A novel role of serum cytochrome c as a tumor marker in patients with operable cancer

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Abstract

Purpose This study aimed to evaluate serum cytochrome c (cyto-c) levels as a novel role of tumor marker in patients with operable malignant tumors.

Methods Serum cyto-c levels and lactate dehydrogenase (LD) activity were measured in a total of 257 cases (232 malignant and 25 benign). To identify the relationship between serum cyto-c and current tumor markers, 6 variables, such as gender, age, invasion, lymph node metastasis, distant metastasis, and LD, were analyzed by uni- and multivariate regression analysis methods. The test performance of serum cyto-c for the prediction of malignant behavior was evaluated by receiver operating characteristic (ROC) curves.

Results The serum cyto-c level was significantly higher in patients with malignant tumors than patients with benign tumors (20.6 ng/mL versus 15.5 ng/mL; $P = 0.017$, Mann-Whitney *U* test). No difference in the levels among subtypes of cancer was found, indicating that the change in serum cyto-c levels reflect cancer individually and not specific subtypes of cancer. The survival in patients with serum cyto-c levels over 40 ng/mL was poor (Kaplan-Meier test, $p < 0.0001$, Hazard ratio 16.76, 95% confidential interval 4.45-63.04). Multiple linear regression analyses disclosed the close association of serum cyto-c levels with invasion ($P = 0.0004$), metastasis ($P = 0.0262$) except for regional lymph node metastasis, and activity of serum LD ($P < 0.0001$), all of which are well known to represent malignant behavior. Conversely, the measurement of serum cyto-c was verified to have excellent diagnostic accuracy of 0.802 and 0.781 for the detection of invasion and metastasis (the area under curves of the constructed ROCs).

Conclusion Serum cyto-c is a potent tumor marker as a predictor for malignant potential in cancers.

Keywords Cytochrome c・Prediction・Tumor marker・Metastatic potential

Introduction

Circulating tumor markers are used to define a particular disease entity in clinical settings for diagnosis, staging, and monitoring. Tumor markers are usually proteins released from dying tumor cells or elaborated by neoplastic cells. There are two subcategories of the proteins, specific and non-specific. Tumor specific proteins are expressed only in tumor cells, such as chimeric fusion proteins associated with malignant processes, leading to the development of a malignant clone. These protein markers are very useful for the detection and diagnosis of specific malignant tumors. On the other hand, non-specific proteins or markers related to malignant cells are onco-fetal or carcinogenic antigens, such as carcinoembryonic antigen (CEA), alphafetoprotein, prostate specific antigen (PSA), CA15.3, and CA19-9, all of which are still useful for screening, monitoring, staging, and prognosis, although their diagnostic specificity is not especially high. Recently, besides these classical tumor markers, cell specific proteins over-expressed in malignant cells have become available because differentiated wild-typed cells normally express these markers at a subtle and constant level but the corresponding tumor cells express it at a high level. For example, soluble IL-2 receptors, VEGF, and so on, are closely associated with the cell cycle, apoptosis, signal transduction, angiogenesis, and invasion (Holdenrieder and Stieber 2004).

Cancer is characterized by cell division and cell death. However, there are no appropriate cell death markers available for use in a clinical setting, although the activity of serum LD is employed as a universal death marker. Recently, cytokeratine 18, nucleosomes, fragmented DNA, and cyto-c in serum have been discussed as novel markers for the evaluation of apoptotic death (Beachy and Repasky 2008).

It has been reported that cytochrome c (cyto-c) is released into the culture medium after activation of the intrinsic apoptotic caspase pathway in vitro and some patients with hematological malignancies have high levels of serum cyto-c. Barczyk et al. (2005) also showed that the change in serum cyto-c levels is useful for monitoring of

therapeutic responsiveness and prognostic prediction upto 3 years later. Therefore, this study addressed the potential use of serum cyto-c as a surrogate marker alternative to pathological assessment in patients with operable malignant tumors.

Patients and Methods

Serum specimens

Serum specimens and clinical information were collected under the regulations of the Ethical Board criteria defined by the Japanese Association of Laboratory Medicine. A total of 257 patients (232 malignant and 25 benign) was enrolled in this study. Serum cyto-c and LD were measured in all cases, and CEA and CA-19-9 were measured in 28 cases of gastric cancer and in 37 cases of colorectal cancer (CRC). All sera were stored at -40℃ until measurement. Pretreatment serum cyto-c levels were measured using serum samples collected from individuals diagnosed as having lung, gastro-intestinal, colorectal, breast, uro-genital, ovaro-uterine cancers, and benign tumors. Benign tumors consisted of tubular adenoma of the colon, serous cystadenoma, thymoma, severe dysplasia of the uterus, moderate dysplasia of the urinary bladder, and prostatic hyperplasia. Almost all patients were operable status but 9 were inoperable. No patient was treated preoperatively with either chemotherapy or radiation therapy. The median follow-up period was 6 months, ranging from 1 to 19 months. Of 249 cases available for histological examination, invasion and metastasis were evaluated according to the tumor-node-metastasis (TNM) staging system. The presence of distant metastasis was detected by image analysis.

Cytochrome c quantification

Human cyto-c was quantified using an in-house electrochemiluminescence immunoassay method. Twenty µL of serum sample diluted 1:11 with 200 µL of reaction buffer was incubated with 3 x 10^7 micro magnetic beads (Invitrogen, Carlsbad, USA)

coated with anti-cyto-c monoclonal antibody for 9 min at 30°C. After the beads were washed two times with washing buffer, 200 μL of another anti-cyto-c monoclonal antibody coupled with ruthenium(II) Tris (bipyridyl)-N-hydroxysuccinimide ester [Ru(bpy)32+] (BioVeris, Gaithersburg, USA) was added to the beads and incubated for 9 min at 30°C. After the beads were washed three times with washing buffer, the beads were conducted into the electrode and the photons (wavelength, 620 nm) emitted from the Ru(bpy) 32+ coupled to the anti-cyto-c monoclonal antibody were counted with a photo-multiplier tube. To quantify the cyto-c present in the serum sample, purified human cyto-c (R & D Systems, Minneapolis, USA) as a standard antigen was assayed in parallel. The above ECLIA procedures were carried out with an automatic ECLIA analyzer (Picolumi 8220; Sanko Junyaku, Tokyo, Japan). Then, serum LD was measured by the International Federation of Clinical Chemistry (IFCC)-recommended method. CEA and CA19-9 were measured based on chemiluminescence enzyme immunoassay technology using commercially available kits, LUMIPULUSEpicorna-CEA and PULUSE-picorna-CA-19-9 (SRL Inc, Tokyo, Japan)

Statistical analysis

Differences in the distribution of cyto-c with clinical and pathological feautures were used for univariate and multivariate analysis. Mann-Whitney *U* test and Kruskal-Wallis *H* test were used for univariate analysis. To compare the abilities of tumor markers to distinguish patients with r/o metastasis and invasion, receiver operating characteristic (ROC) curves were constructured. In addition, areas under the ROC curves (AUCs) with 95% confidence intervals (CI) were calculated for cyto-c, LD, CEA, and CA19-9. The optimal cut-off value for cyto-c was determined by Youden's index. Survival rates were calculated by the Kaplan-Meier method, and were compared with log-rank test. For all analyses, a two-tailed *P* value of < 0.05 was considered statistically significant. Statistical analyses were performed using "StatFlex Ver.5.0" software.

Results

Distribution of serum cyto-c levels

The basic patient characteristics of enrolled patients are summarized in Table 1. Of a total of 257 cases, the median cyto-c levels were 20.6 ng/mL (IQR;16.2-29.2, range 7.2-629.3) in 232 patients with malignant tumors and and 15.5 ng/mL (IQR; 11.8-19.4, range 8.5-52.5) in 25 patients with benign tumors, respectively (P = 0.002, Mann-Whitney U test). Individual data were displayed as plots of the median and 5^{th}, 25^{th}, 75^{th}, and 95^{th} percentiles of the preoperative cyto-c levels in each group, as shown in Figure 1. No difference in each cyto-c level was observed among the 10 subtypes of malignant tumor (P = 0.362, Kruskal-Wallis H test), revealing that serum cyto-c was not specific for tumor types. Diagnostic sensitivity and specificity to discriminate malignant from benign tumors were 55.6% and 76.0% when the cut-off was 19.4 ng/mL based on the maximization of Youden's index.

Twelve patients died during this study period, and 6 of them had cyto-c levels over 100ng/mL, which indicates the potent prognostic value of cyto-c. Survival curves were compared among patient groups with different serum cyto-c levels, < 40, 40–99, and > 100 ng/mL. The Kaplan-Meier curve revealed that survival was significantly poorer in patients with the serum cyto-c level over 40 ng/mL ($p < 0.0001$, Hazard ratio 16.76, (95% confidential interval 4.45-63.04) (Figure 2)

Performance of cyto-c in predicting the presence of invasion and metastasis

The results are shown in Table 2. The cyto-c level was statistically associated with invasion ($P < 0.0001$), metastasis ($P < 0.001$), and high LD activity ($p < 0.0001$, Mann-Whitney U test). Multiple linear regression analysis also showed significant association of the elevation of serum cyto-c levels with invasion (P = 0.0004), metastasis (P = 0.0262), and LD ($P < 0.0001$). The performance characteristics of serum cyto-c as a laboratory test were analyzed using the ROC curve for two predictive

factors (metastasis and invasion) in comparison with LD, CEA, and CA19-9. The preoperative ROC-AUC of cyto-c was higher than those of the other markers, as shown in Figure 3 (AUC for metastasis = 0.781 (95% CI = 0.711-0.852) and AUC for invasion = 0.802 (95%CI = 0.730-0.873)). Based on the maximization of Youden's index, the optimal cut-off values for cyto-c in preoperative patients with cancer were 22.7 ng/mL (sensitivity, 81.6%; specificity, 68.9%) for metastasis, and 22.3 ng/mL (sensitivity, 86.5%; specificity, 66.9%) for invasion.

Validation of cyto-c for subtypes of tumor

To examine the usefulness of cyto-c in respective cancer types, the same analyses were conducted for 31 patients with gastric cancer and 44 patients with CRC. In gastric cancers, as shown in Table 3, cyto-c at the preoperative stage was significantly associated with advanced stage ($P < 0.001$), the presence of distance metastasis ($P = 0.009$), and progression ($P = 0.031$), CEA, CA19-9 and LD did not show statistically significant associations. Moreover, the ROC-AUC for advanced stages was 0.913 for cyto-c, 0.677 for CEA, 0.792 for CA19-9, and 0.507 for LD.

Next, for patients with CRC, as shown in Table 4, the test performance characteristics of cyto-c as a tumor marker were equivalent to both CEA and LD, but higher than CA19-9.

Discussion

This study presents for the first time the possibility of the use of serum cyto-c as a surrogate marker alternative to pathological assessment in patients with operable malignant tumors. The present study clarified that cyto-c levels at the preoperative stage vary widely regardless of the different subtypes of cancer and stratification by serum cyto-c level can predict advanced status. Using uni- and multivariable analyses, this study also found that the elevation of serum cyto-c was closely associated with

serum LD activity and the presence of invasion and metastasis equivalent to the advanced status classified according to the TNM classification. Preoperative serum cyto-c levels were highly predictable for invasion and metastasis in patients diagnosed as having operable cancers. The test performance of cyto-c by ROC-AUC analysis was superior to those of the current tumor makers, LD, CEA, and CA19-9.

Increased serum cyto-c has already been reported by *in vivo* and *in vitro* studies in hematological malignancies (Barczyk et al. 2005) and benign disorders involving systemic cell death (Hosoya et al. 2005). However, little is known about serum cyto-c associated with malignant solid tumors. The present findings are noteworthy to understand the origin of serum cyto-c in solid tumors and its usefulness as a tumor marker in patients with operable tumors. In addition, a strong association was evident between serum cyto-c and serum LD as well as the presence of invasion and distant metastasis. First of all, in terms of the origin of serum cyto-c, a previous study (Osaka et al. 2008) showed that hematological cell lines undergoing apoptosis by TRAIL can release cyto-c and LD into the culture medium, suggesting that both cyto-c and LD are mainly derived from dying tumor cells, and LD is employed as a universal death marker. Actually, on the origin of serum cyto-c, recent studies provide a significant suggestion of a close relationship between apoptosis and tumorigenesis and especially concerning the grade of malignancy of individual tumors common to any subtype of cancer. For example, the growth of malignant tumors was shown to be under an imbalanced homeostasis of cell proliferation and cell death by apoptosis/necrosis (Harris 2002). Manjo et al. (1995) reported that cells in the center of solid tumors mainly die via necrosis and cells at the margins are preferentially eliminated by apoptosis. Such cell death was documented to be spontaneous apoptosis by TUNEL methods and to have a role in the development and progression of cancer. Such spontaneous apoptosis is also reported to be involved in mitochondrial cyto-c release and the activation of caspase-3 (Meggiato et al. 2000). Thus, it was concluded that higher levels of apoptosis reflect

higher grade of malignancy, which results in elevated serum cyto-c and becomes a negative prognostic index. Referring to these current concepts, the present results appear to be reasonable and valuable. Firstly, the elevation is demonstrable in only one-third of patients in any subtype of cancer, indicating that the elevation is not a common feature in cancers and results from individual cancer behavior, such as malignancy. On the other hand, serum cyto-c levels were independent of the size of the primary tumor and lymph node metastasis but dependent upon invasion and distant metastasis. This may represent only one facet of malignancy, such as a tumor type predisposed to metastasis being characterized by high apoptosis and high density of micro-vascularity (Tanaka et al. 2003). Secondly, the elevation of 40ng/ml or more at the preoperative time predicted poor prognosis, and the elevation of cyto-c levels was closely associated with invasion and metastasis. All of these findings support the close association of cyto-c with the biological property of high grade malignancy in tumors.

The diagnostic sensitivity of cyto-c for the presence of cancers was low, 55.6%, so that it is unlikely to be recommended for screening of cancers. However, for preoperative patients who have a cancer, the ROC-AUCs of cyto-c for invasion and metastasis were superior, 0.802 to 0.781, compared to those of LD, CA19-9, and CEA. The diagnostic sensitivities of cyto-c for invasion and metastasis in patients with operable tumor were 81.6% and 86.5%, respectively. These values are probably tolerable for the clinical setting as a new tumor marker. To our knowledge, this is the first report that cyto-c can predict malignant behavior.

This unique feature of cyto-c as a tumor marker was confirmed in patients with gastric cancer and CRC. Of not, the cyto-c levels were associated with the advanced stage classified by the presence or absence of distant metastasis and progression according to the TNM classification system. In particular, the ROC-AUC of cyto-c for metastasis in gastric cancer was high, 0.913. On the other hand, in patients with CRC, the test performance characteristics of cyto-c as a tumor marker were equivalent to

both CEA and LD but higher than CA19-9.

The present study demonstrated that cyto-c is useful as a biomarker for pathological assessment involved in invasion and metastasis in patients with operable cancers. However, it must be repeated that cyto-c is useful as a tumor marker only in patients bearing cancer because serum cyto-c is also elevated in patients with non-malignant diseases, such as hepatitis, myocardial infarction (Alleyne et al. 2001), systemic inflammatory response syndrome (Adachi et al. 2004), and influenza-associated encephalopathy (Hosoya et al. 2006). Accordingly, to further validate the usefulness of cyto-c as a a novel tumor biomarker, we need a large-scale prospective study.

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Figure 1

Serum specimens from 257 patients were evaluated for cyto-c levels. Box and whisker plots of median (horizontal line) and 5%, 25%, 75%, and 95% percentiles of serum cyto-c levels in malignant and benign tumors, **a:** (number) Lung cancer, **b:** Gastric cancer, **c:** Colorectal cancer, **d:** Hepatobilliary and pancreatic cancer, **e:** Breast cancer, **f:** Ovaro-uterine cancer, **g:**uro-genital cancer, **h:** Skin cancer, **i:** Other malignant tumor, **j**: Benign tumor. The medians of malignant tumors and benign tumors were 20.6 ng/mL and 15.5 ng/mL (P = 0.020, Mann-Whitney U test). There were no significant differences in malignant tumor types (P = 0.362, Kruskal-Wallis H test).

Figure 2

Kaplan-Meier overall survival curves based on serum cytochrome c level in 232 patients with malignant tumors (** p < 0.0001, * P = 0.001; Wilcoxon's test). The Hazard ratio was 16.76 (95% Confidence interval 4.45-63.04; P < 0.0001 (Serum cyto-c (≧ 40 versus < 40 ng/mL)).

Figure 3

Receiver operating characteristics (ROC) curves for the prediction of metastasis and invasion in patients with malignant diseases. Area under the ROC curves (AUCs) showed higher predictive accuracy for cyto-c levels than LD, CEA, or CA19-9.

Table 1. Comparison of clinical characteristics and concentrations of serum cyto-c in each cancer category

	N	Age (mean)	M:F	LN	M	I	Concentration of cytochrome c in serum	
							Median	Range
Lung cancer	40	67.0	24:16	7	6	5	20.3	10.4-161.4
Gastric cancer	32	62.4	18:14	8	5	3	22.6	10.7-629.3
Colorectal cancer	43	69.1	27:16	8	9	9	20.7	7.2-135.0
Hepatobilliary and pancreatic cancer	25	65.0	17:8	3	10	5	22.8	12.3-116.0
Breast cancer	10	60.9	0:10	3	0	2	17.9	11.3-36.4
Ovaro-uterine cancer	23	57.2	0:23	1	7	5	22.3	11.0-189.5
Uro-genital cancer	27	63.5	22:5	0	4	2	20.7	11.5-179.6
Skin cancer	17	70.9	5:12	4	4	0	18.7	7.2-51.6
Other	15	63.1	9:6	6	3	6	17.4	10.8-220.4
Benign tumor	25	65.1	9:16	0	0	0	15.5	7.2-2107.6

LN; Lymph node metastasis, M; Other metastasis (intracanalicular , distant and disseminated), I; Invasion (extra organ extension)
Invasion was not available in 30 patients. Lymph node status was not available in17 patients. Distant metastasis was not available in 17 patients.

Table 2. Association of preoperative serum cytochrome c levels with clinical and pathological feautures of 232 patients with malignant diseases

		No (%)	Median serum cytochrome c level, ng/mL (Range 10-90%)	*P*	
				Univariate[1]	Multivariate[2]
Age (mean)	> 65	123 (53.0)	19.2 (12.3-42.3)	0.3955	0.6375
	≤ 65	109 (47.0)	22.7 (13.7-66.3)		
Gender	M	122 (47.4)	22.6 (12.3-52.7)	0.2031	0.5843
	F	110 (52.6)	19.3 (13.2-42.7)		
Invasion¶	Positive	37 (15.9)	29.3 (19.9-52.0)	< 0.0001*	0.0004*
	Negative	165 (71.1)	18.7 (12.3-36.0)		
Metastatic lymph node†	Positive	40 (17.2)	19.7 (11.2-29.8)	0.5745	0.3080
	Negative	175 (75.4)	20.7 (13.1-51.1)		
Metastasis‡	Positive	48 (20.7)	31.4 (16.6-72.1)	< 0.0001*	0.0262*
	Negative	167 (72.0)	18.8 (12.2-33.1)		
LD (median) §	> 170	117 (50.0)	24.3 (13.1-47.2)	< 0.0001*	< 0.0001*
	≤ 170	114 (49.1)	18.8 (12.0-31.5)		
Total (%)		232 (100)	20.6 (12.6-50.3)		

* Statistically significant.
¶ Invasion was not available in 30 patients.
† Lymph node status was not available in 17 patients.
‡ Metastasis was not available in 17 patients.
§ LD was not available in 1 patient

Table 3. Serum cytochrome c in gastric cancer

		N	Cytochrome c (ng/mL)		CEA (ng/mL)		CA19-9 (U/mL)		LD (IU/L)	
			Median	*P*	Median	*P*	Median	*P*	Median	*P*
Gender				0.095		0.487		0.515		0.324
	M	16	26.1		3		8		152	
	F	15	19.1		1.6		10		170	
Age				0.144		0.307		0.093		0.345
	≧70	9	19.1		1.5		13		161	
	<70	22	23.9		2.5		8		162	
Stage				<0.001*		0.173		0.296		0.385
	Ⅰ-Ⅱ	21	18.8		1.6		8.5		154	
	Ⅲ-Ⅳ	10	35.0		3.4		14		181	
Lymph metastasis				0.274		0.097		0.183		0.626
	N0	19	19.1		1.3		8		151	
	N1	11	25.6		2.8		12		165	
Distance metastasis				0.009*		0.257		0.063		0.902
	M0	25	19.1		1.6		8.5		162	
	M1	6	40.6		3.4		33		159	
Progression				0.031*		0.337		0.878		0.256
	T1, T2	24	19.1		1.6		9.5		158	
	T3, T4	5	29.3		3.6		8		125	

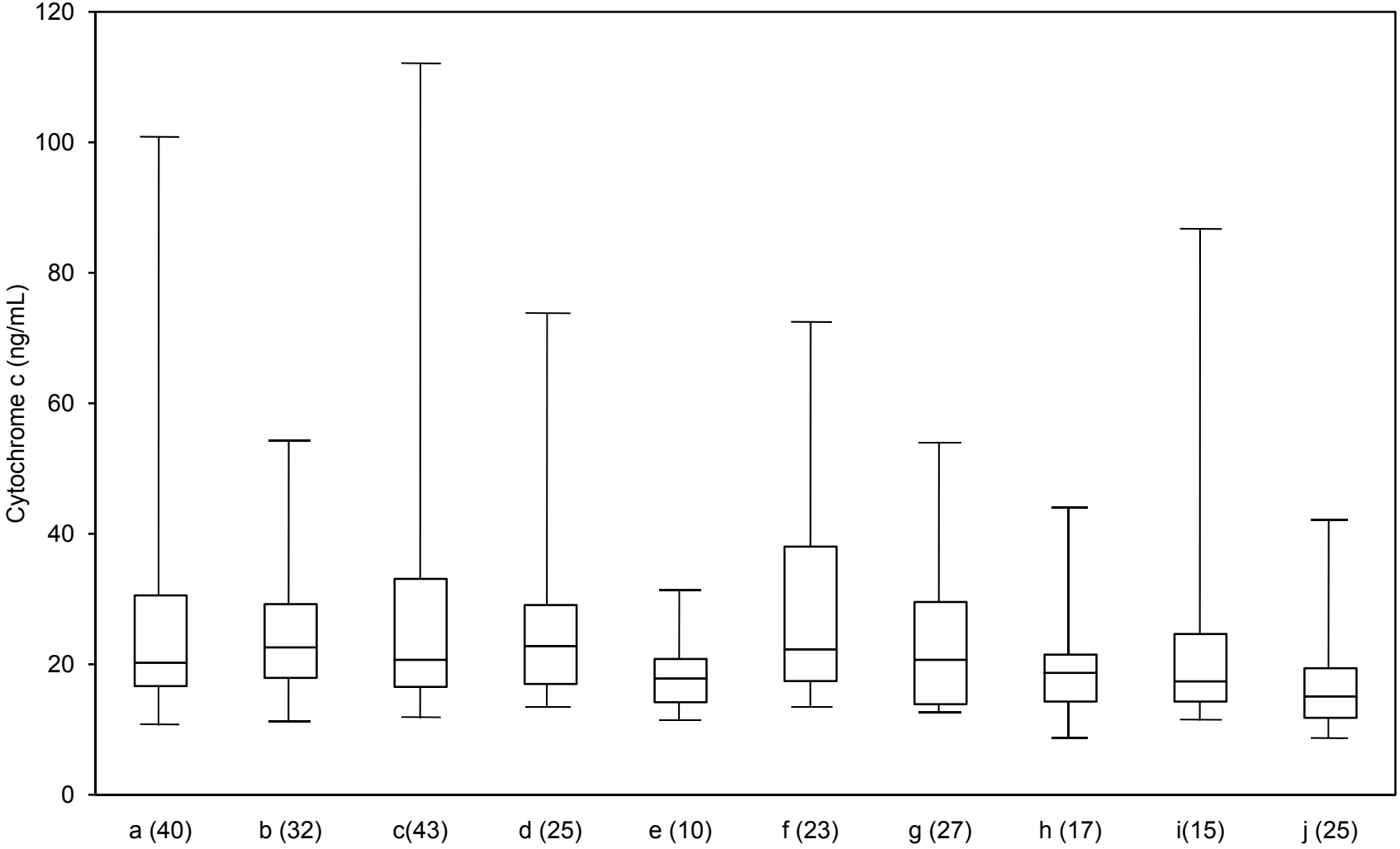
* Statistically significant.

Table 4. Serum cytochrome c in colorectal cancer

	N	Cytochrome c (ng/mL)		CEA (ng/mL)		CA19-9 (U/mL)		LD (IU/L)	
		Median	*P*	Median	*P*	Median	*P*	Median	*P*
Gender			0.757		0.825		0.152		0.052
M	27	20.7		3.2		11.5		158	
F	17	24.8		2.7		14		183	
Age			0.027*		0.617		0.375		0.669
≧70	22	19.1		3.1		13		172	
<70	22	26.8		3		11.5		167	
Stage			0.008*		0.002*		0.200		0.011*
O-Ⅱ	25	18.8		2.7		11		158	
Ⅲ-	19	30.6		13.6		13		197	
Distance metastasis			0.005*		0.009*		0.596		0.003*
M0	26	18.4		2.7		10.5		155	
M1	18	29.7		10.3		11		216	
Lymph metastasis			0.120		0.050*		0.493		0.117
N0	30	19.5		2.7		11		165	
N1	12	26.9		6.7		11		194	
Progression			0.022*		0.004**		0.289		0.021*
T1, T2	22	18.6		2.6		9.0		155	
T3, T4	18	33.1		10.9		11.5		187	

* Statistically significant.

Figure 1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100
80
60
40
20
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Cytochrome c (ng/mL)
a (40)
b (32)
c(43)
d (25)
e (10)
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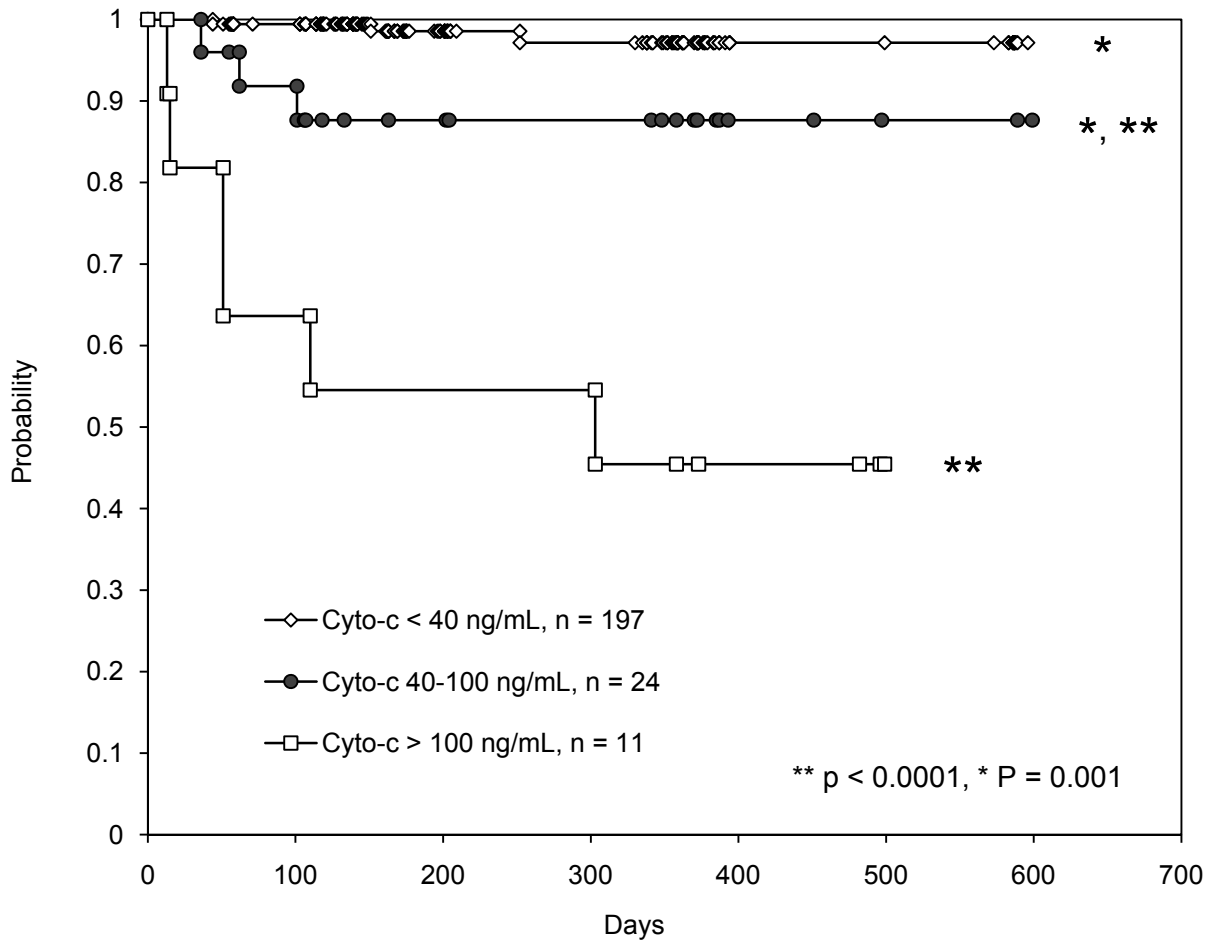
g (27)
h (17)
i(15)
j (25)

Figure 2


Probability
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0.8
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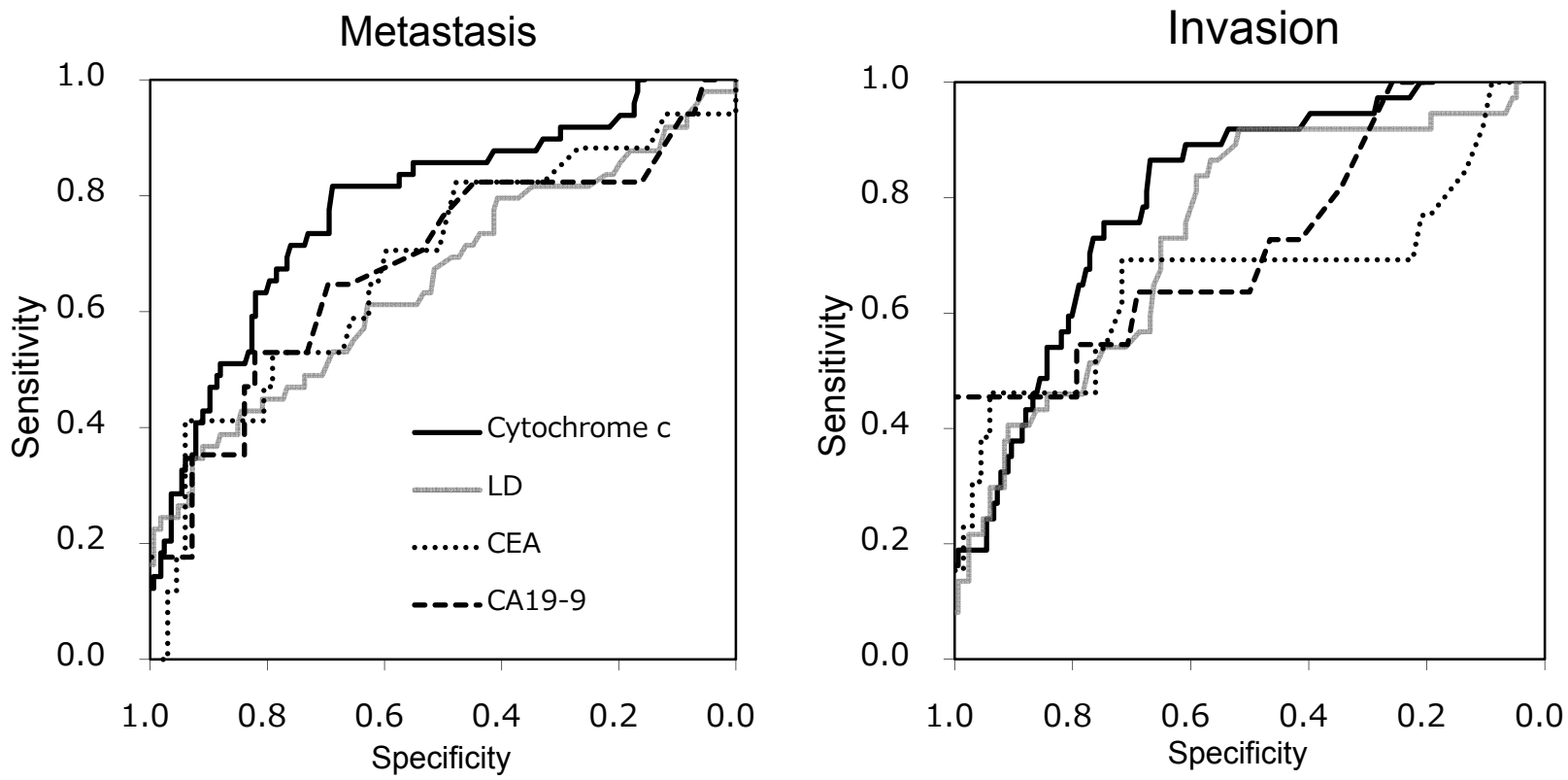
**
Cyto-c < 40 ng/mL, n = 197
Cyto-c 40-100 ng/mL, n = 24
Cyto-c > 100 ng/mL, n = 11
** p < 0.0001, * P = 0.001

Figure 3



	Area under curves (95% CI)	
	Metastasis	Invasion
Cytochrome c	0.781 (0.711-0.852)	0.802 (0.730-0.873)
LD	0.651 (0.563-0.740)	0.738 (0.654-0.822)
CEA	0.676 (0.550-0.802)	0.660 (0.528-0.793)
CA19-9	0.671 (0.508-0.834)	0.710 (0.532-0.888)