Clinical significance and therapeutic implication of CD200 in pancreatic cancer

Shoichi Kinoshita
Nara Medical University
Masayuki Sho (m-sho@naramed-u.ac.jp)
Nara Medical University https://orcid.org/0000-0002-6208-7661
Takahiro Akahori
Nara Medical University
Satoshi Nishiwada
Nara Medical University
Minako Nagai
Nara Medical University
Kazuhiro Migita
Nara Medical University
Shinsaku Obara
Nara Medical University
Noriko Ouji-Sageshima
Nara Medical University
Toshihiro Ito
Nara Medical University
Naoya Ikeda
Nara Medical University

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Abstract

Background CD200, a negative T cell regulator as well as a cancer stem cell marker, is a significant prognostic factor and potential therapeutic target in specific cancers. However, the clinical significance of CD200 is unknown in pancreatic ductal adenocarcinoma (PDAC). Methods CD200 was evaluated in 220 resected PDAC patients. Surgery was performed with or without neoadjuvant chemotherapy (NACRT), and adjuvant therapy was administered with systemic therapy or systemic therapy added with hepatic arterial infusion (HAI) therapy. We investigated the clinicopathological outcomes associated with CD200, in relation to the administered multimodal treatment. We further evaluated the impact of the immunological and cancer stem cell properties associated with CD200. Results NACRT patients had a lower average age, lower lymph node metastasis, higher negative surgical margins, and higher HAI administration rate, compared to upfront surgery (US) patients. NACRT was associated with better OS, and higher CD200 expression (66.4% vs. 32.2%, P<0.001) compared to US. CD200 was an independent poor prognostic factor in NACRT (hazard ratio 2.51; 95% confidence interval 1.35-4.66; P = 0.004), but not in US patients. In NACRT patients, the hepatic recurrence rate was relatively high in CD200+ cases despite HAI therapy. CD200 was associated with significantly lower CD4+, CD8+, and CD45RO+ tumor-infiltrating lymphocyte levels. Furthermore, the correlation of CD200 with pancreatic cancer stem cell markers CD44/CD24/ESA was stronger in irradiated human pancreatic cancer cells. Conclusions Our data highlight novel roles for CD200 in immune evasion as well as therapy resistance in pancreatic cancer.

Background

Pancreatic ductal adenocarcinoma (PDAC) remains to be one of the most intractable cancers. Standard treatment by upfront surgical resection followed by adjuvant chemotherapy [1] has shown little improvement through decades [2], especially in the more locally advanced subtype of borderline PDAC [3, 4]. Therefore, recently, efforts have become shifted to evaluate the efficacy of neoadjuvant therapy (NAT). Although NAT presents promising results [5, 6], high-level evidence is lacking, and upfront surgery is still supported as the first-choice treatment in resectable PDAC. Furthermore, it is still unknown whether chemotherapy or chemoradiotherapy is the optimal modality for NAT. Therefore, NAT is a strategy undergoing substantial investigation in the treatment of PDAC.

In some cancers, immunotherapy, namely immune checkpoint blockade (ICB) therapy, has shown remarkable clinical efficacy [7, 8] and is becoming to be a crucial therapeutic modality in cancer treatment. However, pancreatic cancer is widely an immune insensitive cancer, and to date, ICB monotherapy has failed to show clinical activity [9, 10]. Therefore, the methods to fully utilize the therapeutic potential of ICB therapy needs further investigation. Recently, studies have shown synergistic effects of ICB with conventional treatment modalities, including chemotherapy and radiotherapy [11, 12], and has led to remarkable clinical results in lung cancer [13, 14]. Thus, combination immunotherapy may be a possible method to utilize ICB therapy in PDAC. However, the optimal combinations and potential clinical impact are still unknown.

CD200 is a transmembrane protein of the immunoglobulin superfamily, related to the B7 family, which acts as a T cell costimulatory ligand [15-17]. In addition to its potential role in maintaining self-tolerance [18], CD200 was found to modulate immune-suppression in tumors [17]. Furthermore, since CD200 was found to co-express with cancer stem cell (CSC) markers, it was assumed that CD200 played an essential role in cancer progression by enabling CSCs to evade the immune system [19]. CD200 was reported to be overexpressed in several cancers, such as breast, colon cancer, and melanoma [20], and was also reported to be a significant prognostic factor in some hematologic malignancies [21, 22]. However, to date, the clinical significance of CD200 expression is still unknown in PDAC.

Taken together, we speculated that the investigation of immunological factors in patients who received multimodal therapy might provide valuable insights into the utilization of combination ICB therapy in PDAC. In this study, we aimed to clarify the clinical significance of a negative T cell regulator CD200 in PDAC. We investigated the prognostic impact of CD200 in PDAC patients who received multimodal therapy and assessed the interaction between CD200 expression and the treatment modalities. We further investigated the influence of the immunological and cancer stem cell properties of CD200 in clinical and pre-clinical settings.

Methods

Patients and clinical factors

Two hundred thirty-one patients who underwent surgery for PDAC at Nara Medical University Hospital, from Jan 2006 to April 2015, were reviewed. Surgery was performed with or without NACRT, and adjuvant therapy was administered with systemic therapy or systemic therapy added with HAI therapy. Among patients who received NACRT, 6 patients with high histological response who showed few or no residual cancer, 1 patient who had premature discontinuation of pre-operative treatment, and 2 patients that were found unresectable at laparotomy were excluded from this study. Among patients with upfront surgery (US), 1 patient with in-hospital mortality, and 1 patient with insufficient follow-up data were excluded from this study. Cancer staging was classified according to the UICC TNM classification of malignant tumors, 7^{th} edition [23]. Resectability status was classified according to the NCCN Guidelines for Pancreatic Adenocarcinoma [24]. Histological response for NACRT was classified according to the Evans grading system [25]. Written informed consent was obtained from all patients before treatment, according to our institutional guidelines. This study was approved by the institutional review board (Nara Medical University Ethics Committee).

NACRT protocol

NACRT was applied as previously reported [26]. Briefly, radiotherapy of 50 Gy in 25 fractions or 54 Gy in 27 fractions by the intensity-modulated radiation technique, with concurrent weekly systemic gemcitabine

(GEM) (1,000 mg/m^2) was administered. After re-evaluation of resectability upon completion of NACRT, surgery was performed within 3 to 4 weeks.

Patients who did not consent to NACRT, as well as those who participated in other clinical studies which did not involve NACRT, received upfront surgery.

Post-operative therapy

Patients were treated with systemic chemotherapy added with HAI, or standard systemic therapy alone. Adjuvant therapy by HAI therapy with concurrent systemic chemotherapy was administered as previously reported [27]. In brief, after full recovery from surgery, intervention radiologists placed a percutaneous trans-femoral catheter-port system. The tip of an anticoagulant-coated tapered 5F indwelling catheter (Anthron PU catheter; Toray Medical, Tokyo, Japan; or W-Spiral catheter; Piolax Medical Devices, Tokyo, Japan) was introduced into either the gastroduodenal artery or hepatic artery, and a manually made side hole was positioned in the common hepatic artery. The catheter was connected to a port (Celsite Port; Toray Medical) that was embedded subcutaneously in the right lower quadrant. In order to maximize hepatic drug delivery and minimize gastrointestinal toxicity, collateral blood flow was embolized to unify the hepatic artery blood supply, as described previously [28]. Continuous HAI of 5-FU (1,000 mg/m^2 / 5 hours) with concurrent bolus systemic GEM (1,000 mg/m^2) was administered weekly for 3 weeks in a 4-week cycle (HAI/GEM). Patients were monitored regularly for hepatic artery angiopathy and hepatic adverse events. Three courses of HAI/GEM were followed by 3 courses of systemic GEM or 4 courses of S-1 (80 to 120 mg daily, according to body surface area, for 4 weeks followed by 2 weeks rest).

Patients who were not amenable to HAI therapy due to either physical or intervention-associated technical issues [28, 29], or who did not consent to therapy, received standard systemic therapy only. Standard systemic chemotherapy was administered with GEM or S-1 for 6 months post-surgery. Additionally, some patients participated in clinical studies which required systemic adjuvant therapy as protocol treatment.

Immunohistochemistry

CD200 and CD44s antibodies were purchased from R&D Systems, Minneapolis, Minnesota, USA. CD4, CD8, CD45RO antibodies were purchased from DAKO, Tokyo, Japan. Immunohistochemistry (IHC) staining was performed by hand, as previously reported [30]. Primary antibody dilution was 1:40, 1:800, 1:40, 1:1, and 1:1 000 for CD200, CD44s, CD4, CD8, and CD45RO, respectively. Detection was performed with ImmPRESS polymer detection kit (Vector Laboratories, Burlingame, California, USA), according to the manufacturer's instructions, then counterstained with hematoxylin. CD200 and CD44s expression were evaluated by inspection of 10 random fields in ×200 magnification within the proximity of cancerous areas. A small, oval or spindle-shaped cell with a large nucleus, which showed cytoplasm staining of

CD200 with an intensity similar to islet cells, was observed in the peripheral of some cancer cell lesions. The intensity of this cell was uniformly strong. There were differences in the ratio of positive fields, but regardless of distribution, patients who displayed these positive cells were considered as CD200 positive PDAC cases. For evaluation of CD44, the variant isoform CD44s was selected. CD44+ PDAC was defined with similar criteria as CD200+ PDAC. A cell with a shape similar to CD200 positive PDAC, which displayed membranous staining was observed in some cases. Were considered these cases as CD44 positive PDAC. For tumor-infiltrating lymphocyte (TIL) evaluation, 5 random fields in ×200 magnification with clustered lymphocytes in the proximity of cancerous lesions were investigated, and the mean lymphocyte count was documented.

Cell lines and culture

The human pancreatic cancer cell lines PANC-1 and Capan-2 were obtained from Riken BioResource Research Center (Tsukuba, Japan) and DS Pharma Biomedical (Suita, Japan) respectively, and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum.

Irradiation

4.0×10^5 cells were seeded in 10 cm culture dishes. After overnight incubation, the monolayer cultures were irradiated with 10 Gy in a single fraction using a 150-KVp X-ray generator (MBR-1520R, Hitachi, Kashiwa, Japan) at a dose rate of 1.0 Gy/min. Following irradiation, cells were incubated for 7 days in the same culture medium.

Flow cytometry analysis

The expression of CD200, CD44, CD24 and EpCAM of irradiated and control cells were analyzed by FACSCalibur and CellQuest Pro software (BD Biosciences, Franking Lakes, New Jersey, USA). The staining of these markers was performed following the manufacturer's protocol. Briefly, a single-cell suspension was prepared by trypsinization, and then cells were incubated with monoclonal antibodies (mAbs) for 25 min at room temperature after fixation with 4% paraformaldehyde for 5 min. The following mAbs were used; fluorescein isothiocyanate (FITC)-labeled anti-CD24, allophycocyanin (APC)-labeled anti-CD44, APC-labeled anti-epithelial cell adhesion molecule (EpCAM) (Biolegend, San Diego, California, USA), phycoerythrin (PE)-labeled anti-CD200 (Beckman Coulter, Brea, California, USA), and isotype controls for each mAbs. The viability of cells was tested by 7-amino-acitomycin D staining and was approximately 98% in each experiment. The isotype control staining defined the quadrant area.

Statistics

Survival time was calculated from the date of surgery in upfront surgery patients, and the date of neoadjuvant treatment start in NACRT patients. Patients alive at the time of follow-up point were censored. The final date of follow-up was July 2016. The average follow-up period was 32.1 months (M) in upfront surgery patients, and 34.5 M in NACRT patients. Survival curves were estimated using the Kaplan-Meier method. The log-rank test was used to detect differences between curves. Chi-square and Fisher's exact test was used for categorical variables, as appropriate. Student's t-test was used for continuous variables. Univariate and multivariate analysis of prognostic factors were calculated with the Cox proportional hazards model. Differences were considered significant when $P < 0.05$.

Results

Patients

We investigated 115 US and 105 NACRT patients. Among the baseline clinicopathological characteristics of US and NACRT patients, there was a significant difference between the age, regional lymph node metastasis, and resection margin status. For adjuvant therapy, significantly greater patients received HAI after NACRT than US patients (75.2 % vs. 51.3 %, P = 0.0002; Table 1). Moreover, the overall survival (OS) of NACRT was better than US patients (37.1 M vs. 27.3 M, P = 0.018). OS was better after HAI than systemic therapy in both NACRT (57.8 M vs. 23.0 M, P = 0.0001) and US patients (41.2 M vs. 19.4 M, P = 0.0003). In a univariate and multivariate analysis of all patients, HAI administration (hazard ratio (HR) = 0.514, 95% confidence interval (CI) 0.297-0.892, P = 0.018) and distant metastasis were found to be independent prognostic factors (Additional file 1: Table S1).

Prognostic impact of CD200 in PDAC in association with pre-operative treatment

First, we evaluated CD200 expression in whole tissue specimens by IHC. The staining of CD200 was consistently intense in a small, round to spindle cell found in the peripheral of cancer lesions (Fig. 1a). Patients positive of this cell were defined as CD200+ PDAC. The CD200 positivity rate was significantly higher in NACRT (n = 67, 66.4%), compared with US patients (n =37, 32.2%) (P < 0.001; Fig. 1b). Furthermore, CD200 positive cells detected in half or more of the inspected fields were significantly greater in NACRT (n = 31, 46.3%) than US (n = 3, 8.1%) (P < 0.0001). In US patients, there was no difference in postoperative survival between CD200+ and CD200- cases (21.9 M vs. 30.6 M, P = 0.31; Fig. 1c). On the contrary, in NACRT patients, CD200+ cases had a significantly poorer prognosis than CD200- (33.4 M vs. 57.8 M, P = 0.03; Fig. 1d). Subsequently, we investigated the clinicopathological factors associated with poor prognosis in NACRT patients. Among the baseline patient demographics, RECIST response was the only significant difference associated with CD200 expression (Table 2). A univariate and multivariate Cox regression analysis of NACRT patients showed CD200 expression to be an independent poor prognostic factor (HR = 2.51, 95% CI 1.35-4.66, P = 0.004), as well as resection status,

post-NACRT CA19-9, and distant metastasis (Table 3). However, in univariate and multivariate analysis of all patients, CD200 was not a prognostic factor.

To evaluate why CD200 lacked prognostic value in US patients, we assessed the differences in patient background. Since HAI was an independent prognostic factor in all patients, the significant difference in HAI administration rate between US and NACRT patients needed consideration. Therefore, we evaluated the prognostic impact of CD200 in a subgroup of patients only which received HAI therapy. In this subgroup, there was no significant difference between the OS of US (n = 59) and NACRT (n = 79) patients (41.2 M vs. 57.8 M, P = 0.19). Moreover, CD200 was nonetheless associated with poor prognosis in NACRT patients (37.1 M vs. 65.4 M, P = 0.0064), but not in US (28.5 M vs. 41.2 M, P = 0.29) (Fig. 1d,e).

Potential immunological impact of CD200

To investigate the mechanisms of poor prognosis in CD200+ patients after NACRT, we first looked into the association between CD200 and immunological factors.

We and others have reported TILs as an important immunological factor, and also a prognostic factor in PDAC [30-33]. In this study, we evaluated CD4, CD8, CD45RO positive TILs (Fig. 2a). The CD4+, CD8+ and CD45RO+ TIL count of CD200+ versus CD200- patients were 24.84 ± 11.31 vs. 31.81 ± 14.68 (P = 0.021), 32.69 ± 19.65 vs. 44.48 ± 23.30 (P = 0.019), and 52.71 ± 26.28 vs. 68.61 ± 29.88 (P= 0.016), respectively in NACRT patients (Fig. 2b). Prognosis did not differ in groups dichotomized by median or quartile TIL counts.

Correlation of CD200 with pancreatic cancer CSC marker

Since CD200 has been reported to co-express in CSCs [19], we next considered whether CSC properties were associated with the poor prognosis in CD200+ NACRT patients. However, to the author's knowledge, there have been no reports regarding the association of CD200 with PDAC CSC markers, including CD44/CD24/epithelial specific antigen (ESA) [34], or CD133 [35]. Thus, we first investigated the correlation of CD200 with recognized PDAC CSC markers. CD44 is reported to have an essential role in tumor progression [34], as well as radiotherapy resistance [36]. CD44s, a subtype of CD44, is expressed on tumor-initiating cells [37] and is reported to have a significant role in regulating stemness in pancreatic cancer cells [38]. Therefore, as an initial investigation, we evaluated the correlation of CD200 with CD44s expression by IHC in whole tissue samples of NACRT patients.

CD44s membranous staining was observed in some cells in the peripheral of cancer lesions, similar to those with CD200 staining (Fig. 3a). CD200 and CD44s positivity significantly correlated, with CD200+ found in 65.7 % of CD44s+ , and 23.7% in CD200- (P<0.0001; Fig. 3b). Since we found the significant

correlation of CD200 and CD44s expression, we hypothesized that CD200 was associated with CD44+/CD24+/ESA+ pancreatic CSCs.

Influence of irradiation to CD200 and CSC marker expression in human pancreatic cancer cells

Next, since the CD200 expression rate increased after NACRT in the clinical setting, we investigated the role of irradiation on CD200 expression in an *in vitro* model. Also, we sought to verify our hypothesis of CD200 and CSC marker correlation by cell-surface marker analysis of CD200, CD44, CD24, and ESA with flow cytometry in human PDAC cells.

After a single-fraction irradiation of 10Gy, the CD200 positivity rate increased from 0.5% in control to 13.5% in irradiated PANC-1 cells, and from 1.7% in control to 17.5% in irradiated Capan-2 cells (Fig. 3c). Regarding CSC markers, post-irradiation CD200+ cells showed a stronger association with CSC markers after irradiation, compared with post-irradiation CD200- or control cells (Fig. 3d). The CD44+/CD24+ double-positive rate was higher after irradiation in CD200+ cells in both PANC-1 and Capan-2 cells. Although ESA expression was high (>96 %) regardless of irradiation in both PANC-1 and Capan-2 cells, CD200+ had a higher ESA+ rate compared to CD200-, in both control and irradiated cells (data of ESA expression from control cells not shown).

Impact of CD200 on the post-operative recurrence patterns

Finally, we investigated the association between CD200 and post-operate recurrence patterns. Since we have reported the preventive effect of HAI against hepatic recurrence [27], and also, since hepatic recurrence generally has a more unsatisfactory clinical outcome compared with other recurrence types, we especially paid attention to the hepatic recurrence patterns. The total hepatic recurrence rate did not differ between US (19.1%, n = 22) and NACRT (22.9%, n = 24) (P = 0.51). Among the patients who received post-operative treatment, the hepatic recurrence rate was lower after HAI (6.1 %, n = 7) than systemic therapy (11.3 %, n = 13) in US (P = 0.05), but was similar (15.2 %, n = 16 vs. 5.7 %, n = 6) in NACRT (P = 0.37). The rate of other failure patterns did not differ between HAI and systemic therapy in either NACRT or US patients (Additional file 2: Table S2).

First, we investigated the recurrence patterns, according to CD200 expression. In US recurrence patients, the hepatic recurrence proportion was similar in CD200+ (n = 7/26; 26.9%) and CD200- (n = 15/53; 28.3%) (P = 1.0). In contrast, in NACRT recurrence patients, the proportion of hepatic recurrence was significantly higher in CD200+ (n = 19/45; 42.2%) than in CD200- (n = 5/27; 18.5%) (P = 0.043). Interestingly, pulmonary recurrence proportion was significantly higher in NACRT CD200- (n = 14/27; 51.9%) than in CD200+ (n = 8/45; 17.8%) (P = 0.0036). There were no other distinct recurrence patterns associated with CD200 expression.

Next, we further investigated the association between CD200 and hepatic recurrence according to the adjuvant therapy regimen in NACRT. NACRT patients were stratified into 4 groups according to CD200 expression and treatment regimen (Fig. 4a). For this analysis, 6 patients who could not receive any adjuvant therapy were excluded. As a result, in NACRT CD200- patients, hepatic recurrence proportion was lower in HAI (n = 2/18; 11.1%) than systemic therapy (n = 3/8; 37.5%). In contrast, in NACRT CD200+ patients, hepatic recurrence proportions were similar in both HAI (n = 14/34; 41.2%) and systemic therapy (n = 3/8; 37.5%). Furthermore, the post-HAI hepatic recurrence onset was significantly earlier as well as higher in CD200+ (14.1 M, 27.5%) than CD200- (26.6 M, 7.1%) (P = 0.022, Fig. 4b).

Likewise, the association between CD200 and hepatic recurrence according to adjuvant therapy regimen was investigated in US patients. As a result, the proportion of hepatic recurrence was lower after HAI compared to systemic therapy, regardless of CD200 expression (Fig. 4c). Hepatic recurrence occurred in 1 out of 9 (11.1%) of HAI/CD200+, 6 out of 30 (20.0%) of HAI/CD200-, 5 out of 16 (31.3%) of systemic/CD200+, and 8 out of 21 (38.1%) of systemic/CD200- patients with recurrence.

Discussion

Immunotherapy, namely ICB therapy, in combination with conventional modalities, such as chemotherapy, radiotherapy (RT) or surgery, is a potential novel treatment strategy. RT is considered as an appropriate candidate for combination immunotherapy [11], as the concept of immunoradiotherapy is to augment proimmunogenic effects by enhancing antigen presentation with RT, while checkpoint inhibitors concomitantly expand the population of effector T cells. However, on the other hand, some contrary reports show RT to suppress anti-tumor immunity [39]. Furthermore, multiple co-existing checkpoint inhibitors interact to complement immune evasion and may gain resistance to ICB therapy [40]. Therefore, although some preclinical studies have shown remarkable results, the complex mechanisms of how immunotherapy and other conventional anticancer treatment interact with the tumor microenvironment as well as systemic immunity, and how it can ultimately impact the clinical outcome, are still widely unknown.

We have been treating borderline resectable PDAC, as well as resectable PDAC with multimodal treatment by NACRT and adjuvant HAI therapy [26]. In this study, to identify insights into novel combination immunotherapy strategies, we set to retrospectively investigate the clinical impact of a T cell negative-regulator CD200 under the various circumstances of multimodal therapy in our series. As a result, CD200 seemed activated by NACRT and displayed association with suppression of local tumor immunity and therapy resistance to HAI.

From the results of IHC evaluation, we have shown for the first time that CD200 had a significant prognostic impact on PDAC. CD200 was associated with poor prognosis in post-NACRT patients, independent of widely known clinical factors including NCCN resectability status, pre-operative CA19-9 levels, and distant metastasis. We suspected that the suppressive immunological effect of CD200 was a potential mechanism of poor prognosis. By evaluation with IHC, NACRT CD200+ cases were indeed

associated with significantly lower TILs levels. This suggested the association of CD200 with suppressed immunity in the local tumor environment. However, we could not show the prognostic impact of CD4+, CD8+, or CD45RO+ TILs in our study cohort. Further investigation of the prognostic role of other tumor-infiltrating immune-suppressing cells, such as T regulatory cells, tumor-associated macrophages, and myeloid-derived suppressive cells may be warranted in future studies.

There are conflicting reports regarding the immune-modulating effects of RT. Although RT has been previously regarded as a modality for local control, the effect to enhance systemic antitumor immunity is becoming recognized [41]. RT is reported to up-regulate tumor immunity by mechanisms such as enhancing tumor-specific antigen–MHC complexes [42] or diversifying the T cell receptor repertoire of TILs [40]. On the other hand, it has also been regarded to suppress TILs [43], as well as promote the accumulation of regulatory T cells [44]. Furthermore, pre-clinical evidence showed the up-regulation of immune checkpoint inhibitors by irradiation [45]. From our clinical and pre-clinical data, it was suggested that irradiation negatively modulated local tumor immunity through the mediation of CD200. Therefore, CD200 may have a significant role in immune evasion when administrating RT in PDAC.

CD200 co-expression with CSC markers has been reported in prostate, breast, brain, and colon cancer [46]. In this study, we have shown for the first time that CD200 expression correlated with pancreatic cancer CSCs. As a result of irradiation, CD44+/CD24+/ESA+ expressing cell were enriched, suggesting that cells presenting CSC properties were activated or derived from irradiation stimulus. Since the correlation with CSC markers in both CD200+ and CD200- cells changed after irradiation, we assumed that the cells presenting PDAC CSC markers were not the result of the expansion of cells which were resistant to irradiation, but rather, that they were derived from irradiation stimulus.

Data from preclinical studies show that CSC properties such as tumor initiation, therapy resistance, and metastasis promotion are not unique to CSCs, but are also seen in other cells such as those that underwent epithelial-mesenchymal transition (EMT). Mani et al. showed that a subgroup with CSC-properties could be induced from neoplastic cells by a forced EMT state [47]. Pre-clinically, radiation stimulus has been reported to induce EMT and promote a motile phenotype [48-51]. Furthermore, EMT per se is involved with fundamental mechanisms in cancer progression, such as resistance to cell death and senescence, therapy resistance, and immune evasion [52]. Such mechanisms may have been involved in the acquisition of CSC properties in the irradiated PDAC cells in our study.

From our clinical data, CD200+ NACRT patients appeared to be refractory to HAI therapy, suggesting that therapy resistance resulting from CSC properties of CD200 may have contributed to the poor prognosis. Furthermore, the survival of CD200- post-NACRT patients was significantly better, while the survival of CD200+ post-NACRT patients was similar to the SF patient cohort. Therefore, in CD200+ NACRT patients, although NACRT was associated with higher local tumor control, the resistance to post-operative therapy may have caused a trade-off in prognostic benefit, to result in survival similar to the US approach. In the present study, CD200 was not evaluated before NACRT administration, so the direct change of CD200 expression could not be observed in the individual patients. In future studies, elucidation of the prediction

of CD200 expression after NACRT could help to predict the optimal indication for NACRT in PDAC. Furthermore, since CD200- NACRT patients showed a remarkable prognosis, it was suggested that therapy resistance associated with CD200 expression might have a vital clinical significance in PDAC with chemoradiation, and strategies to eradicate CD200 may be a key step to improve the efficacy of multidisciplinary therapy in pancreatic cancer.

Conclusions

In summary, we have shown the novel roles of CD200 in immune evasion and therapy resistance in PDAC. CD200 may play a critical role in the modulation of tumor immunity and therapy resistance under irradiation stimulus; therefore, it was suggested that CD200 might be a potential target in combination with RT in PDAC. Although further mechanistic investigations are required, this study provides valuable insights into establishing novel methods to improve the efficacy of multidisciplinary treatment against human pancreatic cancer.

Abbreviations

PDAC: Pancreatic ductal adenocarcinoma; NAT: Neoadjuvant therapy; NACRT: Neoadjuvant chemoradiotherapy; CSC: Cancer stem cell; HAI: Hepatic arterial infusion; US: Upfront surgery; UICC: Union for International Cancer Control; NCCN: National Comprehensive Cancer Network: GEM; Gemcitabine; IHC: Immunohistochemistry; TIL: Tumor-infiltrating lymphocyte; mAbs: Monoclonal antibodies; FITC: Fluorescein isothiocyanate; APC: Allophycocyanin; EpCAM: Epithelial cell adhesion molecule; PE Phycoerythrin; M: Months; OS: Overall survival; MST: Median survival time; HR: Hazard ratio; CI: Confidence interval; ESA: Epithelial specific antigen; RT: Radiotherapy

Declarations

Ethics approval and consent to participate: This study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before treatment, according to our institutional guidelines. This study was approved by the institutional review board (Nara Medical University Ethics Committee).

Consent for publication: Not applicable

Availability of data and materials: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Tables

Table 1 Patient characteristics of all patients

Characteristic	Upfront Surgery		NACRT		p-value
	(N = 115)		(N = 105)		
Median age, years (range)	69	(33-87)	69	(36-78)	0.0170
Sex, n (%)					0.6844
Male	62	(53.9%)	60	(57.1%)	
Female	53	(46.1%)	45	(42.9%)	
Median pre treatment CA19-9, U/mL (range)	91	(1-6757)	103	(1-4581)	0.9165
Resectability status (NCCN Guidelines) , n (%)					0.1357
Borderline resectable with arterial involvement	9	(7.8%)	15	(14.3%)	
Borderline resectable with venous involvement / resectable	106	(92.2%)	90	(85.7%)	
T (TNM), n (%)					0.1988
3/4	93	(80.9%)	92	(87.6%)	
1/2	22	(19.1%)	13	(12.4%)	
N (TNM), n (%)					< 0.0001
1	70	(60.9%)	28	(26.7%)	
0	45	(39.1%)	77	(73.3%)	
M (TNM), n (%)					0.4490
1	5	(4.3%)	2	(1.9%)	
0	110	(94.7%)	103	(98.1%)	
Resection margin[a], n (%)					0.0025
R1	25	(24.3%)	9	(8.6%)	
R0	78	(75.7%)	96	(91.4%)	
Tumor grade, n (%)					0.8878
High	41	(35.7%)	36	(31.3%)	
Low	74	(64.3%)	69	(68.7%)	
Adjuvant therapy, n (%)					0.0002

HAI therapy	59	(51.3%)	79	(75.2%)
Systemic therapy	48	(41.7%)	20	(19.0%)

NACRT: neoadjuvant chemoradiotherapy; NCCN: National Comprehensive Cancer Network; CA19-9: carbohydrate antigen 19-9; HAI: hepatic arterial infusion. [a]Insufficient pathological data in 12 upfront surgery patients.

Table 2 Patient Characteristics of NACRT according to CD200 status

Characteristic	CD200+		CD200-		p-value
	(N = 67)		(N = 38)		
Median age, years (range)	68	(36-78)	69	(47-78)	0.7689
Sex, n (%)					0.6832
Male	37	(55.2%)	23	(60.5%)	
Female	30	(44.8%)	15	(39.5%)	
Median pre NACRT CA19-9, U/mL (range)	101	(1-4581)	108	(1-1903)	0.7543
Median post NACRT CA19-9, U/mL (range)	38	(1-977)	28	(1-872)	0.3990
Resectability status (NCCN Guidelines) , n (%)					0.1551
Borderline resectable with arterial involvement	7	(10.4%)	8	(21.1%)	
Borderline resectable with venous involvement / resectable	60	(89.6%)	30	(78.9%)	
RECIST					0.0406
PR	9	(13.4%)	12	(31.6%)	
≤SD	58	(86.6%)	26	(68.4%)	
T (TNM), n (%)					0.2177
1/2	6	(9.0%)	7	(18.4%)	
3/4	61	(91.0%)	31	(81.6%)	
N (TNM), n (%)					0.6442
0	51	(76.1%)	27	(71.1%)	
1	16	(23.9%)	11	(28.9%)	
M (TNM), n (%)					0.5337
0	65	(97.0%)	38	(100.0%)	
1	2	(3.0%)	0	(0.0%)	
Resection margin, n (%)					0.7198
R0	62	(92.5%)	34	(89.5%)	
R1	5	(7.5%)	4	(10.5%)	

Histological response (Evans grade), n (%)					0.6843
≤IIA	35	(52.2%)	22	(57.9%)	
IIB	32	(47.8%)	16	(42.1%)	
Tumor grade, n (%)					0.2847
High	20	(29.9%)	16	(42.1%)	
Low	47	(70.4%)	22	(57.9%)	

NACRT: neoadjuvant chemoradiotherapy; NCCN: National Comprehensive Cancer Network; CA19-9: carbohydrate antigen 19-9; RECIST: Responce Evaluation Criteria in Solid Tumors; PR: partial responce; SD: stable disease

Table 3 Univariate and multivariate analysis of risk factors for overall survival in NACRT patients (n = 105)

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
Age (≥70 vs. <70)	1.061	0.626-1.799	0.826			
Sex (Female vs. Male)	0.736	0.432-1.257	0.262			
Resectability Status (NCCN Guidelines BR-A vs. BR-P/R)	3.358	1.765-6.389	<0.001	5.057	2.412-10.60	<0.001
post NACRT CA19-9 (≥37 U/mL vs. <37 U/mL)	2.194	1.285-3.748	0.004	2.578	1.489-4.463	0.001
RECIST (SD vs. PR)	0.811	0.436-1.508	0.507			
T (3/4 vs. 1/2)	3.241	1.012-10.37	0.048	2.400	0.739-7.798	0.145
N (1 vs. 0)	1.158	0.641-2.092	0.627			
M (1 vs. 0)	6.577	1.540-28.09	0.011	8.156	1.746-38.09	0.008
Resection Margin (R1 vs. R0)	2.531	1.058-6.052	0.037	1.463	0.581-3.683	0.419
Histological Response (Evans grade IIB vs. ≤IIA)	1.107	0.651-1.883	0.707			
Tumor Grade (Low vs. High)	1.572	0.896-2.759	0.115			
CD200 (+ vs. -)	1.869	1.052-3.319	0.033	2.513	1.354-4.664	0.004

NACRT: neoadjuvant chemoradiotherapy; HR: hazard ratio; CI: confidence interval; NCCN: National Comprehensive Cancer Network; BR-A: borderline resectable with arterial involvement; BR-P: borderline resectable with protal vien involvement; R: resectable; CA19-9: carbohydrate antigen 19-9; RECIST: Responce Evaluation Criteria in Solid Tumors; PR: partial responce; SD: stable disease

Figures

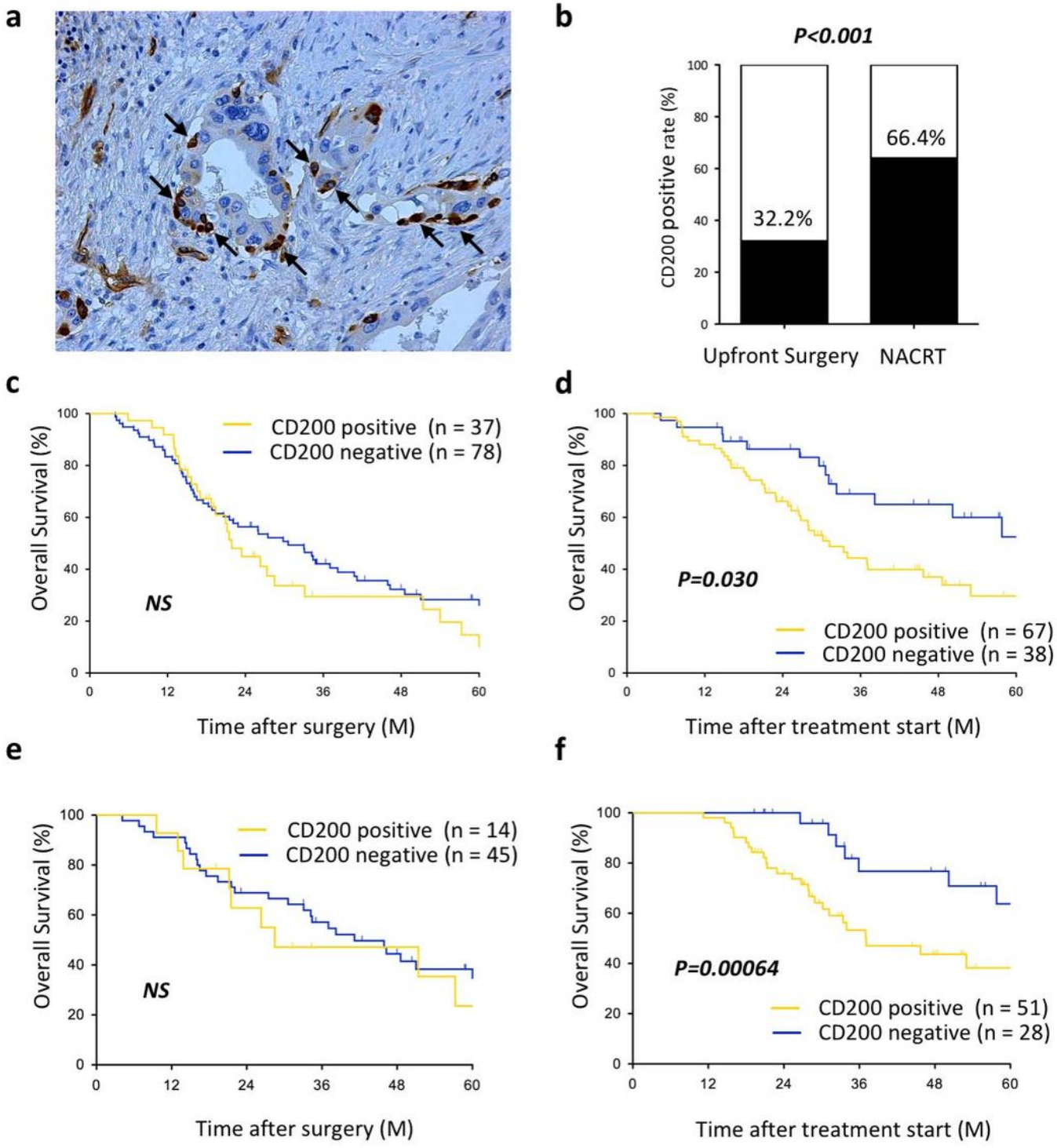

Figure 1

Clinical impact of CD200 expression in pancreatic cancer, in correspondence with pre-operative therapy administration. a Representative stain of CD200 positive pancreatic cancer in post NACRT patient. Original magnification ×200. b CD200 positivity rates in US and NACRT patients. Kaplan-Meier curves for overall survival according to CD200 status in c 115 US patients and d 105 NACRT patients. Kaplan-Meier

curves for overall survival in patients with HAI, according to CD200 status in e 59 US patients and f 85 NACRT patients

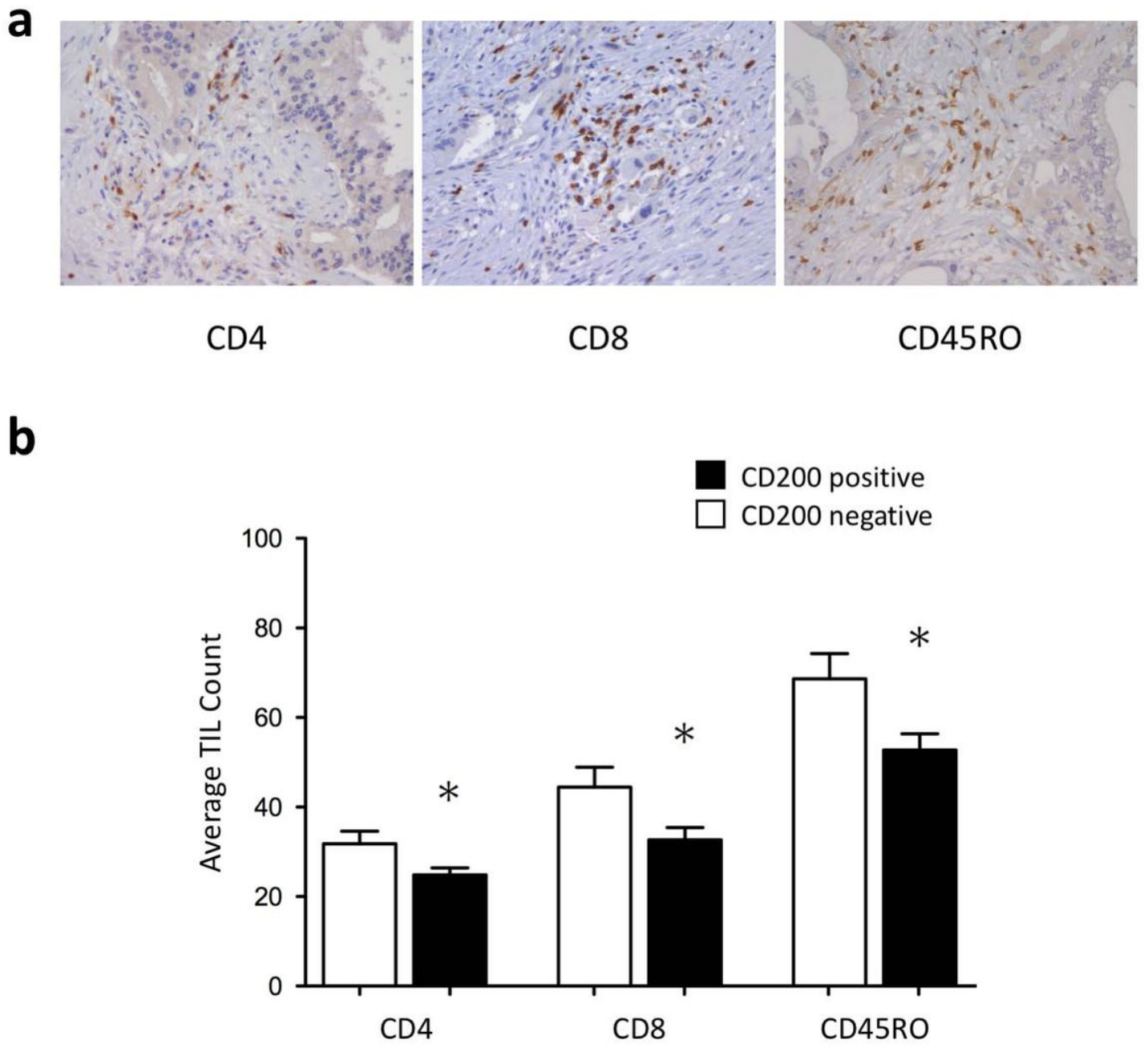

Figure 2

Association between CD200 and TILs. a Representative stains of CD4, CD8, CD45RO positive TILs in NACRT patients. Original magnification ×200. b Average count of CD4+, CD8+, and CD45RO+ TIL in NACRT patients, according to CD200 status

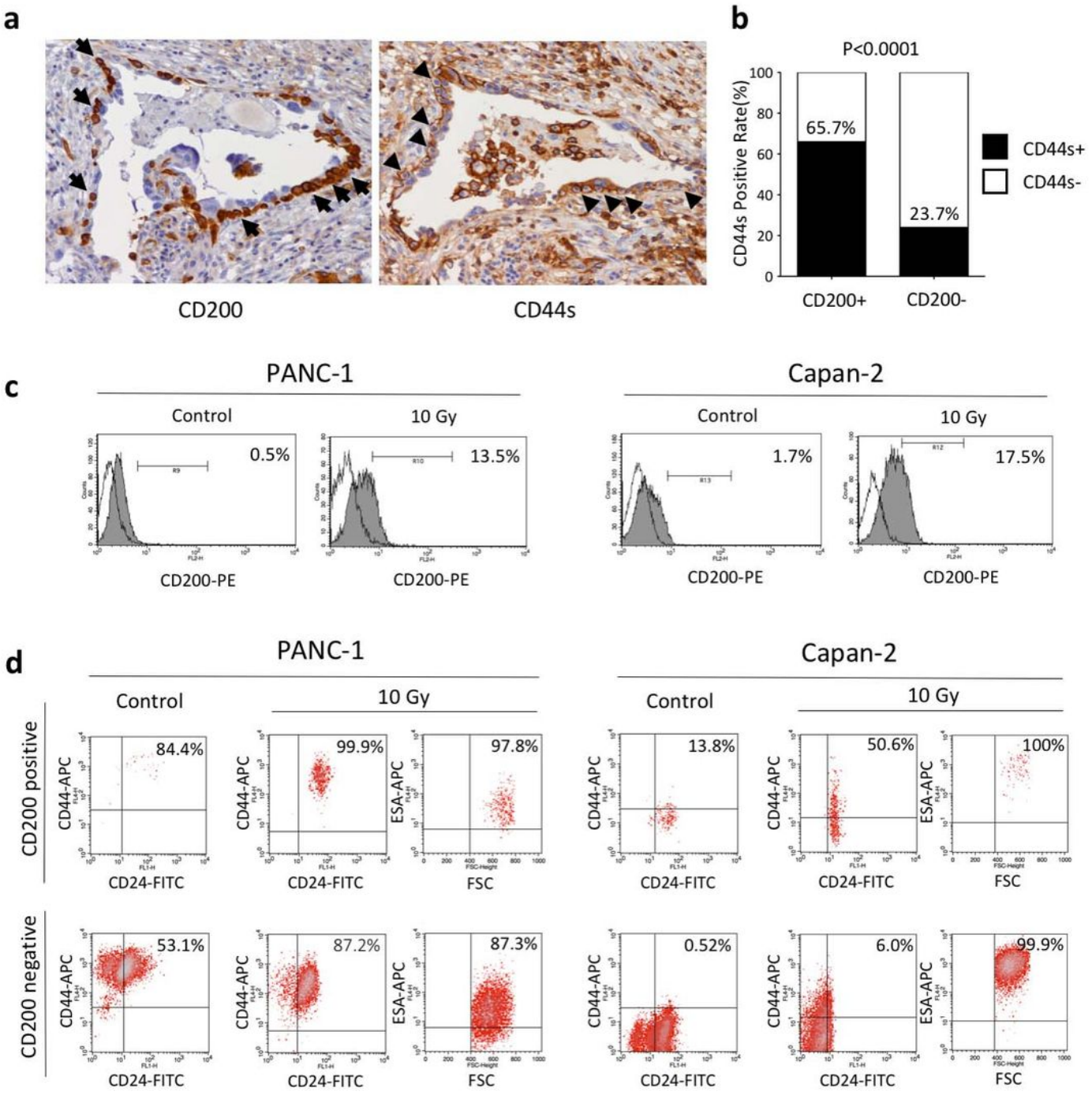

Figure 3

Impact of irradiation on CD200 expression and correlation with pancreatic cancer stem cell markers. a Representative stains of CD200 (arrows) and CD44s (arrowheads) positive pancreatic cancer in NACRT patients. Original magnification ×200. b Correlation between CD200 and CD44s expression rates in 105 NACRT patients. c Flow cytometry analysis of the effect of irradiation on CD200 expression in human pancreatic cancer cell-lines PANC-1 and Capan-2. d Flow cytometry analysis of the correlation between

CD200 and pancreatic cancer stem cell markers CD44, CD24, and ESA in PANC-1 and Capan-2 with irradiation. Flow cytometry analysis is representative data from three independent experiments

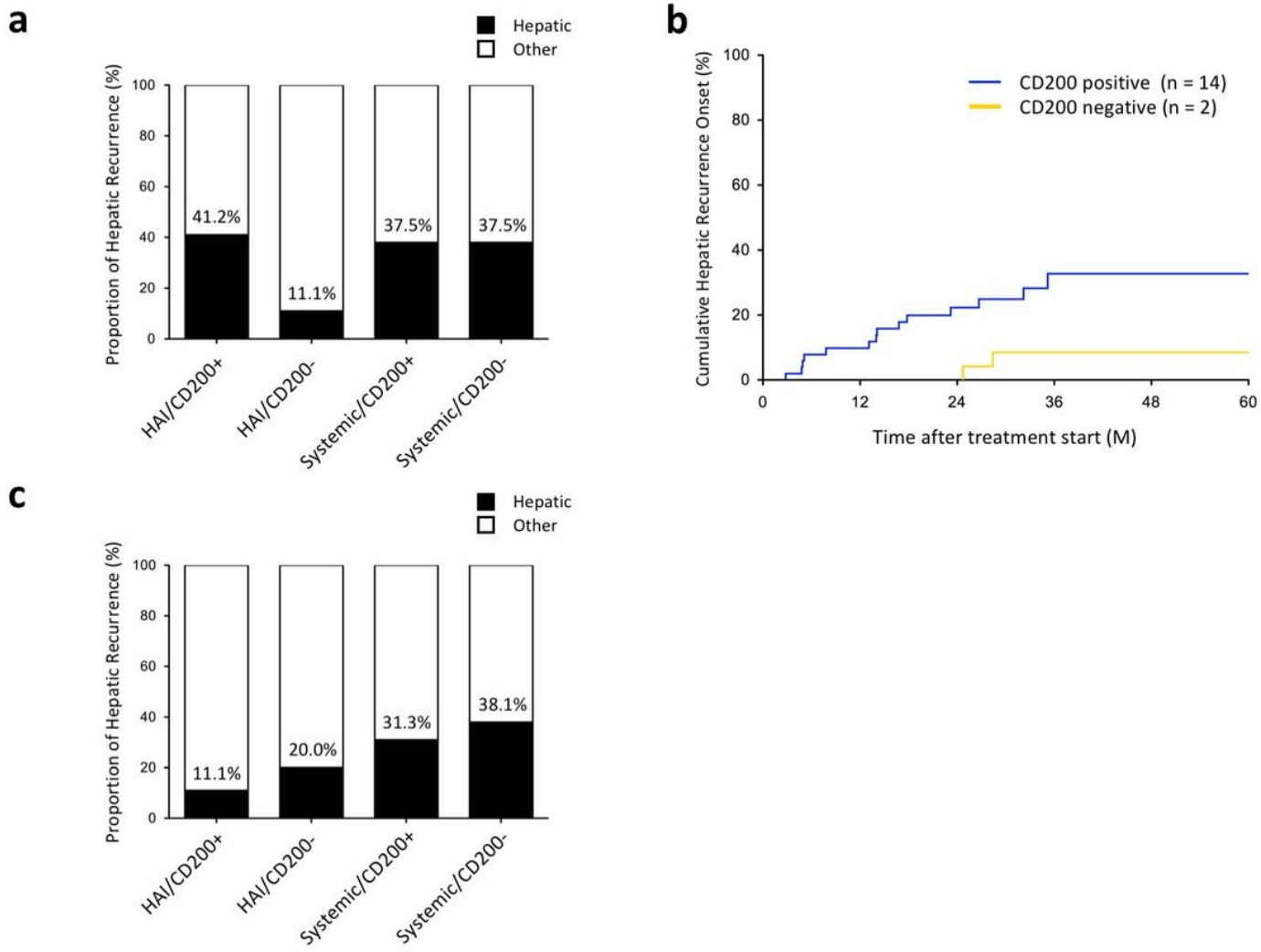

Figure 4

Clinical impact of CD200, according to the adjuvant therapy regimen. a Proportions of hepatic recurrence in post-operative recurrent NACRT patients, in groups stratified by adjuvant therapy regimen and CD200 expression. b Kaplan-Meier curves of cumulative post-HAI hepatic recurrence onset in NACRT patients, according to CD200 status. c Proportions of hepatic recurrence in post-operative recurrent US patients, in groups stratified by adjuvant therapy regimen and CD200 expression

Supplementary Files

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