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# Metastasis-promoting role of extravasated platelet activation in tumor

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ABSTRACT

*Background:* The last decade has focused attention on the central role of platelets interacting with the tumor cells and the immune system in promoting tumor progression and distant spread through release of growth factors, such as transforming growth factor beta, vascular endothelial growth factor A, and plasminogen activator inhibitor 1, into the tumor microenvironment. We focused on the potential metastasis-promoting role of extravasated platelet aggregation in pancreatic cancer and stroma.

*Materials and methods:* Resected pancreatic cancer specimens from 40 patients were used in this study. To examine the expression and localization of platelet aggregation in the epithelial–mesenchymal transition (EMT) region in cancer and stroma, CD42b, Snail1, and E-cadherin were assessed using immunohistochemistry. We determined the correlation of these expressed proteins with clinical features.

*Results:* CD42b expression was detected at the invasive front of the tumor, which was in 73% of the EMT portion, but not in the region of tubular formation. Increased Snail1 and reduction and/or loss of E-cadherin expressions were noted in 85% and 75% of the EMT portion, respectively. There was a significant correlation between CD42b and Snail1 expressions ($P = 0.02$) and CD42b and reduction and/or loss of E-cadherin expressions ($P = 0.008$).

*Conclusions:* We demonstrate that extravasated platelet aggregation is associated with the first step in the formation of the EMT. These data suggest a potential role for antiplatelet agents to suppress EMT and metastasis by changing the tumor microenvironment.



## 1. Introduction

Pancreatic ductal adenocarcinoma (PDAC), one of the most lethal of all human cancers, is a common cause of cancer mortality in the United States and Japan [1]. PDAC ranks as the fourth most common cause of cancer death, and its incidence is increasing worldwide [1,2]. The overall 5-y survival rate among patients with PDAC is still <5% [3,4]. The lethal nature of PDAC is attributed to its high metastatic potential to the lymphatic system and distant organs [5]. Lack of effective

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therapeutic options contributes to the high mortality rate of PDAC.

Recent evidence suggests that the epithelial–mesenchymal transition (EMT) region plays an important role in disease progression and development of drug resistance in PDAC [2,6]. EMT is defined as the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype [7]. EMT endows cells with migratory and invasive properties, induces stem cell properties, prevents apoptosis and senescence, and contributes to immunosuppression [8]. In this process, cells acquire molecular alterations, such as loss of E-cadherin, fibronectin, vimentin, and others that facilitate dysfunctional cell–cell adhesive interactions and junctions [8,9]. E-cadherin is a single-spanning transmembrane glycoprotein that maintains intercellular contacts and cellular polarity in epithelial tissues. Loss of E-cadherin is associated with tumor invasiveness, metastatic dissemination, and poor prognosis in several solid tumor cancers [10,11]. Members of the Snail family of transcription factors have been shown to induce EMT, a fundamental mechanism of embryogenesis and progressive disease [12]. EMT and Snail have been related to cancer hallmarks, such as the gain of unlimited replication potential, a greater resistance to apoptosis, and evasion of immunosurveillance [13].

Some of the main drivers of EMT in PDAC are the transcription factors Snail, Slug, and Zeb1, which are in turn regulated by cytokines such as tumor necrosis factor α and growth factors such as transforming growth factor beta (TGF-β). TGF-β is known to promote metastasis by enhancing EMT and invasiveness in primary carcinomas [14]. Also, vascular endothelial growth factor (VEGF) receptor 1 activation led to an increase in expression of the EMT-associated transcription factors Snail, Twist, and Slug [9]. VEGF-A, known as a vascular permeability factor, is a secreted protein that plays a pivotal role in hyperpermeability of vessels in addition to angiogenesis [15,16]. Furthermore, plasminogen activator inhibitor 1 (PAI-1) is known to play a paradoxical positive role in tumor growth and metastasis [17,18] as an important mediator of mesenchymal stem cell/colon cancer cell interactions [19].

Platelets contain storage pools of growth factors such as platelet-derived growth factor, TGF-β, VEGF, thrombospondin 1 (TSP-1), platelet factor 4, and matrix metalloproteinases [20–22]. Furthermore, PAI-1 in microvascular endothelial cells has been shown to be regulated by platelets and TSP-1 [21]. Thus, platelet-derived factors could potentially be involved in the promotion of a metastatic phenotype. The balance of platelet-secreted molecules such as TGF-β, VEGF, hepatocyte growth factor, and PAI-1 may have important therapeutic implications in the control of angiogenesis and fibrosis [23].

Aggregated platelets coat tumor cells during their transit through the bloodstream and mediate adherence to vascular endothelium, protection from shear stresses, evasion from immune molecules, and release of an array of bioactive molecules that facilitate tumor cell extravasation and growth at metastatic sites [24]. Although the intravasated platelet aggregation focused attention on EMT, extravasated platelet aggregation has been less noticeable. Hematoxylin and eosin staining could not be used to confirm the presence of extravasated platelet aggregation (EPA) in the tissue because platelets lack a nucleus.

Recent reports of large randomized trials suggested that aspirin prevented distant metastasis, and this could account for the early reduction in cancer deaths in trials of daily aspirin *versus* control [25]. Aspirin use has been associated with reduced risk of cancer progression and metastasis [26,27] by potentially reducing the propensity of cancers to metastasize through a direct platelet-mediated effect.

However, there is no report regarding the role of EPA in primary tumors. In this study, we investigated the association between EMT and EPA in the PDAC microenvironment.

## 2. Materials and methods

### 2.1. Patients and tissue samples

Between January 2006 and December 2009, 40 PDAC patients who had been diagnosed radiologically underwent surgery at the Department of Gastroenterological Surgery of Kanazawa University Hospital. Among them, 25 patients had pancreatic head cancers and 15 had body and tail cancers. They included 25 men and 15 women with an average age of 64 y (range, 37–78 y).

### 2.2. Ethics statement

The study was approved by the Ethics Committee of the Kanazawa University Hospital. The written informed consent was obtained from each patient enrolled in the study.

### 2.3. Pathologic specimens

Paraffin-embedded tissue samples of 40 patients with PDAC were obtained from the Institute of Pathology for immunohistochemical analysis. The specimens were previously fixed in 10% formalin and embedded in paraffin. Several 3-μm-thick sections were cut from each paraffin block; one was stained with hematoxylin and eosin, and three were subjected to immunohistochemical staining for CD42b, Snail1, and E-cadherin.

### 2.4. Immunohistochemical examination

For immunohistochemical staining, the Dako Envision system, which uses dextran polymers conjugated to horseradish peroxidase (Dako, Carpinteria, CA), was used to avoid any endogenous biotin contamination. Sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase was blocked by immersing sections in 3% $H_2O_2$ in 100% methanol for 20 min at room temperature. Antigen retrieval was achieved by microwaving sections at 95°C for 10 min in 0.001 M citrate buffer (pH 6.7). After blocking the endogenous peroxidase, sections were incubated with Protein Block Serum-Free (Dako) at room temperature for 10 min to block nonspecific staining. Subsequently, sections were incubated for 2 h at room temperature with 1:100 diluted mouse or rabbit monoclonal antibodies against CD42b (anti-CD42 rabbit monoclonal, EPR6995; Abcam, Cambridge, MA), Snail (anti-SNAIL1 rabbit polyclonal antibody, sc-28199; Santa Cruz Biotechnology, Santa Cruz, CA), or E-cadherin (anti-E-cadherin mouse