Significance of *ELF3* mRNA Expression for Detection of Lymph Node Metastases of Colorectal Cancer

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Abstract. *Background: Lymph node (LN) evaluation is an important factor for the prognosis of colorectal cancer (CRC). The purpose of our study was to investigate the effectiveness of E74-like factor 3 (ELF3) and carcinoembryonic antigen (CEA) as useful markers to detect LN metastases in CRC. Materials and Methods: We examined the mRNA expression of ELF3 and CEA in LNs and tissues from 22 patients with CRC and in controls with ulcerative colitis (UC) by real-time quantitative reverse transcription polymerase chain reaction, as well as by hematoxylin–eosin staining. Results: ELF3 and CEA expression showed statistically significant differences among four LN groups: LNs from patients with CRC categorized into three Dukes' stages and LNs from patients with UC ($p<0.001$ and $p<0.001$, respectively). We found a statistical correlation between the expression levels of both markers in patients with CRC compared with each Dukes' stage. Conclusion: ELF3, as a gene marker, may be sufficiently practical to detect LN metastases of CRC, rather than CEA.*

Lymph node (LN) evaluation is an important factor for the prognosis of colorectal cancer (CRC). LN metastases might cause recurrence of CRC, and are related to prognosis and survival (1). Carcinoembryonic antigen (CEA) was first described as a gastrointestinal oncofetal antigen, and is now known to be overexpressed in most carcinomas (2). CEA is generally used for the detection of LN metastases of CRC (3, 4). Several studies have reported that *CEA* mRNA quantification by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) is a reliable method for the detection of metastases of CRC (5, 6).

Our study focused on E74-like factor 3 (ELF3) (also called as ESE-1, ESX, ERT, and jen), which was first described in breast cancer cells and has been used to detect LN metastases in breast cancer (7, 8). ELF3 is an epithelium-specific E-twenty six (ETS) transcription factors, a family of procceses consisting of approximately 30 members related to each other by a conserved DNA-binding domain (DBD) (9, 10). ETS factors exhibit altered expression in colon cancer, by which they regulate pathways that are relevant to tumor progression (11). The *ELF3* gene is localized on human chromosome 1q32.1-2. It contains nine exons that encode a 371-amino acid protein (9, 12). Recently, the structure and function of ELF3 was described; ELF3 contains a helix-loop-helix motif that consists of three α-helices, four β-sheets, and a turn that connects helices 2 and 3; the third helix is a DNA recognition helix (10, 13). mRNA expression of *ELF3* is limited to epithelial cells and is involved in tumorigenesis (14). ELF3 controls the intestinal epithelial differentiation during development by regulation of the expression of transforming growth factor β receptor type II (TGFβR II), which behaves as a tumor suppressor, in epithelial cells (11). ELF3 activates the TGFβR II promoter and regulates TGFβR II, which is related to extracellular matrix remodeling and tumorigenesis (10). The $ELF3^{-/-}$ embryonic phenotype is associated with diminished

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Key Words: ELF3/ESE-1/ESX/ERT/jen, lymph node metastasis, colorectal cancer, real-time quantitative PCR.

Table I. *Primer sequences and polymerase chain reaction (PCR) conditions.*

Primer		Sequence	Length[a]	Annealing temperature
ELF3	F	5'-CTCATGCCAGGCACTGTGCTA -3'	120	61˚C
	R	5'-GAATCAAGGCACACCTGTGGAA -3'		
CEA	F	5'-CATCATGATTGGAGTGCTGGTTG-3'	115	60˚C
	R	5'-GCTGTTGCAAATGCTTTAAGGAAGA-3'		
GAPDH	F	5'-GCACCGTCAAGGCTGAGAAC-3'	138	61˚C
	R	5'-TGGTGAAGACGCCAGTGGA-3'		

F, Forward; R, reverse; [a]expected product size (bp).

epithelial expression of TGFβR II, and lack of TGFβR II leads to impaired enterocyte and goblet cell differentiation (15). A previous report has shown that *ELF3* is expressed in colonic mucosa, but not in hematopoietic cells and peripheral blood lymphocytes (14). In addition, it has been reported that *ELF3* is expressed in normal colonic mucosa and carcinoma, but not in normal LNs (16). For these reasons, we investigated whether *ELF3* compared to *CEA* could be used as a biomarker for detecting LN metastases of CRC by using real-time quantitative RT-PCR (qRT-PCR).

Materials and Methods

Patients. Twenty-two specimens of tumor tissues, 19 specimens of non-tumor tissues and 123 LNs were dissected from 22 patients with CRC. Eleven specimens of inflammatory tissues and 11 LNs, serving as controls, were dissected from 11 patients undergoing surgery for ulcerative colitis (UC). Eight patients with CRC were enrolled and the excision of 34 LNs was carried out in the Department of Surgery, Kansai Rosai Hospital between April and July 2001. Eighty-nine LNs and all tissue specimens were obtained from surgical resection performed at the Department of Surgery, Hyogo Collage of Medicine between September 2009 and March 2010. The study design was approved by the Ethics Review Committee on Genetic and Genomic Research, Kobe University Graduate School of Medicine.

Tissue preparation. Each LN was cut into halves under sterile conditions to prevent RNA cross-contamination between specimens. One half of the node was fixed in 10% buffered formalin and embedded in paraffin for hematoxylin–eosin staining (HES). The other half was stored in RNA Later™ solution (Ambion, Austin, TX, USA) at –20˚C until RNA extraction.

RNA extraction and cDNA synthesis. Total cellular RNA was extracted from LNs and tissues using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Purified RNA was quantified and assessed for purity by UV spectrophotometry. To eliminate genomic DNA, RNA samples were optimized using DNase I (Deoxyribonuclease I Amplification Grade, Invitrogen) before RT-PCR.

cDNA was synthesized using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The reaction mixture containing 1 μg RNA was incubated at 37˚C for 15 min and at 98˚C for 5 min, and was then immediately frozen.

Real-time qRT-PCR. One microliter of cDNA was used as the template in real-time qRT-PCR amplification with newly designed primers for *ELF3* (GenBank Acc: NM_004433), *CEA* (GenBank Acc: NM_004363) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (GenBank Acc: NM_002046), as shown in Table I. *GAPDH* was used as housekeeping gene to calculate the relative level of expression of each gene (7, 16). qRT-PCR was performed in a MyiQ real-time PCR system (Bio-Rad, Hercules, CA, USA) using the SsoFast EvaGreen Supermix (Bio-Rad), according to the manufacturer's recommendation. The protocol was as follows: initial denaturation at 95˚C for 30 s, 40 cycles of amplification; denaturation at 95˚C for 5 s; annealing at the temperature suitable for each gene marker for 10 s, and extension at 72˚C for 10 s. Each sample was assayed in duplicate. A control and two references were included in every run to confirm each examination.

Histological examination. Sections of formalin-fixed, paraffin-embedded LNs were examined by HE staining at the Department of Surgical Pathology, Hyogo College of Medicine. All LNs from patients with CRC were categorized into Dukes' stages. Extramural cancer deposits (EX) are defined as cancer foci which are not adjacent to the primary tumor and not associated with LNs (17).

Statistical analysis. Statistical analysis was performed with PASW for Windows version 17.0 (SPSS Japan Inc., Tokyo, Japan). To set cut-off values for each gene marker, receiver operating characteristic (ROC) curve analysis was performed by plotting the true-positive fraction (sensitivity) and false-positive fraction (specificity) pairs with area under the curve (AUC) values for LNs, dichotomized according to the presence of CRC metastasis diagnosed with HE staining (18, 19). Data were evaluated using the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test with Bonferroni correction for multiple groups. Analyses of correlations between levels of different mRNA species were performed using a two-tailed Spearman rank correlation test. Differences were considered statistically significant at $p<0.05$.

Results

The clinicopathological characteristics of patients with CRC are shown in Table II, including location, histological grade, depth of invasion, and status of pathological metastasis in LNs. According to Dukes' staging, patients were categorized into three groups: A (n=4), B (n=9) and C (n=9). Almost all cases had lymphatic invasion and/or venous invasion regardless of

Table II. *Clinical and pathological characteristics of patients with colorectal cancer.*

Case	Location	Dukes' stage	Histology	Depth	Histological LN metastasis	EX
1	R	A	tub1	sm	–	–
2	S	A	tub1	sm	–	–
3	R	A	tub1	mp	–	–
4	S	A	tub2	mp	–	–
5	R	B	tub1	ss	–	–
6	R	B	tub1	ss	–	–
7	D	B	tub2	ss	–	–
8	A	B	poor1	ss	–	–
9	A	B	tub1	ss	–	–
10	A	B	poor1	ss	–	–
11	S	B	tub2	ss	–	–
12	A	B	tub2	ss	–	–
13	A	B	muc	se	–	+
14	S	C	tub1	mp	–	–
15	D	C	tub2	ss	–	–
16	S	C	tub2	ss	–	–
17	T	C	tub2	ss	+	–
18	D	C	poor1	ss	+	+
19	D	C	tub2	ss	+	–
20	Rb	C	tub2	mp	+	–
21	R	C	tub2	ss	+	+
22	S	C	tub1	se	+	–

D, Descending colon; A, ascending colon; R, rectum; Rb, rectum below peritoneal reflection; S, sigmoid colon; T, transverse colon. tub1, Well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; poor1, poorly differentiated solid adenocarcinoma; muc, mucinous adenocarcinoma. sm, Submucosa; mp, muscularis propria; ss, subserosa; se, serosa-exposed. EX, Extramural cancer deposits without lymph node structure.

LN metastasis. Routine HE staining diagnosis of LNs revealed metastasis in 6 (27.2%) out of 22 patients, lymphatic invasion in 16 (72.7%), and venous invasion in 20 (90.9%). In almost all cases, invasion reached the subserosa. EX were detected in four patients. Case 13 was EX-positive diagnosed with metastasis-negative LNs on conventional pathological staging.

qRT-PCR was performed to quantify *ELF3*, *CEA* and *GAPDH* in tumor tissues (n=22), non-tumor tissues from patients with CRC (n=19), and inflammatory tissues from patients with UC, serving as controls (n=11). The results are shown in Figure 1. Relative mRNA expression of *ELF3* did not exhibit any significant differences among these tissues: tumor tissues, mean=5033.42; non-tumor tissues, mean= 6037.6; and inflammatory tissues, mean=1723.6. mRNA expression of *CEA* was found significantly differing among these tissues ($p<0.05$, Kruskal–Wallis test): tumor tissues, mean=127264.8; non-tumor tissues, mean=256710.1; and inflammatory tissues, mean=11712.5. Subsequent Mann–Whitney *U*-tests with Bonferroni correction showed that *CEA* expression was significantly higher in non-tumor tissues than in inflammatory tissues ($p<0.05$).

ROC analysis was performed using relative expression of LNs from patients with CRC, according to LN metastases diagnosed with *HES*, to set the best cut-off values in qRT-PCR. The cut-off values are shown in Figure 2. AUC values were as follows: *ELF3*=0.955 with standard error (SE)=0.018, 95% confidence interval (CI)=0.919–0.990, $p=6.9\times10^{-7}$ and *CEA*=0.903 with SE=0.043, 95%CI=0.818–0.987, $p=0.00001$. The best cut-off values of *ELF3* and *CEA* were set at 27.5 with 100% sensitivity and 91.1% specificity rates, and 26.9 with 81.8% sensitivity and 90.2% specificity rates, respectively.

To investigate whether each gene was overexpressed in metastatic LNs from CRC, we measured their mRNA expression in 12 LNs from patients categorized into Dukes' stage A, 67 LNs from patients categorized into Dukes' stage B and 44 LNs from Dukes' stage C. As a control, we also measured the expression in 11 LNs dissected from patients with UC. As shown in Figure 3, the mRNA expression of *ELF3* and *CEA* was statistically significantly different in Dukes' stage A, B and C, and in the control groups ($p<0.001$ and $p<0.001$, respectively, Kruskal–Wallis test). Subsequent the Mann–Whitney *U*-test with Bonferroni correction indicated significantly higher expression of *ELF3* in Dukes' stage C (mean=149.6) compared to Dukes' stage B (mean=86.7) ($p<0.001$), and in Dukes' stage C compared to controls (mean=2.2) ($p<0.001$). There was also a significant difference in *CEA* mRNA expression in Dukes' stage C (mean=3914.0) compared to Dukes' stage B (mean=9116.8) ($p<0.001$), in Dukes' stage C compared to controls (mean=0.3) ($p<0.001$), in Dukes' stage B compared to controls ($p<0.001$), and in Dukes' stage A (mean=817.1) compared to controls ($p<0.05$), shown by the Mann–Whitney *U*-test as presented in Figure 3B.

Furthermore, in order to investigate the correlation between the mRNA levels for the two biomarkers, we compared their mRNA expression in LNs from patients with CRC and controls (Table III). LNs from each stage and control group were analyzed separately. There were significant correlations between *ELF3* and *CEA* mRNA expression overall (r=0.680; $p<0.001$), and in Dukes' stage A (r=0.853; $p<0.001$), Dukes' stage B (r=0.591; $p<0.001$), and Dukes' stage C (r=0.774; $p<0.001$), but not in the controls (r=–0.127; $p=0.709$).

The relationship between the qRT-PCR results and the histological examination are shown in Table IV. The results can be summarized as follows: there were 11 out of 11 true-positives for *ELF3*; and 9 out of 11 for *CEA* (statistical analysis was omitted due to low case numbers).

Discussion

To our knowledge, this is the first study of LN metastases of CRC focused on *ELF3*. In this study, we evaluated *ELF3* and *CEA* as gene markers for the detection of LN metastases from CRC by qRT-PCR. We found that the mRNA

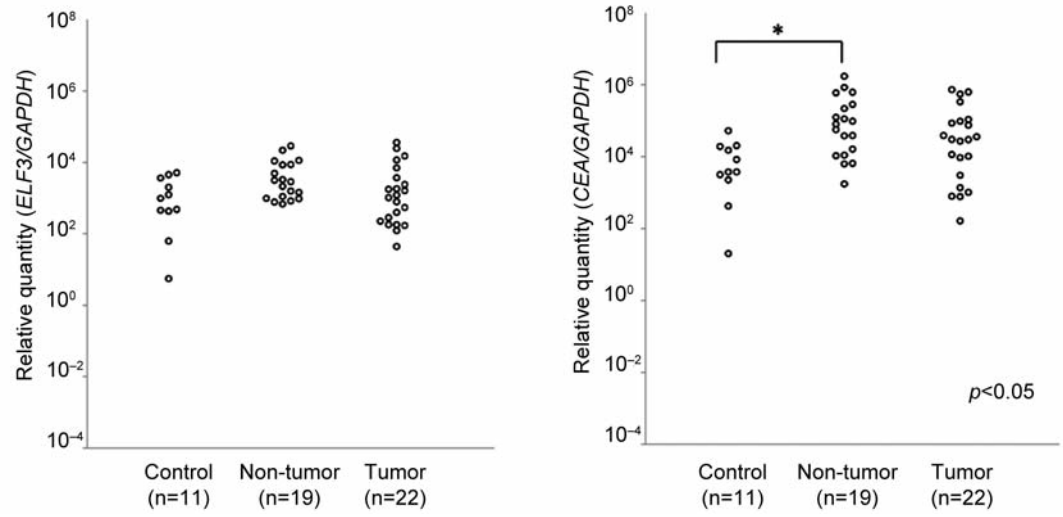

Figure 1. *Relative mRNA expression of E74-like factor 3 (ELF3) (A) and carcinoembryonic antigen (CEA) (B) in tissues from patients with colorectal cancer (CRC) determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Dots show mRNA levels in 19 non-tumor tissues and 22 tumor tissues from patients with CRC, compared with 11 inflammatory tissues from patients with ulcerative colitis as controls. p-Values are based on Kruskal–Wallis tests. *p<0.05, based on Mann–Whitney U-test with Bonferroni correction.*

expression of *ELF3* did not differ in primary tumor tissues, non-tumor tissues and inflammatory tissues. On the other hand, we found significant differences in CEA expression among these tissues. It has been reported that *ELF3* expression is increased in large cell carcinoma and adenocarcinoma in lung cancer, as compared to normal tissues (14). A previous study has also reported that expression of epithelium-specific genes such as *ELF3* are also increased in inflammatory disease (11). In that study, inflammation was related to the expression of *ELF3*, which probably also acts as an important modifier of non-neoplastic intestinal disease by regulating pathways that are relevant to tissue injury and repair. According to our results, there were no differences in the expression of *ELF3* among tissues. Therefore, we suggest that *ELF3* may be a gene marker for metastasis in LNs rather than in other tissues.

In our ROC analysis, AUC values for *ELF3* and *CEA* expression were 0.955 and 0.903, respectively. A previous study has reported that AUC values >0.9 indicate high accuracy, and a range of 0.7–0.9 indicates moderate accuracy (19). As a result of our ROC analysis, we conclude that *ELF3* expression is more accurate for the diagnosis of LN metastases than is *CEA*.

ELF3 and *CEA* expression significantly differed among LNs from Dukes' stage A, Dukes' stage B, Dukes' stage C, and controls. Moreover, we found statistically significant differences between the expression levels of both markers in Dukes' stage C as compared with Dukes' stage B and controls. This confirms that *ELF3* and *CEA* expression in CRC is sufficiently high to distinguish patients with from patients without LN metastases. In addition, the correlation of *ELF3* and *CEA* expression was highly significant in patients with CRC compared with controls. *CEA* is already known as a

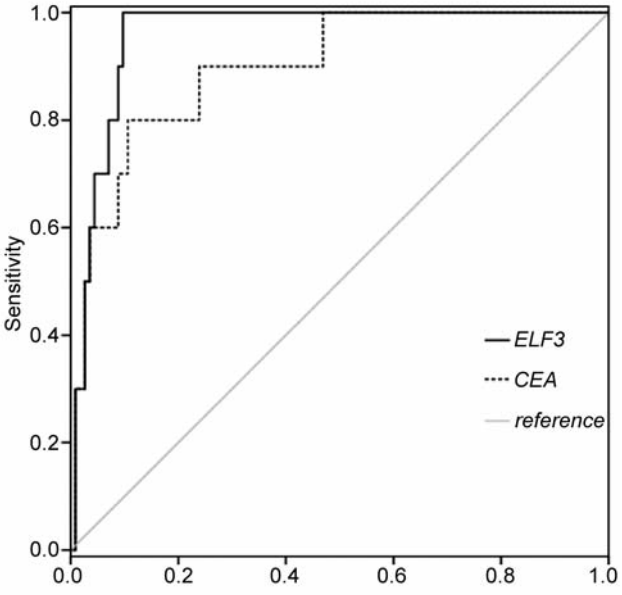

Figure 2. *Receiver operating characteristic (ROC) curves for E74-like factor 3 (ELF3) and carcinoembryonic antigen (CEA) to distinguish lymph node (LN) metastases in patients with colorectal cancer (CRC). Area under the curve (AUC) values for ELF3=0.955 with standard error (SE)=0.018, 95% confidence interval (CI)=0.919-0.990, $p=6.9\times10^{-7}$; and CEA=0.903, SE=0.043, 95%CI=0.818-0.987, p=0.00001. Cut-off values were set at 27.5 and 26.9, respectively.*

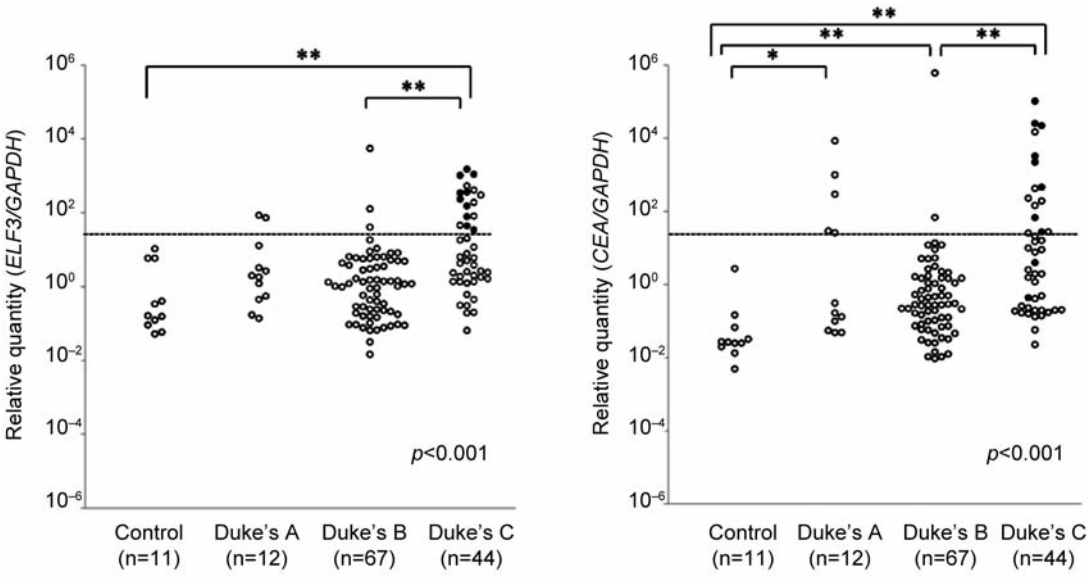

Figure 3. *Relative mRNA expression of E74-like factor 3 (ELF3) (A) and carcinoembryonic antigen (CEA) (B) in lymph nodes (LNs) from patients with colorectal cancer (CRC), as categorized by Dukes' classification. Dots show mRNA levels in 123 LNs from patients with Dukes' stage A, B and C CRC, compared with 11 LNs from patients with ulcerative colitis serving as controls. Black dots indicate LNs with tumor cells, and grey dots indicate LNs without tumor cells, as identified by hematoxylin–eosin staining. Bars show cut-off values for ELF3 and CEA, which were set at 27.5 and 26.9, respectively. p-Values are based on Kruskal–Wallis tests. *p<0.05 and **p<0.001, are based on Mann–Whitney U-test with Bonferroni correction.*

useful marker for detecting metastasis in LNs and blood samples (3-6). We suggest that *ELF3* is an equally useful marker for the detection of metastasis in patients with CRC.

Furthermore, *ELF3* was successful for detection of all histologically-positive LNs, whereas *CEA* was not. Indeed, one study has shown that *CEA* was not detected in a breast cancer cell line (4), and another report has shown that the expression of CEA in CRC is lower than that in benign LNs (20). From this point of view, *ELF3* seems to be a more useful marker than *CEA*.

Case 13 was EX-positive diagnosed with negative LNs on conventional pathological staging. EX are also named mesenteric implants, tumor deposits, and isolated tumor deposits (ITDs) (21-23). The presence of EX was an independent prognostic factor affecting overall survival and related to poor prognosis in colon cancer (24, 25). In our study, *ELF3* and *CEA* expression exceeded cut-off values in case 13. Our finding might contribute to the detection of EX.

In conclusion, our results suggest that the expression of *ELF3* in LNs alerts us to the possibility of metastases. *ELF3* may be more suitable than *CEA* as a gene marker for the detection of LN metastases from CRC and requires further verification as a biomarker in a larger population study.

Conflicts of Interest Statement

No conflicts of interest exist in the submission of this manuscript.

Table III. *Correlation between expression levels of biomarker E74-like factor 3 (ELF3) and carcinoembryonic antigen (CEA) mRNAs in lymph nodes of patients with colorectal cancer and controls.*

ELF3 vs. CEA	r[a]	*p*-value[a]
All lymph nodes	0.680	<0.001
Control	–0.127	0.709
Dukes' A	0.853	<0.001
Dukes' B	0.591	<0.001
Dukes' C	0.774	<0.001

[a]r and *p*-values obtained using two-tailed Spearman rank correlation test.

Table IV. *Lymph node metastases detected by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and histological examination.*

Marker	Histological metastasis	qRT-PCR	
		Positive[a] n (%)	Negative n (%)
ELF3	Positive	11/11 (100)	0/11 (0)
	Negative	10/112 (8.9)	102/112 (91.1)
CEA	Positive	9/11 (81.8)	2/11 (18.2)
	Negative	11/112 (9.8)	101/112 (90.2)

[a]Cut-off values as indicated in ROC analysis: *ELF3*>27.5; *CEA*>26.9.

Acknowledgements

The Authors are grateful to Dr. Usami, Dr. Shintani and Dr. Aoyama for their valuable comments and for providing technical assistance at the Faculty of Health Sciences, Kobe University Graduate School of Health Sciences.

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Received May 6, 2012
Revised July 13, 2012
Accepted July 16, 2012