Dual antiplatelet therapy inhibits neutrophil extracellular traps to reduce liver micrometastases of intrahepatic cholangiocarcinoma

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Running title: DAPT reduces cancer liver micrometastasis via suppressing NETs

Abbreviations: NETs, neutrophil extracellular traps; iCCA, intrahepatic cholangiocarcinoma;; MPO, myeloperoxidase; citH3, citrullinated histone 3; DAPT, dual antiplatelet therapy; ASA, aspirin

Abstract

The involvement of neutrophil extracellular traps (NETs) in cancer metastasis is being clarified, but the relationship between intrahepatic cholangiocarcinoma (iCCA) and NETs remains unclear. The presence of NETs was verified by multiple fluorescence staining in clinically resected specimens of iCCA. Human neutrophils were co-cultured with iCCA cells to observe NET induction and changes in cellular characteristics. Binding of platelets to iCCA cells and its mechanism were also examined, and their effects on NETs were analyzed in vitro and in in vivo mouse models. NETs were present in the tumor periphery of resected iCCAs. NETs promoted the motility and migration ability of iCCA cells in vitro. Although iCCA cells alone had a weak NET-inducing ability, the binding of platelets to iCCA cells via P-selectin promoted NET induction. Based on these results, antiplatelet drugs were applied to these cocultures in vitro and inhibited the binding of platelets to iCCA cells and the induction of NETs. Fluorescently labeled iCCA cells were injected into the spleen of mice, resulting in the formation of liver micrometastases coexisting with platelets and NETs. These mice were treated with dual antiplatelet therapy (DAPT) consisting of aspirin and ticagrelor, which dramatically reduced micrometastases. These results suggest that potent antiplatelet therapy prevents micrometastases of iCCA cells by inhibiting platelet activation and NET production, and it may contribute to a novel therapeutic strategy.

(222 words)

Keywords

Aspirin, Ticagrelor, P-selectin, Platelet, Time-lapse imaging

1. Introduction

Cholangiocarcinoma is the second most common primary malignancy affecting the liver[1]. Although surgery is the only curative therapy, most patients have metastatic or locally advanced disease at first diagnosis, and only about 25% of patients are eligible for resection[2]. Even in surgically resected intrahepatic cholangiocarcinoma (iCCA), postoperative outcomes are poor, and recurrence has been reported to occur in up to 50-60% of patients, with a median disease-free survival of 26 months and 5-year survival after surgical resection ranging from 15% to 40%[3]. Even worse, in metastatic iCCA patients, the most effective first-line chemotherapy with gemcitabine and cisplatin remains unattractive, with OS of less than 1 year[4]. Salvage therapies are scarce and limited. Thus, there is an urgent need to elucidate the mechanism of tumor progression and distant metastasis of iCCA and develop therapies to prevent them.

The initial step of metastasis must be the arrival of circulating tumor cells (CTCs) from the primary site to the distant site to form an early metastatic niche. Recent studies have suggested that platelets play crucial roles in this niche formation[5, 6]. Once cancer cells migrate into the circulation from the primary site, they are promptly attacked by immune cells. However, circulating platelets quickly adhere to CTCs via adhesion receptors expressed on their surface, such as GPIIb/IIIa or CD62P (P-selectin)[7]. By this platelet shield, CTCs are

able to not only avoid stress from the circulation or natural killer cells, but it also promotes their adhesion to endothelium and subsequent extravasation[8, 9]. Furthermore, it has been shown that cytokines derived from platelets, such as TGF-β, facilitate the epithelial-to-mesenchymal transition (EMT) of cancer cells[10]. Therefore, it is presumed that platelets must be among the most critical factors in metastasis, but much remains unclear.

Neutrophil extracellular traps (NETs), which is the phenomenon of neutrophils extruding their nucleotides on stimulation, was initially found to entrap microorganisms[11, 12]. Mounting evidence suggests that NETs appear to have a role in cancer metastasis, because NETs ensnare CTCs in experimental models of sepsis, promoting early adhesive events and metastatic progression[13]. It is important to note that platelets are involved in the induction of NETs in various circumstances, including bacteria in septic blood, ischemic stroke, systemic sclerosis, or transfusion-related acute lung injury[14-17]. From these findings, we hypothesized that platelets are also involved in the process of cancer metastasis via the induction of NETs.

Since little has been known about the interaction between iCCA and NETs, our research started with an investigation of the interaction between NETs and iCCA, and further addressed the role of platelets in iCCA-induced NETs. By forming a complex with platelets, iCCA cells activated platelets, which in turn induced NETs. Furthermore, it was found that

NET production by platelets together with iCCA cells was suppressed by preventing platelet activation using antiplatelet agents. These results suggest that iCCA cells in the circulating blood may cooperate with platelets and NETs, thereby forming micrometastatic foci and improving motility. Platelet-targeted therapy may be one therapeutic strategy to prevent micrometastasis formation in cholangiocarcinoma.

2. Materials and Methods

2.1 Cell culture and reagents

The human iCCA cell lines HuCCT1 and HuH28 were obtained from the Japanese Collection of Research Bioresources Cell Bank. The human pancreatic cancer cell lines, PANC-1 and MIAPaCa-2, and the human colon cancer cell line HCT116 were obtained from the American Type Culture Collection. Cell line cultures were incubated in RPMI, DMEM, or McCoy's media with 10% fetal bovine serum (FBS) and maintained at 37 °C in a humidified incubator infused with 20% O_2 and 5% CO_2. Phorbol 12-myristate 13-acetate, PMA (#P1585, Sigma-Aldrich), the peptidyl arginine deiminase 4 (PAD4) inhibitor, Cl-amidine (#10599, Cayman), deoxyribonuclease, DNase (#10104159001, Roche), and human thrombin (#7009, Sigma-Aldrich) were used.

2.2 Isolation of neutrophils and platelets

Neutrophils were isolated from a healthy volunteer. Whole blood was collected by venipuncture into blood collection tubes coated with EDTA 2K (#365900, Becton, Dickinson and Company). Then, 5 mL of whole blood were layered over 5 mL of Polymorphprep (#114683, Abbott Diagnostics Technologies AS) in a 15-mL tube and centrifuged at 500 g for 35 min at room temperature. The lower leukocyte band containing neutrophils was collected, followed by washing twice with PBS. Finally, they were resuspended in RPMI without FBS.

Platelets were isolated from a healthy volunteer. Whole blood was collected by a 21-G needle into an acid citrate dextrose (ACD)-containing tube, and the first 3 mL of blood were discarded. Blood was centrifuged at 200 g for 20 min at room temperature. The platelet-rich plasma was collected, and HEP buffer including prostaglandin E1 (1 μM) was added at a 1:1 ratio (v/v). After being spun at 100 g for 15 min, the supernatant was transferred and centrifuged at 800 g for 15 min to obtain a platelet pellet. The platelet pellet was rinsed with platelet wash buffer and resuspended in Tyrode's buffer.

2.3 Purification of NETs

NETs were isolated using a previously described method with slight modification[18]. Neutrophils were treated with 20 nM PMA for 4 h. After removal of the supernatant containing

PMA, NETs adhered at the bottom were washed down by pipetting 2 ml of cold PBS and centrifuged at 1,000 g at 4 ºC for 10 min. The cell-free supernatant containing NETs was collected. These PMA-induced cell-free NETs were used as NETs to investigate their effect on cells.

To inhibit NET formation, neutrophils were pretreated with Cl-amidine (PAD4 inhibitor: 200 μM) for 30 min before stimulation with PMA. To decompose NETs, DNase (100 U/ml) was reacted for 30 min after stimulation with PMA for 3 h.

2.4 Patients and tissue samples

Immunofluorescence staining for NETs was performed in the tissues of primary tumors of patients with iCCA who underwent surgery at Okayama University Hospital between 2014 and 2019. All samples were collected from patients who had provided informed consent, and all related procedures were performed with the approval of the internal review and ethics board of Okayama University Hospital.

2.5 Immunofluorescence staining

Paraffin-embedded tissue samples were deparaffinized and rehydrated. Non-specific binding was blocked with 10% FBS for 30 min at room temperature. Cells for

immunofluorescence were fixed with 4% paraformaldehyde for 15 min at room temperature or fixed and permeabilized with methanol for 10 min at room temperature. Cells were then blocked with 10% FBS for 30 min at room temperature. Subsequently, the samples were incubated with rabbit anti-citrullinated histone H3 (citH3) (1:500, Abcam, ab5103), mouse anti-myeloperoxidase (MPO) (1:500, Abcam, ab25989), mouse anti-β actin (1:1000, Sigma, A5441), mouse anti-E-cadherin (1:300, Abcam, ab1416), mouse anti-vimentin (1:1000, Abcam, ab8978), mouse anti-CD41-APC (1:100, BECKMAN COULTER, B16894), mouse anti-CD62P (P-selectin)-FITC (1:100, BECKMAN COULTER, A07790), mouse anti-EpCAM (1:800, Cell Signaling, #2929), rat anti-Galectin-3 (1:500, BioLegend, 125401), and rat anti-Mouse-CD41 (1:100, BD Pharmingen, 553847) overnight at 4 °C. The tissues were incubated with Alexa-Fluor-conjugated secondary antibodies in 2% FBS for 1 h at room temperature. DAPI was then used to counterstain the nuclei, and images were obtained by IX83 (Olympus, Tokyo, Japan). The NETs in tissue samples were determined as the percentage of double-positive for citH3 and MPO signals to avoid false-positives due to autofluorescence of the tissue. The NETs in in vitro experiments were determined as the percentage of the positive citH3 signal[19]. For NET quantification in tissue samples, NETs were counted in at least 3 fields per section, and 3 sections per sample were evaluated. The accuracy of automated measurements was confirmed by two independent

investigators who were unaware of the patients' clinical information. For other quantification of the immunofluorescence area or intensity in vitro, at least 5 fields per 3 independent investigations were investigated.

2.6 Transwell migration assay

The in vitro migration assay was examined in the chambers of 8-μm transwell inserts (BD Falcon™). Cancer cells were cultured with neutrophils or NETs for 48 h. After trypsinization and centrifugation, cancer cells were added to serum-free medium in the upper chamber of each well insert, and RPMI supplemented with 2% FBS was added into the lower chamber. After incubation for 24 h at 37 °C, the non-invading cancer cells were wiped off, and cells on the bottom side of the upper chamber were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The stained cells were counted under a light microscope at a magnification of ×200.

2.7 Interactions between cancer cells, platelets, and neutrophils

Interactions between cancer cells and neutrophils were examined in the presence or absence of platelets. Briefly, washed suspensions of cancer cells were seeded with or without platelets under low shear on the rocking table for 30 min. In some examinations, neutrophils were

added to cancer cells and platelets at a ratio of 1:10:200 (cancer cells: neutrophils: platelets). The following experiments using platelets and neutrophils were performed under the above conditions.

2.8 Platelet adhesion assay

Platelet adhesion to cancer cells was measured using a previously described method with slight modification by flow cytometry based on the detection of CD41 on the surface of cancer cells[20, 21]. Cancer cells and platelets were co-incubated as described above. Platelet-cancer cell aggregates were centrifuged at 50 g for 5 min to fractionate platelet-cancer cell aggregates and unbound platelets. Next, platelet-cancer cell aggregates were washed and labeled with mouse anti-human CD41-APC antibody (Beckman Coulter, B16894). Samples were analyzed within 1 h by flow cytometry (Becton Dickinson). Using a log-forward scatter versus log-side scatter dot plot, a two-dimensional analysis gate was drawn around the cancer cell population, and a fluorescence histogram was obtained for 8,000 events for each sample. Platelet aggregate and cancer cell doublets were excluded using size-based gating. Data were analyzed using FlowJo™ v10 software (Becton Dickinson). The percentage of platelet tumor cell adhesion was calculated as the percentage of cells within the tumor cell gate positive for platelets. Unbound platelets were

used in other experiments.

2.9 Time-lapse confocal microscopy

For all video-microscopy experiments, time-lapse videos were taken with a FV10i (Olympus) equipped with a cell culture chamber.

For the motility assay, cancer cells were stained with CellTracker Green (10 μM, Thermo Fisher) and incubated with 10% FBS for 24 h. The medium was discarded, and NETs were added with 2% FBS. Time-lapse videos were taken every 2 min for 12 h. Cells were tracked using semiautomatic cell tracking in Fiji TrackMate (excluding tracks shorter than 8 h).

For the NETosis assay, neutrophils were stained with CellTracker Red (1 μM, Thermo Fisher), and then SYTOX BLUE (1 μg/ml, Thermo Fisher) was added into the medium with or without PMA. For the extracellular DNA assay, cancer cells, platelets, and neutrophils were co-incubated as described above. At the same time neutrophils were added, SYTOX Blue was added, and time-lapse videos were taken every 8 min for 10 h. The areas of stained extracellular DNA were measured.

2.10 Animal experiments

Athymic female BALB/c nu/nu nude mice aged 8-12 weeks were purchased from CLEA Japan. The animal care and experimental procedures were conducted in accordance with the regulations of the Animal Care and Use Committee of Okayama University. In some experiments, CMFDA (CellTracker^{TM} Green CMFDA Dye, Thermo Fisher Scientific)-labeled HuCCT1 or HuH28 cells (5×10^5 cells) in 200 μl of PBS were injected into the spleen. After one day, the mice were sacrificed, and their livers were removed. In another experiment, mice were divided into two treatment groups receiving placebo or medication using a previously described method with slight modification[22, 23]. Before intrasplenic cancer cell injection, one group received aspirin (ASA) (100 mg/kg) for five days and ticagrelor (150 mg/kg) for two days, and the other group received PBS (placebo, 200 μl). After one and three days, the mice were sacrificed, and their livers were removed. The harvested livers were frozen using Optimal Cutting Temperature compound (#45833, Sakura Finetek Japan) and cryosectioned. Then, liver tissue sections were stained as described above. Cancer cells, NETs, and platelets were measured in at least 5 fields per section, and 5 sections per sample were evaluated in a high-power field.

3. Results

3.1 NETs were present in the tumor microenvironment of iCCAs

To investigate the presence of NETs in iCCAs in clinical specimens, primary tumors of 41 patients with iCCA were initially subjected to immunofluorescence staining. NETs were abundant in the iCCA tissues **(Fig. S1)**, and mostly detected at the periphery of tumors rather than at the centers of tumors, presumably due to necrotic and fibrotic changes in the centers of most tumors. The actual presence of NETs in iCCA tumors suggested some possible clinical implications of NETs.

3.2 NETs change the morphology and promote motility and migration ability of cancer cells

To further explore the association between iCCA cells and NETs, two iCCA cell lines, one moderately differentiated HuCCT1 with epithelial characteristics and the other undifferentiated HuH28 with mesenchymal characteristics, were used. Co-culture with NETs had changed the morphology of iCCA cells, as well as other cancer cells **(Fig. 1A)**, suggesting the changes associated with the EMT. To investigate whether these morphology changes are related to the motility of iCCA cells, HuCCT1 cells were co-cultured with neutrophils stimulated by PMA overnight and observed by time-lapse videos. Coculturing with NETs significantly increased the motility of HuCCT1 cells, whereas these effects were suppressed by DNase, suggesting the involvement of NETs **(Fig. 1B, Video 1)**. In addition,

a migration assay was performed with HuCCT1 cells cultured under these conditions. PMA-induced NETs also potentiated the migration ability of HuCCT1 cells **(Fig. 1C, Fig. S2A)**. Whether such an increase in the aggressiveness of HuCCT1 cells by NETs was due to EMT was further examined. HuCCT1 cells incubated with NETs decreased expression of E-cadherin and increased expression of vimentin **(Fig. 1D, Fig S2B)**. Furthermore, western blotting showed downregulated E-cadherin and upregulated vimentin and slug **(Fig. 1E)**.

The ability to induce NETs in iCCA cell lines was next tested by comparison with pancreatic cancer cell lines. HuCCT1 and HuH28 induced NETs, but not as much as Panc-1 and MIA-PaCa2 **(Fig. S2C)**. Although these results showed that NETs affect the malignant properties of iCCA, iCCA cells, unlike other pancreatic cancer cells, may not induce NETs by themselves.

3.3 Platelets were activated by binding to iCCA cells

Having shown that NETs were present in iCCA primary tumors and that NETs induce the EMT of iCCA cells, the amounts of NETs in the primary tumor of iCCA were not associated with overall survival (OS) or recurrence-free survival (RFS) **(Fig. 2A, Table S1).** It has been reported that high pre-surgery serum NET levels were associated with shorter RFS and OS[24]. NETs can entrap circulating tumor cells (CTCs), thus favoring metastatic dissemination[25]. Next, using floating cells as a CTC model, whether iCCA cells as CTCs

induce NETs was investigated. Floating HuCCT1 and HuH28 cells were co-cultured with neutrophils under low shear on a rocking table. However, floating iCCA cells could not induce NETs by themselves, even in direct co-culture **(Fig. 2B)**. We then hypothesized that other factors in the blood circulation, such as platelets, cooperate to promote NET formation in the circulation.

To study the role of platelets in NET induction, platelets were stimulated with thrombin and then co-cultured with neutrophils. Platelets stimulated with thrombin were activated and expressed CD62P (P-selectin) **(Fig. S3A)**. Although resting platelets failed to induce NETs, activated platelets increased NET induction **(Fig. 2C, Fig. S3B)**.

Next, the interaction of iCCA cells with platelets was investigated. Soluble mediators such as ADP[26], thromboxane A2[27], or high-mobility group box 1[28] derived from cancer cells activate platelets[29]. Unlike pancreatic cancer cell conditioned medium, iCCA cell conditioned medium did not activate platelets **(Fig. S3C)**. Then, iCCA cells were co-cultured with platelets directly. Immunofluorescence staining showed platelets clinging to the surface of iCCA cells **(Fig. S3D, left),** and aggregates of iCCA cells and platelets (CD41-positive cancer cells) were also observed on flow cytometry **(Fig. S3D right)**. Interestingly, both iCCA and PDAC cell lines bound to platelets, but the highest binding activity was found in HuH28 in iCCA cell lines **(Fig. 2D)**. In addition, platelets on iCCA cells expressed P-selectin **(Fig.**

5B, non-treated). Notably, in direct co-culture with HuH28, platelets unbound to the cells in the supernatant were also activated **(Fig. 2E)**, while resting platelets did not express P-selectin **(Fig. 2E)**. On the other hand, iCCA cells did not activate platelets in conditions of indirect co-culture **(Fig. 2E)**. It is well known that activated platelets secrete platelet granule content, and secreted granule contents not only enhance platelet activation signaling, but also activate and recruit circulating resting platelets[30]. It is thought that these activated platelets on iCCA cells promote the activation of surrounding non-binding platelets. These results suggest that direct contact between platelets and iCCA cells is important for platelet activation by iCCA cells, as in blood circulation.

Platelets and cancer interactions have been shown to involve platelet-expressing membrane receptors, such as integrin αIIbβ3, P-selectin, integrin α6β1, and glycoprotein VI (GPVI)[31]. Of them, galectin-3 binds to GPVI to activate platelets and mediate P-selection exposure[32], and it is known to be expressed in hepatocellular carcinoma and cholangiocarcinoma[29, 33, 34]. As expected, galectin-3 was expressed on the surface of the cell membranes of iCCA and PDAC cells **(Fig. 2F, Fig. S3E)**. HuH28 cells had the largest area of galectin-3, and these cells were shown to bind platelets via galectin-3, which was demonstrated using an antibody against galectin-3 **(Fig. 2G)**. These results suggest that galectin-3 is one of the key proteins in the interaction between iCCA cells and platelets.

3.4 iCCA cells promote NET induction by binding to platelets, while NETs promote motility of iCCA cells.

Finally, direct co-culture with iCCA cells, platelets, and neutrophils was investigated. As expected, direct co-culture of iCCA, HuCCT1, or HuH28 cells, with platelets significantly increased NET induction compared with iCCA cells alone or platelets alone **(Fig. 3A, Fig. S4A)**. The complex of iCCA cells, platelets, and NETs was also detected. Careful observation showed that platelets not only bound to the iCCA cell surface, but were also trapped by NETs **(Fig. 3A, right)**. NETs induced by the interaction of iCCA cells and platelets may thus promote further complex formation. Since the time-lapse images could capture the dynamic increase in extracellular DNA as NETs are induced by PMA **(Fig. 3B, Video 2)**, NETs induction in the co-culture conditions was also observed in time-lapse imaging. Direct co-culture of iCCA cells, platelets, and neutrophils significantly increased extracellular DNA over time **(Fig. 3C)**. Through time-lapse observations, some floating cells intertwined with the NETs and then became attached to the culture dish **(Fig. 3D, Video 3)**. In addition, enhanced motility of iCCA cells co-cultured with platelets and neutrophils was significantly abolished by Cl-amidine **(Fig. 3E, Fig. S4B)**. This suggested that NETs had a significant role in enhancing the motility of iCCA cells in certain conditions.

3.5 P-selectin expressed on the platelet surface triggers NETs

To address the mechanism of how the cancer-platelet complex induces NETs, the mechanism of how platelets are activated and bound to cancer was explored. To check the effect of soluble factors derived from platelets, the medium collected from direct co-culture of iCCA cells and platelets was added to neutrophils. There were no significant effects to promote NET induction by their conditioned medium **(Fig. S5A)**, suggesting that direct contact with activated platelets and neutrophils is also important for NET induction. Since initial platelet-neutrophil aggregation is mediated primarily by binding of P-selectin on activated platelets to its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), on neutrophils[13, 35], following direct co-culture with iCCA cells and platelets, P-selectin was blocked using an antibody, and then neutrophils were added to this culture. Blocking P-selectin significantly decreased NET induction compared to non-treated platelets **(Fig 4A, Fig. S5B)**. This result showed that P-selectin on platelets is expressed through interactions with iCCA cells and triggers NET induction.

3.6 iCCA cells, platelets, and NETs were co-localized in vivo

iCCA is known to form multiple intrahepatic metastases (IMs) by a transportal pathway that

leads to a poor prognosis[36, 37]. Next, to investigate whether the interaction of iCCA cells, platelets, and neutrophils was observed in vivo and contributes to liver metastasis, tumor cells were injected into the spleen in mice, and their livers were harvested on day 1 and day 3 after injection. Multiple CMFDA-labeled tumor cells were seen in the liver, and along with the tumor cells, the co-localization of platelets and NETs was confirmed **(Fig. 4B)**. As shown in vitro, it was clearly demonstrated that iCCA cells have a close association with platelets and NETs in the blood circulation.

3.7 Dual anti-platelet therapy decreases NET induction by the interaction of iCCA cells with platelets

The data above indicate that platelets play crucial roles in NET induction by iCCA cells, and thus activated platelets might be a target to suppress NET induction. Among potential anti-platelet agents, such as cyclooxygenase (COX) inhibitors, heparin, or adenosine diphosphate receptor antagonists, in cancer treatment[7], two widely used antiplatelet drugs were chosen: ASA with COX-1 inhibition and ticagrelor with P2Y12 inhibition. Moreover, it is known that the effects of these anti-platelet agents can be enhanced when used as dual anti-platelet therapy (DAPT), as in the treatment of coronary artery disease[38]. To investigate the effects of these two agents on NET induction by the iCCA cells and platelet interaction,

neutrophils were co-cultured with pre-cultured iCCA cells and treated with platelets. Interestingly, platelets pre-treated with dual agents were more effective in suppressing NET induction than platelets pre-treated with a single agent **(Fig. 5A, Fig. S6A)**. To clarify why NET induction was suppressed, the status of platelets co-cultured with iCCA cells was confirmed. DAPT significantly reduced P-selectin expression of platelets on the surface of iCCA cells **(Fig. 5B, Fig. S6A)**. Whether such reduced expression of P-selectin was due to reduced platelet binding to cancer cells was examined. Platelets pretreated by DAPT did not show a changed binding capacity to cancer cells (Fig. 5C left). The effect of DAPT on unbound platelets was also examined in the same setting. DAPT tended to suppress the P-selectin expression of unbound platelets, but the difference was not significant (Fig. 5C right). DAPT strongly suppressed P-selectin expression of platelets and consequently suppressed NET induction by P-selectin.

Finally, the effect of daily gavage administration of DAPT (ASA: 150 mg/kg + ticagrelor: 100 mg/kg) on liver micro metastasis was investigated in nude mice. The injected CMFDA-labeled tumor cells were observed in the liver one day after intrasplenic injection, and the tumor cell area was dramatically decreased in the liver by DAPT (P<0.001; **Fig. 5D**). The areas occupied by NETs and platelets were also decreased in the DAPT group (P=0.021, P=0.0015; respectively; **Fig. 5D**). These data suggest that DAPT suppresses NET induction

through inhibition of platelet activation by iCCA cells and decreases tumor cells trapped in the liver.

4. Discussion

The results showed that NETs were present in the resected iCCA tumors, and that NETs increased the migration and motility of iCCA cells, which was attributed to the EMT, suggesting a possible association between the malignant properties of iCCA cells and NETs. However, iCCA cells were found to have a low ability to induce NETs on their own. Of interest, iCCA cells were found to raise the productivity of NETs by binding platelets, which was shown to be mediated by P-selectin, which appears on cancer-bound platelets. In addition, it was shown that the combined use of ASA and ticagrelor suppressed the expression of P-selectin on the platelets, and, as a result, NET induction could also be suppressed in vitro and in vivo. It has been reported that cancer cells themselves induce NETs by releasing several mediators such as granulocyte colony-stimulating factor, IL8, or HMGB1[19, 39], and that the presence of NETs in the tumors themselves is significantly associated with prognosis. We were the first to demonstrate the presence of NETs in iCCA resection specimens, but there was no significant relationship between the presence of NETs and prognosis in our case cohort. These results may be attributed to the fact that NETs in resected iCCA tumors were

not only formed by cancer cells, but also by such conditions as preoperative cholangitis and intraoperative ischemia/reperfusion procedures. On the other hand, plasma NET levels have been reported to correlate with a poor prognosis in lung cancers and primary liver malignancies[24, 40]. NET levels in the plasma rather than in the primary tumor may affect metastasis and prognosis, and the present study thus focused on the interaction between the binding of platelets to cancer cells and NETs.

For cancer cells to metastasize, significant amounts of CTCs are shed from the primary tumor, and these cells must survive in the circulation, though most are immediately destroyed. The mechanisms of CTC survival involve interactions between tumor cells and platelets, shielding tumor cells from tumor necrosis factor α and natural killer cell-induced cell death[41, 42]. Platelets are known to express proteins on the membrane that cancer cells use for binding[31]. It was found that galectin-3 expressed on the cell membrane of cholangiocarcinoma contributes to binding to GPVI on the platelets. Whereas aggregates of iCCA cells, platelets, and NETs were formed by co-culturing in vitro, many aggregates of cancer, platelets, and NETs were also observed in the liver after intrasplenic injection of cancer into mice. These results suggest that iCCA cells may form such aggregates in the circulation, escape attack from immune cells, and increase adhesion to the vascular endothelium.

It has been reported that platelets that interact with cancer cells release platelet transforming growth factor β and increase the proliferation of ovarian cancer cells[43, 44], and that platelet microparticles stimulate mitogen-activated protein kinase in lung cancer cells and increase cell proliferation[45]. As these reports indicate, platelets alone are known to induce the EMT in cancer cells. In the present study, however, the motility of the cancer cell was not enhanced by platelets alone, although this might be due to the short observation time. As we have shown, platelets are activated by cancer, and then they induce NETs. Subsequently, the induced NETs release HMGB1 and thereby induce EMT in cancer cells[46], while they also trap and activate surrounding platelets[47]. It is inferred that such cascading changes in the environment surrounding the cancer may occur. Thus, even brief exposure to high concentrations of mediators from platelets or NETs may increase cancer motility.

The relationship between platelets and NETs has been reported in thrombosis. According to a report by Boer et al., the aggregation of NETs in coronary arteries suggests that NETs may contribute to thrombus growth and stability[48], and Ducroux et al. also reported that NETs are abundant in stroke thrombosis, suggesting that NETs are recognized as a therapeutic target[49]. Even though interactions between NETs and platelets have been known, as described above, the effects of antiplatelet agents on NETs remain to be clarified. In the present experiments, platelets bound to cancer activated and expressed P-selectin. DAPT

suppressed P-selectin expression, but not ASA or ticagrelor alone. Of note, there is no report that ASA and ticagrelor inhibit galectin-3 binding to GPVI. Thus, we hypothesized that DAPT worked, not by inhibiting the binding of platelets to cancer, but by inhibiting the activation of surrounding platelets by mediators released from platelets bound to cancer. Although it was not possible to show that DAPT significantly suppressed the activation of platelets that were not bound to cancer, the combination of ASA and ticagrelor was reported to have a significantly stronger antithrombotic effect compared to a single agent, supporting our view[50].

Despite advances in surgical technique and chemotherapy, the recurrence rate after iCCA resection is reported to be 50%, and DFS is also as short as 26 months[3]. Considering this high recurrence rate, once iCCA is diagnosed, treatment to suppress micrometastases should be considered. We previously reported that thrombomodulin, an antithrombotic drug, prevented liver metastasis of pancreatic cancer by targeting NETs[46]. NETs and platelets as a therapeutic target could be a new therapeutic strategy for iCCA treatment, and existing antiplatelet agents could be very useful candidates.

The present study showed that intrahepatic cholangiocarcinoma may promote micrometastases as an initial step of metastasis by producing NETs through platelet activation. iCCA cells activate platelets by galectin-3 through direct contact, and P-selectin

on activated platelets produces NETs by direct contact with neutrophils. NETs facilitate adhesion and motility of iCCA cells **(Fig. S6B)**. In conclusion, potent antiplatelet therapy may prevent implantation of iCCA cells by inhibiting platelet activation and suppressing NET production, and it may be a new treatment option for intrahepatic cholangiocarcinoma.

CRediT authorship contribution statement

Masashi Yoshimoto: Conceptualization, Methodology, Investigation, Writing - original draft. Shunsuke Kagawa: Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. Hiroki Kajioka: Investigation. Atsuki Taniguchi: Investigation. Shinji Kuroda: Supervision. Satoru Kikuchi: Supervision. Yoshihiko Kakiuchi: Supervision. Tomohiko Yagi: Investigation. Shohei Nogi: Investigation. Fuminori Teraishi: Supervision. Kunitoshi Shigeyasu: Supervision. Ryuichi Yoshida: Resources. Yuzo Umeda: Resources. Kazuhiro Noma: Supervision. Hiroshi Tazawa: Methodology, Supervision. Toshiyoshi Fujiwara: Conceptualization, Writing - review & editing, Supervision.

Declaration of competing interests

The authors report no conflict of interest

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1. NETs promote motility and migration ability of iCCA cells through the EMT.

A. Representative immunofluorescence images show morphological changes of several cancer cells co-cultured with PMA-induced cell-free NETs. Green, β-actin; blue, nuclei. B. Representative images show the motility of HUCCT1 cells. After HuCCT1 cells stained by cell tracker were added NETs, time-lapse videos were recorded for 12 hours (Video 1). Each dot represents the distance traveled by single cells. More than 250 cells were measured in at least 3 fields in each group. Mean ± SD is shown in each graph. C. Each dot on the graph represents the number of migrated cells/field (x 200). NETs promote migration ability, whereas DNase, and protein arginase deiminase (PAD) 4 inhibitor, Cl-amidine, abolishes this effect. D. NETs decrease expression of E-cadherin and increase expression of vimentin in HuCCT1 cells. Expression of E-cadherin or vimentin was quantified by measurement of integrated density. E. Western blot analysis of the expression of E-cadherin, vimentin, and slug. TGF-β was used as the positive control of EMT induction. One-way ANOVA with Tukey's test was used in the figure. *, $P<0.01$

Figure 2. Cholangiocarcinoma cells bind and activate platelets.

A. Kaplan-Meier survival curves of patients with intrahepatic cholangiocarcinoma showing

high and low NET expression in the primary tumors. The presence of NETs in the tumor microenvironment does not affect overall survival and recurrence-free survival. B. Representative images show the NETs induced by floating cancer cells. Red, citH3; blue, nuclei. C. Thrombin-activated platelets increase NET induction (left). Representative image of NETs induced by activated platelets (right). D. The greatest increases of aggregations of iCCA cells and platelets are observed in HuH28 cells. E. Flow cytometry quantifies the percentage of platelets that are activated and express CD62P among platelets unbound to cancer cells. F. The expression area of Galectin3 per cell is quantified. G. Platelet-tumor cell aggregations are suppressed by Galectin3 antibody. One-way ANOVA with Tukey's test was used in the figure. *, $P<0.01$

Figure 3. The interaction with iCCA cells and platelets induces NETs.

A. Representative images show NETs induced by the interaction with HuCCT1 cells and platelets. Red, citH3; green, EpCAM; blue, nuclei. NETs induced by HuCCT1 cells and platelets were quantified in the area of citH3 (middle). In the right image, the small black particles clinging to the colored HCCT1 cells and NETs are platelets. B. NETosis assay (Video 2); Time-lapse videos of neutrophils (red) with or without PMA in the presence of SYTOX blue (blue). NET induction was quantified by measuring the area of extracellular

DNA per field. C. Time-lapse videos of neutrophils co-cultured with HuCCT1 and/or platelets from healthy donors in the presence of SYTOX blue (blue) (Video 3). Quantification of NET induction was performed in the same manner as above. D. Representative frames of videos at different time points of neutrophils co-cultured with HuH28 and platelets (Video 3). White arrows indicate HuH28 that are trapped by NETs and adhere. Green, HuH28; red, neutrophil; blue extracellular DNA. E. HuCCT1 stained with cell tracker was co-cultured with neutrophils and platelets in the presence of DNase or Cl-amidine. Time-lapse videos were recorded for 12 hours. Each dot represents the distance traveled by single cells. More than 250 cells were observed in at least 3 fields in each group. One-way ANOVA with Tukey's test was used in the figure. *, $P<0.01$

Figure 4. CD62P on platelets activated by direct contact with iCCA cells induces NETs.

A. Induction of NETs by direct coculture with iCCA cells and platelets. NETs induction is suppressed by anti-CD62P. More than 10 fields were observed in each group. The Mann-Whitney U test was used in the figure. B. Representative immunofluorescence images showing the co-localization pattern of HuH28, platelets, and NETs at 24 hours post-injection. Green, HuH28; white, citH3; red, CD41; blue, nuclei.

Figure 5. DAPT decreases NETs induction.

A. Representative images show that anti-platelet treatment of platelets reduces NET induction by the interaction with platelets and iCCA cells. Red, citH3; green, EpCAM; blue, nuclei. NETs were quantified in the area of citH3. B. Platelets pretreated with antiplatelet agents were co-cultured with iCCA cells, and the area of CD62P expression was quantified. C. Left. Platelets pretreated with ASA, Ticagrelor, or both were added to HuCCT1 cells. The percentage of CD41-positive, platelet-bound, cancer cells was then analyzed by flow cytometry. Right. Platelets in the supernatant of the above platelet+HuCCT1 co-culture were analyzed. The percentage of platelets expressing CD62P was calculated. One-way ANOVA with Tukey's test was used in the figure. There is no significant difference between the treatments. D. Representative immunofluorescence images showing decreased NETs, platelets, and HuH28 in the liver of mice treated with DAPT for 5 days at 24 hours post-injection. Green, HuH28; white, citH3; red, CD41; blue, nuclei. Mann-Whitney U test was used in the figure.

Supplementary Figures and Videos

Figure S1.

NETs are present in the tumor microenvironment of iCCA. Representative immunofluorescence images of primary tumors of iCCA show the presence of NETs at the periphery. Red, citH3; green, MPO; blue, nuclei.

Figure S2.

A. Cells on the bottom side of the chamber were fixed and stained with 0.5% crystal violet. Representative images of stained migrated cells. B, Representative figure showing E-cadherin and vimentin expression in HuCCT1 cells. C, Representative images show extracellular DNA of neutrophils co-cultured by several cancer cells. Green, extracellular DNA; blue, nuclei.

Figure S3.

A. The enhanced expression of CD62P on platelets by thrombin shows evidence of platelet activation. A representative flow cytometry diagram (left) and graph showing the extent of expression (right). B. NETs are induced only by thrombin-activated platelets. C. The expression of P-selectin on platelets shows evidence of platelet activation. D. Representative images show cancer cells bound to platelets (left). Red, CD41; green, EpCAM; blue, nuclei. CD41-positive cells mean platelet-bound iCCA cells. Representative

flow cytometry showing platelet-tumor cell aggregates with direct co-culture. Increases of aggregations of iCCA cells and platelets are demonstrated (right). E. Representative images show the expression of Galectin3 in several cancer cells. Green, Galectin3; blue, nuclei.

Figure S4.

A. NETs induced by HuH28 cells and platelets are quantified in the area of citH3. B. Representative images show the motility of HUCCT1 cells.

Figure S5.

A. The medium collected from direct co-culture of iCCA (HuCCT1 and HuH28) cells and platelets does not promote NET induction.

B. Representative images show NETs induced by the interaction with iCCA cells and platelets. Red, citH3; blue, nuclei.

Figure S6.

A. Representative images show CD62P expression of platelets pre-treated with or without anti-platelet agents co-cultured with iCCA cells. Green, CD62P; blue, nuclei.

B. Schematic diagram of the interaction between intrahepatic cholangiocarcinoma, neutrophils, and platelets. NETs present in the primary tumor promote EMT of the cancer. Cancer cells that become CTCs bind directly to platelets through Galectin-3, causing platelet activation. Activated platelets express CD 62P and induce NETosis by binding directly to neutrophils. The aggregation of platelets, iCCA cells, and NETs tends to adhere to the vascular endothelium. DAPT suppresses the expression of CD 62P on platelets, thereby reducing the induction of NETs and reducing the number of cancer cells that adhere to the liver.

Table S1.

Correlations between clinicopathological factors and amount of NETs in 41 iCCA cases

Video 1.

HuCCT1 cells co-cultured with NETs observed by time-lapse videos.

Video 2.

Time-lapse videos of neutrophils (red) with or without PMA in the presence of SYTOX blue

(blue).

Video 3.

Time-lapse observations capture floating HuH28 cells (green) intertwined with the NETs (blue) attaching to the culture dish.