

# Serum Extracellular Vesicles Double-positive for CD9 and CD147 or for CD9 and CD63 Decrease After Surgical Resection in Patients With Colorectal Cancer: Potential Biomarkers of Residual Tumor

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## Abstract

**Background/Aim:** Extracellular vesicles (EVs), including exosomes, are abundant in body fluids, and EV surface proteins can be profiled as potential minimally invasive biomarkers. CD147 (EMMPRIN/Basigin) is a tumor-associated surface glycoprotein implicated in cancer progression. CD9 and CD63 are tetraspanin membrane proteins widely used as EV markers. The present study aimed to evaluate the clinical significance of serum EV subsets double-positive for CD9 and CD63, or for CD9 and CD147 in patients with colorectal cancer (CRC) before and after tumor resection.

**Materials and Methods:** Sixteen patients with CRC were recruited, and paired pre- and postoperative serum samples were analyzed for CD9<sup>+</sup> CD63<sup>+</sup> EVs and CD9<sup>+</sup> CD147<sup>+</sup> EVs. Serum EVs were quantified using the ExoCounter system with antibody-conjugated beads. CD147 expression in resected tumor tissue was analyzed by immunohistochemistry.

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**Results:** CD9<sup>+</sup> CD63<sup>+</sup> EVs were significantly reduced in postoperative samples compared with preoperative samples ( $p=0.0151$ ). CD9<sup>+</sup> CD147<sup>+</sup> EVs also showed a significant postoperative decrease ( $p=0.0186$ ). CD147 immunostaining was positive in 13 out of 16 resected tumors (81.3%). In patients with CD147 negative tumors ( $n=3$ ), serum CD9<sup>+</sup> CD147<sup>+</sup> EV levels were low at both pre- and postoperative time points and remained unchanged.

**Conclusion:** Serum CD9<sup>+</sup> CD63<sup>+</sup> and CD9<sup>+</sup> CD147<sup>+</sup> EV subsets decreased after CRC surgery, supporting their potential utility as minimally invasive liquid biopsy biomarkers of postoperative tumor burden. Future prospective clinical trials will determine whether these EV subsets are indeed biomarkers of postoperative residual tumor.

**Keywords:** Exosomes, extracellular vesicles, ExoCounter, colorectal cancer, CD63, CD9, CD147, serum, colectomy, biomarker, tumor burden.

## Introduction

Colorectal cancer (CRC) is the most frequently diagnosed malignancy in Japan and the second-leading cause of cancer-related mortality (1). Although advances in screening and treatment have contributed to a gradual decline in CRC mortality, this decrease has been relatively modest compared with that observed in other countries (2).

Curative-intent surgery remains the cornerstone of treatment; however, biomarkers that enable sensitive postoperative monitoring of residual disease and tumor burden would be clinically valuable for patient management and treatment planning.

Cancer cells secrete extracellular vesicles (EVs) into body fluids, and these lipid bilayer-delimited particles (particularly those within the 30-150 nm size range) carry proteins (including surface proteins), lipids, and nucleic acids (including mRNA, miRNA, and DNA) derived from donor cells (3, 4). EV cargo, including surface components, can indicate the molecular state of the originating cells. The lipid bilayer of EVs protects encapsulated biomolecules and enhances analyte stability in biofluids (5). Cancer-derived EVs can contribute to tumor microenvironment remodeling, cancer invasion, and the formation of pre-metastatic niches (6-8).

EVs are abundant in body fluids such as blood, urine, saliva, tears and vaginal secretions (9-13). EV-based liquid biopsy may enable minimally invasive and repeatable

sampling for longitudinal assessment of treatment response and sensitive surveillance after curative-intent cancer therapy. For example, EVs may be useful for monitoring minimal residual disease and recurrence (14).

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN) or basigin, is a transmembrane glycoprotein that promotes cancer progression by stimulating matrix metalloproteinase production, angiogenesis, and metastasis. Previous studies have shown that CD147 is overexpressed in CRC tissues relative to normal colorectal mucosa (15, 16). CD147 overexpression has also been shown to be associated with prognosis in CRC. Circulating CD147<sup>+</sup> EVs were previously shown to be a serum biomarker candidate of CRC prognosis (17-19). Circulating EV subpopulations with surface markers such as CD147 have been identified in patients with CRC (20, 21). EV surface markers in both serum and tears were identified in patients with breast cancer (22, 23). CD9 and CD63 are tetraspanin membrane proteins widely used as EV markers to quantify serum EVs.

In the present study, we quantified serum CD9<sup>+</sup> CD147<sup>+</sup> EVs and CD9<sup>+</sup> CD63<sup>+</sup> EVs in patients with CRC as potential biomarkers of postoperative tumor burden.

## Materials and Methods

**Clinical samples.** The present study was approved by the Institutional Review Board of Kobe University Graduate

Table I. Clinical characteristics of the 16 patients with colorectal cancer in the present study, including tumor size, pTNM classification (26), pathological stage, and tumor CD147 immunohistochemistry status (see Figure 3).

Patient no.	Age, years	Sex	Tumor size, mm	Histological type	pTNM classification*	pStage	CD147 status
1	75	Male	55×40	muc	pT4aN1aM1	IVc	-
2	49	Male	80×65	muc	pT4bN1aM0	IIIc	+
3	55	Male	55×25	tub2	pT3N0M0	IIa	+
4	51	Female	28×22	pap	pT2N0M0	I	-
5	68	Male	35×25	tub1	pT4aN0M0	IIb	-
6	40	Female	25×20	tub1	pT2N1aM0	IIIa	+
7	59	Male	20×16	tub2	pT1aN0M0	I	+
8	85	Male	58×28	tub2	pT3N1aM0	IIIb	+
9	71	Male	28×22	tub2	pT1bN0M0	I	+
10	24	Female	55×50	tub2	pT3N0M0	IIa	+
11	76	Female	15×10	tub1	pT1bN0M0	I	+
12	54	Male	60×60	tub2	pT3N1bM0	IIIb	+
13	50	Male	78×48	tub2	pT3N0M0	IIa	+
14	65	Female	55×55	tub2	pT3N1bM0	IIIb	+
15	60	Male	43×30	tub2	pT3N2aM0	IIIb	+
16	59	Male	15×13	tub2	pT1bN0M0	Ib	+

Muc: Mucinous adenocarcinoma; Pap: papillary adenocarcinoma; Tub1: well-differentiated tubular adenocarcinoma; Tub2: moderately differentiated tubular adenocarcinoma. According to the Japanese Classification of Colorectal, Appendiceal, and Anal Carcinoma (26).

School of Medicine and was conducted in accordance with the Declaration of Helsinki (1975). Additional approval was obtained from the Ethics Committee of Kobe University (approval no: B220170). Written informed consent was obtained from all patients.

Patients with histologically-confirmed colorectal cancer (CRC) considered resectable and underwent surgical resection at Kobe University Hospital between December 2023 and October 2025, were enrolled in the present study. A total of 16 patients were included (age range= 24-85 years; 11 men and 5 women). Serum samples were collected preoperatively and 11-120 days postoperatively (Table I). All samples were stored at -80°C and thawed on ice before EV analyses. Serum samples were visually inspected for hemolysis, and only hemolysis-free samples were included.

**Immunohistochemistry.** Immunohistochemistry was performed on formalin-fixed, paraffin-embedded resected-tumor sections. Slides were heated at 60°C for 30 min, deparaffinized in xylene, and rehydrated through graded ethanol solutions. Antigen retrieval was carried out in citrate buffer (pH 6.0) using microwave heating,

followed by cooling to room temperature. After washing with Tris-buffered saline containing 0.05% Tween 20 (TBS-T), sections were blocked for 10 min at room temperature. A primary CD147 antibody (1: 200; cat. no. 34-5600; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) diluted in Dako REAL Antibody Diluent (Dako, Agilent, Carpinteria, CA, USA) was applied (100 µl/section), and the slides were incubated overnight at 4°C in a humidified chamber. After washing with TBS-T, the sections were incubated with the Dako EnVision HRP anti-rabbit labeled secondary antibody (cat. no. K4003; Dako) for 1 h at room temperature. Color development was performed using the Dako DAB+ Substrate Chromogen System (cat. no. K3468; Dako, Agilent). The reaction was stopped by washing with TBS-T. Slides were counterstained with Mayer's hematoxylin, washed, dehydrated through graded ethanol, cleared in xylene, and mounted with coverslips (19). The stained histological sections were examined using a light microscope (BZ-X700; Keyence, Osaka, Japan). Immunostaining was independently evaluated by two investigators. CD147 expression was considered positive when ≥5% of tumor cells showed staining.

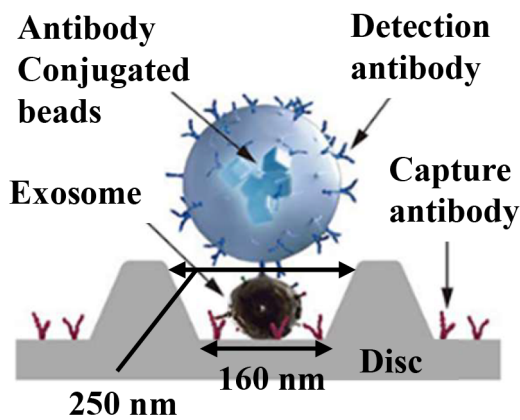


Figure 1. Schematic of the ExoCounter. Cartoon of extracellular vesicles (EVs) labeled with nanobeads on a disc. Each EV is captured into grooves on an optical disc coated with an antibody specific for EVs. The EVs are also recognized by a single bead labeled with another EV-specific antibody. Theoretically, one EV per bead can be counted (24).

**Preparation of CD147 antibody-conjugated nanobeads.** The CD147 antibody (clone HIM6; cat. no. 555961; BD Biosciences, San Jose, CA, USA) was biotinylated using the Biotin Labeling Kit-NH2 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Avidin-conjugated beads were washed with phosphate-buffered saline (PBS) and then incubated with the biotinylated CD147 antibody to allow conjugation *via* avidin–biotin binding. The antibody-conjugated beads were washed again with PBS, and the buffer was subsequently replaced with HEPES buffer prior to use.

**EV quantification with an ExoCounter.** EV quantification was performed using an ExoCounter system (JVC KENWOOD, Kanagawa, Japan), which enables surface marker-based detection without bulk purification, as described previously (22, 24, 25) (Figure 1). Wells containing CD9 antibody-coated capture discs were washed three times with PBS containing 0.05% Tween 20 (PBS-T). The wells were then blocked with either 1% bovine serum albumin in PBS-T or a casein-based blocking solution for 30 min at 37°C and washed three times with PBS-T. Then 10 µl of serum diluted in 40 µl of PBS were

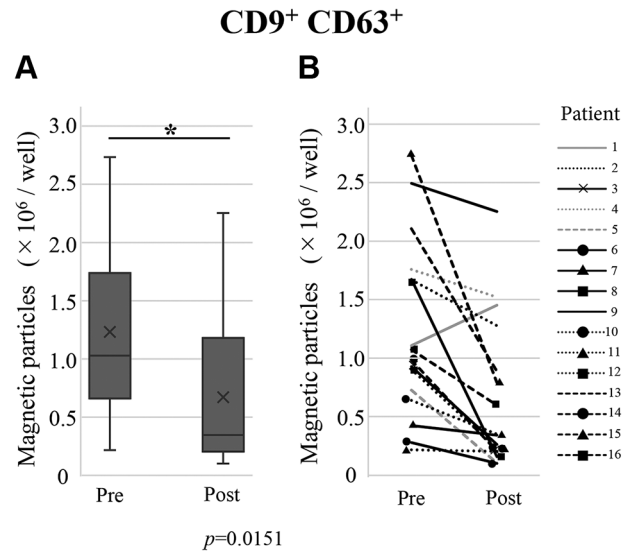


Figure 2. Quantification of CD9<sup>+</sup> CD63<sup>+</sup> extracellular vesicles (EVs) in paired pre- and postoperative serum samples from patients with colorectal cancer. (A) Box-and-whisker plot; the center line indicates the median, the × symbol indicates the mean, and the box represents the interquartile range (25<sup>th</sup>-75<sup>th</sup> percentiles). Each dot represents an individual patient. (B) Paired changes in serum CD9<sup>+</sup> CD63<sup>+</sup> EV levels for each patient. Gray lines indicate cases classified as CD147<sup>-</sup> by tumor immunohistochemistry, whereas black lines indicate cases classified as CD147<sup>+</sup> by tumor immunohistochemistry (Table I). Statistical significance was assessed using the Wilcoxon signed-rank test (\**p*=0.0151).

placed into each well. After incubation at 37°C for 2 h, the wells were washed three times with PBS-T. Then 50 µl of specific antibody-conjugated beads were added to each well, incubated at 37°C for 90 min, and washed three times with PBS-T. For quantification of CD9<sup>+</sup> CD63<sup>+</sup> EVs, commercially available beads pre-conjugated with an anti-CD63 antibody (provided with the assay kit) were used. For quantification of CD9<sup>+</sup> CD147<sup>+</sup> EVs, beads custom-conjugated with CD147 antibody were prepared as described above and used for the assay. Finally, the wells were then washed with deionized water. Beads bound to EVs on each disc were counted with the ExoCounter. The ExoCounter assay was performed using assay kits BX-EAK1JA and BX-EAK2JA. (JVC KENWOOD) (22, 25).

**Statistical analysis.** Comparisons between groups were performed using the Wilcoxon signed-rank test. All

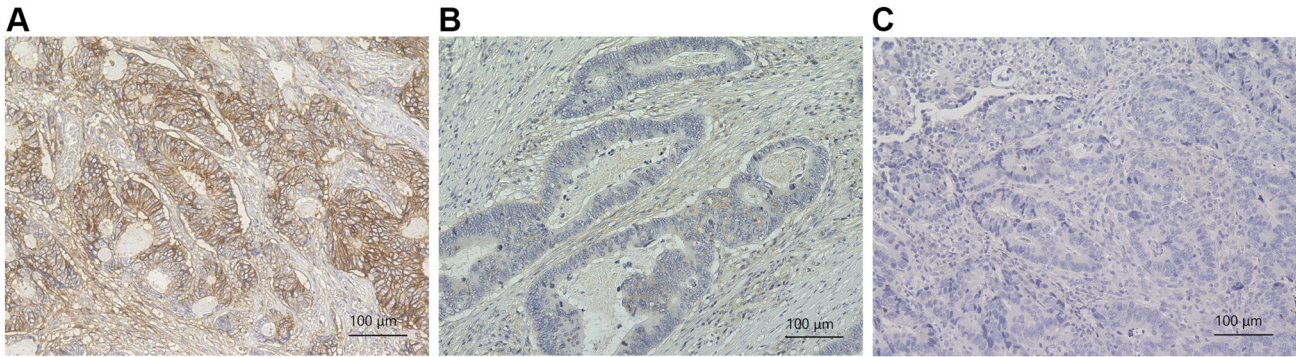


Figure 3. CD147 immunohistochemistry in resected colorectal cancer tissue. Representative images of CD147 immunostaining using a CD147 antibody are shown. Cases were defined as CD147 positive when staining was observed in >5% of tumor cells (A, and B) and as CD147 negative when staining was observed in ≤5% of tumor cells (C) (Scale bar=100 μm).

statistical analyses were performed using EZR version 1.68 (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

## Results

**CD9<sup>+</sup> CD63<sup>+</sup> EVs in pre- and postoperative serum from patients with CRC.** Using the ExoCounter, we quantified CD9<sup>+</sup> CD63<sup>+</sup> EVs in paired pre- and postoperative serum samples (n=16). The abundance of CD9<sup>+</sup> CD63<sup>+</sup> EVs in postoperative serum from patients with CRC was significantly reduced compared with preoperative serum ( $p=0.0151$ ; Figure 2).

**CD147 immunohistochemistry.** In the present study, cases were defined as CD147 positive when CD147 staining was observed in >5% of cancer cells in the resected tumors, with membranous and/or cytoplasmic staining (Figure 3A and B), and as CD147 negative when such staining was observed in ≤5% of cancer cells (Figure 3C). Tumor CD147 immunostaining status is summarized in Table I. Immunohistochemical analysis using a CD147 antibody demonstrated CD147 positivity in 13 out of 16 (81.3%) resected CRC specimens.

**CD9<sup>+</sup>CD147<sup>+</sup> serum EVs decreased after surgery.** CD9<sup>+</sup> CD147<sup>+</sup> EVs were significantly reduced in postoperative

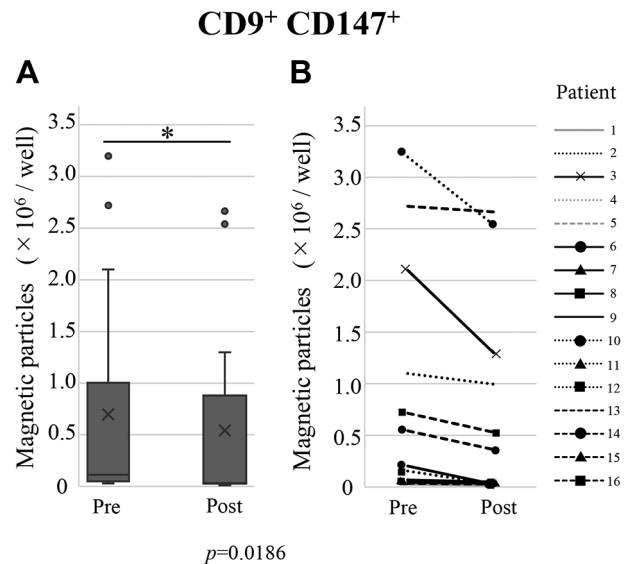


Figure 4. Quantification of serum CD9<sup>+</sup> CD147<sup>+</sup> extracellular vesicles (EVs) in paired pre- and postoperative serum samples from patients with colorectal cancer. Serum CD9<sup>+</sup> CD147<sup>+</sup> EV levels were quantified in patients (n=16) before and after curative-intent tumor resection. (A) Box-and-whisker plot; the center line indicates the median, the × symbol indicates the mean, and the box represents the interquartile range (25<sup>th</sup>-75<sup>th</sup> percentiles). Each dot represents an individual patient. (B) Paired changes in serum CD9<sup>+</sup> CD147<sup>+</sup> EV levels for each patient. Statistical significance was assessed using the Wilcoxon signed-rank test ( $*p=0.0186$ ). CD147 immunohistochemistry (IHC) was negative in tumors from patients 1, 4, and 5 (Table I).

serum compared with preoperative serum ( $p=0.0186$ ) (Figure 4). In the three cases that were CD147 negative by immunohistochemistry (Table I), the serum CD9<sup>+</sup> CD147<sup>+</sup>

EV levels were low at both time points, with minimal postoperative change.

## Discussion

In the present study, we quantified serum EV subpopulations in patients with CRC before and after surgery using the ExoCounter system. Both CD9<sup>+</sup> CD63<sup>+</sup> EVs and CD9<sup>+</sup> CD147<sup>+</sup> EVs significantly decreased after curative-intent tumor resection. In cases with CD147 negative tumors on immunohistochemistry, CD9<sup>+</sup> CD147<sup>+</sup> EV levels remained low, with minimal postoperative change. The present findings suggest that a substantial fraction of circulating CD9<sup>+</sup> CD147<sup>+</sup> EVs may originate from cancer cells. Notably, our cohort included a patient who underwent macroscopically incomplete (R2) tumor resection (patient 1); this patient was also negative for CD147 on immunohistochemistry, and CD9<sup>+</sup> CD63<sup>+</sup> EVs increased postoperatively, while CD9<sup>+</sup> CD147<sup>+</sup> EV levels remained essentially unchanged compared with the preoperative level. Collectively, these findings indicate that ExoCounter-based profiling of serum EV subsets may provide a feasible, minimally invasive approach for postoperative monitoring of tumor burden in CRC. However, CD147 is not exclusively expressed in cancer cells, and circulating CD147<sup>+</sup> EVs may also originate from non-cancer cells. Future clinical trials are needed to determine whether EV surface proteins can serve as accurate tumor biomarkers reflecting postoperative tumor burden in CRC.

*Study limitations.* The present study has several limitations. Firstly, the sample size was relatively small, and the findings should be interpreted with caution. Secondly, postoperative serum samples were collected within a limited time window. Larger studies with extended longitudinal sampling will be required to validate these findings, to assess temporal changes in levels of CD147<sup>+</sup> EVs, and to determine whether EV surface markers can serve as accurate biomarkers reflecting postoperative tumor burden in CRC, including their potential utility for postoperative surveillance and recurrence detection.

## Conclusion

Using the ExoCounter system, we quantified serum EV subsets in patients with CRC and found that both CD9<sup>+</sup> CD63<sup>+</sup> EVs and CD9<sup>+</sup> CD147<sup>+</sup> EVs significantly decreased after surgery. The present findings suggest that monitoring CD147 positive EVs may provide a feasible and minimally invasive approach for postoperative monitoring and for assessing postoperative tumor burden in CRC. Clinical trials are needed to validate the present findings and to clarify their clinical applications.

## Conflicts of Interest

The Authors declare that they have no conflicts of interest.

## Authors' Contributions

Y. Adachi, S. Inubushi, K. Yamashita and T. Matsuda contributed to the conceptualization and study design. Patient samples were collected by Y. Adachi, K. Yasuda, R. Ito, N. Shirakami, T. Tachibana, M. Imai, H. Kagiya, T. Tsuneki, M. Saito, Y. Sugita, T. Ikeda, Y. Koterazawa, T. Aoki, H. Harada, Y. Otowa, N. Urakawa, H. Goto, H. Hasegawa and S. Kanaji. Y. Adachi and S. Inubushi performed the investigation and formal analysis. Y. Adachi, S. Inubushi, K. Yamashita and T. Matsuda wrote the manuscript. R. M. Hoffman revised the manuscript. Y. Kakeji supervised the study. Funding was acquired by K. Yamashita and T. Matsuda.

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## Artificial Intelligence (AI) Disclosure

To improve clarity and readability, language-editing software and AI-assisted tools (ChatGPT-5.2, OpenAI) were employed solely for proofreading, stylistic polishing, and non-substantive suggestions on logical flow.

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