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ORIGINAL PAPER

Glycosylation of ascites-derived exosomal CD133: a potential prognostic biomarker in patients with advanced pancreatic cancer

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Abstract
Cancer cells surviving in ascites exhibit cancer stem cell (CSC)-like features. This study analyzed the expression of the CSC marker CD133 in the ascites-derived exosomes obtained from patients with unresectable pancreatic cancer. In addition, inverse correlation of CD133 expression with prognosis was examined. Of the 133 consecutive patients, 19 patients were enrolled in the study. Exosomes derived from the malignant ascites demonstrated higher density and wider variation in size than those from non-malignant ascites. Western blot revealed enhanced expression of CD133 in exosomes obtained from patients with pancreatic cancer compared to those obtained from patients with gastric cancer or liver cirrhosis. A xenograft mouse model with malignant ascites was established by intraperitoneal inoculation of human pancreatic cancer cells in nude mice. Results obtained from the human study were reproduced in the mouse model. Statistically significant equilateral correlation was identified between the band intensity of CD133 in western blot and overall survival of patients. Lectin microarray analyses revealed glycosylation of CD133 by sialic acids as the major glycosylation among diverse others responsible for the glycosylation of exosomal CD133. These findings suggest that highly glycosylated CD133 in ascites-derived exosomes as a potential biomarker for better prognosis of patients with advanced pancreatic cancer.

Keywords Pancreatic cancer · Exosome · Ascites · CD133 · Glycosylation · Prognostic biomarker

Introduction

Incidence of pancreatic cancer is increasing rapidly and the associated mortality rate predicts it to be the second leading cause of cancer-related death in the United States of America by 2030 [1, 2].

The prognosis of patients with pancreatic cancer is adversely affected because of the rapid progression of the disease often involving large vessels, nerves, and distant organ sites [3]. Histologically, pancreatic ductal adenocarcinoma is characterized by an abundance of extracellular matrix that contributes to resistance towards chemotherapy and radiotherapy, typifying it as a major type of cancer [4]. Consequently, the reported 5-year cancer survival rate was 9% [5]. Patients approaching the end stage of the disease suffer from peritonitis carcinomatosa involving chemorefractory ascites.

Exosomes are lipid bilayer-enclosed extracellular vesicles with a diameter of 50–150 nm [6] containing encapsulated

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nucleic acids and proteins [6]. They are released into the extracellular space and systemic circulation by various cell types including platelets, immune, and endothelial cells [6]. Exosome-derived proteins include members of the tetraspanin family (CD9, CD63, and CD81), endosomal sorting complex required for transport (ESCRT), and heat-shock proteins (Hsp60, Hsp70, and Hsp90) [6].

Cancer cells that survive in ascites exhibit cancer stem cell (CSC)-like features [7, 8]. Exosomes are abundantly secreted by CSC-like cells to maintain a tumor-specific microenvironment in the abdominal cavity and play an important role in tumorigenesis, tumor growth, metastasis, angiogenesis, pre-metastatic niche formation, immunosuppression, drug resistance, and epithelial-to-mesenchymal transition (EMT) [6]. Therefore, analysis of unknown signals associated with CSC-like cells in malignant ascites related to pancreatic cancer is of research interest in predicting treatment refractoriness.

Prominin-1 (CD133), a pentaspan membrane glycoprotein, was initially considered as a specific cell surface antigen expressed on hematopoietic stem and progenitor cells [9, 10]. Recently, CD133 has been reportedly used as a marker to identify CSC population in a variety of solid tumors [11–16], including pancreatic cancer [17]. A previous study significantly demonstrated the role of CD133 in promoting EMT in CSC-like cells [18]; however, information related to the clinical implication of the molecule in malignant ascites is warranted.

Results of our preliminary study indicated abundant expression of CD133 in exosomes derived from malignant ascites of patients with pancreatic cancer. Accordingly, the current study aimed to investigate the relationship between the expression level of exosomal CD133 and prognosis in pancreatic cancer patients. Since it is known that CD133 is highly glycosylated, results of western blotting with densitometry analyses were meticulously scrutinized to verify the glycosylation status.

Materials and methods

Patients and samples

The present study enrolled 133 patients with pancreatic cancer who visited the Center for Multidisciplinary Treatment of Cancer in Kurume University Hospital (Kurume Japan) from June 2014 to June 2017. Malignant ascites was collected from cancer patients who underwent abdominocentesis and/or cell-free and concentrated ascites reinfusion therapy (CART) at the Kurume University Hospital. Ascites derived from patients with benign diseases, including decompensated liver cirrhosis, was used as controls. Informed consent was obtained from all patients in accordance with both, the principles stated in the Declaration of Helsinki and guidelines of the Ethical Committee of Kurume University (Study registration no: 15125). The study has been registered in the UMIN Clinical Trials Registry (Trial ID: UMIN000020296).

Isolation of exosomes

Exosomes were isolated from ascites using the exoEasy Maxi Kit (Qiagen, Hilden, Germany) followed by ultracentrifugation at 45,000 revolutions per minute (rpm) for 5 h for further identification using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Briefly, exosomes were isolated from ascites sample and culture medium using the ExoQuick exosome precipitation solution (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's protocol. Extracted proteins were subjected to western blot and the isolated exosome samples were stored at − 80 °C until further use.

NTA

NTA were performed on a malignant ascites sample obtained from a patient with pancreatic cancer and two non-malignant ascites obtained from patients with alcoholic cirrhosis and hepatitis C-related decompensated liver cirrhosis. Isolated exosomes by ultracentrifugation were diluted in phosphate-buffered saline (PBS) and analyzed using NanoSight LM10 system (Nanosight, Malvern, Worcestershire, UK) equipped with a blue laser (405 nm) as per the manufacturer's instructions. The movement of laser-illuminated nanoparticles under Brownian motion was recorded for 60 s. The acquired video images were processed using the NTA software to determine the particle concentration and size distribution profile. Each sample was measured and recorded five times. Replicate measurements of the particle concentration and size distribution profiles were averaged to yield the representative profile of particle size distribution. NTA was performed in the laboratory of Quantum Design Japan, Inc. (Tokyo, Japan).

TEM analyses of exsosomes

Ascites was fixed with half-Karnovsky fixative (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M PBS, pH 7.4) for 1 h and ultracentrifuged at 45,000 rpm for 5 h. The precipitate was further fixed in phosphate-buffered 1% osmium tetroxide solution, dehydrated in graded series of acetone solution, treated with propylene oxide, embedded in epoxy resin (Quetol 812; Nisshin EM), and ultrathin sections were cut with a diamond knife on an ultramicrotome (Ultracut E, Leica Microsystems, Wetzlar, Germany). Sections were mounted on a copper grid, stained with conventional uranyl acetate and lead citrate solution according to Sato's

improved staining method [19], and imaged using a Hitachi H-7650 TEM operated at an accelerating voltage of 100 kV.

Cell lines and culture conditions

Human pancreatic cancer cell lines, Panc-1 and BxPC-3, hepatocellular carcinoma (HCC) cell line, Huh7, and cervical cancer cell line, HeLa, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human pancreatic neuroendocrine tumor cell line, CM, which is an insulin-secreting line [20], was obtained from Professor Paolo Pozzilli (Rome, Italy). All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% heat-inactivated (56 °C, 30 min) exosome-depleted fetal bovine serum (System Biosciences, Palo Alto, CA, USA), 100 units/mL penicillin, and 100 mg/mL streptomycin (Nacalai Tesque, Kyoto, Japan) at 37 °C in a humidified atmosphere containing 5% CO_2.

Western blotting

Cells were lysed with RIPA buffer (Pierce, Rockford, IL, USA) containing protease inhibitor cocktail (Nacalai Tesque) and Halt phosphatase inhibitor cocktail (Pierce). Total protein concentration was measured using the BCA protein assay kit (Pierce). Samples containing 10 μg, 20 μg or 35 μg of protein were separated on 8.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto equilibrated polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 2% non-fat milk and incubated overnight at 4 °C with the following diluted primary antibodies: anti-CD133 (OriGene, Rockville, MD, USA), anti-CD44 (Cell Signaling Technology, Danvers, MA, USA), anti-double-cortin-like kinase 1 (DCLK1) (Abcam, Cambridge, UK), anti-CD81 (Invitrogen, Carlsbad, CA, USA), anti-CD63, anti-CD9, and anti-HSP70 (System Biosciences), and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA). The bound primary antibodies were detected with horseradish peroxidase (HRP)-labeled secondary antibodies and ImmunoStar® LD (WAKO) detection reagents as per the manufacturer's instructions. Positive signals from the target proteins were visualized using the image analyzer LAS-4000 (Fujifilm, Tokyo, Japan) and densitometry was performed with Multi Gauge version 3.0 imaging software (Fujifilm). To determine the band intensity of glycosylated CD133 obtained in western blot, relative band intensity of both the upper half (representing highly glycosylated (HG) CD133) and lower half of each band was measured using Multi Gauge, and the raw value of the band intensity was normalized by setting the lowest band intensity as 1.000.

Immunohistochemistry for CD133

Tissue samples of primary pancreatic cancer lesions and disseminated peritoneal lesions were prepared from five autopsied patients. Paraffin-embedded tissues were cut into 4-μm-thick sections, mounted onto coated glass slides, and incubated with anti-CD133 (1:300, polyclonal, OriGene) using the Bond-III autostainer (Leica Microsystems, Newcastle, UK). Briefly, tissue sections were subjected to heat-induced epitope retrieval using epitope retrieval solution 2 (pH 9.0) for 30 min followed by incubation with the primary antibody for 15 min. The Bond-III automated staining system used a Bond Polymer Refine Detection System (Leica Microsystems) employing HRP-polymer as the secondary antibody and 3,3′-diaminobenzidine (DAB) as the chromogen. Positive signals were visualized by DAB.

Xenograft model

Panc-1 and BxPC-3 cells (5.0×10^6 per mouse) were percutaneously injected into the abdominal cavity of 5-week-old male BALB/c athymic nude mice (Clea Japan, Inc., Tokyo, Japan). The mice were sacrificed and the ascites were collected at day 60 and day 49 after tumor cell inoculation, respectively. Extracted proteins from the ascites-derived exosomes were subjected to western blotting.

Lectin microarray analysis

Immunoprecipitated CD133 protein concentration of each ascites-derived exosome was determined using the Micro BCA protein assay kit (Thermo Fisher Scientific, Boston, MA, USA). Accordingly, samples were adjusted to a final protein concentration of 50 μg/mL with PBS. Aliquots (20 μL, 1 μg protein) were mixed with 100 μg of Cy3 mono-reactive dye and incubated for 1 h in the dark at room temperature. Cy3-labeled proteins were desalted using Zeba desalt spin columns (Thermo Fisher Scientific) and diluted to a final concentration of 1000 ng/mL with the probing solution. An aliquot of 100 μL was applied onto a lectin microarray chip (LecChip™; GlycoTechnica, Yokohama, Japan) and incubated at 20 °C for 16 h. Fluorescence intensities were measured with Evanescent-field fluorescence scanner (GlycoStation™ Reader 1200; GP Biosciences, Sapporo, Japan) and the data were analyzed using GlycoStation® Tools Pro Suite version 1.5 (GlycoTechnica). Experiments were performed using ascites collected at two time points (on day 20 prior to death and on the day of death) from a patient who underwent abdominocentesis.

Statistical analysis

Survival curves were analyzed using the Kaplan–Meier method. $p < 0.05$ was considered to indicate statistically significant difference in the survival. The Royston–Parmar model was used to evaluate the relationship between the band intensity of CD133 in western blot and overall survival. All experimental analyses were performed using the JMP software version 12.0 (SAS Institute, Inc., Cary, NC, USA). Evaluation of overall comparison of survival curves among patient groups was performed using the log-rank test based on the Kaplan–Meier method. Effect of the band intensity ratio of HG-CD133 was tested after adjusting periods between OS and diagnosis–treatment time using the Royston–Parmar model, which allowed flexible modeling of survival time [21].

Results

Patient characteristics

Flowchart detailing the criteria of patient selection is represented in Fig. 1a. Of the 133 patients with pancreatic cancer enrolled in the current study, 119 patients received systemic chemotherapy. The median survival time (MST) for all the subjects (133 patients) was 12.7 months [95% confidence interval (CI) 10.9–16.6] (Fig. 1b). The MST of 49 patients without ascites was 12.7 months (95% CI 10.1, not available) (Fig. 1c). The MST of 59 patients with malignant ascites was significantly shorter ($p = 0.0059$), corresponding to 13.5 months (95% CI 8.4–18.3) for 40 patients without ascites collection and 8.8 months (95% CI 6.0–13.6) for 19 patients with ascites collection (Fig. 1c). Significant statistical difference was not seen in the MST between the 40 and 19 patients without and with ascites collection, respectively. Clinical characteristics of 19 patients (MA1-19) with ascites collection are presented in Table 1. The mean and median age of 19 patients were 57.1 and 66.0 years, respectively.

Comparative analyses of the characteristic of malignant and non-malignant ascites-derived exosomes

NTA and TEM analyses were performed to elucidate the characteristic shape and size distribution of ascites-derived exosomes. The mode size (± SD) of malignant ascites-derived exosomes was 126.6 ± 10.6 nm (Fig. 2a) compared

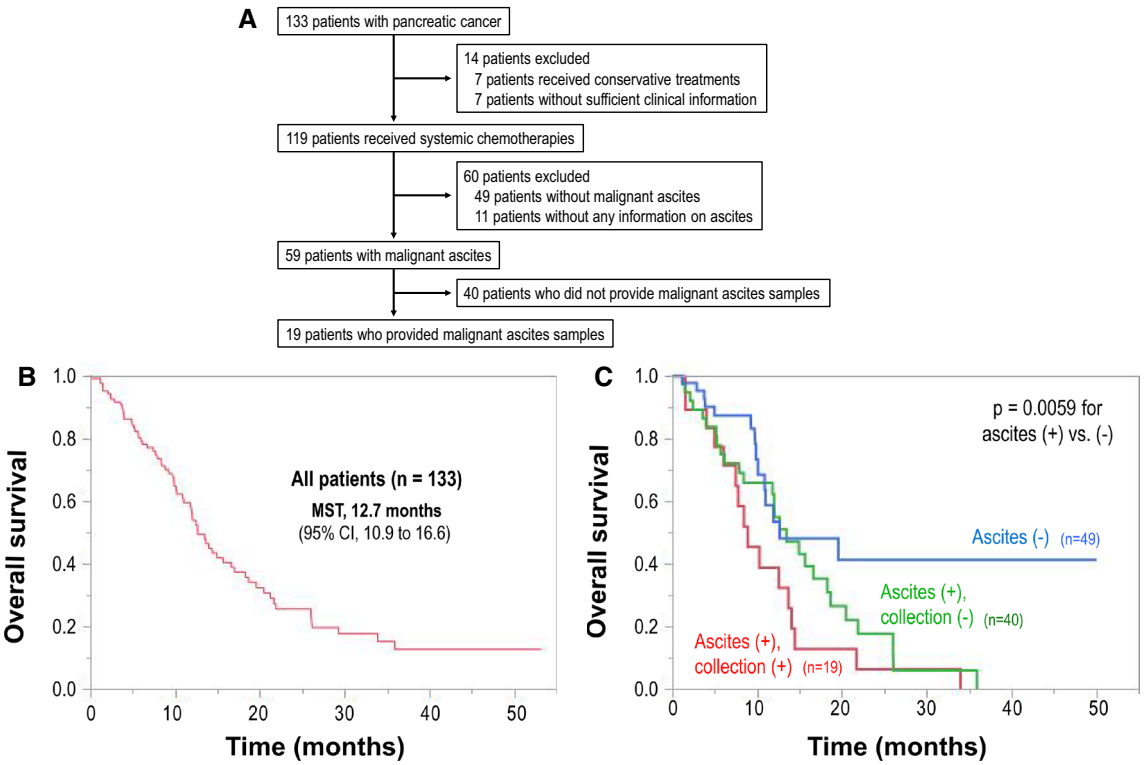

Fig. 1 **a** Flowchart detailing the criteria of patient selection. **b** Kaplan–Meier curve representing overall survival of all patients enrolled in the study ($n = 133$). The median survival time (MST) was 12.7 months [95% confidence interval (CI) 10.9–16.6]. **c** Kaplan–Meier curves representing overall survival of patients without ascites (blue line) and with malignant ascites collected from the abdominal cavity and is represented as two groups: with ascites (red) or without ascites (green)

Table 1 Characteristics of pancreatic cancer patients with malignant ascites

Patient ID	Age	Gender	Cytologic examination of ascitic fluid	Peritoneal dissemination	Overall survival (months)
MA1	53	Male	+	+	12.6
MA2	61	Male	−	+	14.6
MA3	43	Male	+	+	8.5
MA4	35	Male	+	+	7.8
MA5	46	Male	+	−	5.0
MA6	57	Female	+	+	14.2
MA7	55	Female	+	+	1.5
MA8	70	Male	+	+	8.9
MA9	66	Male	+	+	1.5
MA10	48	Male	+	+	4.0
MA11	44	Female	+	+	10.3
MA12	61	Female	+	−	22.0
MA13	64	Male	−	+	6.1
MA14	69	Male	−	+	6.6
MA15	55	Female	+	+	26.0
MA16	80	Female	−	+	34.4
MA17	56	Female	+	+	3.1
MA18	59	Female	+	+	2.9
MA19	63	Male	−	+	7.5

to that of the two non-malignant ascites-derived exosomes, 127.6 ± 7.9 nm (Fig. 2b) and 104.1 ± 0.8 nm (Fig. 2c), respectively. Exosome particle concentration in the samples obtained from the malignant ascites-derived exosomes and the two non-malignant ascites-derived exosomes was 3.34×10^{12}, 4.21×10^{12}, and 1.11×10^{12} particles/mL, respectively. Higher density of exosomes was not detected in malignant ascites, but greater variation in size was seen compared to that in non-malignant ascites. TEM analyses of both, malignant (Fig. 2d) and non-malignant (Fig. 2e) ascites, demonstrated the presence of round-shaped double-membrane enclosed vesicles resembling a typical exosome structure. The expression of exosomal marker proteins CD63, CD9, HSP70, and CD81 was clearly demonstrated in the isolated ascites-derived exosome pellets as indicated by western blot analyses (Fig. 2f). Taken together, these results confirm the purified vesicles obtained by both the methods to be exosomes.

Expression of CD133 in ascites-derived exosomes

Among the CSC-associated proteins CD133, CD44, and DCLK-1, western blot analyses revealed predominant expression of CD133 in exosomes derived from the ascites of patients with pancreatic cancer (patient no. MA11, MA12, and MA18) compared to those derived from the ascites of patients with gastric cancer and decompensated liver cirrhosis (Fig. 3a). Immunohistochemistry comparing disseminated peritoneal cancer lesions (Fig. 3b, c) and its corresponding primary pancreatic cancer (Fig. 3d, e) from five autopsied cases was analyzed to determine the origin of CD133 production. Enhanced expression of CD133 was localized to the cytoplasmic membrane and cytoplasm in cancer cells (Fig. 3c, e).

Expression of exosomal CD133 in pancreatic cancer cell lines and xenografts

Expression of CD133 was detected in the exosomes purified from the culture medium of human pancreatic cancer cell lines Panc-1 and BxPC-3 (Fig. 4a). In an animal model of peritonitis carcinomatosa induced by administration of Panc-1 and BxPC-3 cells (Fig. 4b), presence of CD133 in exosomes derived from the malignant ascites of mouse was reproduced in western blot (Fig. 4c).

Relationship between band intensity of HG-CD133 and overall survival

Ascites samples were collected from 19 of the 59 pancreatic cancer patients with malignant ascites. Purified exosomes derived from the ascites were subjected to western blot (Fig. 5) and densitometry to evaluate the expression and glycosylation levels of CD133. The study focused on the possibility of a relationship between the band intensity of HG-CD133 and overall survival. Table 2 shows the band intensity ratios of HG-CD133 to the low glycosylated (LG)-CD133. An equilateral correlation was found between the band density of HG-CD133 and overall survival ($p = 0.0309$). In the present study, the Royston–Parmar model rather than the Cox proportional hazards model was used for analyzing the data according to the statistical analysis methodology suggested by Adelian et al. [21]. Taken together, results of the present study predict association of enhanced survival with increased band intensity of HG-CD133 in ascites-derived exosomes.

Lectin microarray analysis

Lectin microarray analysis was performed using two comparable ascites samples obtained from a patient with advanced pancreatic cancer (patient No. MA4) (Fig. 6). The results of comparative analysis revealed a significant reduction in the fluorescent intensities of the three lectins, namely SNA (*Sambucus nigra* lectin), STL (*Solanum tuberosum* lectin), and UDA (*Urtica dioica* lectin).

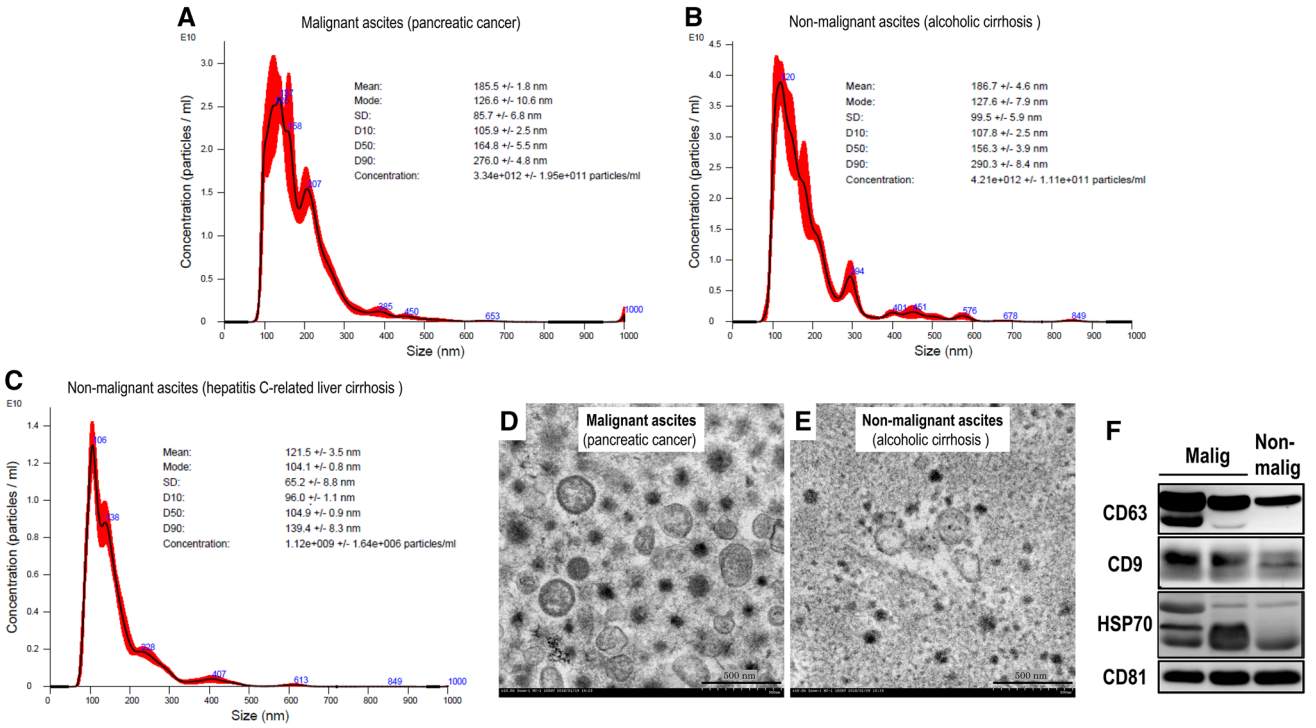

Fig. 2 Characterization of exosomes derived from malignant and non-malignant ascites by nanoparticle tracking analyses (NTA) (**a**–**c**) and transmission electron microscopy (TEM) (**d, e**). **a** Malignant ascites obtained from a pancreatic cancer patient; **b, c** non-malignant ascites derived from patients with alcoholic cirrhosis (**b**) and with hepatitis C-related decompensated liver cirrhosis (**c**). D10, D50, and D90 in the graphs indicate the diameters of nanoparticles (exosomes) after cumulatively measuring 10%, 50%, and 90% of those by NTA, respectively. D50 signifies median diameter. D and E: TEM images of exosomes derived from malignant ascites (**d**) and ascites of an alcoholic patient (**e**). **f** Western blot detection of proteins in ascites-derived exosomes using antibodies against exosomal markers: CD63 (53 kDa), CD9 (28 kDa), CD81 (26 kDa), and HSP70 (53–70 kDa). The samples were obtained from the pancreatic cancer patients MA1 and MA3 and a patient with alcoholic cirrhosis

Discussion

The present study aimed at demonstrating the distinct morphological and biochemical features of exosomes derived from malignant and non-malignant ascites. Accordingly, our novel findings indicate the strong expression of pancreatic CSC marker CD133 in exosomes derived from the ascites of patients with advanced pancreatic cancer. In addition, we observed an equilateral correlation between the band density of HG-CD133 and overall survival of patients.

Previous studies have shown secretion of greater number of exosomes by cancer cells compared to that of non-neoplastic cells [6, 22]. In hepato-pancreato-biliary diseases, the median concentration of exosomes was significantly higher in bile samples obtained from patients with malignant common bile duct (CBD) stenosis than from patients with non-malignant CBD stenosis [22]. However, results of the present study do not indicate consistent increase in the number of exosomes in malignant ascites compared to that in non-malignant ascites. The number of exosomes might be dependent on the fact that exosomes are secreted into the abdominal cavity by diverse kinds of cells such as cancer cells, peritoneal macrophages, and mesothelial cells. The inflammation induced in the abdominal cavity might further influence the number of exosomes released into the ascites. In relation to the size, exosomes derived from the bile of malignant patients were larger than those from non-malignant patients [22]. In the present study, the larger mean size of exosomes detected by NTA was not significantly different between those derived from malignant and non-malignant ascites but wider variation was seen in the size. This wider variation in size might in addition be attributed to the diversity in size of exosomes related to cancer [23, 24]. It should be noted; however, that NTA has limitation in assessing distribution of both the number and the size of exosomes, which may be derived from subjective biases in determining appropriate camera sensitivity, depth of focus, threshold, and particle concentration. Therefore, further investigation with increased number of samples should be needed. Though only a limited number of subjects were analyzed in this study, the fundamental difference in features between malignant and non-malignant ascites confirmed by precise TEM images has not been reported previously.

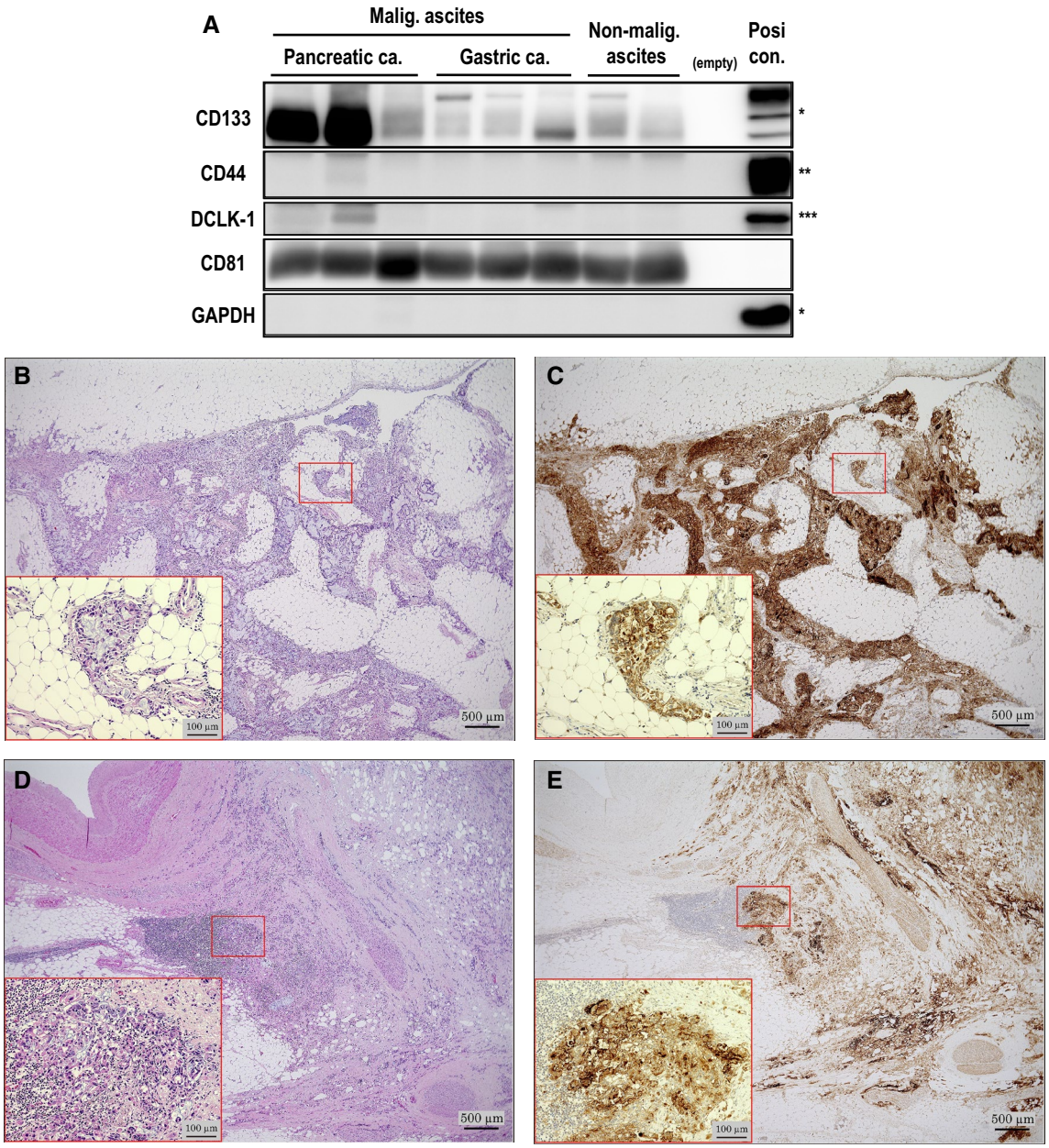

Fig. 3 Western blot of exosomal proteins derived from malignant and non-malignant ascites. **a** Expression of exosomal cancer stem cell (CSC)-associated proteins, CD133, double cortin-like kinase 1 (DCLK-1) and CD44. *, **, and *** indicate positive controls derived from Huh7, HeLa, and CM cells, respectively. **b, c** Hematoxylin and eosin (HE) staining (**b**) and CD133 staining (**c**) of peritoneal disseminated pancreatic cancer lesions in the patient MA9. **d, e**: HE staining (**d**) and CD133 staining (**e**) of the primary tumor of pancreatic cancer in the patient MA9

Further, we focused on the expression of CSC-associated proteins in exosomes derived from malignant ascites, as CSC-like cells are found concentrated in floating cancer cells present in ascites that resemble sphere-forming cancer cells [7, 8]. CSC marker proteins specific for pancreatic cancer include CD133, CD44, CD24, C-X-C chemokine receptor type 4 (CXCR-4), c-MET, epithelial-specific antigen (ESA), DCLK1, aldehyde dehydrogenase isoform 1 (ALDH1), and pancreatic differentiation (PD) 2 [25]. The expression of the above markers in exosomes derived from malignant ascites has not yet been elucidated. In the present study, CD133 was found to be predominantly expressed over the other CSC markers, such as CD44, CD24, and DCLK-1, in exosomes derived from the ascites of pancreatic cancer patients compared to those derived from gastric cancer and decompensated liver cirrhosis patients. To identify the source of exosomal CD133, immunohistochemical analyses of primary lesions

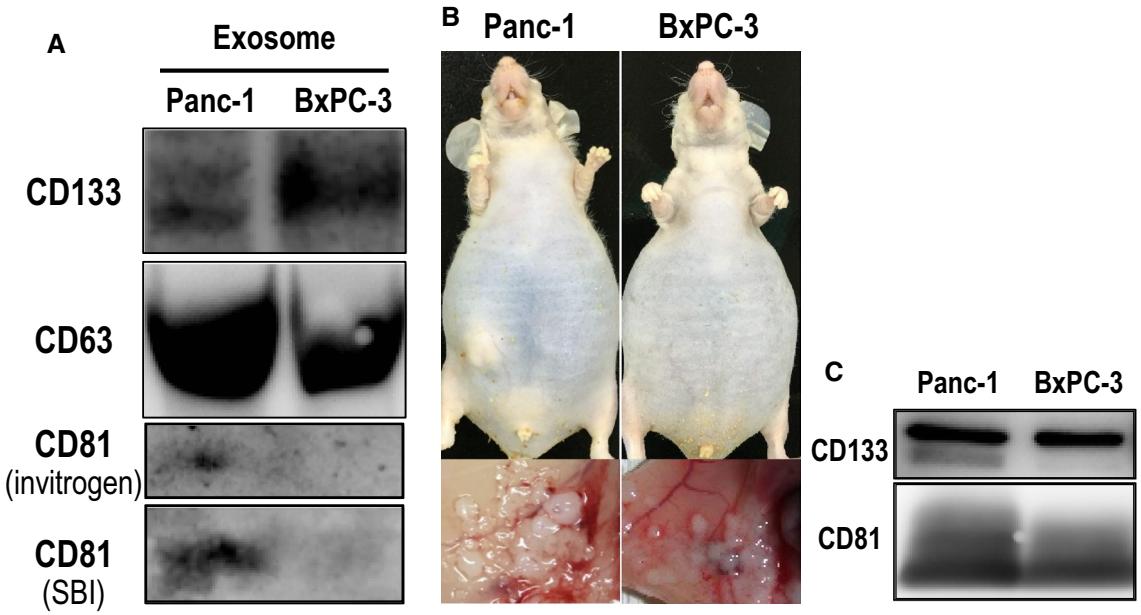

Fig. 4 Expression of exosomal CD133 in human pancreatic cancer cell lines and malignant ascites of mouse. **a** Western blot based expression of CD133 in human pancreatic cancer cell lines Panc-1 and BxPC-3. Two different antibodies for CD81 (from Invitrogen and SBI, respectively) were used. There are faintly positive or negative signals for CD81 in exosomes derived from pancreatic cancer cell lines used. **b** Malignant ascites and disseminated peritoneal cancer lesions (whitish nodules in the bottom picture) in the ascites of mouse. **c** Visible positive signal of CD133 detected in the exosomes derived from the ascites of the mouse model

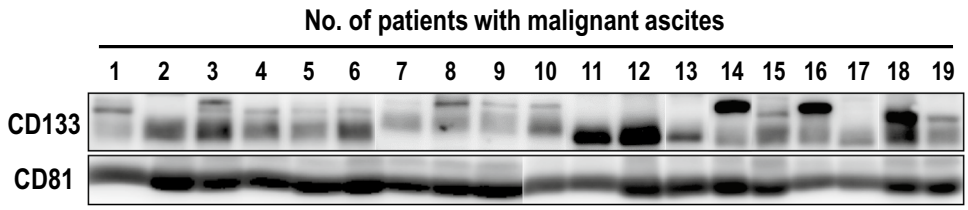

Fig. 5 Western blot panel assessing the exosomal expression levels of highly glycosylated CD133 in the ascites of 19 patients with pancreatic cancer

and peritoneal disseminated lesions from five pancreatic cancer patients who underwent autopsy were performed. Expression of CD133 was localized in the membrane and cytoplasm of pancreatic cancer cells in both the lesions, suggesting its production by peritoneal disseminated pancreatic cancer cells and cells of primary lesions, which are further secreted through exosomes into malignant ascites. Strong expression of CD133 in exosomes derived from the ascites of two out of five autopsied pancreatic cancer patients was confirmed by western blot analyses. Similar high-level expression of CD133 in ascites-derived exosomes was reproduced in a mouse model of peritonitis carcinomatosa induced by xenografting human pancreatic cancer cells. However, the expression of CD133 was found to be lower in exosomes isolated from the culture medium of human pancreatic cancer cell lines Panc-1 and BxPC-3. This might be because of the lack of cell–cell contact and communication with various kinds of non-malignant cells under normal conditions of cell culture. Such difference between in vitro and in vivo might be associated with the expression of the exosomal marker proteins. In our experiments in defining suitable internal control proteins, including CD63 and CD81, band for CD81 was constantly clear (as a bold single band) and reproducible except for that in cell line-derived exosome experiments. Conversely, as shown in Fig. 4a, CD81 expression was faintly positive or negative in pancreatic cancer cells using two different antibodies (from Invitrogen and SBI), while CD63 expression was strong. Based on such results, we concluded that CD81 and CD63 should be applied to in vivo and in vitro experiments, respectively. Therefore, we used CD81 as an internal control in Figs. 2f, 3a, f, 4c, and 5 (except for Fig. 4a). As for CD63, it was detected as a bold single band in cell experiments as shown in Fig. 4a; however, it often exhibited doublet bands in exosomes obtained from in vivo samples, as shown in Fig. 2f. These results encouraged us to use CD63 as a superior internal control to CD81 in western blot using cell-derived exosomes.

Table 2 Densitometric analysis for band intensity ratios of high-glycosylated (HG)-CD133 and low-glycosylated (LG)-CD133

Patient ID	The band intensity ratio of the HG-CD133	The band intensity ratio of the LG-CD133	Overall survival (days)
MA1	2.484	2.091	383
MA2	1.452	4.507	437
MA3	2.762	4.475	256
MA4	1.553	3.641	234
MA5	1.407	3.033	157
MA6	1.687	4.013	424
MA7	1.000	3.383	45
MA8	3.060	3.351	268
MA9	1.528	2.801	45
MA10	1.642	3.942	121
MA11	1.153	7.059	310
MA12	1.789	9.387	659
MA13	1.061	3.864	182
MA14	6.245	2.832	199
MA15	2.704	4.210	779
MA16	5.735	3.120	1031
MA17	1.000	2.938	94
MA18	6.135	4.947	87
MA19	2.469	2.973	225

We further analyzed the clinical implication of HG-CD133 in patients with advanced pancreatic cancer. Significant correlation was found between increased glycosylation of CD133 and enhanced survival of patients as detected by densitometry analyses of western blot. To our knowledge, this is the first report describing the relationship between glycosylation of a specific exosomal CSC marker protein in ascites and patient prognosis. A previous study demonstrated the potential role of serum *N*-glycan profile in predicting the efficacy of chemotherapy and survival of patients with unresectable pancreatic cancer [26]. These results emphasize the importance of high concentration of a certain glycan as an independent risk factor contributing to rapid tumor progression and poor overall survival. However, such studies have not revealed the unique glycosylation status of critical molecules associated with cancer stemness and/or aggressiveness.

To further confirm the alteration of glycosylation in ascites-derived exosomal CD133, lectin microarray analyses were performed using two comparable ascites samples obtained from a patient with advanced pancreatic cancer. Analyses revealed the presence of a dense band representing HG-CD133 in the first-obtained sample and a faint band in the second-obtained sample in western blot. Results of comparative analyses revealed significant decrease in fluorescent intensities of the three lectins, namely SNA (*Sambucus nigra* lectin), STL (*Solanum tuberosum* lectin), and UDA (*Urtica*

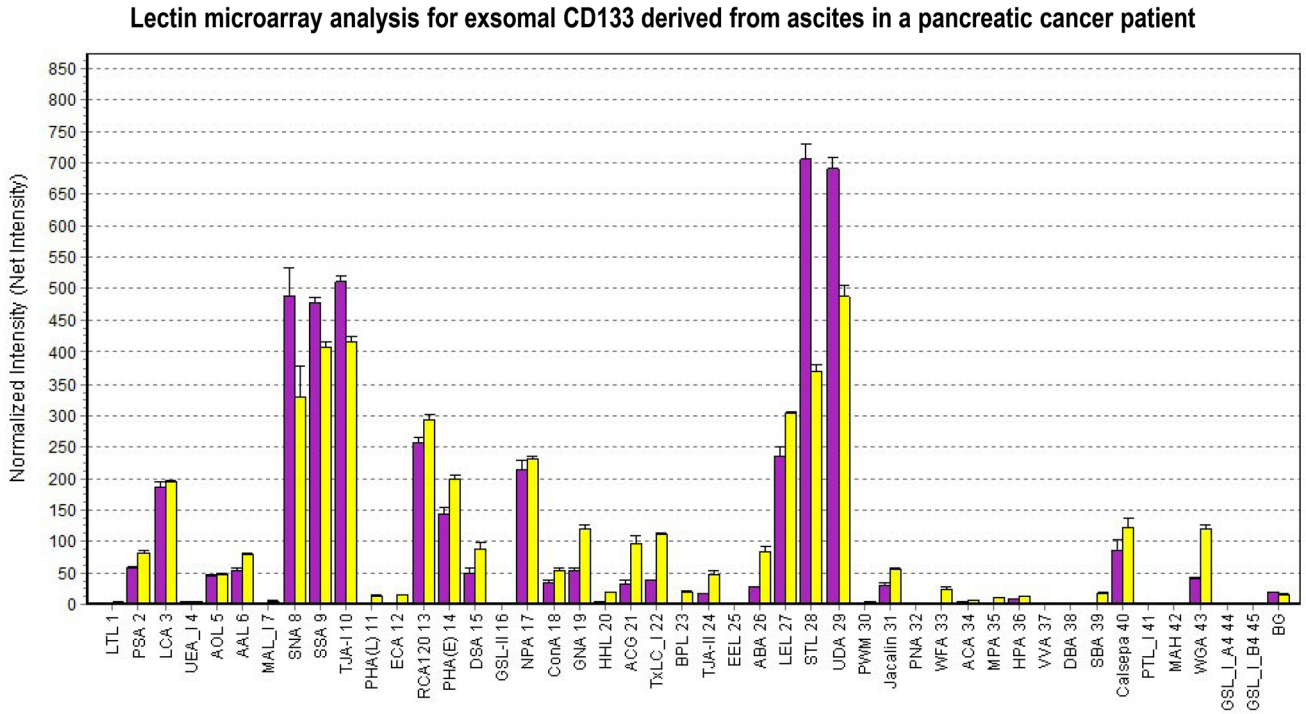

Fig. 6 Lectin microarray analyses of exosomal CD133 purified from the ascites obtained at different time points [20 days prior to (magenta) and on the day of patient's death (yellow)] from a patient (MA4) with pancreatic cancer

dioica lectin). SNA, STL, and UDA bind preferentially to NeuAcα2–3 (sialic acids), *N*-acetyl-D-glucosamine (GlcNAc) β1-4GlcNAc, and GlcNAcβ1-4GlcNAc and mannose (Man), respectively. Thus, the dense band of HG-CD133 reflected its glycosylation status involving several molecules including sialic acids. Although the number of patients enrolled in this study was limited and the generated data were preliminary, we speculate that the glycosylation profile of CD133 in exosomes derived from malignant ascites could be used as a potential biomarker in selecting patients with better prognosis to receive chemotherapy as a part of the advanced pancreatic cancer treatment regime. A cross-validation study, including both training cohort and testing cohort, of a larger number of patients is needed. To further minimize limitation of this study, we should pay careful attention to possible differences in structure and function of exosomes purified by different ways using commercialized kits and ultracentrifugation. Precise study to address this point will be needed.

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Compliance with ethical standards

Conflict of interest The authors have no potential conflicts of interest to be disclosed.

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