Colorectal Cancer-Derived CAT1-Positive Extracellular Vesicles Alter Nitric Oxide Metabolism in Endothelial Cells and Promote Angiogenesis

Atsushi Ikeda1,2, Satoshi Nagayama3, Makoto Sumazaki1,4, Makoto Konishi1, Risa Fuji3, Naomi Saichi1, Satoshi Muraoka1, Daisuke Saigusa5, Hideaki Shimada4, Yoshiharu Sakai2, and Koji Ueda1

ABSTRACT

Accumulating scientific evidences strongly support the importance of cancer-derived extracellular vesicles (EV) in organization of tumor microenvironment and metastatic niches, which are also considered as ideal tools for cancer liquid biopsy. To uncover the full scope of proteomic information packaged within EVs secreted directly from colorectal cancer, we cultured surgically resected viable tissues and obtained tissue-exudative EVs (Te-EV). Our quantitative profiling of 6,307 Te-EV proteins and 8,565 tissue proteins from primary colorectal cancer and adjacent normal mucosa (n = 17) allowed identification of a specific cargo in colorectal cancer–derived Te-EVs, high-affinity cationic amino acid transporter 1 (CAT1, P = 5.0 × 10−5, fold change = 6.2), in addition to discovery of a new class of EV markers, VPS family proteins. The EV sandwich ELISA confirmed escalation of the EV-CAT1 level in plasma from patients with colorectal cancer compared with healthy donors (n = 119, P = 3.8 × 10−5). Further metabolomic analysis revealed that CAT1-overexpressed EVs drastically enhanced vascular endothelial cell growth and tubule formation via upregulation of arginine transport and downstream NO metabolic pathway. These findings demonstrate the potency of CAT1 as an EV-based biomarker for colorectal cancer and its functional significance on tumor angiogenesis.

Implications: This study provides a proteome-wide compositional dataset for viable colorectal cancer tissue–derived EVs and especially emphasizes importance of EV-CAT1 as a key regulator of angiogenesis.

Introduction

Colorectal cancer is the third most commonly diagnosed cancer and responsible for the second largest number of cancer-related deaths (1). The five-year survival rate for localized colorectal cancer is 89.9%, whereas that for colorectal cancer with distant metastasis falls down to 14.2% (2). Therefore, detection of colorectal cancer at the earlier stages is essential for effective improvement of the patients’ survival. Carcinoembryonic antigen (CEA) is the most widely used tumor marker for colorectal cancer, but it is unsuitable for population screening due to its insufficiency in sensitivity (3). As well as CEA, stool-based tests, including immunochemical or guaiac fecal occult blood tests, are not ideal for screening for colorectal cancer because of low sensitivity and specificity (4). On the other hand, colonoscopy is the most promising diagnostic modality for a variety of disorders, including malignant diseases (5). On the other hand, colonoscopy is the most promising diagnostic modality for a variety of disorders, including malignant diseases (5). An unignorable cost (5–9). Moreover, it requires much skill for decent full-bowel examination (10). Thus, development of innovative diagnostics for colorectal cancer has been urgently needed, which satisfies both high sensitivity and less invasiveness.

Extracellular vesicles (EV) are small vesicles released from almost all cell types and play important roles in intercellular communications (11). EVs transport nucleic acids, proteins, metabolites, and other cellular components from donor cells to recipient cells, mediating phenotypic alterations in recipient cells (12–14). Recently, accumulating evidences suggest that EVs can serve as diagnostic modality for a variety of disorders, including malignant diseases (15). We also previously reported a novel strategy to identify EV-based protein biomarkers by comparative proteomic profiling of EVs derived from clear cell renal cell carcinoma (ccRCC; ref. 16).

In this study, we isolated EVs from surgically resected viable colorectal cancer tissues, which we termed as tissue-exudative EVs (Te-EV). We here show proteomic landscape of Te-EVs derived from primary colorectal cancer or adjacent normal mucosa (tumor Te-EVs or normal Te-EVs, respectively), in comparison with quantitative proteome profiles of original tissues. From these datasets, we determined a novel set of EV marker proteins and potential targets for EV-based colorectal cancer liquid biopsy. In particular, physiological roles of CAT1 protein on colorectal cancer–derived EVs as a mediating factor for angiogenesis in tumor microenvironment are demonstrated.

Materials and Methods

Chemicals and antibodies

Monoclonal anti-CD9 (#SHI-EXO-M01), anti-CD63 (#SHI-EXO-M02), and anti-CD81 antibodies (#SHI-EXO-M03) were purchased from Cosmo Bio. Polyclonal anti-rabbit IgG antibody (Alexa Fluor...
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Clinical samples
All clinical samples were obtained at Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo, Japan. The clinical and pathological information of the patients and healthy donors is summarized in Supplementary Table S1. Cancer staging was done according to the seventh edition of the TNM Classification of Malignant Tumors from Union for International Cancer Control. Written informed consents were obtained from all study participants. The procedures involving human subjects are in agreement with the Declaration of Helsinki. This research was approved by the institutional ethics review board.

Isolation of extracellular vesicles
Tissues of primary colorectal cancer and adjacent normal mucosa were freshly obtained from patients with colorectal cancer undergoing colorectal resection in our hospital. Isolation and purification of Te-EVs were performed as follows. The 3 to 5 mm cubes of the excised tissues were rinsed with PBS and incubated in 1.5 mL of RPMI-1640 with gentle rotation at 37°C for 3 hours. The culture medium was centrifuged at 3,000 × g for 5 minutes and then 12,000 × g for 30 minutes to remove larger debris. Te-EVs were isolated with centrifugation of 100,000 × g for 1 hour, and purified by two cycles of washing with 1 mL of PBS and centrifugation at 100,000 × g for 1 hour as a pellet. To generate CAT1-overexpressing EVs (CAT1-EVs), HCT116 cells were transfected with pCAGGS-FLAG-CAT1 vector using Lipofectamine 3000 according to the manufacturer’s instructions, followed by replacement of medium to RPMI-1640 with the Exosome-depleted FBS (Thermo Fisher Scientific, #A2720801) 24 hours after the transfection. For a control, mock-EVs were also prepared from the supernatant of the empty pCAGGS vector-transfected HCT116 cells. Following 48 hours incubation, the culture medium was collected and centrifuged at 2,000 × g for 5 minutes and then 10,000 × g for 30 minutes to remove larger debris. Ultracentrifugal purification of EVs was performed with the same manner as Te-EVs above. Protein concentrations of EV lysates were measured using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, #23235).

Protein extraction from tissues
Proteins were extracted from the original tissues (primary colorectal cancer or adjacent normal mucosa). Approximately 3 mm3 of the tissue was homogenized in 400 μL of phosphate transfer surfactant buffer [20 mmol/L, HEPES-NaOH (pH 7.6), 12 mmol/L sodium deoxycholate, 12 mmol/L sodium deoxycholate, 12 mmol/L sodium-L-arsenate] using a probe sonicator. After centrifugation at 15,000 × g for 15 minutes, the supernatant was quantified with the Micro BCA Protein Assay Kit.

LC/MS analysis
Protein samples (10 μg) were dissolved in Laemmli’s SDS sample buffer, reduced with 20 mmol/L TCEP at 37°C for 30 minutes, and then alkylated with 50 mmol/L iodoacetamide at ambient temperature in a dark for 45 minutes. These samples were subjected to 10% polyacrylamide gel electrophoresis. The electrophoresis was stopped at the migration distance of 2 mm from the top edge of the separation gel. After CBB-staining, protein bands were excised, destained, and cut finely before in-gel digestion with Trypsin/Lys-C Mix (Promega, #V5073) at 37°C for 12 hours. The resulting peptides were extracted from gel fragments and analyzed by Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) combined with UltiMate 3000 RSLC nano-flow HPLC (Thermo Fisher Scientific) equipped with 0.075 × 150 mm C18 tip-columns (Nikkkyo Technos). A two-step linear gradient comprising 2% to 35% acetonitrile for 95 minutes and
35% to 95% acetonitrile for 15 minutes in 0.1% formic acid at flow rate of 250 nl/min was used. The eluates were directly ionized with a spray voltage of 2 kV. Spectra were collected by Full MS ion scan mode over the m/z range 350 to 1500 with 60,000 resolution. CID MS/MS scans were acquired with the Ion trap detector up to 2 seconds for each MS full scan event under data-dependent acquisition mode with the dynamic exclusion function enabled. The raw data are available at a public proteomic database, Japan Proteome Standard Repository/Database (jPOST). ID: JPST000867 (17).

**Identification and label-free quantification of proteins**

Protein identification and label-free quantification were performed on the Proteome Discoverer 2.2 software (Thermo Fisher Scientific). For protein identification, the LC/MS dataset was searched against SwissProt Human Database with Mascot (Matrix Science) or Sequest HT (Thermo Fisher Scientific) database search engine, where FDR <1% was set for peptide identification threshold. For label-free quantification and data normalization, the Minora Feature Detector node in the Processing workflow and the Feature Mapper node followed by the Precursor Ions Quantifier node in the Consensus workflow were used with default parameters in the Proteome Discoverer 2.2 software.

**Statistical analysis**

Proteomic compositions were compared between tumor Te-EVs and normal Te-EVs using the paired t test. Then the P values were adjusted to control FDR less than 5%, using the Benjamini–Hochberg procedure (18). Proteins satisfying the criteria (adjusted PC < 0.05, fold-change >5.0, and valid value >50%) were defined as statistically differential protein cargoes. Box plots, a violin plot, and ROC curves were depicted by R 4.0.0. Principal component analysis was performed by the Analyst module in Expressionist server platform (Genedata AG, Swiss). Continuous variables were analyzed using Student t test, and categorical valuables with Pearson's χ² test or Fisher's exact test, as appropriate. Statistical analysis was performed using JMP software version 13.0 (SAS Institute). A P value of < 0.05 was considered statistically significant.

**Western blotting analysis**

Cells were lysed with RIPA buffer (50 mmol/L Tris-HCl (pH 7.8), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 1 mmol/L EDTA) containing complete protease inhibitor cocktail (Roche Diagnostics, #1187358001). Proteins were separated on 8%–12.5% SDS polyacrylamide gels and transferred onto PVDF membranes (Merck-Millipore, #IPVH00010). Following blocking on 8%–12.5% SDS polyacrylamide gels and transferred onto PVDF membranes (Merck-Millipore, #IPVH00010), protein blotting was performed using 4% Block Ace (Yukijirushi Nyugo, #UK-B80), membranes were incubated with the first antibodies. Membranes were then incubated with horseradish peroxidase (HRP)–conjugated anti-mouse IgG (GE Healthcare, #NA931–1ML) or anti-rabbit IgG (GE Healthcare, #NA934–1ML) and detected with Western Lightning ECL Pro (PerkinElmer, #NEL121001EA). Quantification of band intensity was performed using Image Lab software version 5.2 (Bio-Rad Laboratories).

**IHC analysis**

A total of 80 sets (20 sets for every stage) of colorectal cancer tissue and adjacent normal mucosa were sectioned in 5-μm thick by the microtome system. The deparaffinization, rehydration, and IHC were automatically carried out on the Leica Bond III Automated IHC and ISH system (Leica Microsystems Ltd.) with polyclonal anti-CAT1 antibody (Proteintech) and anti-CD31 antibody. The expression levels of CAT1 were classified into 3 categories: 0, no expression; 1, slightly expressed; and 2, moderately to strongly expressed. Using the categorized data, the expression levels of CAT1 of colorectal cancer tissue were compared with those of adjacent normal tissues.

**Transmission electron microscope analysis**

Transmission electron microscope (TEM) analysis was performed as previously reported (19). Briefly, EV samples (10 μg) were fixed with 2% paraformaldehyde and incubated with polyclonal anti-CAT1 antibody. Immunoactive EVs were visualized with the second antibody preadsorbed with the 20-nm gold (anti-mouse IgG antibody, Abcam, #ab2724) for Te-EVs or 40-nm gold (anti-rabbit IgG antibody, Abcam, #ab19180) for CAT1+−EVs and mock-EVs, and observed with H-7650 (HITACHI).

**EV sandwich ELISA**

After immobilization of the in-house anti-CAT1 antibody (250 ng) on the bottom of Nunc-immuno plate II (Thermo Fisher Scientific, #442404), surface of wells was blocked with 5% BSA in PBS for 30 minutes. Following 3 hours incubation of plasma samples (10 μL) with PBS (90 μL), wells were washed with PBS 3 times and incubated with 100 ng of biotinylated anti-CD81 antibody in 1% BSA for 1 hour. After 3 times wash steps, 100 μL of 30,000-fold diluted streptavidin poly-HRP40 (Stereospecific Detection Technologies, #SP40D30) was added and incubated for 45 minutes, followed by PBS washing. HRP activity was detected with 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, #34028). The absorbance at 450 nm was read by Sunrise microplate reader (Tecan). All procedures were done at room temperature. To normalize the absorbance data among the plates, we prepared standard curves by plotting different concentrations of purified CAT1+−EVs. In each plate, we defined the absorbance signal produced by 1.0 μg of CAT1+−EVs as 1 U. To construct a combination diagnostic model using EV-CAT1 and CEA, logistic regression model was used for maximization of AUC of the receiver operating characteristics (ROC) curve: log[(1−p)/p] = 1.45 − 14.1x1 − 0.56x2 (x1: EV-CAT1 concentration, x2: CEA concentration).

**EV incorporation assay**

To monitor a short-term EV incorporation, HUVECs (3.0 × 10⁴ cells/well) were seeded on 8-well chamber slides (0.7 cm²/well, Thermo Fisher Scientific) and cultured for 12 hours. PKH26GL-labeled (Sigma-Aldrich, #PKH26GL) CAT1+−EVs (6 μg) or PBS (as control) were added and incubated for 4 hours. Following 3 times washes by PBS, the cells were fixed with 4% paraformaldehyde. The slides were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, #H1500) and observed with fluorescent microscope (IX83, Olympus). To confirm a long-term EV-mediated transfer of CAT1 protein, HUVECs (5.0 × 10⁵ cells/well) were seeded on 24-well plates and cultured for 12 hours. CAT1+−EVs (5 μg)/PBS (as control) were then added and incubated for further 12, 24, or 36 hours. After blocking with 1% BSA and fixing with 4% paraformaldehyde, cells were incubated with polyclonal anti-CAT1 antibody (Abcam) for an hour, followed by subsequent staining with anti-rabbit IgG antibody (Alexa Fluor 488) for an hour. Finally, the slides were mounted with VECTASHIELD Mounting Medium with DAPI and observed with fluorescent microscope IX83.
Cell growth assay  
HUCVECs (2 x 10^3 cells/well) were seeded on 96-well plates and cultured for 24 hours. CAT1^-EVs (1 μg), mock-EVs (1 μg), or PBS (as control) were added and incubated for further 24, 48, or 72 hours. After incubation, cells were treated with Cell Counting Kit-8 reagent (Dojindo, #CK04) for 2 hours and the absorbance at 450 nm of the culture medium was read with Sunrise microplate reader.

Tube formation assay  
HUCVECs (8 x 10^5 cells/well) mixed with CAT1^-EVs (10 μg) or mock-EVs (10 μg) were seeded on Gelretex Matrix-coated 24-well plates (Thermo Fisher Scientific, #A1569601) and cultured for 16 hours. HUCVEC cultured in the matrix form structures that mimic a pseudo capillary pattern, leading to meshed tube networks. The cells were stained with 2 μg/mL of Calcein AM (Thermo Fisher Scientific, #C1430) at 37°C for 30 minutes and observed with fluorescent microscope IX83. Images were obtained at four non-overlapping, randomly selected fields at each well. The total length of the tube network was extracted from the images using NIH ImageJ software (ver. 1.52i) and Angiogenesis Analyzer plugin (20), available at the website: http://image.bio.methods.free.fr/ImageJ/Angiogenesis-Analyzer-for-ImageJ&lang=en.

Metabolome analysis  
HUCVECs were transfected with LV-CAT1 or LV-mock (HUCVEC-CAT1 or HUCVEC-mock, respectively) and polybrene (10 μg/mL, final). After serum starvation for 12 hours, HUCVEC-CAT1 or HUCVEC-mock cells (5 x 10^5) were stimulated with the endothelial cell growth medium for 15 minutes. Cells were rinsed with ice-cold PBS twice and immediately scraped off from dishes. For analyses of arginine, citrulline, NG-Hydroxy-L-arginine (NHA), guanosine triphosphate (GTP), nicotinamide adenine dinucleotide phosphate (NADPH), and nicotinamide adenine dinucleotide phosphate (NADP), 200 μL of methanol, including 15C6-Arginine (1 μg/mL) as an internal standard (IS), was added and stirred in an ultrasonic bath. Samples were centrifuged at 16,400 x g, 4°C for 10 minutes, and 130 μL of the supernatants were concentrated to 40 μL by centrifugal evaporator. Finally, 3 μL of the concentrated sample was subjected to LC-MS/MS analysis. Analytical methods for multiple reaction monitoring (MRM) were described previously (21). Analysis of cyclic guanosine monophosphate (cGMP) was performed as reported previously with several modifications (22, 23). The cell pellet was resuspended in 100 μL homogenization buffer containing 10 mmol/L Tris-HCl (pH 7.4), 10% glycerol, and 10 mmol/L citrulline-2,4,15C6,15N6 as an IS. After homogenization, proteins were removed by adding 350 μL of acetonitrile, followed by centrifugation at 16,400 x g, 4°C for 15 minutes. Subsequently, 405 μL of the supernatant was vacuum-dried and dissolved in 90 μL of 0.1% (v/v) formic acid in water. The injection volume was 10 μL. The metabolites were measured using the LC/MS system (TSQ Quantiva, Thermo Fisher Scientific) equipped with NANOSPACE SI-2 (OSAKA SODA). Chromatographic separation was performed using ZIC-PhILIC column (2.1 x 100 mm, Sequant) for arginine, citrulline, GTP, NADPH, NHA, and NADP analyses or Discovery HS F5 column (2.1 x 150 mm, Sigma-Aldrich) for cGMP analysis. Data acquisition and analysis were done by Xcalibur software (Thermo Fisher Scientific). All analyses were done in triplicate.

cGMP ELISA  
HUCVECs (1 x 10^5) were incubated with 80 μg of CAT1^-EVs or mock-EVs for 24 hours. After serum starvation for 12 hours, HUCVECs were stimulated with the endothelial cell growth medium for 15 minutes. Collected cells were lysed and subjected to cGMP ELISA. The assay was performed using cGMP ELISA Kit (Cayman Chemical, #581021) according to the manufacturer’s instruction with acetylating protocols.

Results  
Isolation of extracellular vesicles from viable-resected tissues  
Figure 1A shows a schematic overview illustrating isolation of Te-EVs from surgically resected colorectal cancer or adjacent normal mucosa (n = 17). Three hours incubation of tissues with gentle rotation secured a sufficient amount of Te-EVs (98.7 ±76.7 μg total EV proteins). When the efficiency of EV enrichment was assessed with western blotting analyses, expression level of typical EV marker proteins, CD63 and CD61, were significantly higher in Te-EV samples than those in total tissue lysates (Fig. 1B and C). These molecular features and results of subsequent proteome profiles (Fig. 2B, C and D) indicated that EVs were efficiently released and collected from viable colorectal cancer tissues as Te-EVs.

Comprehensive proteome analysis of Te-EVs and original tissues  
From LC/MS analyses, 8,565 tissue proteins and 6,307 Te-EV proteins (Supplementary Tables S2 and S3, respectively) were identified with peptide FDR <1% (9,854 nonredundant proteins in total). Among them, 5,018 proteins were commonly identified in both tissues and Te-EVs (Fig. 2A), for which, interestingly, only limited quantitative relationship was observed (R^2 = 0.185, Fig. 2B). This fact represented a large compositional difference between EV proteome and the original cell proteome, suggesting that highly selective loading of protein cargoes may occur during construction of EVs. Indeed, as reported previously, tetraspanin family proteins were significantly enriched in EVs compared with intracellular levels (Fig. 2C). Notably, we found for the first time that vacuolar protein sorting (VPS) family proteins were more characteristically loaded in EVs than those in total tissue lysates. As reported previously, tetraspanin family proteins were significantly enriched in EVs compared with intracellular levels (Fig. 2C). Notably, we found for the first time that vacuolar protein sorting (VPS) family proteins were more characteristically loaded in EVs (Fig. 2D), which would be useful as specific EV luminal protein markers. As for the identified 6,307 total Te-EV proteins, 3,620 proteins (57.3%) were already cataloged in the most common EV database, ExoCarta, whereas we further added 2,687 new entries as EV cargo proteins (Fig. 2E). When focused on colorectal cancer cell–derived EVs, 6,166 proteins were found in Te-EVs from tumor regions of colorectal cancer tissues (tumor Te-EVs), in which 4,877 proteins (79.1%) were newly identified in this study (Fig. 2F). Thus, in addition to thoroughly optimized proteomic analysis by the high-end LC/MS system, our strategy for isolation of the high-purity EVs in serum-free media allowed such in-depth analysis of EV proteins (>10^6 of dynamic range, Supplementary Fig. S1), including discovery of a new class of EV marker proteins, the VPS family. Indeed, to our knowledge, the dataset to 6,307 protein identification is the largest one ever as a report of single set of EV proteomics analysis.

Specific protein cargoes on colorectal cancer cell–derived EVs  
To clarify specific molecular signatures in viable colorectal cancer tissues–derived EVs, which should serve as ideal targets of colorectal cancer diagnosis or therapy, a paired t test was used for comparison of proteome in normal Te-EVs with that in tumor Te-EVs, then the P values were adjusted to control FDR less than 5%. As the result, 487 proteins (Supplementary Table S4) were found to be significantly enriched in tumor Te-EVs (adjusted P < 0.05 and fold-change >5.0), whereas 88 proteins were diminished (adjusted P < 0.05 and fold-
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Figure 1.
Extraction of Te-EVs from colorectal cancer tissues. A, Schematic illustration for preparation and proteomic analysis of Te-EVs is shown. Te-EVs were isolated from freshly resected primary colorectal cancer tissues or adjacent normal mucosa (n = 17). Comprehensive proteome analysis was performed with LC/MS for Te-EVs and also original tissues. B, Evaluation of molecular characteristics of Te-EVs by western blotting of EV marker proteins, CD63 and CD81. 10 μg of protein was loaded to each lane. C, Relative protein abundances of CD63 or CD81 in (B) were shown in the bar charts. N, adjacent normal mucosa; T, primary colorectal cancer.

change <0.2; Fig. 3A). Principle component analysis confirmed that top 100 of the upregulated proteins in tumor Te-EVs were sufficient to classify 17 pairs of samples into two groups, normal mucosa tissue–derived EVs or colorectal cancer tissue–derived EVs (Fig. 3B). Interestingly, gene ontology (GO) analysis of the 487 proteins revealed that regulators of gene expression were highly loaded into tumor Te-EVs, whereas response elements against external stimulations were purged from them (Supplementary Fig. S2). Such characteristic selection of protein cargoes may define the modes of functional manipulation of the colorectal cancer microenvironment by EVs.

For the purpose of establishing a novel tool for colorectal cancer liquid biopsy based on the dataset above, we further focused on 11 cell surface proteins out of 487 cargoes specifically loaded in tumor Te-EVs (Supplementary Table S5), for which direct detection with “EV sandwich ELISA” is applicable. The extraction of the cell surface proteins was done by selecting proteins that were annotated to contain a transmembrane domain, available at the Uniprot database. Particularly, solute carrier family 7 member 1/high-affinity cationic amino acid transporter 1 (SLC7A1/CAT1) was estimated to be a solid candidate of a colorectal cancer biomarker due to its overexpression in colorectal cancer tissues (24). As the result of our LC/MS-based label-free quantification, the expression levels of CAT1 were significantly higher on tumor Te-EVs compared with those on normal Te-EVs (P = 5.0 × 10^-8, fold change = 6.2, Fig. 3C). The expression of CAT1 on the surface of tumor Te-EVs was ensured by the gold-labeled anti-CAT1 antibody and TEM in Fig. 3D. The expression of CAT1 was observed in 32.4% of tumor Te-EVs, whereas none of normal Te-EVs expressed CAT1 on their surfaces. The result of LC/MS analysis was confirmed by western blotting analysis using Te-EVs from the independent sample set (n = 10, Fig. 3E), showing significantly higher expression of CAT1 on tumor Te-EVs (P = 0.01, Fig. 3F).

To figure out the origin of CAT1++-EVs, we next evaluated CAT1 expression levels in tissue samples by IHC staining analysis of the colorectal cancer tissue array slide (n = 75). As shown in the representative images (Fig. 3G), significantly higher expression of CAT1 was observed in colorectal cancer cells, whereas none or weak expression was only detected in normal colon mucosa (P = 6.4 × 10^-8), although stage-dependent tendency was not observed (Fig. 3H). This result could support an idea that the CAT1-overexpressed EVs (CAT1++-EVs) might be biogenerated and released from colorectal cancer cells.

A diagnostic potential of plasma EV-CAT1 for detection of colorectal cancer

To evaluate a diagnostic power of EV-CAT1 as a peripheral blood biomarker for colorectal cancer, we constructed a high-throughput EV sandwich ELISA (Fig. 4A) that allowed simultaneous quantification of CAT1 abundances on EVs from 96 crude plasma samples. The result of EV-CAT1 measurement from 119 cases (25 health donors, 23 colorectal cancer stage I patients, 25 stage II patients, 25 stage III patients, and 21 stage IV patients) was displayed as a box plot in Fig. 4B. Statistical assessments revealed that the concentrations of EV-CAT1 in
colorectal cancer patients’ plasma (0.22± 0.24 U) were significantly elevated compared with those in healthy donors’ plasma (0.082 ± 0.10 U/mL; \( P = 3.8 \times 10^{-7} \)). Importantly, the escalation of EV-CAT1 level was observed even in the stage-I group (0.19 ± 0.17 U; \( P = 1.8 \times 10^{-6} \)), indicating high potency for detection of colorectal cancer.

In comparison with the existing biomarker CEA in the same cohort (\( n = 119 \); Supplementary Table S1) by ROC curve analysis, the detection efficacy of EV-CAT1 was slightly better [AUC, 0.821; 95% confidence interval (CI), 0.732–0.909] than CEA (AUC, 0.780; 95% CI, 0.690–0.870; Fig. 4C). But, due to independent tendency of positivity (Supplementary Fig. S3), a combination diagnostic model using EV-CAT1 and CEA demonstrated much better detection

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**Figure 2.** Proteomic overview of colorectal cancer Te-EVs and tissues. A, The Venn diagram shows number of identified proteins in colorectal cancer tissues or Te-EVs by LC/MS analysis. B, Quantitative relationship was assessed between tissue proteome and Te-EV proteome. The plots show proteins that were commonly identified both in Te-EVs and tissues by LC/MS analysis. The x and y axes represent averaged relative protein abundances. The right shows the magnified view around the origin. Coefficient of determination (\( R^2 \)) is shown. C, Representative EV marker proteins, CD9, CD63, and CD151 were highly enriched in Te-EVs compared with tissues. D, A newly identified class of EV luminal markers, vacuolar protein sorting (VPS) family proteins, also demonstrated significantly higher expressions in Te-EVs than those in tissues. E, The Venn diagram shows comparison between the deposited proteins in the ExoCarta database and the identified total Te-EV proteins in this study. F, The Venn diagram shows comparison between the deposited proteins as colorectal cancer EV proteins in the ExoCarta database and the identified proteins from tumor region–derived Te-EVs in this study.
Figure 3.
Identification of CAT1 as a specific surface antigen on CEC-derived EVs. A, The result of differential analysis based on paired t test between proteome from tumor region-derived EVs (tumor Te-EVs) and that from normal mucosa-derived EVs (normal Te-EVs) is displayed as the volcano plot. Significantly upregulated 487 proteins (adjusted P< 0.05 and fold-change >5.0) or downregulated 88 proteins (adjusted P< 0.05 and fold-change <0.2) were indicated in red or blue dots. B, Principal component analysis was performed for top 100 upregulated proteins in (A). C, The line chart shows LC/MS-based relative protein abundances of CAT1 on EVs in 17 paired samples. D, A representative image of transmission electron microscopy (TEM) for tumor Te-EVs. The black dot on the surface of the EV indicates the signal of the gold particle-labeled anti-CAT1 antibody; bar, 200 nm. The expression of CAT1 was positive in 24 out of 74 (32.4%) of tumor Te-EVs whereas none (0/66) of normal Te-EVs were CAT1 positive. E, Expression of CAT1 and CD9 in normal (N) and tumor (T) Te-EVs was confirmed by western blotting. F, The CD9-normalized protein abundances of CAT1 in (E) were displayed with the box plot. G, Representative images of IHC staining of CAT1 in colorectal cancer tissues. The expression levels of CAT1 were classified into 3 groups as indicated; bars, 50 μmol/L. H, The result of IHC staining of CAT1 for 75 samples was summarized with the violin plot.

N, normal colon mucosa tissue; I–IV, tissues of pathological stage I–IV colorectal cancer.
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Figure 4.
The diagnostic potential of plasma EV-CAT1 for detection of colorectal cancer. A, The framework of anti-CAT1 and anti-
CD81 EV-sandwich ELISA is shown. B, biotin; SA, streptavidin; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-tetramethylben-
zidine. B, The result of EV-CAT1 sandwich ELISA measuring 119 plasma samples is displayed as the box plot. N, normal
donors; I–IV, plasma from pathological stage I–IV patients with colorectal cancer. The P values were calculated by
Student t test. C, The diagnostic potential to distinguish patients with colorectal cancer (n = 94) from normal donors (n = 25) was assessed by ROC curve analysis. In addition to the single usage of EV-CAT1 or CEA, the combination bio-
marker model, EV-CAT1 + CEA, was also evaluated based on logistic regression. AUC, area under the curve.

EV-mediated transfer of CAT1 promotes growth of vascular endothelial cells and enhances tubule formation in colorectal cancer microenvironments

According to the datasets above, it was a fact that CAT1 protein was overexpressed on both colorectal cancer cells and colorectal –
derived EVs. However, a physiological relevance of CAT1 overexpression on EVs to colorectal cancer development was still unclear.
Therefore, to explore preferential recipient cells of CAT1++-EVs, CAT1-upregulated cells (except for cancer cells) were searched in the
microenvironments of colorectal cancer tissues using IHC staining analysis (Fig. 5A). From this observation, interestingly vascular endothelial cells near or inside colorectal cancer tissues showed significantly stronger signals of CAT1 than those in adjacent normal tissues (Fig. 5B). Considering high expression of CAT1 on tumor Te-
EVs, we speculated that colorectal cancer cells might transfer CAT1 protein to vascular endothelial cells via EVs.

To clarify this functionally, we at first prepared CAT1++-EVs from CAT1-overexpressed HCT116 cells (Fig. 5C), for which specific expression of CAT1 was confirmed by Western blotting analysis (Fig. 5D). Moreover, TEM images depicted the CAT1 expression on surface of EV membrane (Fig. 5E). Then we treated vascular endo-
thelial cell HUVEC with CAT1++-EVs and investigated their behavior and influence on HUVEC in vitro. After 4 hours exposure of fluo-
rescence-labeled CAT1++-EVs to HUVEC, lots of sporadic fluorescent signals were observed in the cells (Fig. 5F), showing that CAT1++-EVs were incorporated into intracellular region of HUVEC. From an elongated period of observation (after 3 days), we found a dotted pattern of CAT1 expression on the surface of cells (Fig. 5G).

This image clearly demonstrated that an amino acid transporter protein CAT1 was finally reconstituted to its innate subcellular location after EV-mediated transportation. The transportation of CAT1 via EV was supported by higher expression of CAT1 observed in CAT1++-EV-treated HUVECs compared with those treated with mock-EV (Fig. 5H).

Importantly, the CAT1++-EV-treated HUVECs, which showed ectopic CAT1 expression (Fig. 5H), exhibited significantly upregulated growth speed compared with those treated with mock EVs (n = 3, P = 0.02; Fig. 5i) Western blotting analysis of HUVEC used in the cell growth assay showed high expression of CAT1 in HUVEC treated with CAT1++-EV compared with HUVEC treated with PBS or mock-EV. When the CAT1++-EV-treated HUVECs were placed in thin 3D Matrigel culture plates, these cells also showed faster formation of tubular structures (Fig. 5J) and K. P = 0.01). These data suggested that EV-mediated excessive supply of CAT1 to vascular endothelial cells might promote angiogenesis in colorectal cancer microenvironment.

Overexpressed CAT1 promotes angiogenesis by modulating cGMP metabolism

Considering CAT1’s well-known function of transporting extracellular arginine, which is a substrate for the synthesis of key angiogenic modulator NO in the vascular endothelium (25, 26), we focused on NO-signaling pathways to clarify the molecular mechanism of pro-
angiogenic effect exerted by CAT1++-EVs. For functional profiling of tumor Te-EVs, we performed GO-based analysis on biological process and molecular function, using the lists of 487 upregulated and 88 downregulated proteins in tumor Te-EVs. The results revealed that proteins associated with regulation of nitrogen compound metabolic process (GO:0010127) and organonitrogen compound biosynthetic process (GO:1910566) were significantly enriched (Supplementary Table S6A–S6D). For instance, key regulators of the NO synthesis pathway, such as NAD(P)H dehydrogenase [quione] 1 (NQO1; fold efficiency (66.7% sensitivity, 92.0% specificity, and AUC, 0.907; 95% CI, 0.850–0.963). These data could encourage us to conduct further efforts about EV-CAT1 using a larger cohort for more solid confirmation as a clinically-applicable biomarker in the future study.
Figure 5.
CAT1-dependent promotion of cell growth and tubule formation of HUBEC via EVs. A, Representative images of IHC staining for CAT1 (A, C, E, G, I, K) or CD31 (B, D, F, H, J, L) are shown. CD31 (PECAM1) was stained as a marker for vascular endothelial cells. Serial sections were used for the comparison between the IHC staining of CAT1 and CD31. B, The rate of moderate to strong-CAT1 expression in vascular endothelial cells of or near colorectal cancer tissues was significantly higher than that of adjacent normal tissues. C, The high expression of CAT1 in HCT116 cells transfected with pCAGGS-CAT1-FLAG (CAT1-overexpressing HCT116) compared with those transfected with pCAGGS-FLAG (Control HCT116) was observed. D, The expression level of CAT1 or CD9 was examined for EVs purified from CAT1-overexpressed HCT116 cells (CAT1++-EVs) or mock-transfected cells (mock-EV). E, A representative image of TEM for mock-EV or CAT1++-EV is shown. The black dot on the surface of the EV indicates the signal of the gold particle-labeled anti-CAT1 antibody; bar, 50 nm. F, Fluorescent microscopic images of HUVEC 4 hours after treatment with PBS or fluorescence-labeled CAT1++-EV; bar, 5 μm. G, Fluorescent microscopic images of HUVEC 72 hours after treatment with PBS or CAT1++-EV. The expression of CAT1 was detected by the Alexa 488-labeled secondary antibody; bar, 5 μmol/L. H and I, The expression of CAT1 (H) or the growth activity (I) was measured in HUVEC 72 hours after treatment with PBS, mock-EV, or CAT1++-EV. Each error bars are presented as the mean SE (n = 3). J, Representative images of tube formation assay. 72 hours after treatment with PBS, mock-EV, or CAT1++-EV, HUVEC was cultured in Matrigel plates for 24 hours. K, The relative total tube length in (J) was measured by ImageJ. Each error bar is presented as the mean SE (n = 3).
change = 19.5; ref. 27), Dihydrololate reductase (fold change = 5.9; ref. 28), FAD synthase (fold change = 6.3; ref. 29), and Seliapirin reductase (fold change = 5.7; ref. 30) were linked to these GO terms, indicating that NO production in the EV-targeted cells would be promoted concurrently with CAT1 (31). To confirm NO signaling pathway initiated by CAT1 in vascular endothelial cells, MRM-based targeted metabolome analysis was performed using CAT1-overexpressed HUVEC (HUVEC-CAT1) or mock vector-transfected HUVEC (HUVEC-mock; Fig. 6A). Absolute quantification measurements for NO-related metabolic pathways revealed that arginine, nicotinamide adenine dinucleotide phosphate (NADP), and cGMP were significantly upregulated in HUVEC-CAT1 after serum stimulation (Fig. 6B). On the contrary, GTP was downregulated in HUVEC-CAT1 compared with HUVEC-mock. Direct measurement of cGMP in HUVEC treated with CAT1-+-EVs revealed significantly higher concentration than in HUVEC treated with mock-EVs, which confirmed the metabolic alteration via EVs in physiologic context (Fig. 6C). Considering that cGMP activates cGMP-dependent protein kinase (PKG) that was reported to promote angiogenesis through activation of both ERK and p38 signaling pathways (32), it is suggested that overexpressed CAT1 in HUVEC, brought by CAT1-+-EV, may contribute to angiogenesis in the microenvironments of colorectal cancer (Fig. 6D).

Discussion

In this study, 6,307 protein components of EVs were cataloged, which directly derived from resected fresh tissues of patients with colorectal cancer (Te-EVs). In fact, both the comprehensiveness, protein IDs, and the quantitative depth of this EV proteome analysis (×106) are maximum at present (Supplementary Fig. S3). It is notable that the number 6,307 protein IDs exceeded the ExoCarta repository (5,875 proteins) composed of previously published EV proteome datasets (33). For in-depth omics analysis of EVs, thorough purification of EVs, as well as optimal analytical technologies, is indispensable. From this point of view, the unique characteristics of Te-EVs fit the requirement well. Because the viable tissue blocks are cultured in serum-free medium for a short term, only minimal amount of free proteins (such as albumin, IgG, IgA, transferrin, and so on) are included, meaning that excessive purification procedures are not needed for obtaining high-purity EVs. More importantly, in contrast with blood- or urine-derived EV samples, Te-EVs do not contain whole-body-derived EVs, allowing specific molecular profiling of disease site-derived EVs. It is also critically important that a pair of tumor- and normal tissue-derived EVs with an identical genetic background is available individually. This fact facilitates precise identification of cancer-specific cargoes in tumor tissue-derived EVs regardless of noises of individual variations. In addition, Te-EVs are amplifiable by elongated culture duration, suggesting that a wide range of EV analyses would be practicable even from tiny biopsy samples or precious clinical specimens. Indeed, all the advantages above lead our first challenge about the study of Te-EVs from ccRCC to successful identification of a potential diagnostic biomarker on EV (EV-AZU1) that showed specific load on the surface of ccRCC-derived EVs (n = 20, P = 2.85 × 10⁻⁹). Fold change = 31.6; ref. 16). Furthermore, an EV-mediated key regulator of Akt signaling in ccRCC, EV-LAIR1, was also found, which would possess a great potential as a target of ccRCC therapy (34). Thus, including the present study about colorectal cancer, application of Te-EV could accelerate biological elucidation of molecular characteristics of physiologically secreted EVs from any solid tumors and also clinical development of EV biomarkers and therapeutics.

In addition to 6,307 EV proteins, here we acquired qualitative profiles of 8,565 proteins in original tissues (in total 9,984 nonredundant proteins). Quantitative and qualitative comparison between EV proteome and original tissue proteome could help to settle the important question of whether a subset of protein cargoes would be actively transported into EVs or passively distributed to EVs (Supplementary Fig. S4; Supplementary Table S7). The proteomic distribution of subcellular location in Te-EVs is similar with that in original tissues (Supplementary Fig. S5). Considering that the quantitative expressions of Te-EV proteome are not relative to that of original tissue proteome (Fig. 2B), there should be a mechanism of active transportation of certain proteins into EVs regardless of their subcellular locations. In addition to identification of a new class of EV luminal markers, the VPS family (Fig. 2D), this knowledge base would make a large contribution to unravel the biogenesis of EVs and the biological significance of their existence.

In the present study, we showed involvement of CAT1 in activation of the NO metabolic cascade. Considering the function of NO as a direct effector for tumorangiogenesis (25, 26), tumor Te-EVs may play a role to promote angiogenesis in the tumor microenvironments. Colorectal cancer cells might secrete “customized” EVs for transferring such proteins to mediate the phenotype of EV-targeted cells, leading to microenvironment alteration favorable for their survival, development, or metastasis. On the other hand, KEGG pathway analysis for 487 proteins above demonstrated enrichment of pathways related to viral infections (Supplementary Table S8A. For analysis of down-regulated proteins, see Supplementary Table S8B). Previous study reported high prevalence of colorectal cancer in virus-infected patients including human papilloma virus and Epstein-Barr virus (35). This fact may reflect the passive transport of proteins related to viral infections, transforming the environment into favorable one for colorectal cancer expansion.

Recently, EVs have been attracting a fair amount of interest from increasing researchers as a promising tool for diagnostic and prognostic biomarkers (36, 37). In this study, the plasma EV-CAT1 measurement demonstrated a good potential to be used for detection of earlier stages of colorectal cancer cases (Fig. 4B). Indeed, EV-CAT1 complemented the sensitivity of CEA at the earlier stages of colorectal cancer, resulting in reinforcement of diagnostic power in the combination model of EV-CAT1 and CEA, compared with the single use of CEA (Fig. 4C). Importantly, the experimental procedures of the EV sandwich ELISA are identical with those of the traditional sandwich ELISA, which can be smoothly automated with a similar cost of general tumor markers. Given these features and the fact that the detection property of EV-CAT1 was independent of CEA (Supplementary Fig. S3), a complementary use of EV-CAT1 measurement with CEA in a cancer screening would enhance an opportunity of detecting colorectal cancer by a noninvasive blood test, although larger-scaled preclinical validation study should be necessary.

CAT1 is one of the cationic amino acid transporter family proteins and considered as the major carrier of arginine, l-ysine, and ornithine (38–40). CAT1 transfers extracellular arginine directly to the membrane-bound endothelial NO synthase that is co-localized with CAT1 in caveolae (41), leading to production of NO. NO is reported to initiate various intracellular signaling pathways, including activation of soluble guanylate cyclase (sGC) which converts GTP to cGMP (42). Then cGMP binds to four sites on the regulatory subunits of cGMP-PKG, inducing activation of the catalytic subunit of PKG. In the present study, we visually showed EV-mediated transfer of CAT1 from colorectal cancer cells to the surface of vascular endothelial cells (Fig. 5E) and accumulation of arginine, NADP, and cGMP in...
Figure 6.

CAT1-dependent modulation of the NO metabolic pathway in vascular endothelial cells. **A**, Expression level of CAT1 was measured in mock-transfected HUVEC (HUVEC-mock) or CAT1-overexpressed HUVEC (HUVEC-CAT1). **B**, The results of quantitative metabolomic analysis for the arginine/NO/cGMP pathway were illustrated. Absolute quantification of the metabolites was performed for HUVEC-mock or HUVEC-CAT1 after stimulation with arginine for 15 minutes. Each error bar is presented as the mean SE (n = 4). **C**, Cyclic GMP concentrations of HUVEC treated with CAT1++-EVs or mock-EVs were compared. Each error bars are presented as the mean SE (n = 3). **D**, An illustration of CAT1 transfer from colorectal cancer cells to vascular endothelial cells via EVs in tumor microenvironment. Putative signaling pathway in a vascular endothelial cell from the arginine/NO/cGMP pathway to its downstream is shown. Arg, arginine; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide phosphate; NHA, L-n hydroxy-L-arginine; NO, nitric oxide; Cit, citruline; sGC, soluble guanylate cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PRAK, p38-regulated/activated kinase; FAK, Focal adhesion kinase.
CAT1-overexpressed cells after growth stimulation (Fig. 6B). Moreover, cGMP upregulation in vascular endothelial cells mediated by CAT1-overexpressed EVs were observed, which confirmed the impact of CAT1 on EVs in more physiological context. All of these known metabolic pathways and our data suggested that intact colorectal cancer cells might manipulate the cGMP-dependent signals in surrounding vascular endothelial cells. Indeed, PKG is known to strongly activate both ERK and p38 MAPK signaling pathways. Previous studies showed that PKG promotes proliferation of vascular endothelial cells by phosphorylating Raf-1 and activation of the Raf–MEK–ERK signal transduction (32). On the other hand, p38 activates MAPK-activated protein kinase 5 (MAPKAPK5) by phosphorylation of Thr-182, which mediates migration of vascular endothelial cells toward tumors and incorporation of them into a tumor vasculature through activation of Focal adhesion kinase I (43). In consideration of our data that extrinsic CAT1 significantly promoted tube formation of vascular endothelial cells in Matrigel (Fig. 5J and K), colorectal cancer–derived CAT1-TE-EVs may act as an attractant of angiogenic blood vessels by activating the arginine–NO–cGMP metabolic pathway and ERK/p38 phosphorylation signals in vascular endothelial cells. In the biology of colorectal cancer, VEGF is a well-established key regulator of angiogenesis, which induces both ERK and p38 signals (44), indicating that CAT1-TE-EVs and VEGF would coordinately activate different angiogenic pathways around the microenvironment of colorectal cancer.

In conclusion, encyclopedic database about 6,307 Te-EV proteins and 8,565 original tissue proteins were filed for colorectal cancer based on in-depth proteomic analysis, where a novel EV-based biomarker CAT1 was found. Our strategy to use Te-EVs can be applied to other modalities of analyses, such as DNA, miRNAs, metabolites, and so on, for any solid tumors or noncancer diseases. CAT1-TE-EVs promoted cell growth of vascular endothelial cells via arginine-oriented metabolic and phosphorylation pathways, implying that they could also serve as novel druggable pathways in colorectal cancer in the future.

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Atsushi Ikeda, Satoshi Nagayama, Makoto Sumazaki, et al.


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