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ORIGINAL PAPER

Detection of Binding of a Synthetic Granzyme B-like Peptide Fluorescent Conjugate within Platelet-like Structures in Cancer-related Peripheral Blood Specimens and Tissue Sections

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Abstract Platelets and cytotoxic T lymphocytes (CTL) are important whole blood components in peripheral blood. Studies have shown that platelets, from precursor megakaryocytes, are significant factors in cancer prognosis, cancer progression, and metastasis; but a direct platelet-cancer relationship remains unclear. CTL play an essential role in cancer surveillance by inducing cancer cell death with granzyme B. A recent report has shown the presence of binding targets with binding affinity to a synthetic granzyme B-like peptide fluorescent conjugate (GP1R) in different types of cancer cells grown in vitro. It suggests that these binding targets may serve as a "universal-pathologic-biomarker". It is not known if similar biomarkers may be present in platelets of cancer patients. We show with fluoroscopic images that GP1R can bind to binding targets: 1) within platelets in methanol-fixed whole blood smears of patients with breast cancer and lung cancer, and 2) within platelet-like structures in formalin-fixed-paraffin-embedded (FFPE) nude mouse xenogeneic breast tumor tissues. Samples without cancer-association displayed no discernible GP1R-binding in platelet-like structures. Our data demonstrate for the first time that a similar "universal-pathologic-biomarker" detectable by GP1R-binding is present in circulating platelets of cancer patients. The data depict a co-existence of animal-platelets and human-breast cancer cells, both have a common pathologic biomarker detectable by GP1R, in the tumor growth. The fluoroscopic images indicate a visual direct connection between pathologic platelets and cancer. These preliminary results may lead to developments of novel platelet-based cancer diagnostics and therapeutics and a better understanding of the potential multifunction of GP1R and its relationship to megakaryocytes and PD1.

Keywords Biomarker · Cancer · Fluorescence microscopy · Granzymes · Peptide · Platelets

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Introduction

Platelets, derived from the cellular fragments of megakaryocytes, with a disk-shape normally are the smallest (2–3 μm) whole blood components in peripheral blood. Qualitatively, platelets have been suggested to play a supporting role as facilitator in cancer progression and metastasis [1–3]. Quantitatively, thrombocytosis has been regarded as an adverse prognostic factor [4–8] in cancer patients. Despite these proposed associations with cancer, platelets have not yet become a major pharmaceutical target in cancer drug development especially when a visible link to show a direct connection between platelets and cancer remains unknown. As part of the whole blood components, platelets (both qualitatively and quantitatively) are mostly known for the role they play in formation of blood clot. A recent study showed that autologous platelets could be targeted and destroyed by cytotoxic T lymphocytes (CTL) in chronic idiopathic thrombocytopenic purpura [9]. CTL are known to target tumor cells and destroy them with granzyme B enzyme as part of our innate cancer surveillance mechanism.

Lo and Luther first reported [10] the detection of cancer cell death mediated by a synthetic granzyme B-like peptide fluorescent conjugate (GP1R, BioJENC, USA) without the presence of CTL. Their study also showed the presence of a possible "universal- pathologic-biomarker" constitutively

expressed in different types of laboratory maintained cancer cells. The proposed "universal-pathologic-biomarker" was demonstrated using fluorescence microscopy to show intracellular binding of the GP1R peptide fluorescent conjugate in (methanol-fixed) cancer cells grown in vitro. It is not known if a similar biomarker is detectable in whole blood specimens of cancer patients. Our objective is to determine if circulating platelets in peripheral blood of cancer patients have a similar "universal-pathologic-biomarker" that can be used to help make a direct visual connection with the disease condition related to cancer. Another objective is to determine if platelets with similar biomarkers are present in tumor growth. The simple one-step GP1R direct-staining protocol applicable to whole blood specimens (without the need of any cell separation) as reported by Lo and Luther [10] showed that GP1R has no binding in bovine red blood cells (RBC) and other serum proteins, demonstrating a high target-binding specificity and selectivity of GP1R useful for this study. Detection of GP1R binding in tumor growth can be better determined in the tumor tissue from the nude mouse model implanted with xenogeneic human cancer cells to avoid potential interference by the host's own CTL/granzyme B as nude mouse naturally has limited CTL.

Materials and Methods

Whole Blood Smears

The peripheral blood specimens provided by cancer patients (lung cancer and breast cancer) and non-cancer patient with their consent were used for this study. Our institutional review approved the study. Fresh EDTA-peripheral blood samples were smeared on glass slides and allowed to air dry before methanol fixation. Methanol-fixed peripheral blood smears were used in the cell binding experiments that were done twice in duplicate. The cell binding experiments employed the same GP1R staining protocol as reported by Lo and Luther [10].

Formalin Fixed Paraffin Embedded (FFPE) Tissues

FFPE tissue sections of xenogeneic tumor comprised of laboratory-maintained human SKBR3 breast cancer cells implanted in the nude mouse and FFPE tissue sections of human normal lung were used (all FFPE samples were donated via Dr. Luther) for this study. The donor of the FFPE tissue sections used in this study had obtained necessary consent and approval before offering for our research use. Our institutional review approved the study. The donated tissue sections were prepared according to standard pathology laboratory protocol and the tissue sections had been checked with Giemsa stain equivalent for good quality before offering for our research use. Rehydrated and permeated FFPE tissue sections on glass slides were used in the cell/tissue binding experiments that were done twice in duplicate.

Peripheral Whole Blood Smears and FFPE Tissues Binding Experiments

GP1R, a synthetic granzyme B-like peptide conjugated with rhodamine fluorescent dye (BioJENC, USA), was used in staining incubation with: 1) peripheral whole blood smears pretreated with methanol fixation, and 2) FFPE tumor tissue and human normal lung tissue. In the peripheral whole blood smears binding experiments, EDTA-peripheral blood smears of cancer patients and non-cancer patient fixed with methanol on a slide were incubated with GP1R (300–500 μL of 1:1000 dilution in phosphate buffered saline or PBS) for 5 minutes at room temperature. Whole blood smear treated with standard Diff-Quik counter stain was used to demonstrate the presence of platelets with similar morphological features (size and shape) as shown with GP1R fluorescent stain. In the FFPE tissue binding experiments, the FFPE tissue sections (rehydrated and permeated) on a slide were incubated with GP1R (300–500 μL of 1:1000 dilution in phosphate buffered saline or PBS) for 15–30 minutes at room temperature. After rinsing the slides gently twice with distilled water or PBS, the cells on the slides were covered with PBS and a cover slip for visualization by light microscopy and fluorescence microscopy. Microscopic images were captured by AxioCamMR3.

Results

Peripheral Whole Blood Smears and FFPE Tissue Binding Experiments

Using fluorescence microscopy, fluorescent deposits were detected within platelets in the peripheral whole blood specimens of patients with lung cancer and breast cancer as shown in Fig. 1. Control non-cancer patient did not show the same result as shown in Fig. 2. Fluorescent deposits were detected within platelet-like structures in FFPE mouse xenogeneic tumor tissue (tumor growth from human breast cancer cells implanted in the nude mouse) but not FFPE human normal lung tissue as shown in Fig. 3.

Discernible fluorescent deposits were not demonstrated in RBC of all the samples, regardless of their different sources, as shown in Figs. 1, 2, and 3. These samples included different

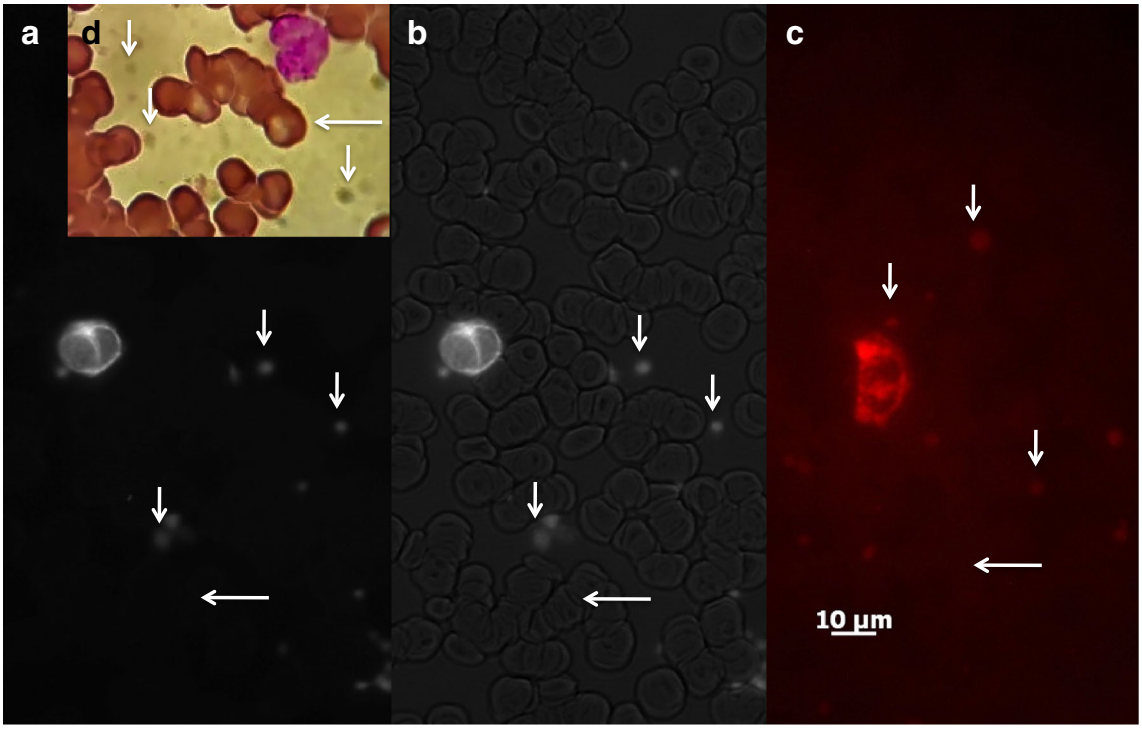

Fig. 1 Fluorescence microscopy of platelets with GP1R-binding in whole blood smears of patients diagnosed with cancer. Fluoroscopic images of platelets in peripheral whole blood smears with methanol pre-fixation of patients diagnosed with lung cancer (**a** and **b**) and breast cancer (**c**) are shown. Microscopic images of platelets in a peripheral whole blood smear with Diff-Quik stain of the same lung cancer patient is shown in **d**. The presence of fluorescent deposits in platelets (small disk- and pleomorphic-shaped structures) indicates binding of GP1R to corresponding cellular binding targets. Some platelets with positive GP1R-binding are shown near or on the large binucleated cells with fluorescent deposits. In **a** and **c** the microscopic images were recorded using fluorescence microscopy; in **b** the microscopic image was recorded using light microscopy and fluorescence microscopy combined of the same field as shown in **a**. In **d** the microscopic image was recorded using light microscopy. Prior to image recording, the methanol-fixed blood smears had a 5-min incubation at room temperature with GP1R or a standard Diff-Quik staining procedure. A similar GP1R-binding pattern was shown in platelets (*down arrows*) of blood smears of patients with lung cancer (same cells in **a** and **b**) and breast cancer (**c**). GP1R unstained RBC (*left arrows*) are shown in the background (**a**, **b**, and **c**). Platelets with similar morphological features (size and shape) as depicted in panels a and b are shown (*down arrows*) in the blood smear of the same patient with lung cancer stained with counter stain Diff-Quik (**d**), which also shows numerous stained RBC (*left arrow*). The scale bar for each panel is the same as shown in **c**. A representative of about 10 platelet-like structures in each field examined (n=5) in duplicates is shown

human blood specimens of patients with different types of cancer (patients with lung cancer and breast cancer), different disease conditions (patients with and without cancer), and different sample preparations (methanol-fixed blood smears and FFPE tissue sections).

Discussion

We demonstrate here with fluoroscopic images that circulating platelets in cancer patients show the presence of binding targets with binding affinity to GP1R, a synthetic granzyme B-like peptide fluorescent conjugate. The detection of GP1R fluorescent deposits in these methanol-fixed platelets indicates that GP1R-binding-targets are constitutively expressed in platelets and are likely the same as the novel "universal-pathologic-biomarker" detected in different cancer cells grown in vitro as reported by Lo and Luther [10]. Thus, these platelets with GP1R-binding-targets in cancer patients may be targeted as "abnormal or foreign" by CTL. The accumulation of these "abnormal" platelets in circulation may signal problematic CTL, both qualitatively and quantitatively. It is not clear if the cause of such abnormality in circulating platelets might have been originated in the precursor megakaryocytes, as many "abnormal" platelets were detected in circulating whole blood.

Finding the presence of GP1R-binding in platelet-like structures within the FFPE mouse xenogeneic tumor tissue but not the FFPE human normal lung tissue is significant. The lack of discernible GP1R-binding in circulating whole blood components within the blood mass (including platelets) as shown in the FFPE human normal lung tissue appears to provide supporting evidence that GP1R-binding-target or "universal-pathologic-biomarker" may not be present normally in peripheral blood unrelated to the disease of cancer. It is

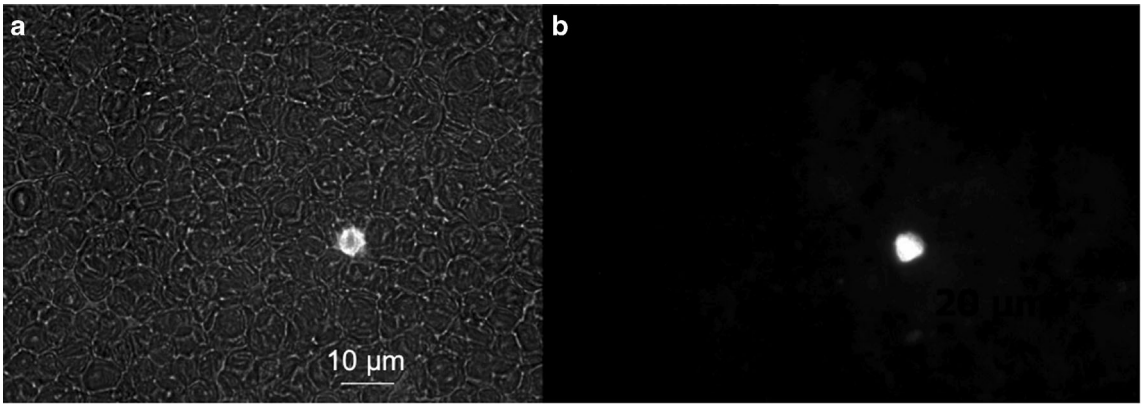

Fig. 2 Fluorescence microscopy of whole blood components in peripheral blood smear of patient with no known cancer. The absence of fluoroscopic images of platelets in the control peripheral whole blood smear (with methanol pre-fixation) of a patient with a medical condition unrelated to cancer is shown. The absence of fluorescent deposits in platelet-like structures and RBC indicates a lack of binding of GP1R to corresponding cellular binding targets. In Fig. 2a the microscopic image was recorded using light microscopy and fluorescence microscopy combined; in Fig. 2b the microscopic image of the same cells as shown in Fig. 2a was recorded using fluorescence microscopy. Prior to image recording, the methanol-fixed blood smear had a 5-min incubation at room temperature with GP1R. Unlike the images shown in Fig. 1, there is no similar GP1R-binding pattern shown in the methanol-fixed peripheral whole blood smear of the non-cancer patient (**b**) to indicate the presence of platelets with GP1R-binding-targets. Using light microscopy, only microscopic images of many unstained RBC are discernible (**a**). The scale bar for each panel is the same as shown in **a**. A representative of about 100 RBC in each field examined ($n=5$) in duplicates is shown

further supported by the demonstration of ample GP1R-binding within platelets in the peripheral whole blood specimens of a lung cancer patient. Thus, detection of the presence of the "universal-pathologic-biomarker" by GP1R-binding in platelets of peripheral blood may signal an abnormal condition linked to cancer. Further validation in FFPE human tumor tissues of the presence of GP1R-positive platelets would help determine what roles these "abnormal" platelets might play in different stages of cancer development.

The absence of discernible GP1R-binding-targets within RBC in all of the binding experiments regardless of the different sources of the tested samples indicates that RBC may serve as an intrinsic negative control for GP1R fluorescent stain. The negative GP1R-binding results in RBC of cancer patient whole blood specimens indicate that RBC in circulation despite their closeness to "abnormal" platelets do not acquire the "universal-pathologic-biomarker" and may not be targeted by granzyme B or granzyme B-like peptides. Lo and Luther reported that the GP1R-binding-target may be associated with a glycosylation product in target cells of CTL and no discernible GP1R-binding was detected in bovine RBC even after a 24 h-incubation with GP1R [10]. Our results showing a lack of GP1R non-specific binding in RBC appeared to agree with theirs and depicted a high target selectivity of GP1R in whole blood.

Finding the co-existence of mouse platelets and human breast cancer cells that share the same "universal-pathologic-biomarker" in the mouse xenogeneic tumor tissue is significant given the presence of a dysfunctional immune system with limited CTL (qualitatively and quantitatively) in the nude mouse. It appears to provide a visible link to show a direct connection between platelets and tumor growth. The presence of platelet-like structures detected in the mouse capillary blood vessel, within the FFPE tumor tissue, with the same pathologic biomarker as shown in the human breast cancer cells, further demonstrates a direct connection. This may help shed some light on thrombocytosis (i.e. platelet counts above the normal threshold) as an adverse prognostic factor in cancer patients. Platelet derived tumor growth factor beta (TGFβ) has been the focus of thrombocytosis as the contributing factor for poor prognosis in cancer patients by promoting metastasis [11] and tumor cell malignancy [12, 13]. Our data may suggest that thrombocytosis could be the result of increasing demand of "abnormal" platelets with the pathologic biomarker to support the unrestricted tumor growth. This could be a strong indication of problematic CTL in the host causing poor prospect of controlling cancer growth and spread. It is not known if the life expectancy of these "abnormal" platelets in circulation might exceed the 10-day time anticipated for normal platelets. This could provide a mechanism for the uninterrupted supply of "abnormal" platelets to help sustain the tumor growth and further contribute to thrombocytosis indicating an adverse prognosis.

The positive GP1R-binding data on the tumor tissue provide the first visual support that platelets may play a significant role in cancer development, cancer progression, and metastasis founded on the presence of a "universal-pathologic-biomarker" they have in common with cancer cells grown inside the host with a weak immune system supported by limited CTL. This common biomarker has binding affinity to a synthetic granzyme B-like peptide, which like the native granzyme B enzyme used by CTL has been shown to induce cancer cell death [10] upon binding. Thereby, it may lead to

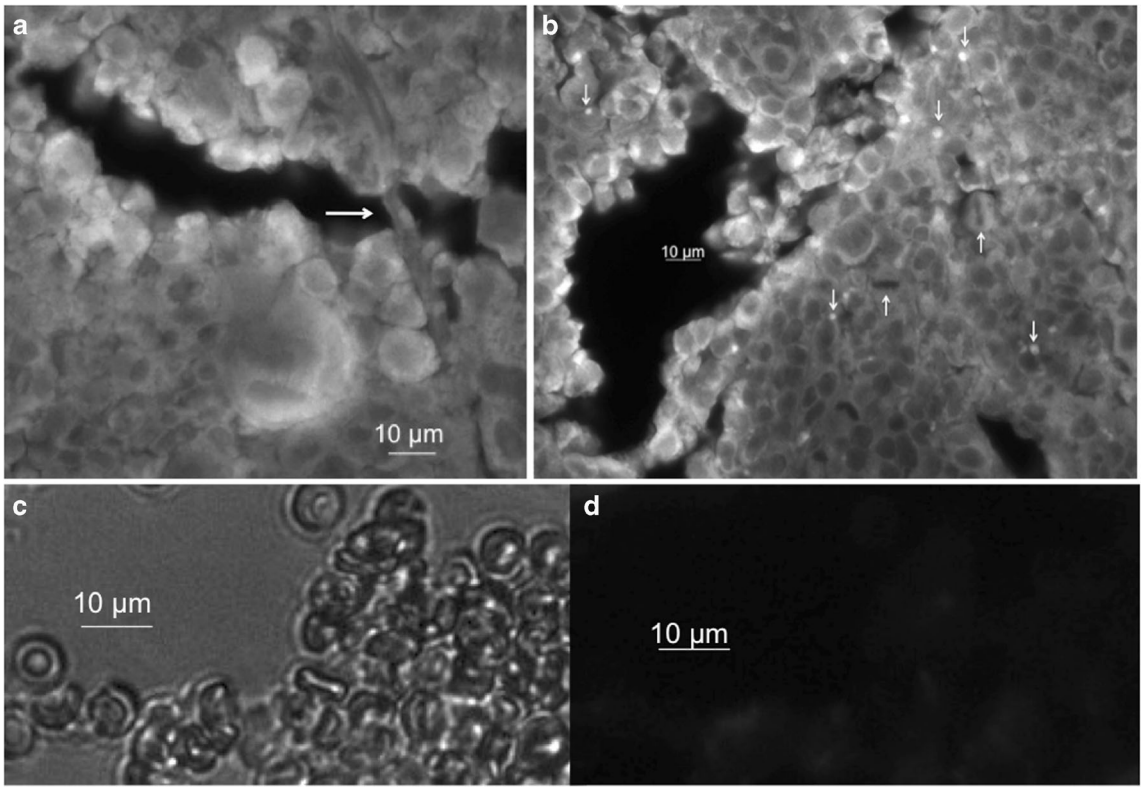

Fig. 3 Fluorescence microscopy of platelet-like structures with GP1R-binding in FFPE mouse xenogeneic tumor tissue (tumor growth from SKBR3 human breast cancer cells implanted in the nude mouse). Microscopic images of platelet-like structures and breast cancer cells in FFPE tumor tissue (**a** and **b**) are shown in comparison to microscopic images of a blood mass in FFPE human normal lung tissue (**c** and **d**). The presence of fluorescent deposits within platelet-like structures and cancer cells as shown indicates binding of GP1R to corresponding cellular binding targets. The lack of discernible fluorescent deposits within a blood mass presented with numerous unstained RBC (**c** and **d**) indicates an absence of GP1R-binding targets within any whole blood components including platelets found in this FFPE human normal lung tissue. In **a**, **b**, and **d** the microscopic images were recorded using fluorescence microscopy; in **c** the microscopic image was recorded using light microscopy and fluorescence microscopy combined (same field as **d**). Prior to image recording, the FFPE tissues (rehydrated and permeated) had an incubation of 15–30 min at room temperature with GP1R. Positive GP1R-binding in platelet-like structures (activated platelets change shape from a general disk-shape to amorphous form with pseudopods) within a capillary blood vessel (*right arrow*) of FFPE tumor tissue is shown in **a**. The FFPE tumor tissue that shows a similar GP1R-binding pattern within platelet-like structures (*down arrows*) as seen within platelets in the peripheral whole blood smears of cancer patients is shown in **b**. Mitotic figures (*up arrows*), indicative of active cell growth, nearby the platelet-like structures with positive GP1R-binding are shown in **b**. Cancer cells at different growth phases with various GP1R-binding intensity are discernible (**a** and **b**). In the FFPE human normal lung tissue sample, the absence of GP1R-binding within a blood mass, applicable to all whole blood components including platelets, is demonstrated when its fluoroscopic image in **d** is compared to the light microscopic image of the same in **c**. Unstained RBC within the same blood mass are depicted in the image recorded with light microscopy and fluorescence microscopy combined (**c**). The scale bar for each panel is as shown. A representative of about 50 stained and unstained cells in each field examined (n=5) in duplicates is shown

new approaches of platelet-based cancer detection and treatments as discussed in the following.

Cancer patients detected to have positive GP1R-binding in platelets and cancer cells are likely suitable candidates to receive cancer immunotherapy treatments that aim to strengthen or restore functional CTL for effective cancer cell killing. The same cancer immunotherapy may also be effective in reducing the supply of "abnormal" platelets by killing the source of such abnormality, which may have originated from the precursor megakaryocytes. Individuals detected to have GP1R-binding-positive platelets are candidates for further investigation of a disease condition related to cancer. This would be particularly beneficial to patients whose treatment plan often is mostly based on positive radiographic images of mammography screening or CT/MRI scan alone. The negative GP1R-binding platelet test may help these patients to avoid unnecessary treatments due to false-positive radiographic images.

Treatments that aim to impair the blood supply or prevent new blood vessel growth in tumors may have effectiveness in limiting the supply of circulating "abnormal" platelets with the "universal-pathologic-biomarker" to fuel the tumor growth, but these treatments often may result in serious adverse side effects in vivo. Unconventional treatments such as repeated platelet transfusions that aim to replenish the patients with normal platelets (i.e. without the GP1R-binding-targets) from a non-cancer donor may be a potentially helpful anti-cancer regimen when other cancer treatments have failed. A

novel cancer treatment approach comprising the synthetic granzyme B-like peptides that aims to inhibit the "universal-pathologic-biomarker" in platelets and cancer cells without the adverse side effects on RBC may produce a desired outcome for cancer patients. Since the same granzyme B-like peptide could also target the platelet precursor megakaryocytes in the bone marrow if these giant cells indeed show the presence of "universal-pathologic-biomarker" in cancer patients as suspected, conceivably eliminating both the underlying cause of abnormality in circulating platelets and supply of "abnormal" platelets to the sites of tumor growth. A treatment approach as such could also help minimize the spread of cancer cells to the bone, the most common site of metastasis, where megakaryocytes may also be present. Could this lead to finding of the cancer gene in megakaryocytes and a cancer cure? Could "abnormal" platelets be used as a vehicle to help deliver cancer drugs to the sites of tumor growth? Answers to these questions may lead to more cancer breakthrough.

The overall new information in this report based on GP1R-binding offers more insight into the complicated task undertaken by the CTL of our innate cellular immune system and the possible multifunction of granzyme B in the fight against cancer. To guard the host against cancer development, both CTL and granzyme B are needed and likely have multiple targets including but not necessarily limited to tumor cells and their supporters (such as platelets as shown in this report). When CTL and granzyme B are problematic or limited in the host (like the nude mouse), both tumor cells and "abnormal" platelets can grow in large quantities and their "universal-pathologic-biomarker" remains targetable by granzyme B related peptides. The undesirable binding interaction of PD-1 (Programmed death-1) on the activated CTL with PDL-1 (Programmed death-1 ligand-1) on the tumor cells has been shown to be a solvable problem of CTL and can be blocked to restore their effective killing activity, thereby becoming the focus of recent new cancer immunotherapy [14–16]. Such interaction may not only allow tumor cells to grow, but it may also allow accumulation of circulating "abnormal" platelets. Consequently, detection of abundant circulating "abnormal" platelets with GP1R-binding-targets not only may signal a disease condition related to cancer but may also signal problematic CTL resulted from PD-1 and PDL-1 interaction. It led us to question if granzyme B might have a hidden task of protecting CTL from tumor cell's PDL1 interference, such as binding to PD1 of CTL in vivo. Would it be an unfulfilled task in the presence of limited functional CTL especially during advanced cancer progression? Thus, it is interesting that GP1R-binding was detected in small lymphocyte-like cells of a patient with advanced pancreatic cancer in a separate study. If so, this suggests that CTL, to sustain an effective anti-cancer immune system, may use native granzyme B (in whole or in part) to play multiple roles including a potential role of PD1 binding to protect the CTL. Hence, synthetic granzyme B-like peptides (like the one used in GP1R) may also have multifunction in bringing the tumor growth under control even in the presence of problematic CTL in vivo. What would the "universal-pathologic-biomarker" and PD1 have in common to attract the same granzyme B-like peptide? Finding the answer to this may help reveal the identity of the "universal-pathologic-biomarker" detected in "abnormal" platelets and cancer cells grown in vivo as shown in this report, and bacteria as reported by Lo and Luther [10].

In conclusion, we show binding of a synthetic granzyme B-like peptide fluorescent conjugate within platelets in methanol-fixed peripheral whole blood specimens of cancer patients, indicating the presence of a constitutively expressed "universal-pathologic-biomarker" that was previously reported in methanol-fixed cancer cells grown in vitro [10]. The same peptide fluorescent conjugate binding shown within platelet-like structures and cancer cells in FFPE mouse xenogeneic tumor tissue provides a visual direct connection between platelets and cancer due to the presence of a common pathologic biomarker, which may become a potentially useful pharmaceutical target for cancer therapy.

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