Original article

Clinical Impact of Carbonic Anhydrase 9 Expression on Neoadjuvant Chemoradiotherapy in Pancreatic Ductal Adenocarcinoma

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ABSTRACT

Background: PDAC cells upregulate carbonic anhydrase 9 (CA9) expression in order to survive in hypoxic tumor environments, which plays a key role in tumor progression. However, the relationship between CA9 expression and preoperative treatment has not been clarified. We evaluated the clinical impact of CA9 expression on the efficacy of neoadjuvant chemoradiotherapy (NACRT) in pancreatic ductal adenocarcinoma (PDAC).

Methods: We investigated CA9 expression in 273 surgical specimens and 20 serum samples obtained from patients with PDAC and evaluated their clinical outcomes. We analyzed the function of CA9 using human pancreatic cancer cell lines.

Results: CA9 was positively expressed in 36.2% of patients who underwent NACRT, which was significantly lower than those who underwent upfront surgery (US) (58.9%, $p < 0.001$). Interestingly, patients who were CA9-positive in the US group had a significantly poorer prognosis than that of those in the NACRT group (median survival time [MST], 21.5 months vs. 49.2 months, $p < 0.001$), while there was no significant difference between patients who were CA9-negative in the US and NACRT groups (MST, 45.8 months vs. 46.3 months, $p = 0.357$). Moreover, serum CA9 levels tended to correlate positively with CA9 expression in cancer tissues. In-vitro experiments demonstrated that CA9 expression was reduced after treatments with radiation and chemoradiation therapy (RT/CRT), and that CA9 knockdown suppressed the impact of RT/CRT on cancer cell proliferation.

Conclusions: CA9 may act as a target molecule for RT/CRT, highlighting its clinical importance as a valuable biomarker for more stringent indications for NACRT.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies and has an extremely poor prognosis [1,2]. Although surgical resection remains the only opportunity for a potential cure or long-term survival in patients with PDAC, only 20% of patients are diagnosed with anatomically resectable disease [3,4]. Furthermore, despite recent advancements in treatments for cancers, even after curative radical resection, most patients with PDAC experience recurrences, resulting in a 5-year survival rate of 10-25% [5,6]. Since surgery alone can have only an insufficient impact on oncological outcomes, multidisciplinary treatment strategies, including chemotherapy, radiation therapy (RT), and immunotherapy, have been actively explored and investigated to improve this unfavorable prognosis [7]. However, PDACs create a characteristic tumor microenvironment for therapeutic resistance around themselves, which plays an essential role in their intractability.

The tumor microenvironments of many solid tumors, including PDAC, exhibit hypoxic conditions because of extraordinary hyperproliferation of cancer cells and relatively insufficient blood supply [8,9]. To adapt to the cruel environment, cancer cells upregulate carbonic anhydrase 9 (CA9), an oncoprotein and surrogate marker of tumor hypoxic conditions (hypoxia), which can maintain a normal pH value under hypoxic conditions through the stabilization of hypoxia-inducible factor 1 subunit alpha (HIF1A) by KRAS signaling [10,11]. CA9 is highly overexpressed in a large number of cancers, including PDAC, and plays a key role in tumor progression, metastases, and patient prognosis [12-14]. Moreover, CA9 is scarcely presented in normal tissues, making it an attractive potential biomarker and therapeutic target [11]. However, although hypoxia and the stabilization of HIF1A play critical roles in the resistance to chemotherapy and radiotherapy in cancers, the mechanism by which

CA9, downstream of the pathway, affects resistance to pancreatic cancer therapy has not been investigated [15-18].

Neoadjuvant therapy (NAT) has become an increasingly common option in modern multidisciplinary treatment strategies for PDAC worldwide. RT is an effective treatment tool for many cancers and can alter the local tumor environment. In fact, Chen et al [19] reported that low-dose RT improved pancreatic tumor oxygenation by inducing vascular normalization in the microenvironment. We have performed neoadjuvant chemoradiotherapy (NACRT) for patients with PDAC since 2008 and have reported the pathological and clinical impact[20,21]. However, optimal patient indications and guidance for NAT regimens, including neoadjuvant chemotherapy (NAC) or NACRT, remain controversial [22]. This clinical question highlights the lack of clinically-useful biomarkers that can predict response to NACRT and facilitate more informed decision-making for NAT in PDAC.

Based on these clinical experiences and questions, we aimed to investigate the clinical impact of CA9 expression on oncological outcomes and the effect of NACRT in PDAC. This is the first such investigation to our knowledge. To this end, we focused on multifaceted translational research combining *in-vitro* and clinical sample analyses.

2. Methods

2.1. Data Source

The Cancer Genome Atlas (TCGA) public gene-expression profiling data set was used to interrogate the expression of CA9 in patients with PDAC. The primary and processed data were downloaded from the UCSC Xena Browser (https://xenabrowser.net) in May 2020, along with the associated clinical information [23]. Cases in which patients had distant metastasis or insufficient survival information were excluded. Data from 182 patients from TCGA were used to validate the prognostic significance of CA9 expression in the internal validation phase of this study. The cutoff value for CA9 was set at the median.

2.2. Patients

This study was approved by the Institutional Review Board of the Nara Medical University (approval number: 3379). We enrolled 273 patients who underwent curative resection of PDAC at Nara Medical University Hospital (Nara, Japan) between January 2006 and December 2017. All patients are Asian. The exclusion criteria included tumor histology other than PDAC; no viable tumor cells; patients who did not undergo NACRT, but underwent NAC; and patients who died within 30 days following surgery. The primary treatment choice was NACRT. The most common reason for choosing upfront surgery is the patient's preference. Additionally, preoperative radiation was not suitable in some cases due to tumor conditions, such as intestinal involvement. Oncological data for each patient were collected from the medical records. The resectability status of tumors evaluated by computed tomography was defined according to the 2021 National Comprehensive Cancer Network Guidelines, version 2. Stage classification for the evaluation of tumors was categorized according to the 7th edition of the American Joint Committee on Cancer and the Union for International Cancer Control TNM classification

of malignant tumors. All the resected specimens were fixed in 10% phosphate-buffered formalin and embedded in paraffin. As previously reported, the NACRT regimen included gemcitabine and concomitant three-dimensional radiation of 54 Gy [20].

2.3. Immunohistochemistry (IHC)

IHC was performed on 4-mm-thick, routinely processed formalin-fixed paraffin-embedded (FFPE) sections of tumor specimens. Briefly, the slides were dewaxed, and heat-induced antigen retrieval was performed with target retrieval solution (pH9; DAKO, Tokyo, Japan) for 10 min at 120℃. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide for 10 min. The sections were labeled for overnight at 4℃ with primary antibodies against human CA9 (diluted 1:100, mAb#5649; Cell Signaling Technology Danvers, MA, USA). After incubation with the secondary antibody (ImmPRESS HRP Anti-rabbit IgG Polymer Detection Kit; Vector Laboratories, Newark, CA, USA), staining was performed using the ImmPACT DAB Kit (Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and mounted. Positive controls (tonsil tissues) were included in each staining experiment. Membrane staining of lymphocytes was verified by staining with an internal positive control. Slides were examined at 200× magnifications. Five differently stained fields in each section were assessed by observers blinded to patient information. Scoring was based on the percentage of tumor cells with membranous staining, with 40% or more being positivity. The cutoff was set at the value that maximized the prognostic difference between positive and negative in the upfront surgery group.

2.4. Enzyme-linked Immunosorbent Assays (ELISAs)

Soluble CA9 levels in serum samples and cell culture media were determined using the Human Carbonic Anhydrase IX Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. Calibration curves were prepared using the purified standards.

2.5. Serum Samples

Serum samples were collected from a total of 20 patients with PDAC at Nara Medical University. To evaluate the NACRT-induced changes in the expression levels of each molecule, we collected serum specimens at two time points during the treatment course, before and after NACRT.

2.6. Cell lines and reagents

For in vitro studies, Pancreatic carcinoma cell lines (BxPC-3 and PANC-1) were obtained from RIKEN BioResource Research Center, Ibaraki, JAPAN. All tumor cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Under normal circumstances (normoxia), cells were incubated at 37℃ with 5% CO_2 and 21% O_2. Under hypoxia conditions, cells were incubated at 37℃ with 5% CO_2 and 1% O_2 balanced with N_2.

2.7. Transient expression

CA9 expression was silenced in pancreatic cancer cell lines using small interfering RNA (siRNA). The CA9 siRNA (s224790) was purchased from Thermo Fisher Science, MA, USA. Cells were seeded into six-well plates at a density of 5×10^4 cells/well. When the cells reached cultured to 30–40% confluency, cells were transfected with 20nM of CA9 or control siRNA using Lipofectamine (Invitrogen, CA, USA). After 48h of incubation, cells were harvested for polymerase chain reaction (PCR) and Fluorescence-activated cell sorter (FACS).

2.8. The treatment protocols for the vitro

The cell treatment protocol in vitro assay is shown in Supplemental Figure. Briefly, the cells were plated at 1.0×10^5 cells/well under normoxia conditions, and incubation under hypoxia was started 24 hours later, which served as the control. After 24h of incubation under hypoxia, the cells were transfected with CA9 or control siRNA. After 48h of incubation under hypoxia, gemcitabine (Wako, Osaka, Japan) was added to cell culture media at a concentration of 0.1μM to the chemoradiotherapy (CRT) groups. Similarly, after 48 hours of incubation under hypoxia, the RT groups were irradiated with 2 Gy of ionizing radiation every 24 hours for five consecutive days. All cells were harvested for PCR and FACS or some assays described below.

2.9. Quantitative real-time PCR analysis

Total RNA from cell lines was isolated using the miRNeasy Micro Kit (Qiagen, MD, USA). Complementary DNA synthesis from 1mg RNA was performed using the PrimeScriptTM RT Master Mix (Takara,

Shiga, JAPAN). Real-time PCR was then performed with a StepOne Plus Real-Time PCR System (Applied Biosystems, MA, USA) using TaqMan Gene Assays (Applied Biosystems) for CA9 (Hs00154208_m1), HIF-1A (Hs00153153_m1), actin beta (ACTB) (Hs01060665_g1). The PCR thermal cycle conditions were as follows: initial step at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. The relative abundance of target transcripts was evaluated and normalized to the expression level of ACTB as an internal control using the 2-ΔDCt method, where ΔDCt is the difference in Ct values between the mRNAs of interest and the normalizer. Normalized values were further log2 transformed.

2.10. Flow cytometry analysis (FCM)

For determining human CAIX expression, after being treated with flow cytometry staining buffer (R & D Systems Inc., MN, USA), the cells were incubated with 10 μL/10^6 cells anti-human PE-conjugated anti-CAIX (R&D Systems Inc.) at 4°C for 30 min in the dark. Measurements were obtained via Moxi GO II flow cytometer (Orflo Technologies, ID, USA).

2.11. Proliferation assays

Proliferation assays were done with the CellTiter-Blue® cell viability kit (Promega, WI, USA), as manufacturer's instructions. Briefly, the cells were plated at 10 × 10^3 cells/well in 96-well plates. After each treatment shown in Supplementarl Figure, Cell Titer-Blue® Cell Viability Reagent (Promega) was added to each well, and the cells were incubated for an additional 1 h. Cell Titer-Blue® fluorescence was detected with a

SoftMax® Pro 5 device (Molecular Devices, CA, USA), using an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

2.12. Apoptosis assays

FACS analysis using the Annexin V/PI staining method was used to determine the apoptotic rate in control and treated cells. Briefly, the cells were washed with Annexin V Binding Buffer (Biolegend, CA, USA) and double-stained with FITC-Annexin V conjugate (Biolegend) and Propidium Iodide staining solution (Thermo Fisher Science). Measurements were obtained via Moxi GO II flow cytometer (Orflo Technologies).

2.13. Statistical Analysis

Statistical analyses were performed using the JMP 14.0, a statistical software program (SAS Institute, Inc., Cary, NC, USA). Overall survival (OS) was calculated from the date of the start of treatment to the date of death from any cause or the last follow-up date. Survival was analyzed using Kaplan–Meier curves, and differences between groups were assessed using the Gehan–Breslow–Wilcoxon test. The parameters were compared using the chi-squared and Fisher's exact tests between the two variables. Continuous variables were evaluated using t-tests. Significance was set at $p < 0.05$.

3. Results

3.1. High-CA9 expression demonstrated a significantly poorer prognosis in patients with PDAC in TCGA Datasets

As a preliminary evaluation, we first assessed the performance of CA9 in predicting the prognosis in patients with PDAC using messenger (m) ribonucleic acid (RNA) expression data from an independent public dataset. First, the RNA sequencing profile and clinical data of patients with PDAC were extracted from TCGA datasets. None of the patients in this cohort underwent NAT. We divided the patients into two groups according to the median value of the expression levels of CA9 and compared the OS using the Kaplan–Meier method (Fig. 1A). The median survival time (MST) of cases in the high-CA9 group was 17.5 months, which was significantly poorer than that in the low-CA9 group (MST, 30.0 months, $p = 0.013$), highlighting the importance of CA9 expression in predicting prognosis in patients with PDAC who undergo US.

3.2. Differences of CA9 Expression in Pancreatic Cancer Cells in the US and NACRT Group

In total, 273 patients who underwent curative resection of PDAC at our institution were included. Among them, 124 patients who underwent curative-intent surgery alone were classified as the US group, whereas 149 patients who underwent curative-intent surgery following NACRT were classified as the NACRT group. We summarized clinicopathological data of each group in Supplemental Table. The clinicopathological results of the two groups did not differ from our previous publications [21]. First, we compared the OS of the two groups using the Kaplan–Meier method, which demonstrated that the 5-year OS rate of patients in the NACRT group was 37.8%, which was significantly better than that of patients in the US group (26.6%, $p < 0.001$, Fig. 1B). Next, to

evaluate the significance of CA9 expression in human pancreatic cancer, we performed IHC analysis of FFPE tissues. CA9 was mainly expressed in the cell membrane of cancer cells (Fig. 1C), whereas its expression was almost absent in non-cancerous tissues, including islet cells. The positive rate of CA9 in the 273 surgical specimens was 46.5%. The positive rate of CA9 in the NACRT group was 36.2%, which was significantly lower than that in the US group (58.9%; $p < 0.001$; Fig. 1D).

3.3. The Prognostic Impact of CA9 Expression According to NACRT and CA9 Status

Subsequently, we divided the patients into two categories based on the expression level of CA9 and compared their prognoses using the Kaplan–Meier method. Although the rate of patients who were CA9-positive with R1 resection is significantly higher than that of patients who were CA9-negative in the NACRT group ($p = 0.012$; Table 1), there were no other statistical differences in the oncological characteristics between patients who were CA9-positive and -negative in either group. In the US group, consistent with the TCGA results, the MST of patients who were CA9-positive was 21.5 months and significantly poorer than that of patients who were CA9-negative (MST, 45.8 months; $p=0.030$; Fig. 2A). In contrast, there was no significant difference between patients who were CA9-positive and CA9-negative in the NACRT group (MST, 49.2 months vs. 46.3 months; $p = 0.938$; Fig. 2B).

We next evaluated the patient's prognosis in the US and NACRT groups according to CA9 status. Interestingly, patients who were CA9-positive in the US group had a significantly poorer prognosis than those in the NACRT group ($p < 0.001$; Fig. 2C), whereas there was no significant difference between patients who were

CA9-negative in the US group and those in the NACRT group (p = 0.357; Fig. 2D). In summary, these results demonstrated that CA9 expression defined prognosis after curative surgery in patients without NAT and that patients who were CA9-positive may be the most clinically optimal subgroup for NACRT, highlighting the utility of CA9 in predicting the prognostic value of NACRT.

3.4. Hypoxia Induced CA9 in Pancreatic Cancer Cell Lines

To elucidate the interactions between CA9 and CRT/RT in PDAC cells, *in-vitro* assays were performed using human pancreatic cancer cell lines. We first assessed CA9 expression levels in BxPC-3 and PANC-1 cells using real-time PCR and FCM under normoxic and hypoxic conditions. CA9 mRNA and protein expression levels were significantly increased by hypoxic in both the cell lines (Fig. 3A and B).

3.5. RT and CRT Reduced CA9-expression Levels in Pancreatic Cancer Cell Lines

Next, we evaluated CA9 expression levels in pancreatic cancer cell lines after RT and CRT compared to the control without treatment under hypoxic conditions. Each treatment schedule is shown in the Supplemental Figure. CA9 mRNA and protein expression levels after each treatment were significantly lower than those in the control (Fig. 3C to F). Importantly, these results were compatible with our IHC findings using actual clinically resected specimens and suggested that the expression of CA9 on cancer cells is inhibited by RT/CRT.

3.6. CA9 Knockdown Suppressed the Impact of Treatments of RT/CRT for Pancreatic Cancer Cell Proliferation

To make the experimental results more comprehensible, we used BxPC-3 cells in later experiments, which markedly increased CA9 expression under hypoxic conditions. In the proliferation assay under hypoxic conditions, the proliferation rate of BxPC-3 cells with CA9 knockdown was 39.2% of that observed in BxPC-3 cells without CA9 knockdown. In BxPC-3 cells without CA9 knockdown, RT/CRT reduced the growth of cancer cells; however, interestingly, the treatment effects of RT/CRT were significantly inhibited in cancer cells with CA9 knockdown (control siRNA + RT, 37.1% vs. CA9 siRNA + RT, 50.8%, $p < 0.001$; control siRNA + CRT, 10.8% vs. CA9 siRNA + CRT, 32.1%, $p < 0.001$, Fig. 4A). Each of these proliferation rates was calculated using the proliferation rate of the untreated cell line as 100%. These findings indicated that the cell-killing effect of RT/CRT in cancer cells may target CA9 expression.

3.7. CA9 Knockdown Suppressed Treated Pancreatic Cancer Cell Apoptosis

Based on these results, we investigated the impact of CA9 expression on apoptosis induction by RT/CRT treatments. Transient CA9 knockdown in pancreatic cells decreased apoptotic cell numbers induced by RT and CRT compared to that of control siRNA-treated cells. (early apoptosis: control siRNA, 33.7%; control siRNA + RT, 49.3%; control siRNA + CRT, 60.5%; CA9 siRNA, 33.1%; CA9 siRNA + RT, 32.8%; and CA9 siRNA + CRT, 35.3%, late apoptosis: control siRNA, 3.53%; control siRNA + RT, 5.82%; control siRNA + CRT, 13.1%; CA9 siRNA, 4.93%; CA9 siRNA + RT, 8.41%; and CA9 siRNA + CRT: 8.03%; Fig. 4B). This underlying experiment paradoxically suggested that the importance of CA9 expression in cancer cells for the apoptosis-inducing effects of RT/CRT.

3.8. Additional Evaluation of CA9 Levels Utilizing Matched Serum Specimens Collected Pre- and Post-NACRT

Although validation steps using surgically resected specimens and pancreatic cancer cell lines are necessary to prove the importance of CA9, we believe that the verification of this molecule in preoperative blood specimens will play a key role in facilitating translation to clinical settings. Based on this logic, we collected matched serum specimens before and after NACRT from a subset of 20 patients with PDAC and evaluated serum CA9 levels using ELISA to compare the values between before and after NACRT. The serum CA9 levels after NACRT were elevated in 18 of 20 patients compared to before, with significant differences between pre- and post-NACRT values (the serum CA9 levels before NACRT, 109.2 ± 98 pg/mL; after NACRT, 235.1 ± 195 pg/mL; p=0.004; Fig. 5A). This result demonstrated the interesting finding that CA9 was decreased by NACRT in surgically resected tissues; in contrast, it was elevated in serum.

3.9. CRT Induced Significantly Increased CA9 Levels in Cell Culture Medium

To clarify this paradox of CA9 expression between the tissue and the serum, we evaluated CA9 levels in the cell culture medium after CRT compared with that before. The CRT schedule was the same as that used for PCR and FCM analyses. The CA9 level after CRT was significantly elevated in the medium compared with that before CRT, despite its decrease in the cells (the CA9 levels of the medium before CRT, 6.8 ± 0.7 pg/mL; the CA9 levels of the medium after CRT, 45.2 ± 5.0 pg/mL; p = 0.002; Fig. 5B). The results of these serum/tissue

and medium/cell experiments led us to hypothesize that the therapeutic effect of NACRT involves the release of CA9 from cancer cells into the extracellular liquids.

3.10. Serum CA9 Levels After NACRT Correlates with CA9 Expression in Surgically Resected Specimens

Finally, to investigate the applicability of CA9 as a biomarker in clinical settings, we evaluated the correlation of CA9 expression between serums and surgically resected tissues. Accordingly, IHC for CA9 was performed on surgical specimens from 20 patients with matched serums evaluated above, resulting in 10 patients being classified as CA9 negative, and the other 10 as CA9 positive. Interestingly, when we examined the relationship of CA9 expression between serums and tissues, we observed that the positive cases of CA9 expression in cancer tissues tended to have higher serum CA9 than that of the negative cases (CA9 negative, 157.5 ± 111 pg/mL; CA9 positive, 312.7 ± 234 pg/mL; $p = 0.075$; Fig. 5C), suggesting the potential of CA9 as a noninvasive biomarker.

4. Discussion

As cancer treatment reaches the threshold of a new era of precision medicine, further development of personalized treatment strategies is essential. In this context, the guidelines for RT/CRT in patients with PDAC remain ambiguous, despite the fact that RT/CRT generally provides robust local cancer control and plays an important role in multimodality treatment strategies for other cancers. Our study provided a novel step in this

direction, wherein we carried out a comprehensive and systematic investigation of the role of CA9 and, for the first time to our knowledge, reported that CA9 had a clinical impact, especially on RT/CRT-sensitivity in PDAC.

As we have previously reported, our updated data consistently showed that the NACRT group had a more favorable prognosis than the US group [21]. Interestingly, this study indicated that NACRT can offer prognostic benefits only for a limited subgroup, namely CA9-positive patients. Based on the IHC results of the surgically resected specimens, we hypothesized that CRT targets CA9 in cancer cells and attenuates its expression and oncological function. To confirm this hypothesis, we analyzed the relationship between CRT and CA9 using pancreatic cancer cell lines and obtained results consistent with those of IHC. Moreover, the inhibition of CA9 expression by siRNA attenuated the apoptotic and tumor growth inhibitory effects of CRT. These findings indicated that CA9-positive tumor cells have more malignant features but are more sensitive to chemoradiotherapy and that apoptosis is easily induced by the therapeutic effect of CRT. Taken together, these findings highlighted the clinical importance of CA9 as a potential biomarker for predicting sensitivity to CRT and the potential role of a target molecule for CRT.

To the best of our knowledge, the present study is the first to report a relationship between CA9 and NACRT in PDAC. Kudo et al. [24] previously described that CA9-expression on cancer cells was upregulated after NAT, but 93% of the patients in their study had received only chemotherapy. This discrepancy between chemotherapy alone and RT/CRT underscores the important association of CA9 with radiotherapy, but not chemotherapy. Considering that radiotherapy focuses on the local effects on cancer, the local hypoxic microenvironment induced by CA9 in cancer tissues may interact closely with radiotherapy. Although the detailed

mechanisms remain unknown and require further investigation, our results suggested that CA9 expression promotes radiation-induced cancer cell death. Although further study is needed, this suggests that the currently controversial indications for adjuvant radiation/chemoradiotherapy may be of great benefit.

To apply the present results in clinical practice as predictive biomarkers of radiosensitivity, it is essential to understand the dynamics of CA9 levels before and during preoperative treatment. Recently, exosomal CA9 mRNA and serum CA9 have been suggested as potential prognostic markers in other cancer types [25,26]. In this study, we found that serum CA9 levels tended to correlate positively with CA9 expression in cancer tissues; however, we could not detect significant differences because of the small number of cases that matched preoperative serum and surgically resected tissue samples. Nonetheless, this promising finding indicated that pretreatment serum CA9 levels may facilitate informed decision-making by physicians and patients in determining the indications for NACRT.

Furthermore, we discovered that serum CA9 levels were dynamically increased by NACRT, which was reproduced and validated by *in-vitro* experiments using cell lines and culture medium. The mechanism of this phenomenon is expected to be that CA9 is a transmembrane protein that exists on the cell surface, and the extracellular portion of CA9 is shredded and released into the serum [10,27]. These results demonstrated the transferability of CA9 from tissue to blood and the potential of CA9 as a non-invasive biomarker for PDAC. However, further investigations are essential regarding the correlation of CA9 between tissue and blood samples in independent larger patient cohorts and the association of radiation-induced changes in blood CA9 levels with clinical outcomes.

Although our results highlighted the clinical importance of CA9 in PDAC, this study had a few limitations. First, we conducted a retrospective study and analyzed moderately sized clinical cohorts that potentially included associated risks of bias. Second, we were unable to confirm the expression status of CA9 before and after NACRT in the same patients using IHC. To investigate this, we performed IHC for CA9 using endoscopic fine-needle biopsy specimens before treatment. However, because the biopsy samples were usually too small, a detailed evaluation and classification of the CA9 status by IHC was not possible. Third, this study focused solely on comparing the NACRT and US groups. We could not include patients who received adjuvant chemotherapy without radiotherapy due to the very limited number of such patients and the scarcity of available samples. Despite these limitations, the present study provided promising evidence of the clinical impact of CA9 on NACRT sensitivity and an initiative for designing genome-guided clinical trials for patients with PDAC in the future.

In conclusion, we described, for the first time to our knowledge, the clinical impact of CA9 expression in PDAC, especially in NACRT. CA9 may act as a target molecule for radiotherapy, highlighting its clinical importance as a useful biomarker of more stringent indications for RT. Further studies on CA9 are warranted to develop novel therapeutic strategies in the era of individualized multidisciplinary treatment for PDAC.

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Declaration of competing interest

The authors declare that they have no confict of interest.

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Fig. 1. No correlation between CA9 expression and prognosis is observed after NACRT

(A) Comparison of overall survivals between high- and low-CA9 expression groups from TCGA datasets. (B) Kaplan–Meier curves for all patients. (C) Immunohistochemistry for CA9 expression in PDAC tissues (original magnification, ×200). (D) The positivity rate of CA9 in the upfront surgery group and NACRT groups is evaluated using IHC.

Fig. 2. Kaplan-Meier curves for each group

(A) Kaplan-Meier curves for patients who underwent upfront surgery. (B) Kaplan-Meier curves for patients who underwent NACRT. (C) Kaplan-Meier curves for patients with CA9-positive tumors. (D) Kaplan-Meier curves for patients with CA9-negative tumors.

Fig. 3. RT and CRT reduces CA9-expression levels in pancreatic cancer cell lines

BxPC-3 and PANC-1 cells cultured in the indicated condition. (A), (C), and (E), real-time PCR analysis of CA9 expression. (A) Normoxia vs. Hypoxia. (C) control vs. RT. (E) control vs. CRT. (B), (D), and (F), FCM analysis of CA9 expression. (B) Normoxia vs. Hypoxia. (D) control vs. RT. (F) control vs. CRT.

Fig. 4. CA9 knockdown suppresses treatment effect on pancreatic cancer cell proliferation through reducing apoptosis

BxPC-3 cells cultured in the indicated condition. (A) After CA9 inhibition, the growth inhibitory effect of each treatment on CA9-positive BxPC-3 cells is significantly reduced. (B) Annexin V/PI double staining indicates CA9 knockdown suppresses treated pancreatic cancer cell apoptosis.

Fig. 5. CA9 levels in the liquid

(A) Serum CA9 levels of patients who underwent NACRT for pancreatic cancer. (B) CA9 levels in cell culture medium before and after CRT. (C) Relationship between the CA9 level in serum and CA9-expression evaluated by IHC.

Table 1 Clinicopathological characteristics classified by CA9 expression

Characteristic		Upfront surgery					NACRT				
		CA9-positive n=73		CA9-negative n=51		p value	CA9-positive n=54		CA9-negative n=95		p value
Gender, n, %	Male	42	57.5	31	60.8	0.853	26	48.2	61	64.2	0.060
	Female	31	42.5	20	39.2		28	51.9	34	35.8	
Age, n, %	<74	42	57.5	38	74.5	0.059	49	90.7	81	85.3	0.446
	≥75	31	42.5	13	25.5		5	9.3	14	14.7	
Resectability[a], n, %	Resectable	60	82.2	44	86.3	0.543	34	63.0	64	67.4	0.297
	Borderline/URLA	13	17.8	7	13.7		20	37.0	31	32.6	
CA19-9, U/mL, range	Median (range)	80	(1-19296)	97	(1-1714)	0.490	97	(1-4956)	109	(1-3335)	0.310
DUPAN-2, U/mL, range	Median (range)	146	(25-1600)	91	(25-1600)	0.977	157	(25-3700)	160	(25-3980)	0.525
Operation time, min, range	Median (range)	278	(118-587)	308	(107-433)	0.350	311	(121-630)	289	(144-642)	0.278
Blood loss, mL, range	Median (range)	380	(10-3520)	546	(60-2150)		388	(10-3310)	345	(10-6730)	0.436
Pancreatic fistula[b], n, %	≥ grade B[a]	19	26.0	15	29.4	0.688	10	18.5	14	14.7	0.644
pT status[c], n, %	T1/T2	8	11.0	12	23.5	0.061	13	24.1	26	27.4	0.660
	T3/T4	65	89.0	39	76.5		41	75.9	69	72.6	
LN metastasis, n, %	Absent	30	41.1	28	54.9	0.130	39	72.2	75	79.0	0.352
	Present	43	58.9	23	45.1		15	27.8	20	21.0	
Resection status[a], n, %	R0	60	82.2	40	78.4	0.648	47	87.0	93	97.9	**0.012**
	R1	13	17.8	11	21.6		7	13.0	2	2.1	
Adjuvant chemotherapy, n, %	Induction	59	80.8	46	90.2	0.207	50	92.6	89	93.7	0.798
	Completion	35	48.0	32	62.8	0.142	40	74.1	72	75.8	0.816

[a]NCCN Guidelines Version 2.2021. [b]Defined by International Study Group of Pancreatic Fistula.

[c]7th edition of the Union for International Cancer Control TNM classification of malignant tumors.

NACRT indicates neoadjuvant chemoradiotherapy; CA19-9, carbohydrate antigen 19-9; DUPAN-2, Duke pancreatic mono-clonal antigen type 2c; pT status, pathological T status; LN, lymph node.

Supporting imformation
Supplemental Figure: The cell treatment protocol in vitro assay
Supplemental Table: Clinicopathological characteristics in 273 patients