# Serum LOX-1 is a novel prognostic biomarker of colorectal cancer
# （大腸癌の新規予後マーカーである血清 LOX-1）

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学位論文の関連論文の研究背景及び要旨

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〔題名〕
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〔研究背景〕
大腸癌は世界的に、最も頻度の高い癌のひとつであり、日本においては癌関連死亡原因の第2位を占める。過去20年間の化学療法や分子標的薬治療の進歩により進行大腸癌の5年生存率は飛躍的に伸びたが、未だ十分でないと考える。予後バイオマーカーの意義は、より強力な抗癌剤治療が必要となる人を選別しうること、今後の治療ターゲットになりうることである。バイオマーカーを腫瘍組織から得るよりも、血液検査から得ることができれば、非侵襲的で簡便であり、メリットが大きいと考える。リキッドバイオプシーから得られる大腸癌の予後バイオマーカーにはcell-free DNA、microRNAsや炎症性分子、タンパク質等の報告がある。本研究ではタンパク質に着目し、蛋白網羅的解析装置（Somascan）を用いて大腸癌の予後バイオマーカー候補を選定し、バリデーションを行いバイオマーカーとなりうるか検証した。

〔要旨〕
バイオマーカーの選定のため、StageIV大腸癌症例の血清20検体でSomascanを用いて蛋白網羅的解析を行った。Somascanは血液等の微量生体サンプルから、細胞プロセスと病理生態学に関連する1,129種類のタンパク質を一度に定量することができる高感度なアッセイ技術である。予後3年以上の良好群9例と2年以下の不良群11例に分けて比較し、統計学的に差があるタンパク質の中から、新規性のあるタンパク質として、LOX-1（Lectin-like oxidized low-density lipoprotein receptor-1）に着目した。LOX-1は膜通過型のタンパク質で、酸化LDLのレセプターである。酸化LDLと結合して活性酸素やアルギナーゼIの産生を増やし遺伝子の突然変異や発癌に関与するといわれている。また、myeloid-derived suppressor cells（MDSC）の表面マーカーとして、癌の免疫を負にし、腫瘍増殖を促すと

の報告があるが、血清LOX-1値が癌の予後バイオマーカーになるという報告は現在までにない。そこでLOX-1が予後バイオマーカーとなりうるか、一般的なタンパク質の検出方法であるELISAを用いてバリデーションを行うこととした。Somascanで測定した20例の血清検体をELISAでも測定したところ、正の相関を得たことで、測定方法の正当性を示した。バリデーションのため、当科で治療を行ったStageⅢ, Ⅳ大腸癌の血清検体238例をELISAで定量した。LOX-1のカットオフ値を時間依存性ROC曲線から538.67 pg/mLに設定すると、血清LOX-1高値群で有意に予後不良となった。LOX-1と臨床病理学的背景の関連を検討すると、血清LOX-1高値群でリンパ節転移が多く、遠隔転移のある症例が有意に多かった。血清から得られる因子だけで多変量解析を行ったところ、CEAとLOX-1が独立した予後因子であることがわかった。腺癌の腫瘍マーカーとしてはCEAやCA19-9が知られているが、本研究ではCA19-9は独立した予後因子とはならなかった。独立した予後因子であったCEAとLOX-1の組み合わせで予後を検討すると、CEA低値群の中ではLOX-1の高低で予後に差はなかったが、CEA高値群の中では、LOX-1の高値群が低値群よりも有意に予後不良であり、組み合わせにより予後をより層別化できる可能性がある。また、血清LOX-1値が高値群と低値群から各々50例の症例において腫瘍組織でのLOX-1の発現をみるため、免疫染色を行った。腫瘍の細胞質が染まっており、染色の評価は文献と同様に、染色の強度と範囲から算出するcomposite expression score （CES）を用いて行った。カットオフ値を時間依存性ROC曲線からCES8に定め、100例の検討では、高発現群で有意に予後不良の結果が得られた。この結果は、近年文献で報告されている胃癌や大腸癌、膵癌での癌局所のLOX-1の高発現が予後不良に関与するとの結果と同様であり、LOX-1はEMT（上皮間葉転換）を制御するPI3K/Akt/GSK3β経路を通じて腫瘍の浸潤や転移を促進することを示していた。また、LOX-1を制御することで癌化現象や転移を抑制したとの報告があり、LOX-1は腫瘍の進行や転移を制御するターゲットになりうる。組織中に発現したLOX-1は、CRPやIL-18などの炎症性物質の刺激を受けて、血中へ放出されると報告されており、本研究の血清LOX-1高値群で、WBC、CRP、NLRやMLRが有意に高値であり、そのような炎症性マーカーの中ではLOX-1だけが独立した予後因子であった。本研究では血清LOX-1が大腸癌の予後不良を示すバイオマーカーになりうることを示した。血清LOX-1の癌に対する直接的な作用は示せておらず、さらなる研究が必要である。

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ORIGINAL ARTICLE

# Serum LOX-1 is a novel prognostic biomarker of colorectal cancer

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## Abstract

**Background** Colorectal cancer is the third most common cancer worldwide. If biomarkers can be identified in liquid biopsy, diagnosis and treatment can be optimized even when cancerous tissues are not available. The purpose of this study was to identify proteins from liquid biopsy that would be useful as markers of poor prognosis.

**Methods** First, we comprehensively analyzed serum proteins to identify potential biomarkers and focused on serum lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). The relationship between LOX-1 and the prognosis of patients with colorectal cancer has not been reported. Next, we validated this marker using serum samples from 238 patients with colorectal cancer by ELISA and 100 tissue samples by immunohistochemical staining.

**Results** The optimal cut-off value of serum LOX-1 was 538.7 pg/mL according to time-dependent receiver operating characteristics curve analysis. The overall survival of patients with high levels of serum LOX-1 was significantly poorer than that of individuals with low levels of LOX-1 in the training and test datasets. In multivariate analysis for overall survival, serum LOX-1 was an independent prognostic factor identified in liquid biopsy (hazard ratio = 1.729, $p = 0.027$). The prognosis of patients with high LOX-1 expression in tumor tissues was significantly poorer than that of individuals with low expression ($p = 0.047$). Additionally, inflammatory factors such as white blood cell count, C-reactive protein level, neutrophil/lymphocyte ratio, and monocyte/lymphocyte ratio were significantly higher in the group with high serum LOX-1 levels.

**Conclusions** Serum LOX-1 might be a useful biomarker of poor prognosis in colorectal cancer.

**Keywords** Colorectal cancer · LOX-1 · Liquid biomarker · Prognosis

## Introduction

Colorectal cancer (CRC) is the third most common cancer and second leading cause of cancer-related death in industrialized countries [1]. In the past decade, combination treatment comprising fluorinated-pyrimidine with irinotecan or oxaliplatin (FOLFOX, XELOX, FOLFILI), with or without

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monoclonal antibodies such as anti-vascular endothelial growth factor antibody or anti-epidermal growth factor receptor antibody, has markedly improved the prognosis of patients with stage IV metastatic advanced CRC [2–6]. Additionally, it has been recommended that patients with high-risk stage II and III CRC should be administered adjuvant chemotherapy with FOLFOX or XELOX to prevent recurrence. Hence, predictive biomarkers of poor prognosis are crucially needed [7]. If these biomarkers can be identified in liquid biopsy, which analyze cell-free DNA [8], microRNAs [9], and inflammatory molecules [10, 11] in the peripheral blood, diagnosis and treatment could be optimized even when cancerous tissues are not available. To detect novel biomarkers, we performed comprehensive proteome analysis and found that lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was a candidate biomarker for patients with CRC.

LOX-1 is the major receptor for oxidized low-density lipoprotein (ox-LDL) in endothelial cells [12], macrophages [13], smooth muscle cells, and monocytes [14]. This protein is a type II transmembrane glycoprotein [15]. The binding of ox-LDL to LOX-1 increases reactive oxygen species (ROS) formation and strongly contributes to oxidative DNA damage [16–18]. A strong correlation between the serum level of ox-LDL and risk of CRC was described in a large-scale Japanese cohort [19]. However, the relationship between serum levels of LOX-1 and cancer is unknown.

We focused on the protein LOX-1 based on the results of a comprehensive analysis of serum proteins. The aim of this study was to investigate the relationship between serum LOX-1 and CRC prognosis to determine whether LOX-1 can be used as a liquid prognostic biomarker for CRC. We found that serum LOX-1 is a potential biomarker of poor prognosis in CRC.

## Materials and methods

### Comprehensive analysis of serum proteins

We comprehensively analyzed serum proteins using the SOMAscan (SomaLogic, Inc., Boulder, CO, USA) to measure 1129 proteins related to the disease. Serum samples were obtained on the day of admission immediately before chemotherapy from 24 consecutive patients with CRC treated with FOLFOX as first-line chemotherapy at our hospital between February 2009 and November 2012. Serum samples were stored at − 80 °C until use. We analyzed 20 samples from the patients including nine patients who survived for more than 3 years and 11 patients who survived for less than 2 years, excluding four patients who survived for 2–3 years (Fig. 1). All patients had unresectable metastatic lesions, adequate functions of critical organs, and an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1. Written informed consent was obtained from each patient at the time of enrollment. The study was carried out in accordance with the Helsinki declaration on experimentation in human subjects and was approved by the Institutional Ethics Review Boards of Yamaguchi University (H20-102 and H23-135).

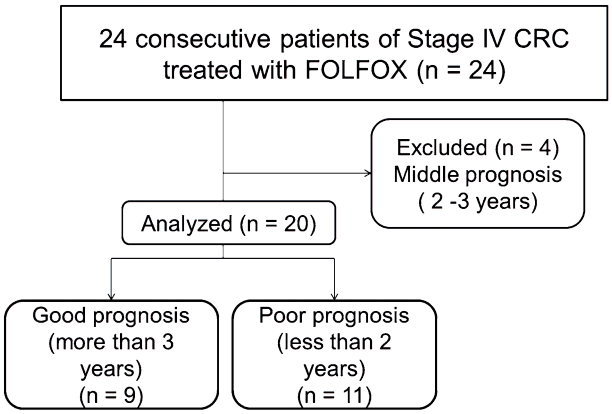


**Fig. 1** CONSORT diagram of the comprehensive analysis of serum proteins. We measured 24 serum samples using SOMAscan and analyzed 20 samples. *CRC* colorectal cancer

### Sample collection for validation

From January 2004 to December 2015, 238 serum samples were obtained from consecutive patients with stage III and IV CRC, excluding the samples obtained for the comprehensive proteome analysis for validation, in our hospital and stored at − 80 °C until use. These were obtained at the time of admission, which was soon followed by surgery or chemotherapy. In this study, the candidate biomarkers were selected from stage IV CRC and hence might be biomarkers for patients with advanced cancer. Concurrently, the prognosis of stage I and II CRC was quite favorable compared to that of stage III and IV CRC. Therefore, we analyzed patients with stage III and IV CRC and excluded those with stage I and II CRC. This study was approved by the Ethics Committee of Yamaguchi University Hospital (H17-83 and H23-135). All samples were obtained with the patients' written informed consent. For immunohistochemical staining, 100 tissue samples were collected from 238 stage III and IV patients with CRC who underwent surgery. Fifty tissue samples were selected from the high-serum LOX-1 group, and another 50 samples were from the low-serum LOX-1 group.

### Measurement of serum LOX-1 levels

Serum LOX-1 concentrations were determined using a Human LOX1 ELISA Kit (ab212161; Abcam plc, Cambridge, UK). This kit employs the sandwich ELISA

principle. The samples were collected in sterile tubes, centrifuged at 2000 × *g* for 10 min, aliquoted into 1.5-mL tubes, and preserved at − 80 ℃ until analysis. They were assayed according to the manufacturer's instructions. Briefly, 50 µL of standard, undiluted sample, or blank was added into each well, and 50 µL of the Antibody Cocktail (capture antibody and detector antibody) was added to each well and incubated for 1 h at room temperature. Then after removing the antibody by washing, 100 µL of TMB substrate solution was added and incubated for 10 min in the dark. Finally, 100 µL of stop solution was added to each well. The absorbances of samples and standards at 450 nm were measured immediately using EnVision Multilabel Plate Readers (PerkinElmer, Shelton, CT, USA). A standard curve was constructed for the stock standard. The sensitivity of the assay was 15.625 pg/mL.

### Immunohistochemical staining (IHC)

IHC was performed on 4-µm-thick formalin-fixed paraffin-embedded sections. Antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6.0 (Dako, Carpinteria, CA, USA) by heating at 121 °C in an autoclave for 10 min. After blocking endogenous peroxidase activity with Peroxidase-Blocking Solution (Dako S2023) for 5 min, the sections were incubated with serum-free protein block (Dako) and with 1% skim milk for 10 min each and then with an anti-LOX-1 antibody (Proteintech Group, Inc., Chicago, IL, USA; dilution 1:600) at 4 °C overnight. Labeling was detected with the Envision Plus Detection kit (Dako) following the manufacturer's protocol, and staining was visualized by incubating the samples with DAB (Dako) for 3 min followed by counterstaining with hematoxylin. Photographs were obtained using the All-in-one fluorescence microscope BZ-X710 (KEYENCE, Tokyo, Japan). To estimate LOX-1 expression in the tumor tissue, the slides were assessed by an experienced and independent pathologist blinded to the patient's status to determine the composite expression score (CES) as described previously [27]. The CES was calculated from staining intensity and frequency measurements. The intensity scores were divided into four levels, specifically 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The frequency scores were also divided into four levels as 1 (5–24%), 2 (25–49%), 3 (50–74%), and 4 (75–100%). When the intensity was 0, CES was determined to be 0. When the intensity was 1 or more, the CES was calculated according to the following formula: CES = 4 × (intensity score − 1) + frequency score.

### Statistical analysis

The cut-off value of serum LOX-1 was determined by time-dependent receiver operating characteristic (ROC) curve analysis, twofold cross-validation, and ROC curve analysis using a certain time period. Differences between groups were estimated by Welch's *t* test or the Mann–Whitney *U* test. Categorical variables were compared by the Chi-squared test. Overall survival (OS) was measured from the day of the first visit to the day of patient death. Survival curves were estimated by the Kaplan–Meier method and analyzed by the log-rank test. Cox's proportional hazard model was used to estimate the hazard ratios (HRs) to determine the relationship between OS and protein expression, other biomarkers, and prognostic clinical information. Statistical analyses were performed with JMP Pro14 (SAS Institute, Inc., Cary, NC, USA) and R language (version 3.5.2). $p < 0.05$ was considered to indicate statistically significant results.

## Results

### Selection of candidate protein for the prediction of CRC prognosis

We first performed a comprehensive analysis using the 20 serum samples, which were divided into two groups as follows: nine cases with good prognosis with an OS of more than 3 years and 11 cases with poor prognosis with an OS of less than 2 years. We ranked the obtained proteins for candidates of liquid biomarkers according to their Fisher ratio (Table 1) and focused on the protein LOX-1. The candidate biomarkers from comprehensive analysis should be novel and potential serum biomarkers for CRC. Hence, the exclusion criteria for further analysis were that the serum analysis had been previously reported for colorectal cancer and there was no reported association with any cancer. Although some previous studies reported that LOX-1 function is related to cancer progression and that LOX-1 expression in tumors might be a poor prognostic biomarker [20–22], there is no report concerning the serum level of LOX-1 as a liquid prognostic biomarker for patients with CRC. Myeloperoxidase, the number one candidate, was reported in 1983 as a marker of phagocytic activity of neutrophils [23]. Numerous reports have been published on the relationship between myeloperoxidase and colorectal cancer [24]. Hence, it might be difficult to find novel roles for this marker in CRC. Proteinase 3, the number two candidate, was purified as a distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters in 1988 [25]. Recently, proteinase 3 was reported as a cancer-associated antigen to which $CD8^+$ T cell responds in patients with hematological malignancies [26]. Since there is no report of proteinase 3 as a target molecule of CRC, it was excluded as a candidate liquid biomarker for CRC. Next, using the same 20 serum samples, we measured the levels of LOX-1 by ELISA. We found a strong correlation between the levels of LOX-1 measured by

**Table 1** Predictive markers from comprehensive analysis of serum proteins in colorectal cancer

| Rank | Target protein | Good prognosis ($n=9$) | | Poor prognosis ($n=11$) | | $Log_2$ ratio | Fisher's ratio | Welch's *t* test |
|---|---|---|---|---|---|---|---|---|
| | | Avg | SD | Avg | SD | | | *p* value |
| 1 | Myeloperoxidase | 29407.4 | 8857.3 | 51014.8 | 10610.8 | −0.8 | 4.65 | < 0.001 |
| 2 | Proteinase-3 | 7979.2 | 2821.1 | 15841.6 | 4687.9 | −1.0 | 3.44 | < 0.001 |
| 3 | LOX-1 | 460.7 | 108.0 | 752.5 | 257.5 | −0.7 | 2.74 | 0.004 |
| 4 | IL-8 | 3318.9 | 943.6 | 7096.9 | 3785.3 | −1.1 | 2.59 | 0.008 |
| 5 | Lactoferrin | 8649.9 | 2371.3 | 14674.9 | 5112.1 | −0.8 | 2.26 | 0.003 |
| 6 | BPI | 3032.8 | 1023.0 | 5904.1 | 2985.4 | −1.0 | 2.03 | 0.011 |
| 7 | hnRNP A2/B1 | 4424.2 | 1835.8 | 7377.9 | 3115.2 | −0.7 | 1.94 | 0.018 |
| 8 | PGRP-S | 948.4 | 328.4 | 1503.8 | 489.8 | −0.7 | 1.79 | 0.008 |
| 9 | VEGF121 | 4635.7 | 1890.0 | 11089.3 | 7703.0 | −1.3 | 1.55 | 0.021 |
| 10 | MCP-3 | 2773.6 | 862.2 | 5455.0 | 4965.8 | −1.0 | 1.21 | 0.107 |

Good prognosis: survived more than 3 years, poor prognosis: survived less than 2 years

*Avg* average, *SD* standard deviation, *LOX-1* oxidized low-density lipoprotein receptor 1, *IL-8* Interleukin-8, *BPI* bactericidal permeability-increasing protein, *hnRNP A2/B1* heterogeneous nuclear ribonucleoproteins A2/B1, *PGRP-S* peptidoglycan recognition protein 1, *VEGF121* vascular endothelial growth factor A, isoform 121, *MCP-3* C–C motif chemokine 7

SOMAscan and ELISA ($r=0.926$, $p<0.001$: Fig. 2a). Thus, the measurement of SOMAscan was reliable.

## Validation of serum LOX-1 level as a poor prognostic biomarker

All 238 samples were used to determine the best cut-off period (months) by conducting time-dependent ROC curve analysis. The comprehensive analysis of biomarkers in this study was based on the survival of stage IV patients with CRC, and prognoses were divided into categories of less than 2 years and more than 3 years. Clinically, the median OS of stage IV CRC is approximately 3 years; therefore, we focused on the range of 30–36 months on the time-dependent ROC curve and selected 35 months as the best cut-off period for further analysis, as indicated by the arrow (Fig. S1). Next, twofold cross-validation was performed; specifically, all samples were randomly divided into two subsets while maintaining the ratios of patients with stage III and IV disease in each dataset. One subset was used for training ($n=120$), and the remaining subset was used for testing ($n=118$). Then the cut-off value of serum LOX-1 was selected by conducting ROC curve analysis at 35 months using the training dataset (Fig. 2b). These processes were repeated 5000 times, and the most frequently detected cut-off value of 538.7 pg/mL was selected as the cut-off value for serum LOX-1. The OS of patients in the group with high serum levels of LOX-1 in a training dataset was significantly poorer than that in the group with low LOX-1 levels (log-rank test, $p=0.0196$; Fig. 2c). Finally, the OS of patients with high levels of serum LOX-1 in a test dataset was a significantly poorer than that of patients with low LOX-1 levels (log-rank test, $p=0.0376$; Fig. 2d).

## Clinicopathological characteristics and relationship with LOX-1

The clinicopathological characteristics of the patients are shown in Table 2. The LOX-1-high group contained significantly more cases of lymph node metastasis ($p<0.001$) and distant metastasis ($p<0.001$) than the LOX-1-low group (Table 2).

## Relationship between serum LOX-1 levels and peripheral blood examination in usual clinical practice

In the LOX-1-high group, the levels of carcinoembryonic antigen (CEA), white blood cell counts, C-reactive protein, neutrophil-to-lymphocyte ratio, and monocyte-to-lymphocyte ratio were significantly higher than those in the LOX-1-low group ($p=0.008$, $p=0.001$, $p=0.012$, $p<0.001$, $p=0.004$, respectively; Table 3).

## Univariate and multivariate analyses of the association between various clinical data and overall survival

Univariate analysis revealed that the levels of LOX-1, CEA, and carbohydrate antigen19-9 (CA19-9), white blood cell, neutrophil, and lymphocyte counts, the neutrophil–lymphocyte and monocyte-to-lymphocyte ratios, and C-reactive protein were significantly associated with OS as liquid predictive biomarkers (Table 4). By multivariate analysis, using the results of predictive biomarkers that had significant values based on univariate analysis, the levels of LOX-1 (HR = 1.729, $p=0.027$) and CEA

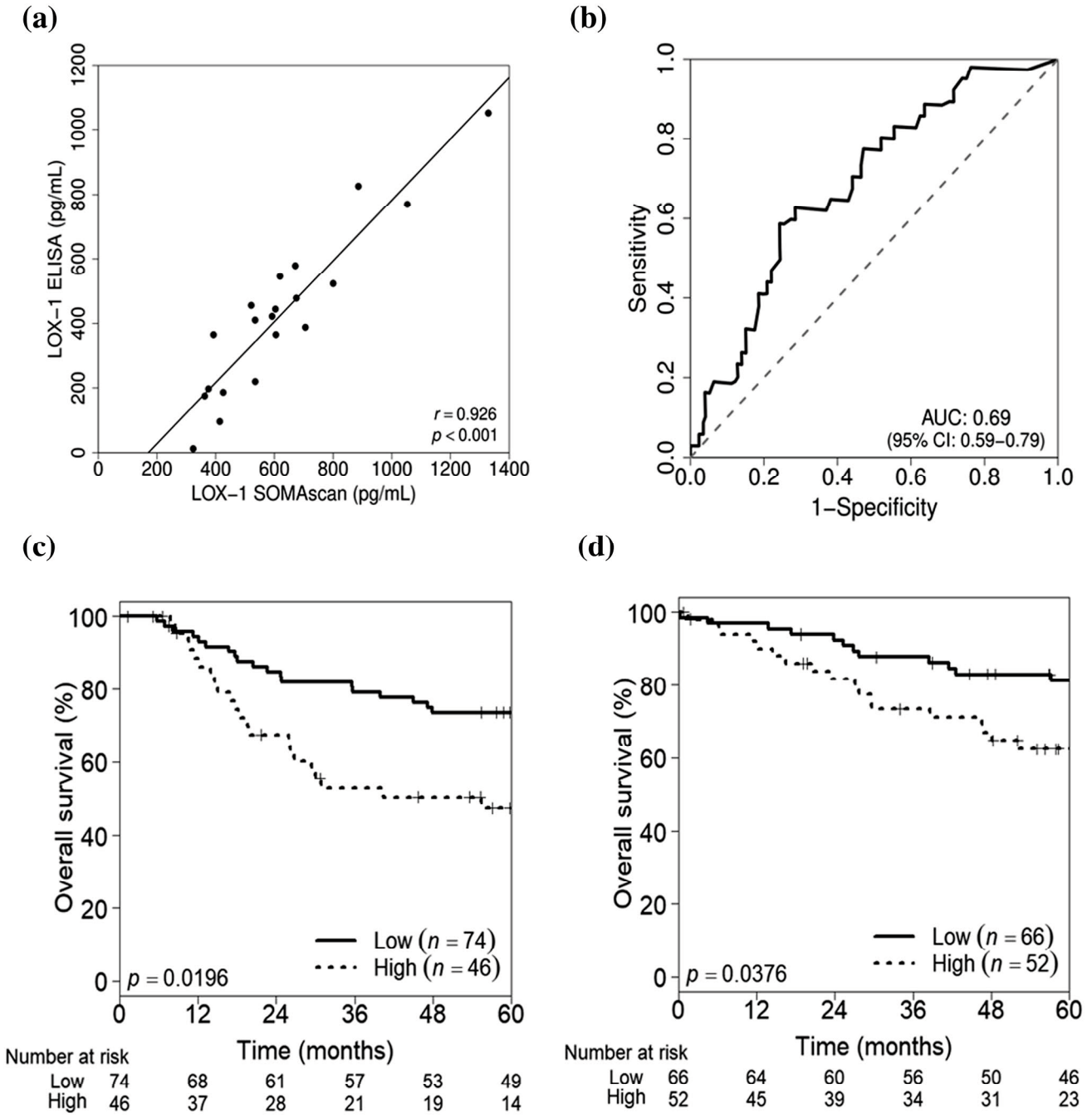


**Fig. 2** Measurement and verification of serum LOX-1. **a** The levels of LOX-1, as measured by SOMAscan and ELISA, were strongly correlated ($n=20$). **b** Determination of the best cut-off value of serum LOX-1 by time-dependent ROC curve analysis in the training dataset ($n=120$). **c** Overall survival in the training dataset ($n=120$). **d** Overall survival in the test dataset ($n=118$). *LOX-1* oxidized low-density lipoprotein receptor 1, *AUC* area under the curve, *CI* confidence interval, *ROC* receiver operator characteristic

(HR = 3.960, $p<0.001$) were confirmed as independent liquid predictive biomarkers of OS (Table 4). Additionally, multivariate analysis including tumor factor, node factor, and metastasis factor was performed (Table 5). As a result, distant metastases (HR = 7.640, $p<0.001$), N2/N3 status, which indicates more than four regional lymph node metastases (HR = 3.367, $p<0.001$), and the monocyte-to-lymphocyte ratio (HR = 2.486, $p=0.006$) were confirmed as independent liquid predictive biomarkers of OS.

**Table 2** Clinicopathological characteristics and serum LOX-1 level in colorectal cancer

| Characteristics | Serum LOX-1 level | | *t* or Chi-squared test |
|---|---|---|---|
| | Low ($n=140$) | High ($n=98$) | *p* value |
| Age | | | |
| Median | 66 | 67.5 | 0.978 |
| Sex | | | |
| Male | 72 | 62 | 0.084 |
| Female | 68 | 36 | |
| Tumor location | | | |
| Right colon | 48 | 29 | 0.484 |
| Left colon | 52 | 44 | |
| Rectum | 40 | 25 | |
| Depth of invasion | | | |
| T1–3 | 118 | 75 | 0.133 |
| T4a, 4b | 22 | 23 | |
| Lymph node metastasis | | | |
| N0, 1 | 106 | 53 | < 0.001 |
| N2, 3 | 34 | 45 | |
| Distant metastasis | | | |
| Absent (M0) | 108 | 51 | < 0.001 |
| Present (M1) | 32 | 47 | |

*LOX-1* lectin-like oxidized low-density lipoprotein receptor-1

## Overall survival analysis according to high and low cut-off values of LOX-1 and CEA

Survival analysis was performed according to the cut-off values of CEA and LOX-1 (Fig. 3). The prognosis of patients with low levels of CEA was favorable regardless of LOX-1 levels. In contrast, the prognosis was significantly poorer for patients with high LOX-1 levels than for those with low LOX-1 levels among the group with high levels of CEA ($p < 0.01$, log-rank test).

## Immunohistochemical staining of LOX-1 and other clinical factors

As shown in Fig. 4a, positive LOX-1 staining was mainly observed in the cytoplasm of tumor cells. The cut-off value, CES 8, was selected by time-dependent ROC curve analysis. The group with higher LOX-1 expression in the tumor tissue showed significantly poor prognosis compared to that in the group with lower expression ($p = 0.047$; Fig. 4b).

## Discussion

This is the first report using the serum LOX-1 level as a significant prognostic biomarker of CRC. First, we found that the OS of patients with CRC with high levels of serum LOX-1 was significantly poorer than that for individuals with low levels of LOX-1 (Fig. 2b–d). Additionally, serum LOX-1, obtained by liquid biopsy, was an independent prognostic factor in multivariate analysis of OS (Table 4). These results indicate that the serum level of LOX-1 can be used as a prognostic biomarker of CRC.

LOX-1 is one of the main receptors of ox-LDL [13], also known as ox-LDL receptor 1 (OLR1), and is overexpressed in polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs). LOX-1, encoded by *OLR1*, is nearly undetectable in neutrophils in the peripheral blood of healthy donors, whereas 5–15% of total neutrophils from patients with cancer and 15–50% of neutrophils in tumor tissues are LOX-1-positive [28]. LOX-$1^+$ PMN cells show significantly higher expression of arginase1 and ROS production, which cause immune suppression in the peripheral blood and tumor microenvironment [28]. As a result, the number of LOX-$1^+$ PMN-MDSCs is negatively related to the OS of patients with hepatocellular carcinoma [29].

LOX-1 can be stimulated by various inflammatory factors such as C-reactive protein [30] or interleukin 18 [31],

**Table 3** Serum LOX-1 and peripheral blood markers in colorectal cancer

| Serum prognostic marker | Lox-1 expression (median [IQR]) | | Mann–Whitney *U* test |
|---|---|---|---|
| | Low ($n=140$) | High ($n=98$) | *p* value |
| CEA (ng/mL) | 6.75 [3.27, 17.85] | 11.30 [4.05, 47.02] | 0.008 |
| CA19-9 (U/mL) | 8.00 [3.77, 37.16] | 11.80 [4.30, 46.30] | 0.215 |
| WBC (/uL) | 5770.0 [4780.0, 7060.0] | 6580.0 [5442.50, 8852.50] | 0.001 |
| Monocyte (%) | 5.20 [4.00, 6.90] | 5.60 [4.30, 6.50] | 0.738 |
| NLR | 2.30 [1.70, 3.45] | 2.95 [2.10, 4.60] | < 0.001 |
| MLR | 0.19 [0.14, 0.28] | 0.24 [0.17, 0.39] | 0.004 |
| CRP (mg/dL) | 0.13 [0.05, 0.72] | 0.41 [0.07, 1.53] | 0.012 |

*CEA* carcinoembryonic antigen, *CA19-9* carbohydrate antigen19-9, *WBC* white blood cell, *NLR* neutrophil–lymphocyte ratio, *CRP* C-reactive protein, *LOX-1* lectin-like oxidized low-density lipoprotein receptor-1, *IQR* interquartile range

**Table 4** Univariate and multivariate analyses of associations between serum LOX-1 and liquid sample

| Factor | Cut-off | Univariate analysis | | | | Multivariate analysis | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HR | 95% CI | | *p* value | HR | 95% CI | | *p* value |
| | | | Lower | Upper | | | Lower | Upper | |
| CEA (ng/mL) | > 10 | 4.813 | 2.938 | 8.219 | < 0.001 | 3.960 | 2.250 | 7.180 | < 0.001 |
| CA19-9 (U/mL) | > 74 | 2.900 | 1.777 | 4.621 | < 0.001 | 1.431 | 0.824 | 2.449 | 0.200 |
| WBC (/uL) | > 6095 | 1.743 | 1.106 | 2.789 | 0.018 | 0.994 | 0.584 | 1.707 | 0.983 |
| Neutrophils (%) | > 65.2 | 1.595 | 1.009 | 2.545 | 0.047 | 1.128 | 0.481 | 2.628 | 0.782 |
| Lymphocytes (%) | < 26 | 1.745 | 1.100 | 2.812 | 0.019 | 1.052 | 0.382 | 3.276 | 0.926 |
| NLR | > 2.5 | 1.744 | 1.104 | 2.783 | 0.018 | 1.115 | 0.320 | 4.227 | 0.868 |
| Monocytes (%) | > 5.4 | 1.193 | 0.755 | 1.887 | 0.449 | | | | |
| MLR | > 0.214 | 2.000 | 1.258 | 3.236 | 0.003 | 1.556 | 0.885 | 2.763 | 0.125 |
| CRP (mg/dL) | > 0.30 | 2.588 | 1.647 | 4.121 | < 0.001 | 1.530 | 0.912 | 2.582 | 0.107 |
| LOX-1 (pg/mL) | > 538.7 | 1.939 | 1.238 | 3.045 | 0.004 | 1.729 | 1.064 | 3.853 | 0.027 |

*CEA* carcinoembryonic antigen, *CA19-9* carbohydrate antigen19-9, *WBC* white blood cell, *NLR* neutrophil–lymphocyte ratio, *MLR* monocyte-to-lymphocyte ratio, *CRP* C-reactive protein, *LOX-1* lectin-like oxidized low-density lipoprotein receptor-1, *HR* hazard ratio, *CI* confidence interval

**Table 5** Univariate and multivariate analyses of associations between serum LOX-1 and all factors

| Factor | Cut-off | Univariate analysis | | | | Multivariate analysis | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HR | 95% CI | | *p* value | HR | 95% CI | | *p* value |
| | | | Lower | Upper | | | Lower | Upper | |
| Tumor (T4/T1–3) | | 3.054 | 1.863 | 4.876 | < 0.001 | 1.430 | 0.827 | 2.405 | 0.195 |
| Node (N2, 3/N0, 1) | | 3.969 | 2.523 | 6.317 | < 0.001 | 3.367 | 2.004 | 5.741 | < 0.001 |
| Metastasis (M1/M0) | | 10.68 | 6.456 | 18.44 | < 0.001 | 7.640 | 4.113 | 14.72 | < 0.001 |
| CEA (ng/mL) | > 10 | 4.813 | 2.938 | 8.219 | < 0.001 | 1.705 | 0.916 | 3.255 | 0.093 |
| CA19-9 (U/mL) | > 74 | 2.900 | 1.777 | 4.621 | < 0.001 | 1.017 | 0.589 | 1.725 | 0.951 |
| WBC (/uL) | > 6095 | 1.743 | 1.106 | 2.789 | 0.018 | 1.006 | 0.580 | 1.755 | 0.984 |
| Neutrophils (%) | > 65.2 | 1.595 | 1.009 | 2.545 | 0.047 | 0.633 | 0.219 | 1.798 | 0.400 |
| Lymphocytes (%) | < 26 | 1.745 | 1.100 | 2.812 | 0.019 | 1.402 | 0.415 | 4.225 | 0.574 |
| NLR | > 2.5 | 1.744 | 1.104 | 2.783 | 0.018 | 1.174 | 0.278 | 5.349 | 0.831 |
| MLR | > 0.214 | 2.000 | 1.258 | 3.236 | 0.003 | 2.486 | 1.297 | 4.830 | 0.006 |
| CRP (mg/dL) | > 0.30 | 2.588 | 1.647 | 4.121 | < 0.001 | 1.143 | 0.652 | 2.012 | 0.640 |
| LOX-1 (pg/mL) | > 538.7 | 1.939 | 1.238 | 3.045 | 0.004 | 1.034 | 0.618 | 1.734 | 0.900 |

*CEA* carcinoembryonic antigen, *CA19-9* carbohydrate antigen19-9, *WBC* white blood cell, *NLR* neutrophil–lymphocyte ratio, *MLR* monocyte-to-lymphocyte ratio, *CRP* C-reactive protein, *LOX-1* lectin-like oxidized low-density lipoprotein receptor-1, *HR* hazard ratio, *CI* confidence interval

which is followed by hydrolysis and release into the blood by specific proteases; the hydrolysis product, known as the soluble form of LOX-1, was recently reported as a potential biomarker for cardiovascular diseases [32]. LOX-1 receptor, upon activation by its ligand ox-LDL, stimulates the expression of adhesion molecules, pro-inflammatory signaling pathways, and proangiogenic proteins including nuclear factor-κB and vascular endothelial growth factor in vascular endothelial cells and macrophages [33]. These reports support our results showing that a high level of serum LOX-1 is related to the poor prognosis of patients with CRC.

Second, in our study, high LOX-1 expression in the tumor tissue was significantly related to poor prognosis (Fig. 4b). Positive staining for LOX-1, which is a transmembrane receptor [15], was mainly observed in the cytoplasm (Fig. 4a). Some studies have already shown that LOX-1 overexpression in tumor tissue is a significant indicator of poor prognosis and tumor progression in gastric cancer [20], pancreatic cancer [21], and CRC [22], supporting our results. Li et al. [20] found that LOX-1 promotes the migration, invasion, and epithelial–mesenchymal transition of gastric cells through the PI3K/Akt/GSK3β pathway, which regulates epithelial–mesenchymal transition [34], metastasis [35], and progression of various cancers. Indeed, our results indicated that the level of serum LOX-1 was significantly high in patients with lymphatic and/or hematogenous metastases

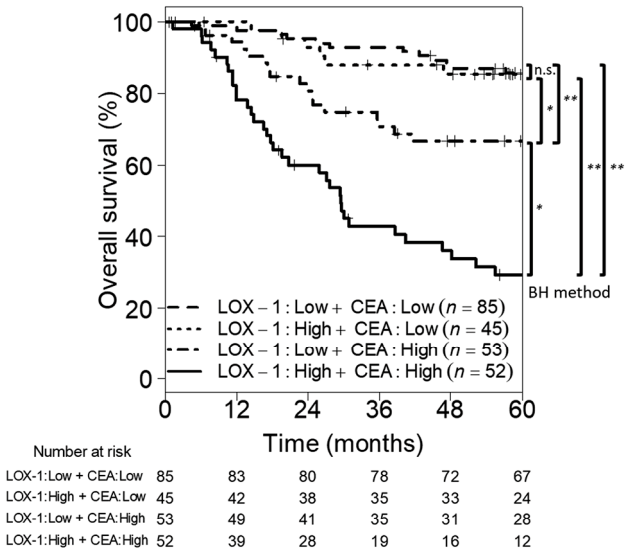


**Fig. 3** Overall survival according to high and low cut-off values of LOX-1 and CEA. The cut-off values to distinguish high and low levels were 538.7 (pg/mL) and 10 (ng/mL) for LOX-1 and CEA, respectively. *LOX-1* Lectin-like oxidized low-density lipoprotein receptor-1, *CEA* carcinoembryonic antigen, *BH method* Benjamini and Hochberg method, * $p<0.01$, ** $p<0.001$, *n.s.* not significant

(Table 2). Although prognostic markers are truly important for stage I and II patients, LOX-1 might be useful only in advanced CRC. LOX-1 was not an independent prognostic factor when analyzed with tumor factors such as lymph node metastasis and distant metastasis, which were very strong indicators of prognosis (Table 5); however, LOX-1 and CEA were independent prognostic factors as peripheral blood markers derived from liquid biopsy (Table 4). CEA and CA19-9 are well-known CRC tumor markers. In this study, CA19-9 was not an independent prognostic factor. In the analysis of OS, prognosis was significantly poorer ($p<0.01$) when LOX-1 was higher in the group with high CEA, but there was no difference in prognosis based on LOX-1 levels in the group with low CEA (Fig. 3). Although CEA is already known as a strong tumor marker, LOX-1 could be an additional prognostic marker. Murdocca et al. showed that LOX-1 expression correlates with the aggressiveness of cancer and that its downregulation diminishes the tumoral phenotype in vitro [22]; moreover, LOX-1 inhibition significantly prevents metastasis formation in vivo [36]. LOX-1 is thus a potential target for the inhibition of tumor progression and metastasis.

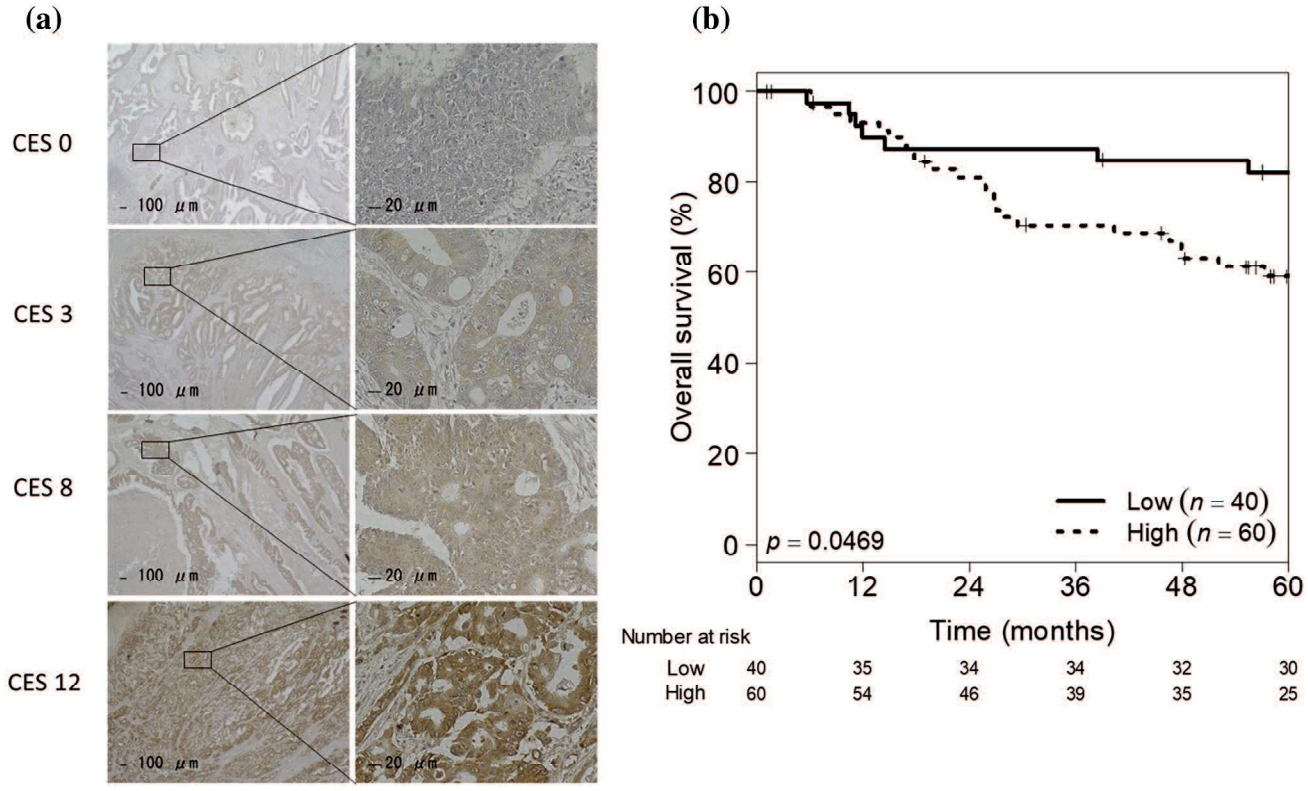


**Fig. 4** LOX-1 expression and its association with CRC prognosis and serum concentrations. **a** Representative IHC staining images of CES 0, 3, 8, and 12, in order. CES 0 (intensity score: 0, frequency score: 4), CES3 (intensity score: 1, frequency score: 3), CES8 (intensity score: 2, frequency score: 4), CES12 (intensity score: 3, frequency score: 4). **b** Overall survival in 100 patients with CRC. *LOX-1* Lectin-like oxidized low-density lipoprotein receptor-1, *IHC staining* immunohistochemical staining, *CRC* colorectal cancer, *CES* composite expression score

Finally, we showed that the white blood cell count, neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, and C-reactive protein level were significantly higher in patients with high levels of serum LOX-1 than in those with low levels (Table 3). These inflammatory factors might be involved in the release of LOX-1 from tissues or immune cells into the serum as previously reported, and all are indicators of immune suppression and poor prognosis in various types of cancer [10]. Among these inflammatory parameters, LOX-1 was the only independent biomarker detected in this study to predict the prognosis of CRC (Table 4).

Limitations of this study were its retrospective, single-institute study design, small number of patients, and the fact that patients were limited to stage III and IV, in addition to a lack of mechanistic studies. In conclusion, serum LOX-1 might be a useful biomarker of poor prognosis for patients with CRC. However, we cannot conclude that LOX-1 is directly responsible for cancer, but rather the results support its role in tumor progression through other molecular pathways. Therefore, further studies are needed to address this.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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