Molecular mechanisms driving the interactions between platelet and gastric cancer cells during peritoneal dissemination

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Abstract. Platelets (PLTs) facilitate tumor progression and the spread of metastasis. They also interact with cancer cells in various cancer types. Furthermore, PLTs form complexes with gastric cancer (GC) cells via direct contact and promote their malignant behaviors. The objective of the present study was to explore the molecular mechanisms driving these interactions and to evaluate the potential for preventing peritoneal dissemination by inhibiting PLT activation in GC cells. The present study examined the roles of PLT activation pathways in the increased malignancy of GC cells facilitated by PLT-cancer cells. Transforming growth factor-β receptor kinase inhibitor (TRKI), Src family kinase inhibitor (PP2) and Syk inhibitor (R406) were used to identify the molecules influencing these interactions. Their therapeutic effects were verified via cell experiments and validated using a mouse GC peritoneal dissemination model. Notably, only the PLT activation pathway-related inhibitors TRKI and PP2, but not R406, inhibited the PLT-enhanced migration and invasion of GC cells. *In vivo* analyses revealed that PLT-enhanced peritoneal dissemination was suppressed by PP2. Overall, the present study revealed the important role of the Srk family in the interactions between PLTs and GC cells, suggesting kinase inhibitors as promising therapeutic agents to mitigate the progression of peritoneal metastasis in patients with GC.

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Abbreviations: CLEC-2, C-type lectin-like receptor 2; EMT, epithelial-mesenchymal transition; Gal-3, galectin-3; GC, gastric cancer; GPVI, glycoprotein VI; NT, non-treatment; PDPN, podoplanin; PLT, platelet; TRKI, transforming growth factor-β receptor kinase inhibitor

Key words: peritoneal dissemination, molecular mechanism, PLT, GC, therapeutic strategy

Introduction

An abundance of blood loss during surgery escalates the likelihood of post-surgical peritoneal metastasis in patients with gastric cancer (GC) (1,2). Platelets (PLTs) support malignant behaviors, especially hematogenous metastases, in various cancer types (3-5). In our prior study, we established that PLTs interact with GC cells, forming complexes that amplify their malignant characteristics through direct contact (6). Surgery is a very effective treatment, but it does cause some bleeding. The PLTs present in this bleeding promote peritoneal metastasis. If we can inhibit PLTs-GC cells interaction with PLTs activation by intraperitoneal administration during or after surgery, we can further improve the prognosis of patients undergoing GC surgery. In this study, we propose that interactions between PLTs and GC cells during surgery intensify the spread of GC cells in the peritoneum. Our objective is to investigate the molecular mechanisms driving this process and evaluate the therapeutic potential of mitigating peritoneal metastasis by inhibiting interactions between PLTs and GC cells.

A multitude of molecular entities and biological pathways are instrumental in the activation of PLTs. In PLTs, the C-type lectin-like receptor 2 (CLEC-2) plays a crucial role in activation, which is mediated by Src and Syk (7-9). Podoplanin (PDPN), a ligand for CLEC-2, is present on renal podocytes, lymphatic endothelial cells, and cancer cells (10,11). Glycoprotein (GP)-VI, a primary collagen receptor, is crucial for PLT activation and function induced by collagen (12,13). Among the various mediators released during PLT activation (14), transforming growth factor (TGF)-β plays crucial roles in the development and progression of various cancer types (15-17). Our previous study demonstrated that blocking the interaction between galectin (Gal)-3 and GPVI reduces the effects of PLTs on GC cells (18).

In this study, we examined the involvement of the PLT activation pathway and TGF-β released from activated PLTs in the enhanced malignancy of GC cells via cancer cell-PLT interactions. Furthermore, we investigated their therapeutic

potential to prevent peritoneal dissemination of GC cells. Our findings indicate that the Src family plays a pivotal role in enhancing the metastasis of GC cells facilitated by PLTs, thereby presenting a potential therapeutic target for mitigating peritoneal metastasis in individuals with GC.

Materials and methods

Reagents. We assessed the inhibitory impacts of various substances on the malignant behaviors induced by PLTs in GC cells. SB-431542, a TGF-β receptor kinase inhibitor (TRKI), was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). SB-431542 (1 μM) was used in the working solution for pre-incubation with GC cells for 1 h before each assay. PP2, an Src kinase inhibitor, was purchased from AdipoGen Life Sciences (San Diego, CA, USA), and its 10 μM solution was used in the working solution for pre-incubation with PLTs for 10 min before each assay. Syk inhibitor R406 was purchased from InvivoGen (San Diego, CA, USA), and its 1 μM solution was used in the working solution for pre-incubation with PLTs for 10 min before each assay.

Cell lines and cell culture. We utilized two GC cell lines, NUGC-3 and MKN74, procured from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). These were propagated in Roswell Park Memorial Institute-1640 medium (supplied by Thermo Fisher Scientific Inc., Waltham, MA, USA), enriched with 100 U/ml of penicillin (obtained from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 100 μg/ml of streptomycin (also from Sigma-Aldrich, Merck KGaA), and 10% fetal bovine serum (FBS; sourced from Thermo Fisher Scientific Inc.). The culture conditions were maintained at 37˚C in a 5% CO_2 environment.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from NUGC-3 cells utilizing the miRNeasy Mini Kit (Qiagen, Hilden, Germany) as per the guidelines provided by the manufacturer. The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) was employed to ascertain the total RNA concentration, and 1 μg of total RNA was converted to cDNA using the HighCapacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.), following the manufacturer's protocol. The levels of transcripts were determined using specific primer sets and SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) via RT-qPCR. The RT-qPCR conditions were set as follows: An initial preheating for 10 min at 95˚C, succeeded by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels served as internal controls for normalization, and Gal-3 mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method (19). Primer sequences were designed using Primer3Plus (https://www.bioinformatics.nl/cgibin/primer-3plus/primer3plus.cgi) with the most conserved region of each sequence obtained from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/nuccore). Each experiment was conducted in triplicate.

The primers used for the RT-qPCR assay were as follows: matrix metalloproteinase (MMP)-9 (forward 5'-CCAACT ACGACCGGGACAAG-3' and reverse 5'-AAGTGAA-GGG GAAGACGCAC-3') and GAPDH (forward 5'-GTCTCCTCT GACTTCAACAGCG-3' and reverse 5'-ACCACCCTGTTG CTGTAGCCAA-3').

PLT preparation. PLTs were sourced from healthy volunteers and isolated from whole blood, following the method outlined in our previous study (20). The PLTs were immediately utilized in a variety of experiments post-extraction. They were adjusted to a final concentration of $1x10^5/\mu$l for use in the subsequent experiments.

Functional assays in vitro. We conducted migration and invasion assays utilizing Falcon Cell Culture Inserts with 8-μm pore membranes (Corning Inc., Corning, NY, USA) and Biocoat Matrigel (BD Bioscience, Franklin Lakes, NJ, USA), following the methodology outlined in our prior study (6). In brief, NUGC-3 ($1x10^5$) and MKN74 ($5x10^5$) cells were placed in the upper chambers of the FBS-free medium, with or without the presence of PLTs, while the lower chambers were filled with 10% FBS medium. For the inhibition experiments, the cells were exposed to a range of inhibitors (SB-431542, PP2, R406) alongside PLTs. Post a 24-h incubation period, cells that failed to migrate or invade through the pores were eliminated using cotton swabs. The cells that had successfully migrated and invaded were stained using the Diff-Quick staining reagent (Sysmex, Kobe, Japan), and the cell count in four separate fields was determined using the BZ-X710 All-in-One fluorescence microscope (Keyence Corp., Osaka, Japan) at 100x magnification and BZ-X Analyzer Software (Keyence Corp.). Each experiment was conducted in triplicate.

Establishment of a peritoneal dissemination mouse model in vivo. In this investigation, male BALB/c-Slc-nu/nu mice of six weeks old (procured from Japan SLC Inc., Shizuoka, Japan) were utilized. These mice were accommodated in a hygienic, temperature-regulated cage setting with a 12-h light-dark cycle. They were given unrestricted access to a laboratory-standard diet and water. The mice were arbitrarily segregated into three clusters, each containing six mice, by animal care specialists not directly participating in the research. The investigators were kept unaware of the group assignments. The exclusion criteria encompassed mortality during the research duration; however, this study did not witness any such instances. On the initial day, NUGC-3 cells ($3x10^6$) in 500 μl of Hanks' balanced salt solution (HBSS(-); sourced from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were administered intraperitoneally into the mice of the non-treatment (NT) group. In the PLT group, PLTs were introduced into NUGC-3 cells. The PLTs were calibrated to a terminal concentration of $1x10^5/\mu$l for subsequent experiments. In the inhibition experiments, the PP2 inhibitor was administered to the mice along with the NUGC-3 cells and PLTs into the peritoneal cavity. Post intraperitoneal administration, the mice were accommodated in individual cages. Weekly monitoring of physical conditions and body weights was conducted. After a span of five weeks, the mice were euthanized and peritoneal dissemination was assessed. For anesthesia induction, a concoction of medetomidine hydrochloride (0.75 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg) was diluted with saline to achieve a dosage of 5 μl/g body weight and

was administered to the mice via intraperitoneal injection, as delineated earlier (21-24). Anesthesia was induced using the aforementioned dosage. However, if the sedation level with anesthesia was deemed insufficient upon evaluation (e.g., loss of postural reaction and righting reflex, eyelid reflex, pedal withdrawal reflex in the fore and hind limbs, and tail pinch reflex), the dosage was escalated or deescalated to ensure adequate sedation in compliance with the Guide for the Care and Use of Laboratory Animals (25). As a measure against hypothermia, we used a circulating warm water blanket as an anesthesia warming device, which maintained a surface temperature of approximately 37.5˚C. In addition, we made efforts to minimize heat loss by using insulation and drapes covering the chest and abdomen. Furthermore, we used atipamezole (1 mg/kg, intraperitoneal injection) to prevent hypothermia (26). Subsequently, peritoneal dissemination was assessed post tumor removal from the host. The count, weight, and maximum diameter of the tumors were recorded. At the culmination of the experiment, all mice were euthanized via CO_2 inhalation (flow rate was regulated at 30% of the cage volume per minute), and death was confirmed by the absence of respiration or cardiac activity. All animal experiments received approval from the Institutional Animal Care and Use Committee of the University of Yamanashi, Japan (approval no. A2-14).

Statistical analysis. Quantitative values are presented as either the mean ± standard error or the median. In cases of multiple comparisons, we employed one-way analysis of variance, followed by the Bonferroni post-hoc test under conditions of equal variance, to draw comparisons between each group and the PLT group. In addition, under conditions of unequal variance, we used the Kruskal-Wallis test, followed by the Bonferroni post-hoc test, to compare each group with the PLT group. The threshold for statistical significance was set at $P<0.05$. All statistical computations were performed using JMP 17 (SAS Institute Inc., Cary, NC, USA), and EZR version 1.54 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (27).

Results

Involvement of Src-dependent PLT activation signaling pathway and TGF-β released from PLTs in the enhanced malignancy by the GC cells-PLT interaction. Several pathways are involved in PLT activation (28). Membrane protein- or extracellular matrix protein-mediated PLT activation, followed by Src family kinase activation, is mediated by integrin, and fibrinogen (5), CLEC-2, PDPN (7,29-31), GPVI and its ligands, Gal-3 (32). Conversely, the activation of PLTs through G-protein-coupled receptors, triggered by soluble PLT agonists, is facilitated by the agonists of G-protein-coupled receptors, which include thrombin, ADP, 5-hydroxytryptamine, PLT activating factor, and thromboxane A2.

We previously reported that the direct contact between GC cells and PLTs promotes the malignant behaviors of cells (6). In our investigation, we concentrated on the PLT activation pathway, particularly the Src family kinase activation pathway, which involves direct contact between GC cells and PLTs (Fig. 1A). To examine this pathway, PP2, a Src kinase inhibitor, was used to investigate whether Src-dependent PLT activation affects the behaviors of NUGC-3 cells co-cultured with PLTs. PP2 significantly inhibited the migration of NUGC-3 cells increased via co-culture with PLTs (50% reduction $P<0.001$; Fig. 1B). PP2 also inhibited the invasion NUGC-3 cells increased by PLTs (48% reduction; $P=0.009$; Fig. 1B). Activated PLTs secrete TGF-β from their alpha granules (33). Here, we examined the inhibitory impact of SB-431542 on PLT-enhanced malignancy of GC cells. SB-431542 effectively suppressed both PLT-induced migration and invasion of GC cells (55 and 45% reduction, respectively; $P<0.001$; Fig. 1B). Images depicting the migration and invasion assays are presented in Fig. 1C and D. Notably, PP2 and SB-431542 individually did not affect the malignancy of GC cells (Fig. S1). The results obtained using NUGC-3 cells (Fig. 1) were consistent with those obtained using MKN74 cells (Fig. S2).

Syk-dependent PLT activation pathway. PLTs contain two primary types of agonist receptors: G-protein-coupled receptors and tyrosine kinase pathway receptors, both essential for PLT activation. Tyrosine kinase pathway receptors, including CLEC-2, FcγRIIA, and GPVI, are linked to Syk activation (34-38). Here, R406, a Syk inhibitor, was used to determine whether the effect of PLTs on GC cells is abolished by suppressing Syk.

R406 failed to suppress the enhanced migration and invasion of NUGC-3 cells in co-culture with PLTs (Fig. 2A). Images depicting the migration and invasion assays are presented in Fig. 2B and C. Similar to PP2 and SB-431542, R406 alone did not affect the malignancy of GC cells.

Therapeutic effect of PLT activation inhibition on peritoneal dissemination. In the mouse model, administration of PLTs with NUGC-3 significantly increased the number, weight, and maximum diameter of peritoneal tumors ($P=0.004$, $P<0.001$, and $P<0.001$, respectively). However, co-administration of PP2 markedly decreased the number, weight, and maximum diameter of peritoneal tumors compared to those in the PLT group [reduction: 38% ($P=0.0013$), 37% ($P=0.0017$), and 34% ($P=0.0031$), respectively; Fig. 3A and B].

Discussion

Peritoneal dissemination in GC leads to a poor prognosis, and many studies are exploring treatment options to prevent it (39,40). Various risk factors for peritoneal dissemination in GC have been identified. Arita *et al* (1) demonstrated that substantial intraoperative blood loss elevates the risk of peritoneal dissemination recurrence in GC patients. Similarly, Kamei *et al* (2), through multivariate analysis, found a significant correlation between the extent of intraoperative bleeding and peritoneal dissemination recurrence, establishing excessive blood loss as an independent risk factor for the recurrence of peritoneal dissemination. Intraoperative blood loss in patients frequently necessitates clinical blood transfusions, potentially resulting in immunosuppressive effects. However, the abovementioned studies clearly demonstrate that intraoperative bleeding is not associated with other recurrence patterns, such as nodal or hematogenous metastases. This may be due

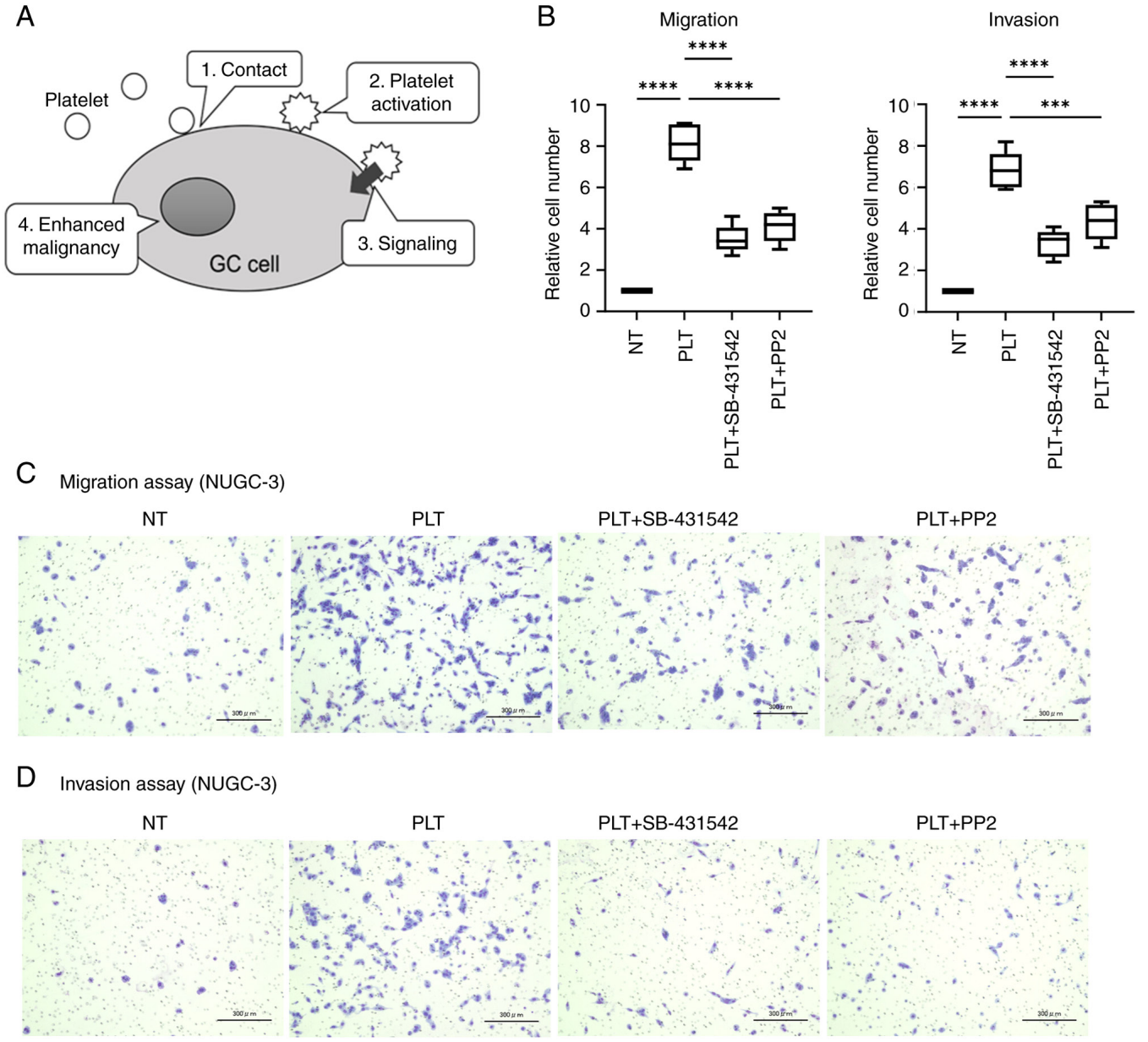

Figure 1. TGF-β receptor kinase and PLT-activation inhibitors suppress the migration and invasion of NUGC-3 cells via PLT contact. (A) Overview of the interactions between GC cells and PLTs. (B) PLT-induced enhanced migration and invasion of NUGC-3 cells was inhibited by SB-431542 (TGF-β receptor kinase inhibitor) and PP2 (Src family kinase inhibitor). Microscopic observations of (C) migration and (D) invasion assays, in the presence and absence of inhibitors, indicated that SB-431542 and PP2 notably inhibit the migration and invasion of NUGC-3 cells. The migration and invasion of NUGC-3 cells were enhanced by co-culture with PLTs. Magnification, x100; scale bar, 300 μm. ***P<0.001, ****P<0.0001 vs. PLT. GC, gastric cancer; NT, non-treatment; PLT, platelet.

to the localized effects of intraoperative blood loss within the peritoneal cavity. A multitude of research has established the existence of free-floating cancer cells within the peritoneal cavity in patients suffering from advanced GC (41,42). During the perioperative period, cancer cells might show enhanced interactions with the components of blood present in the peritoneal cavity. By interacting with circulating tumor cells, PLTs play a role in promoting hematogenous metastasis (43,44). Therefore, we hypothesized that intraperitoneally released cancer cells come in contact with PLTs due to intraoperative bleeding, thereby exacerbating peritoneal metastasis by enhancing cancer malignancy via interactions between cancer cells and PLTs. We previously demonstrated that direct interactions with PLTs significantly promote the malignant behaviors of GC cells, especially migration and invasion occur through mechanisms associated with epithelial-mesenchymal transition (EMT) (6). Furthermore, we revealed that inhibition of such direct interactions completely suppresses PLT-induced peritoneal dissemination in GC (18).

In this study, we examined the specific mechanisms that drive the increased malignant behaviors of GC cells when in direct contact with PLTs. Notably, we identified the involvement of the Src-mediated PLT activation pathway and PLT-released TGF-β in the enhanced malignancy of GC cells. We previously demonstrated that GC cell-PLT contact enhances cell migration and invasion by increasing MMP9 expression via

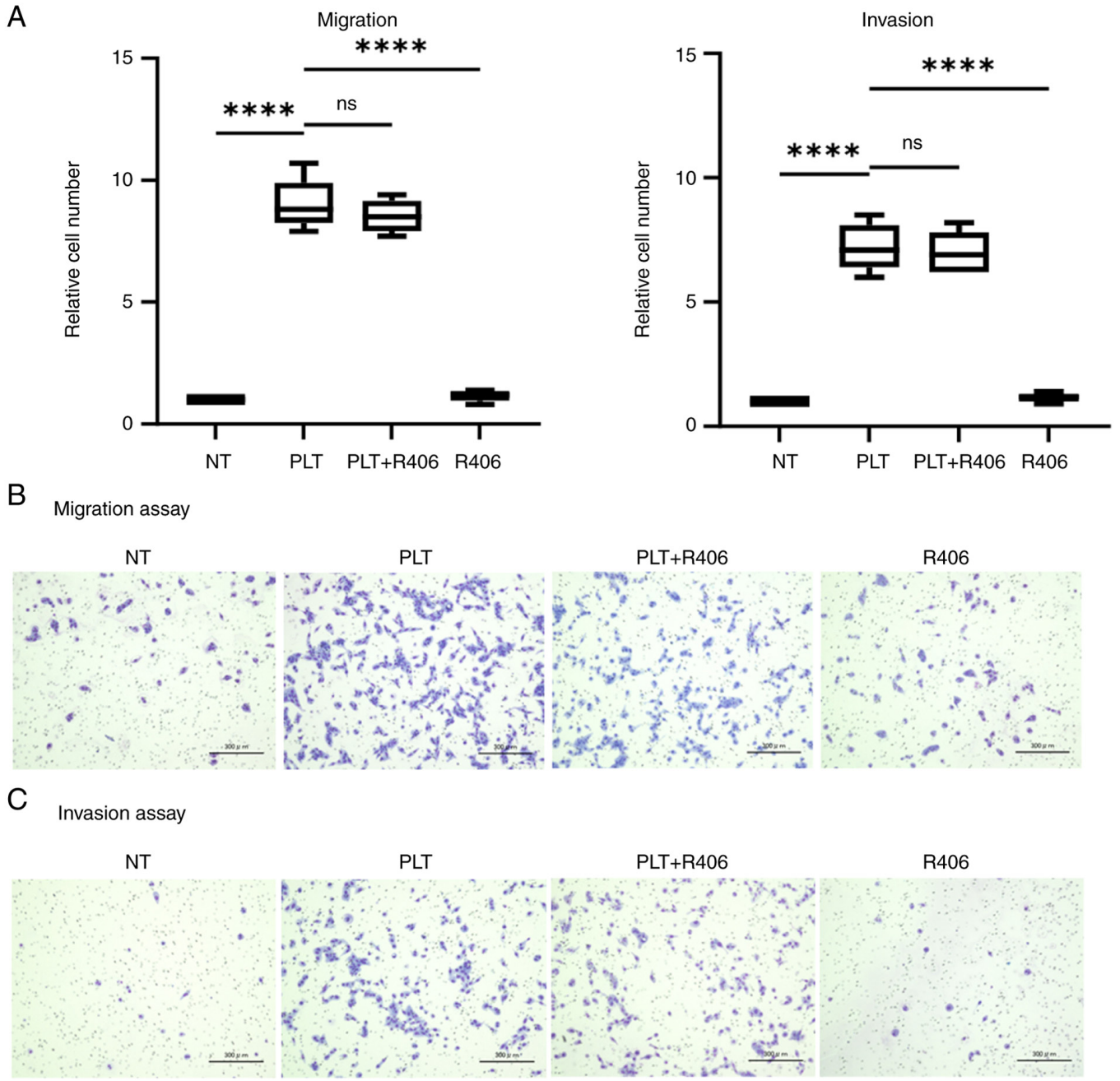

Figure 2. R406 does not suppress the increase in NUGC-3 cell migration and invasion via PLT contact. (A) PLT-induced enhanced migration and invasion of NUGC-3 cells was not inhibited by R406 (Syk inhibitor). Microscopic observations of (B) migration and (C) invasion assays, in the presence and absence of inhibitor, revealed that R406 did not suppress the migration and invasion of NUGC-3 cells, which were enhanced by PLT contact. Magnification, x100; scale bar, 300 μm. ****P<0.0001 vs. PLT. ns, not significant; NT, non-treatment; PLT, platelet.

EMT. In this examination, we assessed the alterations in MMP9 expression levels in GC cells co-cultured with PLTs and treated with PP2 and SB-431542. Our results confirmed that MMP9 expression was significantly suppressed in GC cells (Fig. S3). These results suggest that enhanced migration and invasion of cancer cells can be inhibited via suppression of EMT by inhibiting TGF-β release from PLTs. TGF-β has been reported to induce EMT in cancer cells and promote invasion and metastasis in previous studies (45,46). In fact, our previous study has revealed that various EMT-related molecules, in addition to MMP9, fluctuate in GC cell lines co-cultured with PLTs (6). Also, Wiercinska *et al* (47) established a 3D model of TGF-β-induced invasion in breast cancer. Using this model, they demonstrated that Smad3 and Smad4 are important for TGF-β-induced invasion by inducing MMP2 and MMP9 (47). Based on these reports, TGF-β-Smad2/Smad3-MMP2/MMP9 may also play an important role in GC cells. Therefore, anti-TGF-β strategies targeting the PLT-activating cascade may aid in cancer treatment.

In contrast to PP2, anti-PLT agent R406 did not exert considerable inhibitory effect on cancer cell-PLT interactions in this study. This may be because R406 inhibits the activation signal derived from CLEC-2 more strongly than that derived from GPVI (48). Our findings are consistent with a

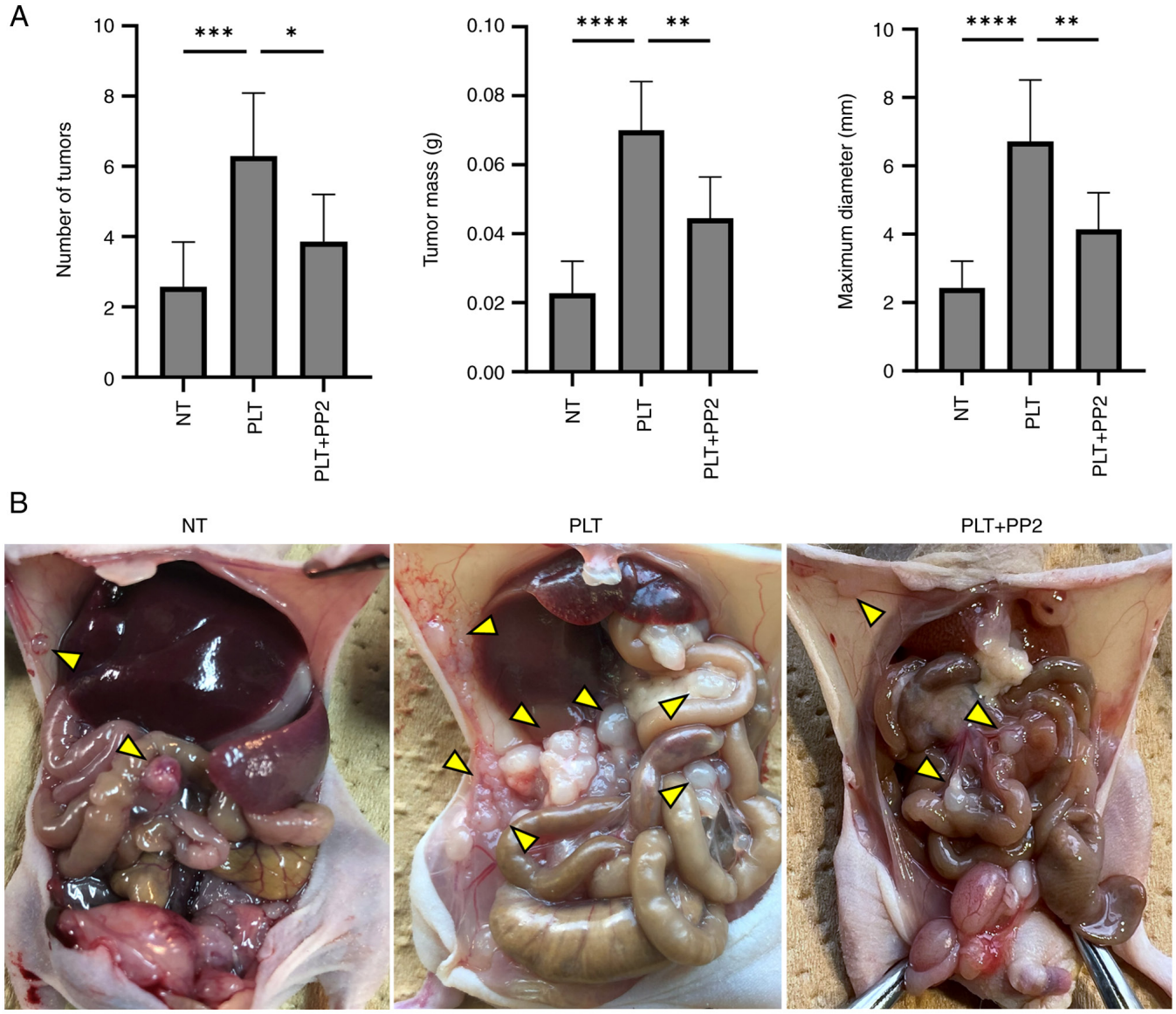

Figure 3. Therapeutic effect of PP2 (Src family kinase inhibitor) against peritoneal dissemination. (A) Number, weight and maximum diameter of peritoneal tumors, and (B) findings from the abdominal cavity (arrowheads indicate peritoneal dissemination). The number, weight and maximum diameter of peritoneal tumors were enhanced by PLT contact (P=0.004, P<0.001 and P<0.001, respectively) and reduced by PP2 [reduction, 38% (P=0.013), 37% (P=0.0017) and 34% (P=0.0031), respectively]. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. PLT. NT, non-treatment; PLT, platelet.

previous report that GPVI is more important than CLEC-2 in GC cell-PLT interactions (18) although the contribution of Src family kinases and Syk in PLT activation mediated by direct interaction between galectin-3 and GPVI remains still unclear. In addition, Src family kinase phosphorylates molecules involved in PLT activation, including those beyond Syk (such as PI3K). It is possible that the lack of significant effects observed with Syk inhibitors may be due to the involvement of a Syk-independent pathway (49).

To validate the results obtained *in vitro*, we extended our investigation to study the suppressive effects of PP2 on PLT-induced GC cells, utilizing a mouse model of peritoneal dissemination. In line with the *in vitro* findings, our *in vivo* analyses indicated a significant augmentation in peritoneal dissemination when PLTs and GC cells were concurrently administered to mice. These observations imply that PLTs may play a role in promoting the peritoneal spread of free cancer cells during intraoperative bleeding. Therefore, necessary precautions must be taken to prevent or limit the interactions between PLTs and GC cells in clinical settings. Additionally, intraoperative bleeding should be mitigated to prevent hemorrhage during surgeries.

Here, *in vivo* analyses demonstrated that co-administration of PP2 significantly attenuated PLT-enhanced peritoneal dissemination compared to single administrations alone. The outcomes of our study underscore the pivotal role of Src family kinases in peritoneal dissemination, thereby spotlighting them as potential therapeutic targets for preventing recurrence in GC patients (Fig. S4). In this study, inhibition of downstream signaling by PP2 and TRKI suppressed PLT-enhanced malignancy *in vitro*. TGF-β signaling has connections to a multitude of diseases such as malignancies and inflammatory and fibrotic conditions (50). Therefore, suppressing TGF-β may attenuate the biological functions of diseased cells. To verify this, we investigated the effects of PP2 on GC cells. Inhibition of PLT activation leads to excessive

bleeding. However, Src knockout mice did not exhibit excessive bleeding, similar to a previous report (51), suggesting that PP2 does not cause bleeding. Here, no adverse events associated with PP2 were observed in *in vivo* experiments.

We demonstrated that PP2 inhibited PLT-enhanced malignancy of NUGC-3, *in vitro* and *in vivo*, however, there remains some issues that needs further investigations about the mechanism of the effect of PP2 in the future. First, we will conduct a quantification experiment to determine the extent to which PLTs are activated and the amount of TGF-β released when NUGC-3 is co-cultured with PLTs. Second, we will analyze in detail the signaling profiles in the NUGC-3 cells when NUGC-3 and PLTs come into direct contact with each other since our previous report has reported that direct contact between NUGC-3 and PLTs is also important for NUGC-3 malignancy (6,18). Finally, we will confirm the synergistic effect of combining the direct contact inhibitor shown to be effective in our previous study with PP2. By verifying these in future experiments, it may be possible to further elucidate the mechanism of enhanced malignant behavior of GC cells induced with interaction between GC cells and PLTs.

In our study, we demonstrated the use of PP2, and we are considering intraperitoneal administration during or after surgery. Suppressing the activation of PLTs, which cannot be completely removed by postoperative washing alone, using PP2, could potentially inhibit the enhancement of malignancy in GC cells and consequently suppress peritoneal dissemination. These new treatment options can be a very attractive therapy that can improve the prognosis of GC patients undergoing surgery, and could potentially be a blessing for patients suffering from refractory cancer.

This study has some limitations. First, the mouse model was intraperitoneally administered with the Src family kinase inhibitor PP2. However, to fully inhibit the PLT activation resulting from PLT-GC cell interactions, administration via oral or intravenous routes would be more appropriate. Second, side effects of PP2, especially its effect on hemostasis, could not be determined in this study, warranting further investigation. Although similar mechanisms may be involved in different cancer types, further exploration of specific molecules and pathways is warranted for each type of cancer.

In conclusion, this study revealed that Src family kinase inhibitors suppressed the PLT-enhanced malignancy of GC cells *in vitro* and that PLT interaction with GC cells markedly increased their peritoneal dissemination. However, inhibition of Src-mediated PLT activation significantly suppressed the PLT-enhanced peritoneal dissemination of GC cells *in vivo*. Overall, our findings suggest that targeting the Src-mediated PLT activation pathway holds promise as a therapeutic strategy to prevent peritoneal dissemination in GC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

TN, RS, SF, YH, KM, KaT, SM, KaS, KoT, KeS, YK, HA, HK, NT, TS, KSI and DI conceived the study idea and designed the study. TN and RS confirm the authenticity of all raw data. KM, SF, KaT, SM, KaS and KeS collected and assembled the data. YH, KoT, YK, HA and HK analyzed and interpreted the data. All authors have made contributions to the composition of this manuscript, and read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethic Committee of the University of Yamanashi (approval no. 2159; Chuo, Japan) for patient experiments. The study was approved by the Animal Care and Use Committee at the University of Yamanashi (approval no. A2-14; Chuo, Japan) for animal experiments. The study adhered to the ethical standards outlined in The Declaration of Helsinki and its subsequent amendments (52). All volunteers provided written informed consent for the use of their samples. All animal procedures were conducted in accordance with the ARRIVE 2.0 guidelines (53) and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (25).

Patient consent for publication

Written informed consent for publication was obtained from all healthy volunteers.

Competing interests

The authors declare that they have no competing interests.

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