Research Article

Intracytoplasmic Lumen in Urine Cytology Predicts Worse Prognosis in Non–muscle-Invasive Bladder Cancers

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Short Title: ICL predicts worse prognosis in non–muscle-invasive bladder cancer

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Abstract (280 words)

Background: Intracytoplasmic lumina (ICL) are observed in several cancers, including urothelial carcinoma. We have reported that ICL in urine cytology (cICL) is more frequent in high-grade urothelial carcinomas than in low-grade urothelial carcinomas; however, the correlation between the presence of ICL and prognosis is unclear.

Objectives: To determine the association between cICL and prognosis in bladder cancer

Method: We investigated retrospectively 87 patients with bladder cancer who received a histological diagnosis within 3 months of urine cytology at Kanazawa Medical University between 2003 and 2007. Cytological diagnosis and the number of cICL, histological diagnosis, tumor grade or variant, pT stage, ICL in histological specimens, and immunohistochemistry for mucins were evaluated. Data on treatment type, recurrence, survival, cause of death, and length of follow-up were collected from electronic medical records.

Results: Muscle invasion, high-grade urothelial carcinoma, lymph node metastasis, distant metastasis, adjuvant therapy, and disease-related mortality were more frequent in patients with cICL-positive bladder cancer than in those without cICL-positive bladder cancer. Immunohistochemistry revealed the expression of Muc-1 and Muc-4 in patients with cICL-positive bladder cancer. Univariate analysis revealed that cytological diagnosis by the Paris system and the 2015 version of the Japanese reporting system, muscle invasion, high-grade urothelial carcinoma, lymph node metastasis, distant metastasis, and adjuvant chemotherapy and/or radiotherapy were significant factors associated with prognosis. Furthermore, survival was shorter in patients with cICL-positive non–muscle-invasive bladder cancer than in those with cICL-negative non–muscle-invasive bladder cancer. In the multivariate analysis, only distant metastasis was significantly associated with survival.

Conclusions: cICL predicted shorter survival in patients with non–muscle-invasive bladder cancer, suggesting that ICL is one of the important diagnostic features of high-grade urothelial carcinoma with worse prognosis in urine cytology.

Introduction

Urothelial carcinoma (UC) of the bladder is characterized by frequent recurrence, and high-grade noninvasive papillary UCs occasionally progress to invasive UCs. Urine cytology is a basic and noninvasive diagnostic procedure for follow-up of patients with bladder cancer. One of the diagnostic issues in urine cytology is the vague diagnostic criterion that is based on the Papanicolaou classification. The Paris system (TPS) for reporting urinary cytology, established in 2016, and the Japan reporting system (JRS) for urinary cytology, established in 2015, comprise criteria with improved objectivity [1,2].

Intracytoplasmic lumina (ICL), a well-known feature in invasive lobular carcinoma of the breast, signet-ring cell carcinoma of the stomach, among others, are sometimes observed in UC. The presence of ICL in UC was previously reported in studies utilizing cytology and/or histology [3–7]. At the ultrastructural level, the ICL contain coarsely granular material and occasionally has microvilli in the lumen [3,4]. Immunohistochemically, ICL were rarely reported to comprise secretory components [3]. Importantly, we previously reported that the ICL in urine cytology (cICL) were more frequent in high-grade UCs (HGUCs) than in low-grade UCs (LGUCs) [8]; however, the correlation between the presence of cICL and prognosis in UC has not been reported to date. In the current study, we present the results of our clinicopathological analysis for the cICL and clarify its association with the prognosis of bladder cancer.

Materials and Methods

The current study included 87 patients with bladder cancer who were treated at Kanazawa Medical University between 2003 and 2007, received histological diagnosis within 3 months after urine cytology, and were followed up for more than 2 months. Patients who died as a result of other cancers were excluded from the study. Clinical data, such as treatment type, recurrence, survival, cause of death, and length of follow-up, were collected from the electronic medical records.

To assess urine cytology specimens, approximately 10 mL of bladder washings or voided urine samples were centrifuged at 1500 rpm for 5 minutes, and the sediments were fixed in 2% carbowax with 70% ethanol. The cells were collected and dried, followed by the Papanicolaou staining. Only decolorized cytological slides for long-term storage were restained with the Papanicolaou stain as follows: the cover glasses were removed by xylene, and the slides were immersed in 100% ethanol for 5 minutes. After placing in running water for 1 minute, the slides were placed in 1% hydrochloride with 70% ethanol for 1 minute for complete decolorization, washed in running water for 10 minutes, incubated in 70% ethanol for 1 minute, and stained by the Papanicolaou stain.

The cytological diagnosis and the presence and the number of cICL were evaluated and reviewed by three cytologists (TT, MT, and SM) and one pathologist (SN). We reviewed all the cytological slides individually, discussed cases involving disagreements while reviewing them under the microscope and ultimately reached an agreement and adjudication with reference to the histology. The cytological diagnosis was evaluated by three systems: the Papanicolaou classification, the TPS [1] and the JRS [2]. The Papanicolaou classification was used at the time of therapy for all patients, and the TPS and the JRS were used for the retrospective review of the cases.

The definition of cICL in the current study was the presence of ICL with mucin in viable urothelial cancer cells (Fig. 1a and 1b). Mucin in ICL were visible as eosinophilic dot-like material in vacuoles in the HGUC by Papanicolaou staining. Positive mucin staining was observed in the cICL on slides restained with Alcian blue (pH 2.5) (Fig. 1c) and periodic acid-Schiff (Fig. 1d). cICL were also observed on the slides restained with Papanicolaou staining (Fig. 1e), which were fainter than those observed in the nonrestained slides but nonetheless allowed evaluation. Degenerated vacuoles or cICL without mucin were excluded because the cICL without mucin could not be differentiated effectively from the degenerated vacuoles. The cICL compressed the nucleus to the periphery, and the cells with the cICL resembled signet-ring cells. The cutoff for the number of cICL was two cICL per high-power field at 40× magnification. Receiver-operating characteristic curve analysis was used to determine the cutoff value for the number of cICL that predicted survival.

The histological diagnosis of the transurethral bladder tumor resection (TUR) or cystectomy specimens, including histological grades or variants and the pT stage based on the WHO classification of tumors of the urothelial tract and TNM classification of carcinomas of the urinary bladder [9], were reviewed. Additionally, the number of ICL in histological specimens (hICL) was counted in the slides stained with hematoxylin/eosin (Fig. 1f), Alcian blue (pH 2.5) (data not shown), periodic acid–Schiff (data not shown), and mucicarmine (Fig. 1g). The number of hICL was counted in the mucicarmine-stain slides.

For immunohistochemistry, formalin-fixed and paraffin-embedded tissue sections were used. The mouse monoclonal antibodies against Muc-1 (Ma695, Leica Biosystems, NCL-MUC-1, New Castle, UK), Muc-2 (Ccp58, Leica Biosystems, NCL-MUC-2), Muc-4 (8G7, Santa Cruz Biotechnology, sc-53945, Texas, USA), and Muc-5AC (CLH2, Leica Biosystems, NCL-MUC-5AC) were used. Immunohistochemistry was performed using an automated immunostainer (Leica Biosystems, Bond-MAX, Nussloch, Germany). Immunohistochemical staining for the mucins was categorized into positive and negative in the hICL and into one of the following five groups in the tumor: negative, few, focal, most, and diffuse.

All statistical analyses were performed using the EZR software program on R commander (version 1.37) [10]. Fisher's exact test was performed to evaluate the association between cICL and variables except age and the length of follow-up. The Mann-Whitney *U* test was used to assess the association between cICL and age and the duration of follow-up. The univariate log-rank test was performed to evaluate the association between survival and specific variables, and the Kaplan–Meier curves were constructed to compare disease-specific survival between muscle-invasive and non–muscle-invasive bladder cancer with and without cICL. Variables with p values < 0.2 in the univariate log-rank test were included in the subsequent Cox multivariate analysis. Adjuvant therapy was excluded from the multivariate analysis because it evident depended on the TNM classification. P values < 0.05 were considered to indicate statistical significance.

Results

Clinical features

In the study cohort of 87 patients with bladder cancer, the median age at the time of histological diagnosis was 71 (range, 46–90) years. There were 21 (24%) female patients and 66 (76%) male patients. TUR and cystectomy were performed in 80 (92%) and 7 (8%) patients, respectively. The median duration of follow-up after the diagnosis was 6.3 (range, 0.2–15.6) years. Regarding adjuvant therapy after surgery, intravesical infusion therapy and systematic chemotherapy and/or radiotherapy were administered in 43 (49%) and 27 (31%) patients, respectively.

The comparison of the clinical characteristics between patients with bladder cancer with and without cICL is summarized in Table 1. The analysis using Fisher's exact test revealed that age at diagnosis, sex, type of surgery, and local recurrence were not significantly different between the two groups. cICL were found in 1 patient with upper tract lesions (1/9, 11 %) and in 19 with local recurrent disease (19/59, 32 %). However, patients with cICL-positive bladder cancer had significantly shorter follow-up duration after diagnosis (p = 0.004); more frequent lymph node metastasis (p = 0.035), distant metastasis (p = 0.028), and systemic chemotherapy and/or radiotherapy (p < 0.001); and a higher rate of death from bladder cancer (p = 0.045) compared with patients with cICL-negative bladder cancer.

Cytological features

cICL were found in bladder washings from 20 bladder cancer patients (20/68, 29 %) and voided urine from 3 patients (3/19, 16%), and this difference was not statistically significant (Fisher test). Based on the Papanicolaou classification, the cICL-positive cases were seen in class II (1/12, 8%), class III (3/23, 13%), class IV (4/15, 27%), and class V (15/31, 48%). Conversely, the cICL were only found in the HGUC and malignancy categories (23/64, 36%) of the TPS and the JRS, respectively. The three cytological classifications were significantly different between the cICL-positive and cICL-negative groups by Fisher's exact tests (p = 0.009, p = 0.004, and p = 0.002 for the Papanicolaou classification, the TPS, and the JRS, respectively). Five cases with suspicious LGUC were cytologically classified as

class II–IV in the Papanicolaou classification, low-grade urothelial neoplasia in the TPS, and atypical cells or suspicious for malignancy in the JRS.

Histological features

Histologically, the cohort included 45 (52%) noninvasive papillary UCs (low grade, n = 25; high grade, n = 20), 9 (10%) urothelial carcinomas *in situ*, and 33 (38%) invasive UCs (high grade, n = 21; with squamous differentiation, n = 6; with glandular differentiation, n = 1; with plasmacytoid variant, n = 2; giant cell variant, n = 2; lymphoepithelioma-like variant, n = 1). There were 76 (87%) non–muscle-invasive bladder cancers (pTis, pTa, and pT1) and 11 (13%) muscle-invasive bladder cancers (pT2–pT4).

The histopathological characteristics comparing bladder cancer patients with and without cICL are summarized in Table 2. By Fisher's exact test, histological classification, histological grade, hICL, pT stage, and muscle invasion were significantly different between the cICL-positive and the cICL-negative groups. There were 7 (13%) patients with cICL-positive bladder cancer among the total of 54 patients with noninvasive bladder cancer, whereas there were 16 (48%) patients with cICL-positive bladder cancer among the total of 33 patients with invasive bladder cancer. In the group of patients with noninvasive bladder cancer, the cICL were more frequent among those with UC *in situ* (3/9, 33%) than those with noninvasive papillary UC (4/45, 9%). In addition, the cICL were more frequent among those with HGUC (22/62, 35%) than those with LGUC (1/25, 4%). Finally, the cICL were more frequent among patients with muscle-invasive tumors (7/11, 64%) than those with non–muscle-invasive tumors (16/76, 21%). Among the 16 patients with ICL-positive non–muscle-invasive bladder cancer, there were 9 (56%), 3 (19%), 3 (19%), and 1 (6%) patient with IUC, UCIS, NIPUC-HG, and NIPUC-LG, respectively; follow-up after diagnosis revealed local recurrence in 12 (75%) patients; muscle-invasive bladder cancer in 5 (31 %) patients; and distant metastasis such as liver, lung, and bone in 4 (25%) patients, whereas 4 (25%) patients, including 3 patients with IUC and 1 patient with NIPUC-HG, died due to bladder cancer.

Immunohistochemistry for mucins

Thirty-one cases were available for additional immunohistochemical analyzes to determine the expression of mucins, which revealed that Muc-1, Muc-2, Muc-4, and Muc-5AC were expressed in tumor cells in 27 (87%), 11 (35%), 19 (61%), and 11 (35%) cases, respectively. The most/diffuse expression levels for Muc-1, Muc-2, Muc-4, and Muc-5AC were observed in 10 (32%), 1 (3%), 6 (19%), and 1(3%) case, respectively. Furthermore, the bladder cancers with cICL or hICL frequently expressed Muc-1 (Fig. 1h) and Muc-4 (Fig. 1i). The mucin in hICL was positive for Muc-1 and Muc-4 in 17 (89%) and 3 (18%) cases, respectively.

Univariate and multivariate analyses for prognosis

The univariate log-rank analysis (Table 3) revealed that cICL ($p = 0.003$), cytological diagnosis by the TPS and the JRS ($p = 0.012$), muscle invasion ($p < 0.001$), lymph node metastasis ($p < 0.001$), distant metastasis ($p < 0.001$), and adjuvant chemotherapy and/or radiotherapy ($p < 0.001$) were significant clinicopathological risk factors for the prognosis of bladder cancer. The Kaplan–Meier curves for survival of the bladder cancer patients with and without cICL and those with and without muscle invasion are shown in Fig. 2. While there was no significant difference in the survival between patients with and without cICL among those with muscle-invasive bladder cancer, there was a significant difference in the survival between patients with and without cICL among those with non–muscle-invasive bladder cancer ($p = 0.009$). The five-year survival rates were 96% and 69% in patients with cICL-negative non–muscle-invasive cancer and those with cICL-positive non–muscle-invasive cancer, respectively. And furthermore, the analysis of patients with non–muscle-invasive HGUC revealed that the 5-year survival rates were 93% and 66% for the ICL-negative and the ICL-positive patients, respectively, which were not significantly different ($p = 0.062$). By the multivariate Cox analysis (Table 4), only distant metastasis exhibited a significant association with prognosis; cICL did not exhibit a significant association with prognosis in the study cohort ($p = 0.258$).

Discussion

The current study elucidating the association of ICL with disease characteristics in patients with bladder cancer revealed that advanced clinical stage, systematic chemotherapy and/or radiotherapy, shorter follow-up duration after diagnosis, lymph node and distant metastases, and death from cancer were more frequent among patients with cICL. Additionally, the univariate log-rank test determined that cICL was a predictor of shorter prognosis in patients with non–muscle-invasive bladder cancer but not in patients with muscle-invasive bladder cancer.

The frequencies of cICL in patients with HGUC and LGUC (35% and 4%, respectively) in the current study were lower than those reported in our previous study (44% and 11.1% in HGUC and LGUC, respectively) [8]. This difference might be due to the evaluation of the specimens by only one cytologist in the previous study [8], whereas three cytologists determined the number of cICL using the common definition for cICL in the current study. Additionally, multiple samples from the same patient were included in the previous report, which might partially explain the discrepancy between the two studies [8].

The frequencies of patients with high pT stage, high histological grade, invasive cancer, and muscle invasion were higher among those with ICL in the current study. Although there was no difference in the prognosis of patients with and without ICL among those with muscle-invasive bladder cancer by the log-rank test, the prognosis was worse in those with ICL-positive bladder cancer than those without ICL-positive bladder cancer among those with non–muscle-invasive bladder cancer. Among patients with non–muscle-invasive HGUC, ICL-positive cancer tended to be associated with poor prognosis, which should be analyzed further in future studies. ICL in UCs might be a cytological feature suggesting a more aggressive tumor phenotype, such as that reported in breast cancer with signet ring cell differentiation[11] or signet-ring cell carcinoma in the stomach[12]. The findings of the current study suggest that bladder cancers with cICL should be followed closely and/or might be candidates for adjuvant therapy after surgical treatment.

The immunohistochemical assessment for mucins revealed that the hICL were positive for Muc-1 and Muc-4. Kaur *et al*. reported that the expression levels of Muc-1 and Muc-4 were significantly higher in bladder cancers that metastasized compared with the localized bladder cancers; however, the authors did not comment on the ICL [13]. Therefore, further investigation is necessary to elucidate the association between mucins and ICL.

We analyzed the clinicopathological features of the patients based on the presence of hICL, which did not reveal any significant associations (data not shown). The hICL were found not only on the surface of tumors but also in deeper, invasive regions. The presence of ICL in invasive regions might not be associated with prognosis in tumors that have already invaded the surrounding tissue. However, the presence of ICL on the surface might affect the prognosis and predict poor prognosis, which remain unclear. Urine cytology appears to be a better and more convenient method to evaluate the presence of ICL compared with the assessment of histological specimens for non–muscle-invasive bladder cancer.

In conclusion, cICL predicted shorter survival in patients with non–muscle-invasive bladder cancer, suggesting that cICL might be an important diagnostic marker that can be utilized to determine HGUC with worse prognosis in clinical practice.

Statements

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Statement of Ethics

This study has been approved by the Research Ethics Committee of Kanazawa Medical University (H190). Formal consent is not required for this kind of study.

Disclosure Statement

The authors have no conflicts of interest to declare.

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None.

Author Contributions

TT and SN designed this study, searched the published work, collected and interpreted data, and prepared the report. SN was responsible for data interpretation and statistical analysis. TT, MT and SM contributed cytological data collection. YO and YT contributed immunohistochemical analysis. KM contributed collection and interpretation of clinical data. MK contributed assistance of design and interpretation of data. AS, XG, and NK contributed data interpretation and statistical analysis. SY was involved in the data collection and interpretation.

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Figure Legends

Figure 1. Intracytoplasmic lumina (ICL) in urothelial carcinoma cells

a. Urine cytology of a representative case. Two ICL (arrows) are observed in the clusters of urothelial carcinoma cells (Papanicolaou stain; original magnification, 20×). b. Urine cytology of a representative case. ICL (arrow) is observed in the urothelial carcinoma cells. Note that the ICL contains light-green mucin (Papanicolaou stain; original magnification, 100×). c–d. Urine cytology of restained images from a representative case. The ICL in high-grade urothelial carcinoma cells are positive for mucin as indicated by Alcian blue (pH 2.5 stain; c; original magnification, 100×) and periodic acid–Schiff (d; original magnification, 100×). e. The nuclear chromatin signal is fainter than in the original image; however, the ICL (arrow) can be observed in the urothelial carcinoma cell. Note that the ICL contains light-green mucin (Papanicolaou stain; original magnification, 100×).

f. Histology of a representative case of noninvasive papillary urothelial carcinoma, high grade. ICL (arrow) is observed in the superficial layer of the tumor (hematoxylin/eosin stain, original magnification 10×). g. Histology of a noninvasive papillary urothelial carcinoma, high grade. ICL (arrows) are positive for mucicarmine (original magnification, 20×). h–i. Immunohistochemistry for Muc-1 (h; original magnification, 20×) and Muc-4 (i; original magnification, 20×) of a representative case of noninvasive papillary urothelial carcinoma, high grade. ICL (arrows) are Muc-1-positive and Muc-4-negative, whereas carcinoma cells exhibit focal positivity for both Muc-1 and Muc-4.

Figure 2. Kaplan–Meier curves for survival in bladder cancer comparing ICL in urine cytology (cICL) and muscle invasiveness

Non–muscle-invasive bladder cancer with cICL is associated with significantly shorter survival than non–muscle-invasive bladder cancer without cICL ($p = 0.009$), although cICL is not associated with survival in muscle-invasive bladder cancer.

Table 1

The comparison of the clinical characteristics between patients with bladder cancer with and without cICL

Variable	cICL		*p* value
	Positive (n = 23)	Negative (n = 64)	
Median age at diagnosis in years (range)	76 (49-89)	69 (46-90)	0.157
Sex			1
Female	5	16	
Male	18	48	
Type of surgery			0.07
TUR	19	61	
Cystectomy	4	3	
Adjuvant therapy			<0.001*
None	1	16	
Intravesical infusion therapy only	6	37	
Systematic chemotherapy and/or radiotherapy	16	11	
Median follow-up duration after diagnosis in years (range)	1.9 (0.3-14.1)	9.3 (0.2-15.6)	0.004*
Local recurrence	19	40	0.801
Lymph node metastasis	7	6	0.035*
Distant metastasis	8	8	0.028*
Death from bladder cancer	7	7	0.045*

cICL: Intracytoplasmic lumina in urine cytology, TUR: transurethral bladder tumor resection. Regarding to *p* values, age and follow-up duration were analyzed in Mann-Whitney *U* test, and sex, type of surgery, adjuvant therapy, local recurrence, lymph node metastasis, metastasis, and death from bladder cancer were analyzed in Fisher's exact test. Asterisk (*) indicates statistical significance.

Table 2

The histological characteristics comparing bladder cancer patients with and without cICL

Variable		cICL		*p* value
		Positive (n = 23)	Negative (n = 64)	
Histological classification				0.002*
NIPUC	Low grade	1	24	
	High grade	3	17	
UCIS		3	6	
IUC	High grade	11	10	
	With squamous differentiation	3	3	
	With glandular differentiation	0	1	
	Plasmacytoid variant	1	1	
	Giant cell variant	1	1	
	Lymphoepithelioma like variant	0	1	
Histological grade				0.002*
	Low grade	1	24	
	High grade	22	40	
hICL				< 0.001*
	Positive	19	19	
	Negative	4	45	
pT stage				< 0.001*
	pTis	3	6	
	pTa	4	41	
	pT1	9	13	
	pT2	6	3	
	pT3	1	0	
	pT4	0	1	
Muscle invasion				0.006*
	Non–muscle-invasive	16	60	
	Muscle-invasive	7	4	

cICL: ICL in urine cytology, NIPUC: non-invasive papillary urothelial carcinoma, UCIS: urothelial carcinoma in situ, IUC: invasive urothelial carcinoma, hICL: ICL in histological specimens.

Asterisk (*) indicates statistical significance by Fisher's exact test.

Table 3. The univariate analysis of risk factors for prognosis

Variable		Death from bladder cancer	Alive	*p* value
Sex	Male	12	54	0.629
	Female	2	19	
Age	<69 y	7	29	0.768
	≥69 y	7	44	
cICL	Negative	7	57	0.003*
	Positive	7	16	
Cytological classification: The Papanicolaou classification	Class I - III	6	35	0.629
	Class IV - V	8	38	
Cytological classifications: TPS / JRS	HGUC / Malignancy	14	50	0.012*
	Others	0	23	
Muscle invasion	Non–muscle-invasive	8	68	< 0.001*
	Muscle-invasive	6	5	
Histological grade	Low grade	0	25	0.010*
	High grade	14	48	
hICL	Negative	5	44	0.057
	Positive	9	29	
Local recurrence	Negative	2	27	0.324
	Positive	12	46	
Lymph node metastasis	Negative	6	68	< 0.001*
	Positive	8	5	
Distant metastasis	Negative	1	70	< 0.001*
	Positive	13	3	
Adjuvant chemotherapy and/or radiotherapy	Negative	2	60	< 0.001*
	Positive	12	14	

cICL: ICL in urine cytology, TPS: the Paris system, JRS: the Japanese Reporting system, HGUC: high grade urothelial carcinoma, hICL: ICL in histological specimens.

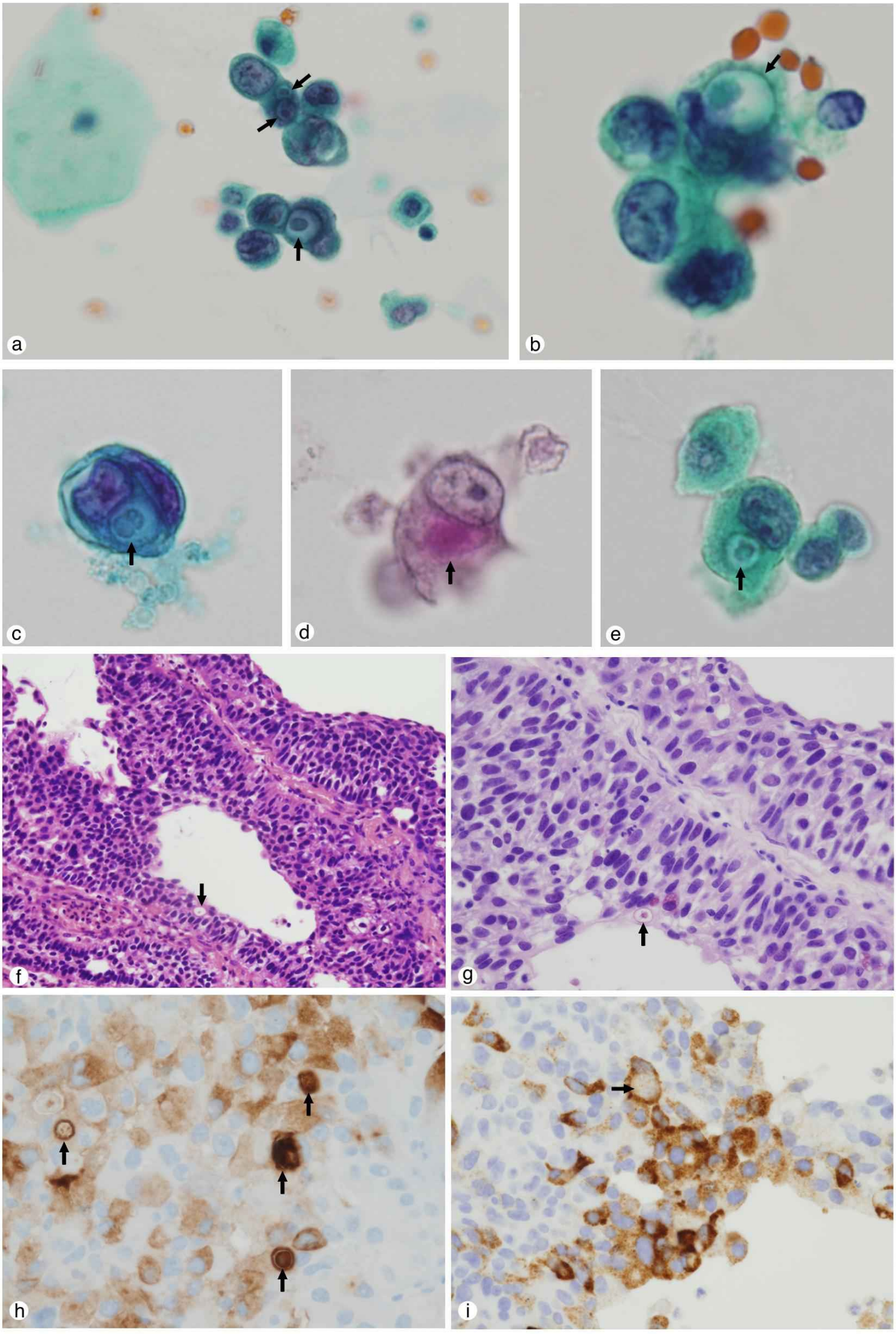
Asterisk (*) indicates statistical significance.

Table 4. The multivariate analysis of risk factors for prognosis

Variables	Hazard ratio	95% CI	P value
cICL	2.26	0.55 – 9.30	0.258
HGUC/ Malignancy by cytological classifications, TPS / JRS	1.95×10^7	0 – INF	0.999
Muscle invasion	2.23	0.55 – 8.99	0.258
High grade variants	2.13×10^7	0 – INF	0.999
Lymph nodes metastasis	2.08	0.61 - 7.07	0.240
Distant metastasis	37.7	4.44 - 320	< 0.001*

cICL: ICL in urine cytology, HGUC: high grade urothelial carcinoma, TPS: the Paris system, JRS: the Japanese reporting system.

Asterisk (*) indicates statistical significance.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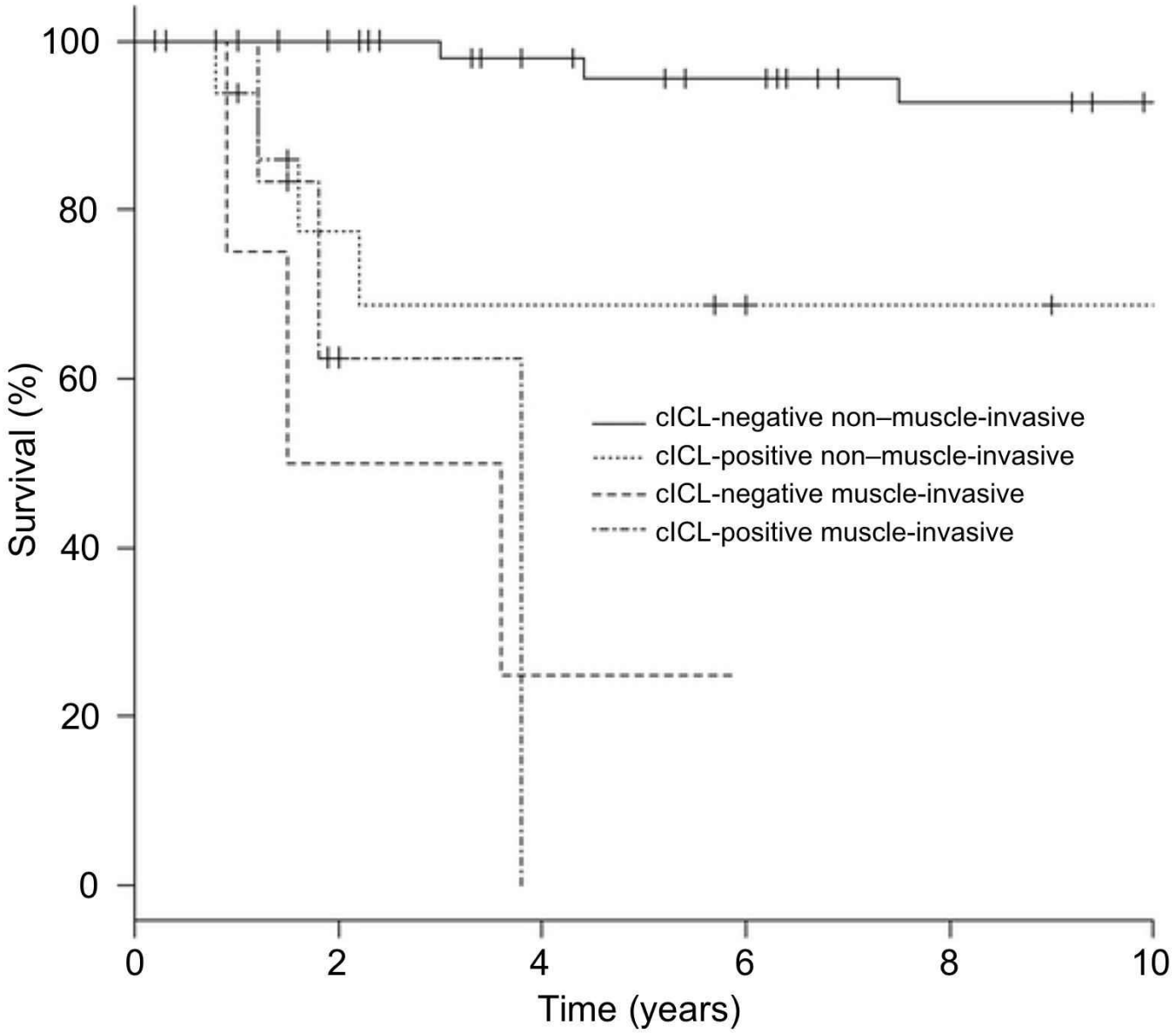
100
80
60
40
20
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Survival (%)
0
2
4
6
8
10
Time (years)
cICL-negative non–muscle-invasive
cICL-positive non–muscle-invasive
cICL-negative muscle-invasive
cICL-positive muscle-invasive