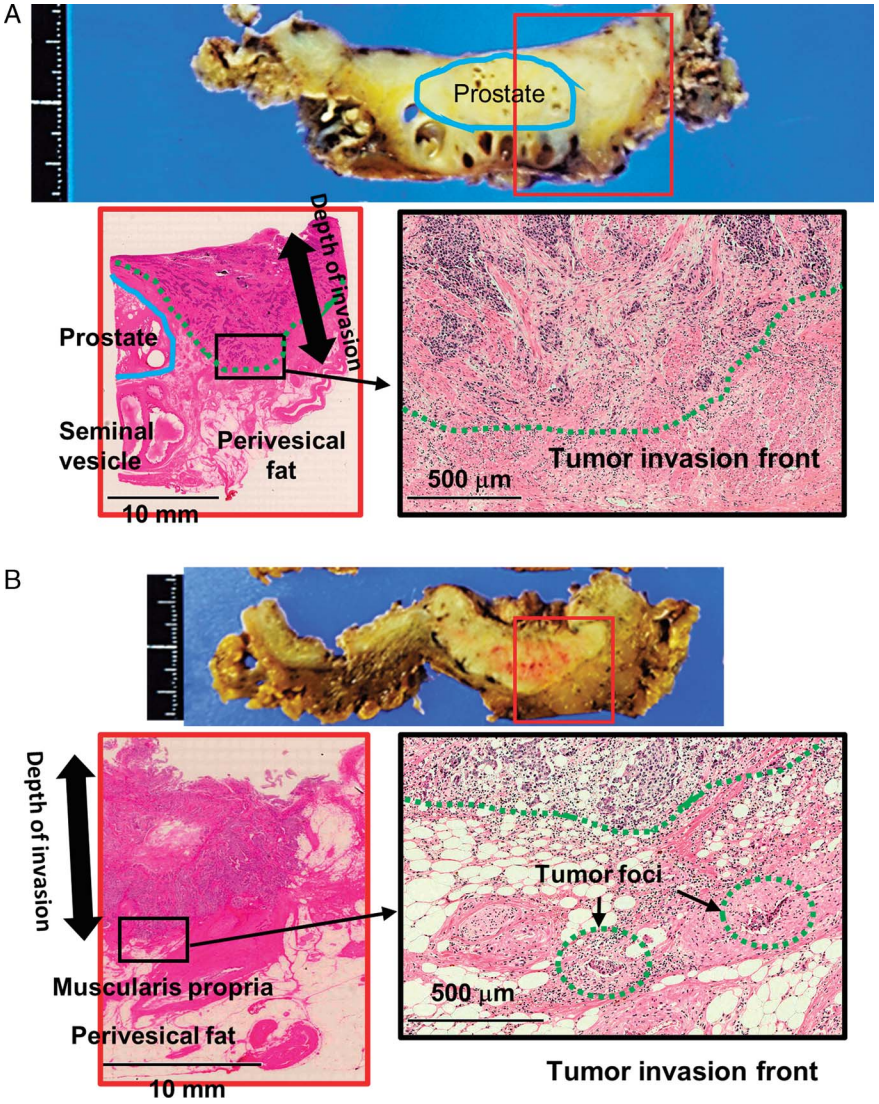

Figure 2. Representative pictures for measuring the depth of invasion. Depth of invasion was measured using the hematoxylin-eosin-stained radical cystectomy specimens. The distance from the bladder mucosa to the tumor invasion front was measured and defined as DOI. If there is a transurethral resection scar, the location of the original mucosa is predicted from the adjacent normal mucosa and measured. (A) A 73-year-old man, pT4, DOI 12 mm. (B) A 69-year-old man, pT3, DOI 15 mm. DOI = depth of invasion.

The subgroup analysis of more extensive MIBC (pT3 and pT4) demonstrated that the long DOI group had significantly shorter PFS (HR, 8.45; 95% CI, 2.57–27.79, p = 0.0004) and CSS (HR, 10.82; 95% CI, 2.48–47.22, p = 0.0015) than short DOI group did.

3.3. Multivariate analysis for CSS

Multivariate analysis of 5 factors (hemoglobin, C-reactive protein, positive surgical margin, lymph node status, adjuvant chemotherapy, and DOI) was used to identify factors that were significantly associated with CSS in BC. Depth of invasion was an independent risk factor for CSS (Table 2; HR, 8.36; 95% CI, 1.32–52.78, p = 0.024).

3.4. Relationship between the DOI and the levels of circulating cfDNA

Circulating cfDNA levels were available for 9 patients with MIBC and 4 patients with Ta NMIBC. Therefore, the relationship between DOI and circulating cfDNA levels was examined in 13 patients with BC. The mean circulating cfDNA level was 29 ng/mL. The DOI of Ta NMIBC was set to 0 mm. Circulating cfDNA levels were significantly higher in patients with long DOI than in those with short DOI (≤17 mm, 20 ng/mL vs >17 mm, 65 ng/mL; p = 0.028; Fig. 4).

4. Discussion

In the management of MIBC, many predictors of poor outcomes, such as age, sex, performance status, preoperative low hemoglobin, C-reactive protein, neutrophil-lymphocyte ratio, pathological T stage, lymph node metastasis, lymphovascular invasion, and tumor growth pattern, have been used.[14–21] In this study, we investigated the DOI in chemotherapy-naive RC specimens from 41 patients with MIBC. To the best of our knowledge, this is the first study to analyze DOI in patients with MIBC.

A previous study reported that the DOI of oral cancer is an independent predictor of CSS in multivariable analyses.[8] In BC, the National Comprehensive Cancer Network dose distinguishes between

Table 1

Clinicopathologic characteristics of the patients.

Variables	Total (n = 41)	DOI ≤ 17 mm (n = 23)	DOI > 17 mm (n = 18)	*p*
Age, median (range)	72 (52–88)	73 (60–84)	71 (52–88)	0.48*
Sex				0.74†
Male	35	20	15	
Female	6	3	3	
Intravesical chemo- and/or immunotherapy prior to RC				0.68†
No	33	18	15	
Yes	8	5	3	
Pathologic tumor stage				0.0069†
pT2	10	9	1	
pT3	14	9	5	
pT4	17	5	12	
Pathologic tumor grade				0.37†
Low	1	1	0	
High	40	22	18	
Concomitant carcinoma in situ				0.013†
Absent	31	14	17	
Present	10	9	1	
Variant histology				0.9†
Absent	30	17	13	
Present	11	6	5	
Lymphovascular invasion				0.68†
Absent	8	5	3	
Present	33	18	15	
Microvessel invasion				0.19†
Absent	11	8	3	
Present	30	15	15	
Lymph node status				0.84†
pN0	28	16	12	
pN1–3	13	7	6	
Tumor budding				0.57†
Absent	18	11	7	
Present	23	12	11	
Positive surgical margin				0.001†
Absent	34	23	11	
Present	7	0	7	
Adjuvant chemotherapy				0.77†
No	31	17	14	
Yes	10	6	4	
DOI, mm				<0.001*
Median (range)	17 (2–75)	10 (2–17)	23.5 (18–75)	
Mean (SD)	16.98 (± 12.73)	9.3 (± 3.8)	26.78 (± 13.39)	

*Man-Whitney *U* test.
†χ^2 test or Fisher exact test.
DOI = depth of invasion; RC = radical cystectomy; SD = standard deviation.

pT2a and pT2b, and it is believed that a long DOI may be associated with prognosis, although the quantification is difficult. Regarding BC, a long DOI was found to increase the risk of cancer infiltration into the surrounding organs and distant metastasis. Thus, the DOI combined with pathological T stage could help predict the outcomes of patients after RC. Subgroup analysis of the pT2-T3 and pT3-T4 groups showed the prognostic value of DOI, suggesting a potential for a postoperative risk stratification tool in clinical practice.

The presence of circulating cfDNA fragments in human blood was first discovered in 1948.[10] In 1977, a study found that compared with healthy individuals, patients with cancer had increased levels of total cfDNA in the serum, showing the potential for therapeutic evaluation.[12]

Several studies have reported higher levels of circulating cfDNA in the plasma and serum of patients with various tumor entities such as ovarian cancer,[22] breast cancer,[23] lung cancer,[24] prostate cancer,[25] and renal cell carcinoma.[26] In addition, many studies have shown that circulating cfDNA in urine has potential value in BC screening.[27] At the end of the 1980s, Stroun et al.[28] described that at least part of the circulating free DNA in the plasma of cancer patients was derived from cancer cells. In 1991, DNA-bearing TP53 mutations were found in the urinary sediments of patients with MIBC, paving the way for the use of genomics in liquid biopsies.[29] Studies based on mutated KRAS sequences in plasma have confirmed the tumor origin of mutant cfDNA.[30] Mutated genes in the plasma were subsequently proposed to represent tumor markers, and the term "circulating tumor DNA (ctDNA)" was coined. Regarding ctDNA detection in patients with BC, several studies have focused on the detection of different DNA alterations in liquid biopsy samples to identify predictive biomarkers. In this study, circulating cfDNA levels were significantly higher in patients with long DOI. This made it possible to predict DOI by measuring the levels of circulating cfDNA preoperatively, suggesting its potential in predicting prognosis in patients with MIBC. We hypothesized that

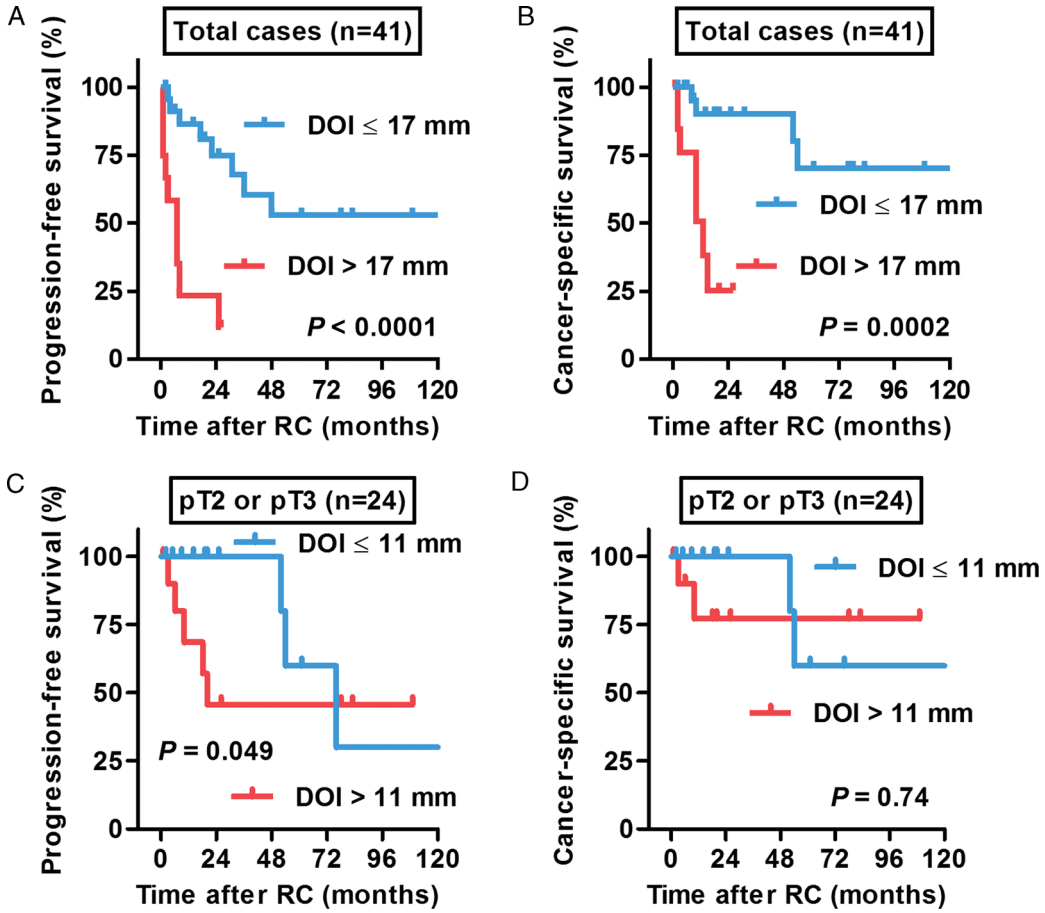

Figure 3. Progression-free survival and cancer-specific survival according to depth of invasion. Progression-free survival and CSS were estimated using the Kaplan-Meier method. Patients with long DOI (>17 mm) are significantly associated with poor PFS and CSS (A, B). In pT2, T3 patients with long DOI (>11 mm) are significantly associated with poor PFS (C, D). PFS = progression-free survival; CSS = cancer-specific survival; DOI = depth of invasion.

cancer infiltration may cause the destruction of normal cells, leading to increased cell death and a subsequent increase in circulating cfDNA levels in serum.

This study has several limitations. First, this was a retrospective analysis, thus, a potential selection bias is inevitable. Second, blood samples of only few patients were available. Therefore, it was not possible to investigate the relationship between circulating cfDNA levels and patient prognosis. However, this study suggests that it could be used to predict prognosis by demonstrating the relationship between circulating cfDNA levels and DOI.

Table 2

Multivariate analysis for cancer-specific survival.

Variables	HR	95% CI	*p*
Hb < 12, g/dL	1.45	0.15–3.24	0.64
CRP > 0.5, mg/dL	3.71	0.61–22.37	0.15
Positive surgical margin	0.7	0.11–4.65	0.72
Lymph node status	1.19	0.52–2.74	0.68
Adjuvant chemotherapy	0.52	0.11–2.42	0.41
DOI > 17 (mm)	8.36	1.32–52.78	0.024

Hb = hemoglobin; CI = confidence interval; CRP = C-reactive protein; DOI = depth of invasion; HR = hazard Ratio.

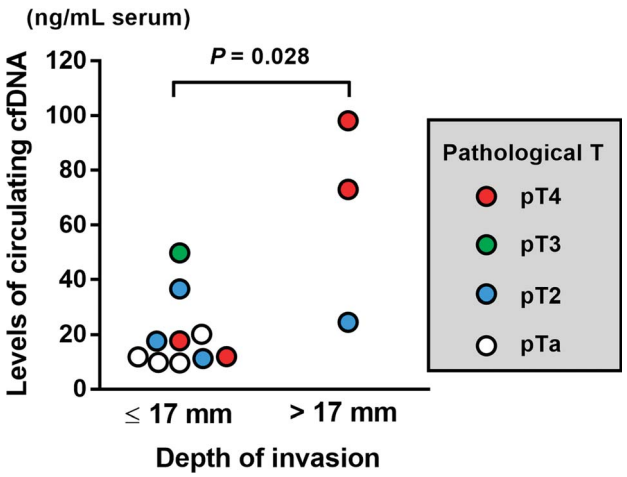

Figure 4. Relationship between levels of circulating cfDNA and DOI. The Mann–-Whitney test was used to examine the relationship between DOI and circulating cfDNA levels. Levels of circulating cell-free DNA are significantly higher in patients with long DOI than in those with short DOI. cfDNA = cell-free DNA; DOI = depth of invasion.

Third, this case group had an extremely poor prognosis because neoadjuvant chemotherapy was not administered. Therefore, there may have been a slight difference from the current clinical case group. Lastly, we were only able to evaluate the levels of circulating cfDNA in this study. Measuring levels of circulating cfDNA concentration is clinically simple and can be easily performed; however, it has not been qualitatively evaluated and has limitations.

This study proved that patients with a long DOI had a poor outcome and showed the possibility of predicting it by measuring the preoperative levels of circulating cfDNA. Therefore, it is necessary to consider additional treatments, such as adjuvant chemotherapy, for patients with long DOI. In addition, when the preoperative levels of circulating cfDNA of the patients are high, the prognosis is predicted to be poor; therefore, it can be considered as an indicator for neoadjuvant chemotherapy.

5. Conclusions

Depth of invasion is a useful prognostic predictor and may be predicted by measuring preoperative levels of circulating cfDNA in the serum. To the best of our knowledge, this is the first report to show an association between DOI and prognosis in patients with MIBC after RC.

Acknowledgments

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Statement of ethics

This study was approved by the Nara Medical University Ethics Committee (reference ID: 1256). Written informed consent was obtained from all the participants. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Author contributions

YI, MM, KF: Made the conceptualization of this study and wrote the original manuscript;
YI, SF, TF, SO, YM, YN, KT, NT: Contributed to the acquisition of patients' data;
YI, SH: Performed the statistical analysis;
KF: Performed the critical review of the original draft.
All authors read and approved the final version of manuscript.

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