Activation of the PX1/IL-1β axis in CD41+/CD62P+ platelets enhances pancreatic ductal adenocarcinoma invasion and metastasis

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Article

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Abstract

The pro-tumor mechanisms of platelets in pancreatic ductal adenocarcinoma (PDAC) are poorly understood. We showed that the count of the CD41+/CD62P+ platelets subtype was significantly elevated in stage III/IV patients. An increased level of CD41+/CD62P+ platelets could serve as an independent risk factor for stage I/II patients after surgery. Furthermore, we found significantly higher PX1 expression in CD41+/CD62P+ platelets than in CD41+/CD62P- platelets in PDAC patients. Mechanistically, PX1 was able to enhance IL-1β secretion in platelets via phosphorylating p38 MAPK and consequently promoted PDAC invasion and metastasis. Finally, we constructed a novel compound named PC63435 by the ligation of carbenoxolone (PX1 inhibitor) and PSGL-1 (CD62P ligand). PC63435 specifically bound to CD41+/CD62P+ platelets and blocked the PX1/IL-1β pathway, which suppressed PDAC tumor invasion and metastasis. Our findings revealed that the activation of PX1 in CD41+/CD62P+ platelets enhanced PDAC cell malignancy and that may be a potent target for PDAC therapy.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive tumor that has an extremely dismal prognosis, with a 5-year survival rate of less than 8%.1 Surgery is currently the only curative treatment for PDAC but is applicable to only 10%–15% of patients.2 Frustratingly, after radical surgery, most patients experience recurrence with inevitable metastasis. The mechanism of PDAC metastasis needs further investigation.

Platelets are not only involved in hemostasis but also contribute to tumor cell invasion and metastasis.3, 4 Increasing evidence indicated that an elevated platelet count predicts poor survival in many types of cancers, such as colorectal cancer, renal cell carcinoma and gastric cancer.5-7 Furthermore, a subtype of blood platelets that are characterized as CD41+/CD62P+ was found to be activated by tumor cells and then promoted the malignant potential of tumor cells.3, 8-10 The percentage of activated platelets (CD41+/CD62P+) was reported to increase in lung cancer and promote tumor metastasis.11 In addition, in colon carcinoma and melanoma, platelets activated by the most effective agonist, thrombin, were found to increase lung metastases by 4- to 413-fold.12 These lines of evidence suggested that activated platelets, but not total blood platelets, may promote tumor metastasis. However, in PDAC patients, the value of platelet counts in predicting prognosis is still controversial,13, 14 and mechanistically the role of activated platelets in PDAC remains elusive.

Current evidence suggests that adhesion of platelets to tumor cells protects tumor cells from recognition and killing by natural killer cells or other immune cells.10 In Addition, angiogenic factors released from activated platelets are able to promote the growth of tumor cells.15 For example, the platelet aggregation-inducing factor podoplanin was found to promote the formation of tumor-platelet aggregates and induce epithelial-mesenchymal transition (EMT) in tumor cells.16 Recently, platelets have been reported to suppress both CD4+ and CD8+ T cells mostly via secreting TGF-β, suggesting the tumor
immunoregulatory role of platelets.\textsuperscript{17} To determine the pro-tumor mechanism of platelets in PDAC, we used RNA-Seq to explore the transcriptomic difference between activated platelets and non-activated platelets. PX1 was observed to be significantly overexpressed in activated platelets.

PX1 is a membrane channel protein and is widely expressed in most mammalian cells or tissues. Recent studies have shown that PX1 promotes breast cancer metastasis and glioma cell proliferation.\textsuperscript{18,19} Our previous study also showed that PX1 promoted hepatocellular carcinoma invasion and metastasis by EMT.\textsuperscript{20} Although the function of PX1 in tumor cells has been studied, the role of PX1 in platelets remains unclear.

In this study, we showed that the counts of activated platelets defined as CD41+/CD62P+, rather than whole-blood platelet counts, are a prognostic risk factor for PDAC. The expression of PX1 in platelets is critical for platelet activation with the interaction of PDAC cells. PX1 expression on activated platelets can promote platelet-derived IL-1\textbeta release via phosphorylating p38 MAPK. The specific blocking of PX1 on activated platelets inhibited platelet activation and PDAC invasion and metastasis. These results suggested that the PX1/IL-1\textbeta axis in platelets can serve as a target for inhibiting PDAC invasion and metastasis.

**Materials And Methods**

**Patients and sample collection**

For the blood platelet count test, we performed a retrospective analysis on data collected from 656 PDAC patients from May 2014 to December 2017. Of these patients, 274 suffered stage I/II PDAC and underwent pancreatectomy with microscopic tumor clearance, and 382 suffered stage III/IV PDAC with metastasis to local lymph nodes, large blood vessels or distant organs. A total of 3105 healthy people from the physical examination center were selected during the same period and matched to PDAC patients by age and sex.

For flow cytometry analysis of platelets, we enrolled two independent cohorts containing 89 patients with stage I/II PDAC and 103 patients with stage III/IV PDAC from January 2018 to March 2018. A total of 95 healthy donors were matched to the PDAC patients by age and sex and selected as the control groups. This study was approved by the Fudan University Shanghai Cancer Center Research Ethics Committee. Informed consent was obtained from all patients prior to the investigation.

**Meta-analysis**

Two independent reviewers searched PUBMED, EMBASE, and MEDLINE databases prior to 30 December 2018. The MeSH (Medical Subject Heading) search included headings that were all combinations of "platelet" and "pancreatic cancer." A pooled risk estimate was calculated with a random-effects model considering both intra-study and inter-study variances. All statistical analyses were conducted using
STATA 11.0 for Windows (Stata, College Station, TX, USA). With strict screening, a total of 12 studies were included for meta-analysis (Supplementary Table 1).

Other assays used in this study are described in the Supporting Information.

Results

**CD41+/CD62P+ platelets are an independent prognostic factor for PDAC**

First, a meta-analysis of 12 published studies was performed to clarify the function of blood platelets in PDAC (Supplementary Table 1). The data showed that a high platelet count is associated with high mortality in PDAC patients (HR = 1.31) (Figure 1A). Next, blood samples from 274 stage I/II patients, 382 stage III/IV patients and 3105 healthy people were collected in our cohort. The three groups were matched in terms of age and sex (Supplementary Table 2). Blood platelets were collected before surgery and anticancer therapy. In contrast to the meta-analysis, we found that the blood platelet count was higher in healthy people (n=3105) than in PDAC patients (n=656) (P < 0.0001) (Figure 1B), while there was no significant difference between stage I/II (n=274) and stage III/IV (n=382) PDAC patients (Figure 1B, C), suggesting that total platelet count is not a valuable predictive marker in PDAC malignancy. Our previous study also showed that total platelet count could not predict PDAC malignancy. The subgroup of CD41+/CD62P+ platelets, representing a group of activated platelets, was reported to promote metastasis in lung cancer. We therefore explored whether the CD41+/CD62P+ platelets is correlated to PDAC malignancy. The flow cytometry analysis of platelets showed that the percentage of CD41+/CD62P+ platelets was higher in stage III/IV PDAC than in stage I/II PDAC (P < 0.001) (Figure 1D). Additionally, the percentage of CD41+/CD62P+ platelets in stage I/II PDAC patients was higher than that in healthy donors (P < 0.0001) (Figure 1D). The stage I/II group of PDAC patients undergoing radical pancreatectomy was divided into two groups according to the median percentage of CD41+/CD62P+ platelets. Elevated CD41+/CD62P+ platelets were significantly associated with several clinical tumor features, such as microvascular invasion (P = 0.002), 8th edition AJCC stage (P < 0.001), and high CA19-9 classification (P = 0.027) (Supplementary Table 3). We also found that elevated level of CD41+/CD62P+ platelets was significantly associated with several clinical tumor features in III/IV stage patients, such as 8th edition AJCC stage (P < 0.001), and metastasis (P < 0.001) (Supplementary Table 4). Kaplan-Meier (K-M) analysis showed that patients with higher CD41+/CD62P+ platelets had a shorter overall survival time (OS) and relapse-free survival time (RFS) than patients with lower CD41+/CD62P+ platelets after surgery (Figure 1E, F). However, higher level of CD41+/CD62P+ platelets was not associated with a shorter OS for those III or IV stage patients (Supplementary Figure 1). Receiver operating characteristic (ROC) curve analysis showed that the area under the ROC curve (AUC) for CD41+/CD62P+ platelets associated with 1-year OS was 0.776 and 1-year RFS was 0.733 in stage I/II patients after surgery (Figure 1G, H). The multivariate analysis indicated that CD41+/CD62P+ platelets represented an independent prognostic risk factor associated with OS (hazard ratio [HR]: 2.180, P < 0.001) and RFS (HR: 3.361, P < 0.001) after surgery (Supplementary Table 5).
PX1 is involved in the activation of CD41+/CD62P+ platelets

Next, we explored critical genes involved in the activation of CD41+/CD62P+ platelets. Platelets isolated from 6 healthy people were treated by thrombin to obtain activated platelets. Then RNA-seq was performed to find differentially expressed genes. We noticed that PX1 transcription level was significantly upregulated in CD41+/CD62P+ platelets (activated platelets) compared to CD41+/CD62P- platelets (non-activated platelets) (Figure 2A). Further validation using qRT-PCR verified that PX1 (not PX2 or PX3) was upregulated in CD41+/CD62P+ platelets than in CD41+/CD62P- platelets (Supplementary Figure 2A). Consistently, western blotting also proved that PX1 expression level was higher in CD41+/CD62P+ platelets than in CD41+/CD62P- platelets (Supplementary Figure 2B). Interestingly, the PX1 expression level in platelets was also higher in stage III/IV patients than in stage I/II patients and healthy people (Figure 2B). We next focused on the role of PX1 in platelet activation. The platelets isolated from PDAC patients were collected for further study. The extended pseudopodia shape of platelets usually represents activated platelets while the round shape represents non-activated platelets. Immunofluorescence (IF) and scanning electron microscopy (SEM) indicated that PX1+ platelets were more easily activated than PX1- platelets (Figure 2C).

To investigate the function of PX1 in platelets in vivo, PX1 knockout (PX1-/-) mice were established as previously described. PX1 expression in platelets was confirmed by western blotting as shown in Supplementary Figure 3A. The blood concentration of platelets between WT (wild-type) and PX1-/- mice was not significantly different, while the bleeding time was significantly extended in PX1-/- mice (Supplementary Figure 3B, C). In addition, the platelet aggregates from the PX1-/- mice were markedly weaker than those of the WT mice (Supplementary Figure 3D-F). These results indicated that PX1 knockout did not affect platelet production but blocked the function of platelets. WT and PX1-/- platelets isolated from WT mice and PX1-/- mice were treated with thrombin for 15min or co-culture with PDAC cells for 24 hours (Panc02), we noticed that WT platelets were more easily activated (higher CD41+/CD62P+ counts) than PX1-/- platelets (Figure 2D). These data suggested that PX1 plays an important role in the activation of CD41+/CD62P+ platelets, and the interaction between tumor cells and platelets may play a role in this phenomenon. However, whether PDAC cells can directly recruit platelets is largely unknown, although our previous study showed that platelet infiltration into tumors was an independent prognostic factor for patients with PDAC. Here, time-lapse live cell imaging revealed that platelets tended to cluster around pancreatic cancer cells (Figure 2E), while in control platelets (without tumor cells), they were evenly distributed (Supplementary Figure 3G). These observations indicated that the communication between platelets and tumor cells may play a role in platelet activation.

PX1 in platelets promoted the migration and invasion of PDAC cells via the EMT pathway

We co-cultured platelets with PDAC cells to determine the effect of PX1 expression in platelets on PDAC migration and invasion. Consistent with previous studies, platelets promoted tumor invasion and metastasis in vitro compared to that without co-culture of platelets (Supplementary Figure 4A-D).
Compared to the co-cultured WT platelets, the migration and invasion of Panc02 cells were significantly inhibited with the co-culture of PX1⁻/⁻ platelets (Figure 3A, B). For further validation, we isolated platelets from PDAC patients and used the PX1-specific inhibitor ¹⁰PX1 to block the PX1 on the platelets before co-culture with PDAC cells. ¹⁰PX1-treated platelets were washed and centrifuged to remove the remaining ¹⁰PX1 to exclude the direct effect of ¹⁰PX1 on PDAC cells (Supplementary Figure 4E, F). We found that platelets with ¹⁰PX1 treatment significantly inhibited the migration and invasion of MIA PaCa-2 cells (Figure 3A, B), suggesting that PX1 expression in platelets determines the pro-tumor role of platelets in PDAC tumor.

In addition, co-culture of PX1⁻/⁻ platelets decreased the PDAC cell-cell adhesion and spindle-like appearance compared to those observed in WT platelets (Supplementary Figure 4G). We then investigated whether PX1 expression could upregulate the EMT pathway in PDAC tumor cells. qRT-PCR showed that WT platelets significantly increased the mRNA levels of snail, vimentin, and N-cadherin, the hallmarks of mesenchymal cells, and reduced the mRNA level of E-cadherin, the hallmark of epithelial cells in PDAC cells, compared to their levels in PX1⁻/⁻ platelets (Figure 3C). Similar results were observed when human platelets were treated by ¹⁰PX1 (Figure 3C). Murine WT platelets and human platelets also increased the protein expression of EMT markers in Panc02 and MIA PaCa-2 cells compared to the loss function of PX1 in platelets (Figure 3D, E).

**PX1 in platelets promotes PDAC invasion and metastasis in vivo**

In agreement with previous studies,²⁴ we found that platelets promoted tumor invasion and metastasis in vivo (Supplementary Figure 5). To confirm the effect of PX1 in platelets on PDAC invasion and metastasis, we established an adoptive platelet transfusion mouse model (Supplementary Figure 6A), in which the endogenous platelets were neutralized and depleted by anti-CD41 antibodies before administration of PX1⁻/⁻ platelets or WT platelets. We found that the number of platelets in mouse was effectively depleted by anti-CD41 injection (Supplementary Figure 6B), and the transfused platelets accounted for 50% of the total platelet counts after exogenous platelet transfusion (Supplementary Figure 6B, C). These data suggested that we successfully established an adoptive platelet transfusion mouse model. In the subcutaneous tumorigenesis model, Panc02 cells co-cultured with PX1⁻/⁻ platelets and WT platelets were implanted subcutaneously into the flank of adoptive platelet transfusion nude mice. The results showed that the tumors co-cultured with PX1⁻/⁻ platelets grew more slowly than those co-cultured with WT platelets (Figure 4A). In WT platelet-educated subcutaneous tumors, more positive vimentin, snail, and N-cadherin staining and less E-cadherin were observed compared to the results in PX1⁻/⁻ platelet-educated tumors (Supplementary Figure 6D). Furthermore, another human PDAC cell line, MIA PaCa-2, was co-cultured with human platelets with/without ¹⁰PX1 blockade and then implanted subcutaneously into the flanks of adoptive platelet transfusion nude mice. Tumors co-cultured with ¹⁰PX1-treated platelets grew more slowly than those without ¹⁰PX1-treated platelets (Supplementary Figure 6E). Similar result was observed when PDAC patient-derived platelets were co-cultured with MIA PaCa-2 cells compared to the platelets isolated from healthy donors (Supplementary Figure 6F). In the
mouse lung metastasis model, Panc02 cells co-cultured with PX1^{-/-} platelets and WT platelets were injected into adoptive platelet transfusion nude mice via the tail vein. We found that more metastatic lung nodules were formed in the WT platelet-injected mice than in the PX1^{-/-} platelet-injected mice (Figure 4C).

To visualize the localization of platelets in lung, WT platelets and PX1^{-/-} platelets were fluorescently labeled by PKH26 and injected into mice via the tail vein. We found that more WT platelets were present in lungs (Figure 4D), while PX1^{-/-} platelets mainly remained in the blood (Supplementary 6G). Interestingly, more Panc02 cells were observed to be present in the lung when mixed with WT platelets compared to that with PX1^{-/-} platelets (Figure 4D). These data suggested that PX1 expression in platelets promotes PDAC tumor metastasis. To further confirm the effect of PX1 in platelets on PDAC metastasis, the adoptive platelet transfusion WT mice was used to study. Compared to the transfusion of WT platelets, transfusion of PX1^{-/-} platelets resulted in less platelet activation and less lung metastasis in the WT mice after Panc02 cell injection with the tail vein (Figure 4E, F). Orthotopic mouse model of pancreatic cancer (Panc02 tumor cells) were established in adoptive platelet transfusion WT mice. After 14 days, all mice were assessed by bioluminescence imaging (BLI). The results showed that the WT mice with the transfusion of PX1^{-/-} platelets had lower average BLI tumor intensities than were found in mice transfused with WT platelets (Figure 4G). In addition, the WT mice transfused with PX1^{-/-} platelets had longer survival times (Figure 4H). Furthermore, PX1^{-/-} platelets alleviated the deterioration of the whole body of mice and bloody ascites caused by tumor invasion in adoptive platelet transfusion WT mice. (Supplementary Figure 7A-C). These data indicated that PX1 expression in platelets promoted the aggressiveness of PDAC tumors.

**PX1 induced the synthesis and secretion of IL-1β in platelets that promoted PDAC cell invasion and metastasis**

Cytokines and chemokines are cell-cell communication factors and essential coordinators of tumor metastasis. We performed a high-throughput multiplex cytokine screening of blood liquid from an established orthotopic PDAC model in WT and PX1^{-/-} mice. The data showed that the protein level of IL-1β was significantly higher in the blood of WT mice compared to that of PX1^{-/-} mice after establishing the PDAC orthotopic model (Figure 5A). Other chemokines, such as MCP-3 (CCL7), MIP-1β (CCL4), MIP-2 (CXCL2) and RANTES (CCL5), were not significantly different between WT and PX1^{-/-} mice after the tumor orthotopic establishment (Supplementary Figure 8A). Several other cytokines, such as IL-10, IL-18, M-CSF, and TGF-β, also did not show any differences (data not shown). In the co-culture of PDAC cells in vitro, the expression of IL-1β was significantly elevated in supernatants of cells when WT platelets rather than PX1^{-/-} platelets were co-cultured with Panc02 or treated by thrombin (Figure 5B), suggesting that PDAC cells stimulates the WT platelet-derived IL-1β secretion. We also observed that the serum IL-1β levels were higher in stage III/IV patients (n=20) than in stage I/II patients (n=20) and healthy people (n=20) (Figure 5C). Furthermore, immunofluorescence and flow cytometry results showed that most of the CD41+CD62P+ platelets isolated from PDAC patients were IL-1β positive, while CD41+CD62P- platelets were IL-1β negative (Figure 5D, E). Next, the wound healing assay and transwell assay were performed to
test the role of IL-1β secreted by platelets in PDAC metastasis. The data revealed that anti-IL-1β antibodies (IL-1βAb) significantly impeded the migration ability and EMT pathway of PDAC cells co-cultured with WT platelets (Figure 5F-I), while recombinant IL-1β (rmIL-1β) significantly promoted the migration ability and EMT pathway in PDAC cells co-cultured with PX1−/− platelets (Figure 5F-I). For further control, we used IL-1βAb and rmIL-1β to treat PDAC cells without the co-culture of platelets. The data showed that rmIL-1β promoted PDAC cell invasion and migration in vitro (Supplementary Figure 8B, C). In vivo, IL-1βAb administration in WT mice suppressed lung metastasis compared to that in WT mice injected with PBS (Figure 5J). In contrast, rmIL-1β increased lung metastasis in PX1−/− mice compared to that in PX1−/− mice injected with PBS (Figure 5J). These results demonstrated that PX1 induced the synthesis and secretion of IL-1β in platelets, which promoted PDAC cell invasion and metastasis.

**PX1 enhanced platelet-derived IL-1β secretion through phosphorylating p38 MAPK**

The p38 MAPK pathway has been reported to regulate NLRP3 and IL-1β expression in macrophages.\(^2^5\) Furthermore, our RNA-Seq data showed that PX1 was associated with the NLRP3/IL-1β and p38 MAPK pathway (Supplementary Figure 9A). Other pathways, such as the WNT/β-catenin, PI3K/AKT, and ERK/MAPK pathways, did not show significant differences between the WT platelets and PX1−/− platelets (Supplementary Figure 9B). We next tried to determine the relationship between PX1 and the p38 MAPK pathway. After 15 min of thrombin treatment, we found that the phosphorylated p38 MAPK (p-p38 MAPK) level was upregulated only in WT platelets but not in PX1−/− platelets (Figure 6A). We found that the p-p38 MAPK level in human platelets was inhibited by the 10PX1 peptide (Figure 6A). Furthermore, the expression of p-p38 MAPK in patients' platelets was significantly higher than that in platelets isolated from healthy people after thrombin treatment (Supplementary Figure 9C). Immunofluorescence also showed a higher p-p38 MAPK expression in WT platelets compared to that in PX1−/− platelets (Figure 6B). After thrombin stimulation for 24 h, P38 MAPK was not upregulated in either WT or PX1−/− platelets. By contrast, only in WT platelets were NLRP3 and IL-1β expression as well as their mRNA levels increased (Figure 6C, D). Consistent with this observation, the expression of NLRP3 and IL-1β was significantly decreased only in human platelets with 10PX1 treatment (Figure 6C, D). After blocking the p38 MAPK pathway by SB203580, the expression of NLRP3 and IL-1β was significantly decreased in WT-derived platelets and human platelets (Figure 6E). These data suggested that PX1 regulated the secretion of IL-1β via activating the p38 MAPK pathway. Consistently, in vivo, the serum IL-1β level in WT mice was significantly reduced by using SB203580 (Figure 6F). Furthermore, SB203580 inhibited the activation of WT platelet and suppressed PDAC cell invasion and metastasis (Figure 6G, H). Of note, SB203580-treated platelets were washed and centrifuged to remove the remaining SB203580 for excluding the direct effect of SB203580 on PDAC cells (Supplementary Figure 9D, E). We also found that PX1 deficiency inhibited ATP release from platelets after the thrombin treatment (Supplementary Figure 9F), which is similar to the findings of a previous study that PX1 is associated with ATP release in macrophages.\(^2^3\) The structure analysis showed that ATP could bind to the ATP binding site on p38 MAPK and thereby promoted the phosphorylation of p38 MAPK (Figure 6I).
**PX1 is a potential therapeutic target for PDAC**

Our above results indicated that the PX1-p38 MAPK-IL-1β axis was activated in CD41+/CD62P+ platelets and promoted PDAC cell invasion and metastasis. Thus, we investigated whether PX1 on CD41+/CD62P+ platelets could serve as a novel therapeutic target for PDAC tumors. P-selectin glycoprotein ligand-1 (PSGL-1) was reported to specifically bind to CD62P, which is a marker of activated platelets and is expressed on their surface. Carbenoxolone is a PX1 inhibitor. These two components were ligated by chemical synthesis to generate the new PX1 inhibitor (Figure 7A). 1H-nuclear magnetic resonance (NMR) and mass spectrometry indicated that PSGL-1 and carbenoxolone formed a perfect combination (Figure 7B, C), and the molecular ion of the newly generated inhibitor was observed at 63435 (Figure 7C). We therefore designated this novel drug as PC63435. Compared to the WT mice injected with PBS, the WT mice injected with PC63435 had a prolonged bleeding time (Supplementary Figure 10A) and impaired platelet aggregation (Supplementary Figure 10B). Flow cytometry showed that the number of CD41+/CD62P+ platelets in the WT mice injected with PC63435 was lower than that in the PBS-injected mice (Figure 7D, E). Additionally, PC63435 reduced serum IL-1β levels in WT mice (Figure 7F). Then, the anti-tumor effect of PC63435 was tested in the orthotopic PDAC mouse model and lung metastasis model in WT mice with Panc02 injection. The data showed that PC63435 suppressed PDAC invasion and metastasis in WT mice compared to those in the control group (Figure 7G-I). In addition, PC63435 significantly prolonged the survival of WT mice by approximately 2-fold compared to that in PBS-treated mice in the orthotopic PDAC mouse model (Figure 7J).

In conclusion, we demonstrated that PX1 expression in platelets activates the p38 MAPK pathway, enhances platelet-derived IL-1β secretion, and promotes PDAC invasion and metastasis. Specifically, we developed a novel drug, PC63435, that is able to block PX1 on CD41+/CD62P+ platelets and therefore suppress the invasion and metastasis potential of PDAC (Figure 8).

**Discussion**

Increasing evidence has shown that platelets contribute to tumor cell invasion and metastasis. To date, the role of platelets in PDAC invasion and metastasis remains unclear. Our study revealed that the CD41+/CD62P+ platelet subtype, rather than whole-blood platelet, was an independent risk factor for poor prognosis in PDAC patients after surgery which could promote the malignant potential of PDAC. We also found that PX1 mediated platelet-derived IL-1β secretion by activating the p38 MAPK pathway as well as the activation of CD41+/CD62P+ platelets. Finally, specifically blocking PX1 in CD41+/CD62P+ platelets with synthetic compounds significantly decreased PDAC invasion and metastasis.

Platelets can be easily acquired from whole blood and are suitable as a predictive biomarker. However, the value of platelet counts in predicting PDAC prognosis is controversial. Preoperative platelet counts were reported to be an independent predictor of survival for PDAC patients after curative pancreatectomy. A meta-analysis even showed that an elevated platelet level was associated with poor outcomes for PDAC patients. However, our previous study failed to identify the role of preoperative blood...
platelet counts in the prognosis of PDAC patients.\textsuperscript{22} In this study, we further expanded the sample size (656 PDAC patients) and selected 3105 healthy people. We found that the platelet count was higher in healthy people than in PDAC patients. This phenomenon can be explained by the fact that many PDAC patients have accompanying hypersplenism, myelosuppression, and other potential causes of thrombocytopenia. However, no significant difference in platelet counts between early-stage PDAC (I/II) and late-stage PDAC (III/IV) was observed. This result indicated that the total blood platelet count is not a reliable indicator for PDAC malignancy. Here, we found that a subgroup of platelets, labeled CD41\textsuperscript{+}/CD62P\textsuperscript{+}, was significantly elevated in PDAC patients compared with the level in healthy donors. The percentage of CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelets in late-stage (III/IV) patients was significantly higher than that in early-stage (I/II) patients. PDAC patients with high CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelet counts had a poor prognosis after radical operation compared to those with low CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelet counts. We thus refer to the CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelet subtype, rather than the total platelet counts, as a reliable biomarker for predicting the prognosis of PDAC.

CD41 represents all platelets, while CD62P is a marker of activated platelet subsets.\textsuperscript{30} CD62P, also known as P-selectin, is an important adhesion molecule and is expressed on the surface of activated platelets. CD62P can mediate platelets and tumor cell adhesion, which are associated with tumor metastasis.\textsuperscript{30} CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelets were significantly higher in lung cancer patients than in healthy populations and promoted tumor metastasis,\textsuperscript{11} supporting our results that the CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelet subtype, but not the whole-blood platelet count, has predictive value in PDAC.

To clarify the pro-tumor mechanism of CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelets, RNA-Seq was performed, and the results showed that PX1 was significantly upregulated in CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelets. PX1, also called Panx1, is a membrane channel protein and mainly involves in participating in intracellular and extracellular signal communication, mediating the release of ATP, and a variety of other physiological and pathological processes. Previously, PX1 on tumor cells has been reported to promote tumor proliferation and metastasis.\textsuperscript{18, 19} However, the mechanism by which PX1 in platelets promotes PDAC invasion and metastasis remains unclear. We found that upregulated PX1 in platelets is indispensable for platelet activation (CD62P expression) and aggregation. This finding is consistent with previous results showing that PX1 is expressed in amplified activated human platelets.\textsuperscript{31} In our study, PX1-upregulated platelets were more susceptible to activation and more likely to promote PDAC invasion and metastasis. Platelet-derived cytokines/chemokines are important mediators of the crosstalk between platelets and cancer cells.\textsuperscript{32} High-throughput cytokine/chemokine detection showed that IL-1β was significantly upregulated in PX1\textsuperscript{+}/CD41\textsuperscript{+}/CD62P\textsuperscript{+} platelets. IL-1β has been shown to be upregulated in solid tumors, is indicative of poor prognosis,\textsuperscript{33, 34} and is required for prostate cancer cell invasiveness.\textsuperscript{35} IL-1β can bind to the IL-1R of tumor cells to promote EMT in tumor cells.\textsuperscript{36} In PDAC, an IL-1 receptor (IL-1R) antagonist inhibited PDAC growth.\textsuperscript{37} The findings from the above studies supported our results that platelet-derived IL-1β secretion enhanced PDAC invasion and metastasis. Therefore, PX1 in platelets promotes PDAC invasion and metastasis by mediating platelet-derived IL-1β release.
Recent studies have shown that PX1 in macrophages mediates IL-1β release by the ATP-gated P2X7 receptor or by activating the NLRP3 inflammasome. After screening several classic pathways in platelets, such as the WNT/β-catenin, PI3K/AKT, and ERK/MAPK pathways, we did not find a significant change in these signaling pathways. However, platelet RNA-Seq gene co-expression network analysis showed that the upregulation of PX1 was associated with p38MAPK, NLRP3 and IL-1β. We found that PX1 promoted platelet-released IL-1β by activating the phosphorylation of the p38 MAPK pathway. p38 MAPK has been reported to regulate NLRP3 and IL-1β expression in macrophages. In our study, blocking p38 MAPK reduced NLRP3 and IL-1β expression in platelets. Another study showed that the inhibition of p38 MAPK decreased IL-1β expression to protect against acute lung injury. The activation of the p38 MAPK pathway augments the production of IL-1β in stimulated monocytes. These studies also support the reliability of our research. p38 MAPK has a domain-active site ATP pocket. ATP can bind to the ATP binding site on p38 MAPK and then promote the phosphorylation of p38 MAPK. We proved that PX1 functions in platelet-mediated ATP release (Supplementary Figure 13). This finding is consistent with the finding from a report by Sandilos that PX1 mediates ATP release in macrophages.

The inhibition of tumor metastasis by targeting platelets has been reported. Li reported that synthetic silica particles functionalized with activated platelet membranes along with the surface conjugation of tumor-specific apoptosis-inducing ligand cytokines dramatically decreased lung metastasis in a mouse breast cancer model. Our results have some obvious advantages compared with Li’s biomimetic synthetic material. First, silica particles are not easily degraded in vivo; in contrast, our carbenoxolone was carried by common drugs, increasing degradability. Second, Li’s methods depend on receptor-ligand interactions for platelet-cancer cell adhesion, where mis-targeting or off-targeting can occur because not all cancer cells have the same receptor-ligand interactions. Our strategy focused on functional platelets that almost always express CD62P, regardless of tumor heterogeneity, to avoid mis-targeting or off-targeting.

The present study is the first to report the pro-tumoral mechanism of PX1 in activated platelets labeled CD41+/CD62P+. This study provides a deeper understanding of platelet subtype function. However, how PX1 amplifies platelet activation is still unclear. Determining whether specifically blocking the PX1 pathway on activated platelets can achieve therapeutic effects in PDAC patients requires further clinical trials. In addition to CD62P, the marker of platelet activation, other sensitive activation markers such as phosphatidylserine (PS) exposure and RCA-1 binding are also worthwhile to be studied for their correlations with PDAC tumor metastasis.

In conclusion, we found a new pathway in which the PX1/IL-1β axis promotes platelet activation and contributes to PDAC cell invasion and metastasis, and this axis can serve as a new therapeutic target for PDAC.

Abbreviations
Declarations

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Contribution: H.L. designed the research and drafted the manuscript. H.L., and W.J. revised the manuscript. H.L., S. R.Z, and H.X.X. performed most of the experiments. J.L., W.J., W.J., P.S.W., Y.M.Y., T.S., and D.X.Q. performed some of the mouse experiments. H.L., W.J., T.J.L., S.L., S.S.X., and W.H.Z. analyzed results and made the figures. P.C.L., H.L.G., H.X.X., and W.Q.W. performed some of the in vitro experiments. L.L. and X.J.Y. supervised the research.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018;68:7-30.
2. Shaib WL, Ip A, Cardona K, et al. Contemporary Management of Borderline Resectable and Locally Advanced Unresectable Pancreatic Cancer. Oncologist 2016;21:178-187.
3. Erpenbeck L, Schon MP. Deadly allies: the fatal interplay between platelets and metastasizing cancer cells. Blood 2010;115:3427-3436.
4. Xu XR, Zhang D, Oswald BE, et al. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Crit Rev Clin Lab Sci 2016;53:409-430.
5. Heras P, Hatzopoulos A, Kritikos N, et al. Platelet count and tumor progression in gastric cancer patients. Scand J Gastroenterol 2010;45:1005-1006.
6. Gogus C, Baltaci S, Filiz E, et al. Significance of thrombocytosis for determining prognosis in patients with localized renal cell carcinoma. Urology 2004;63:447-450.
7. Ishizuka M, Nagata H, Takagi K, et al. Combination of platelet count and neutrophil to lymphocyte ratio is a useful predictor of postoperative survival in patients with colorectal cancer. Br J Cancer 2013;109:401-407.

8. Yang H, Lang S, Zhai ZM, et al. Fibrinogen is required for maintenance of platelet intracellular and cell-surface P-selectin expression. Blood 2009;114:425-436.

9. Nierodzik ML, Karpatkin S. Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype. Cancer Cell 2006;10:355-362.

10. Palumbo JS, Talmage KE, Massari JV, et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. Blood 2005;105:178-185.

11. Gong L, Cai Y, Zhou X, et al. Activated platelets interact with lung cancer cells through P-selectin glycoprotein ligand-1. Pathol Oncol Res 2012;18:989-996.

12. Nierodzik ML, Plotkin A, Kajumo F, et al. Thrombin stimulates tumor-platelet adhesion in vitro and metastasis in vivo. J Clin Invest 1991;87:229-236.

13. Martin HL, Ohara K, Kiberu A, et al. Prognostic value of systemic inflammation-based markers in advanced pancreatic cancer. Intern Med J 2014;44:676-682.

14. Aziz MH, Sideras K, Aziz NA, et al. The Systemic-Immune-Inflammation Index Independently Predicts Survival and Recurrence in Resectable Pancreatic Cancer and its Prognostic Value Depends on Bilirubin Levels: A Retrospective Multicenter Cohort Study. Ann Surg 2018.

15. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell 2011;20:576-590.

16. Takemoto A, Okitaka M, Takagi S, et al. A critical role of platelet TGF-beta release in podoplanin-mediated tumour invasion and metastasis. Sci Rep 2017;7:42186.

17. Rachidi S, Metelli A, Riesenberg B, et al. Platelets subvert T cell immunity against cancer via GARP-TGFbeta axis. Sci Immunol 2017;2.

18. Wei L, Yang X, Shi X, et al. Pannexin1 silencing inhibits the proliferation of U87MG cells. Mol Med Rep 2015;11:3487-3492.

19. Furlow PW, Zhang S, Soong TD, et al. Mechanosensitive pannexin-1 channels mediate microvascular metastatic cell survival. Nat Cell Biol 2015;17:943-952.

20. Shi GJ, Liu CL, Yang YM, et al. Panx1 promotes invasion-metastasis cascade in hepatocellular carcinoma. J Cancer. 2019; 10:5681-5688.

21. Zhang SR, Yao L, Wang WQ, et al. Tumor-Infiltrating Platelets Predict Postsurgical Survival in Patients with Pancreatic Ductal Adenocarcinoma. Ann Surg Oncol 2018;25:3984-3993.

22. Ponomareva AA, Nevzorova TA, Mordakhanova ER, et al. Intracellular origin and ultrastructure of platelet-derived microparticles. J Thromb Haemost 2017; 15:1655-1667.

23. Wang H, Xing Y, Mao L, et al. Pannexin-1 influences peritoneal cavity cell population but is not involved in NLRP3 inflammasome activation. Protein Cell 2013; 4:259-265.
24. Haemmerle M, Taylor ML, Gutschner T, et al. Platelets reduce anoikis and promote metastasis by activating YAP1 signaling. Nat Commun 2017;8:310.
25. Moon JS, Lee S, Park MA, et al. UCP2-induced fatty acid synthase promotes NLRP3 inflammasome activation during sepsis. J Clin Invest 2015;125:665-680.
26. Patel MS, Miranda-Nieves D, Chen J, et al. Targeting P-selectin glycoprotein ligand-1/P-selectin interactions as a novel therapy for metabolic syndrome. Transl Res 2017;8:183.
27. Leblanc R, Peyruchaud O. Metastasis: new functional implications of platelets and megakaryocytes. Blood 2016;128:24-31.
28. Liu Y, Cao X. Characteristics and Significance of the Pre-metastatic Niche. Cancer Cell 2016;30:668-681.
29. Schwarz RE. Platelet counts and prognosis of pancreatic cancer. Lancet 1999;353:2158-2159.
30. Qi C, Li B, Guo S, et al. P-Selectin-Mediated Adhesion between Platelets and Tumor Cells Promotes Intestinal Tumorigenesis in Apc(Min/+) Mice. Int J Biol Sci 2015;11:679-687.
31. Taylor KA, Wright JR, Vial C, et al. Amplification of human platelet activation by surface pannexin-1 channels. J Thromb Haemost 2014;12:987-998.
32. Xu XR, Yousef GM, Ni H. Cancer and platelet crosstalk: opportunities and challenges for aspirin and other antiplatelet agents. Blood 2018;131:1777-1789.
33. Gemma A, Takenaka K, Hosoya Y, et al. Altered expression of several genes in highly metastatic subpopulations of a human pulmonary adenocarcinoma cell line. Eur J Cancer 2001;37:1554-1561.
34. Elaraj DM, Weinreich DM, Varghese S, et al. The role of interleukin 1 in growth and metastasis of human cancer xenografts. Clin Cancer Res 2006;12:1088-1096.
35. Voronov E, Shouval DS, Krelin Y, et al. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci U S A 2003;100:2645-2650.
36. Pantschenko AG, Pushkar I, Anderson KH, et al. The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression. Int J Oncol 2003;23:269-284.
37. Zhuang Z, Ju HQ, Aguilar M, et al. IL1 Receptor Antagonist Inhibits Pancreatic Cancer Growth by Abrogating NF-kappaB Activation. Clin Cancer Res 2016;22:1432-1444.
38. Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J 2006;25:5071-5082.
39. Franchi L, Nunez G. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. Eur J Immunol 2008;38:2085-2089.
40. Zheng DY, Zhou M, Jin J, et al. Inhibition of P38 MAPK Downregulates the Expression of IL-1beta to Protect Lung from Acute Injury in Intestinal Ischemia Reperfusion Rats. Mediators Inflamm 2016;2016:9348037.
41. Lee JC, Laydon JT, McDonnell PC, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994;372:739-746.
42. Kuzmanic A, Sutto L, Saladino G, et al. Changes in the free-energy landscape of p38alpha MAP kinase through its canonical activation and binding events as studied by enhanced molecular dynamics simulations. Elife 2017;6.

43. Sandilos JK, Chiu YH, Chekeni FB, et al. Pannexin 1, an ATP release channel, is activated by caspase cleavage of its pore-associated C-terminal autoinhibitory region. J Biol Chem 2012;287:11303-11311.

44. Li J, Ai Y, Wang L, et al. Targeted drug delivery to circulating tumor cells via platelet membrane-functionalized particles. Biomaterials 2016;76:52-65.