Cholangiocarcinoma Cell Induced Platelet Aggregation Via Activation of Thrombin and Cyclooxygenase

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ABSTRACT

Objective: To investigate whether cholangiocarcinoma (CCA) cells affected platelet aggregation via activation of thrombin and cyclooxygenase.

Methods: Human cholangiocarcinoma (HuCCA) cells were cultured in T-75 Flasks with a standard technique. Cells were grown to confluence until used, after which, cells were detached and then resuspended in DMEM. Resuspended HuCCA cells were assessed for their effects on inducing platelet aggregation of normal volunteers using Born's technique. Hirudin, apyrase, EDTA and indomethacin were used as pharmacological tools to investigate signaling mechanisms.

Results: HuCCA cells can initiate aggregation of platelets in heparinized platelet rich plasma (hPRP). The potency of HuCCA cell induced platelet aggregation depends on the concentration of cell suspension. Interestingly, HuCCA cell induced platelet aggregation was inhibited by hirudin (10, 20 and 40 unit), EDTA (2, 3 and 4 mM) and indomethacin (0.1, 1 and 2 mM) in a dose dependent manner but not by apyrase (5, 10 and 20 units).

Conclusion: HuCCA cells can induce platelet aggregation possibly via activation of thrombin and the cyclooxygenase signaling pathway but not the ADP pathway. Calcium may be an important factor required for this reaction.

Keywords: ADP, calcium, cancer, cholangiocarcinoma, cyclooxygenase, signaling, thrombin, thrombosis

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Most cancer patients normally experience several complications during the course of their illness. Alteration in the hemostatic balance is one of the most common complications in malignant patients. As first reported by Trousseau in 1865, thromboembolic phenomena are frequent and important events in cancer patients.[1] It has been estimated that 15% of patients with cancer will suffer from thromboembolic phenomena during a period of their life.[2] Moreover, thrombotic disorders may be the second most common cause of mortality in cancer patients, as evidenced by autopsy studies.[3] Among these, the most frequent thrombotic complications in patients with cancer are disseminated intravascular coagulation, thrombotic thrombocytopenic purpular and thrombocytosis.[4,5] These complications arise as tumor cells interact with components of the hemostatic system. Platelet activation is one pathway that has been consistently implicated in this pathology.[6,7] There were several types of tumor cell induced platelet aggregation (TCIPA) such as mucin-secreting adenocarcinomas, lung cancer, acute promyelocytic leukemia, and brain tumors.[8-12]

Cholangiocarcinoma (CCA) is a primary intrahepatic cancer of the biliary epithelium. It remains a relatively rare disease, accounting for <2% of all human malignancies,[13] but is commonly found in the northeastern part of Thailand where the liver fluke infection is the highest prevalence.[14] The association between CCA and thrombosis has been reported in many studies.[15-17] However, the mechanism by which CCA mediated platelet function is not known. The study of interaction between CCA and platelets will support a better understanding of the pathophysiology of TCIPA leading to the rationale use of antiplatelet drugs in cancer patients. In this study, the experiments were

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designed to explore the effects of human cholangiocarcinoma (HuCCA) cells on platelet functions of normal volunteers using platelet aggregation determination. The signaling mechanisms on TCIPA will also be elucidated using hirudin (thrombin inhibitor), apyrase (ADP inhibitor), EDTA (calcium chelator) and indomethacin (cyclooxygenase inhibitor) as pharmacological tools.

MATERIALS AND METHODS

Materials

Dulbeco Modified Eargle's Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Hyclone® (Utah, USA). Hirudin was obtained from Fein chemie GmbH (Germany). Apyrase, indomethacin, EDTA and enzyme-free cell dissociation solution (c5914) were purchased from Sigma® (USA).

Subjects

Thirty healthy volunteers age 25-55 years were enrolled into the study. All of them did not ingest aspirin (ASA), any other NSAIDs, or antiplatelet drugs for at least for 2 weeks and they fasted overnight before the day of the study.

Written informed consent was obtained from all subjects. The study protocol was approved by The Ethics Committee for Human Research, Faculty of Medicine Siriraj Hospital.

HuCCA cell culture

Primary HuCCA cells were established in our laboratory. Cells were cultured in T-75 flasks with DMEM containing 15% FBS, 100 unit/ml penicillin G, 100 µg/ml streptomycin and grown to confluence until used at 37°C in a humidified atmosphere of 5% CO_2 in air[18]. Cells were harvested with enzyme-free cell dissociation solution (c5914) and resuspended in DMEM to yield a concentration of 107 cells/ml for platelet aggregation experiments. Viability, as determined by tryptan blue exclusion, was >80%.

Platelet aggregation study

Blood samples were obtained by vein puncture and gently mixed with 8 U/ml heparin as an anticoagulant. Platelet rich plasma (PRP) containing 4.5±1.0 x 10^8 platelets/ml. was prepared to test with each of DMEM (150 µl; control) or HuCCA cell suspensions alone ($1.0x10^7$ cells/150 µl, final concentration = $1.5x10^6$ cells/ml) or HuCCA cell suspensions plus hirudin (10, 20 and 40 unit) or plus apyrase (5, 10 and 20 unit) or plus EDTA (2, 3 and 4 mM) or plus indomethacin (0.1, 1 and 2 mM). Platelet free plasma (PFP) was also prepared for baseline calibration. Platelet aggregatability was determined turbidly according to the method of Born[19] within 2 - 3 hours of blood collection.

Using aggregation & a shape change monitor (BORN-MICHAL, Pharmacological Research, England) the percent maximum aggregation within 7 minutes was evaluated by comparing the amplitudes of the aggregation curves of PRP after being co-incubated with each tested sample to the amplitudes of the aggregation curves of PFP multiplied by 100.

Statistical analysis

Results are shown as mean ± SE from duplicate determinations (samples) of three subjects on separate experimental days (n=3). Student's unpaired t-tests were used to determine the significance of differences between means and p-values of less than 0.05 were accepted as statistically significant.

RESULTS

1. Effects of HuCCA cells on platelet aggregation

HuCCA cells can induce platelet aggregation while conditioned medium 24, 48 and 72 hours (serum free medium from HuCCA cells) cannot (data not shown). The potency of induction activity related to the number of HuCCA cells. Figure 1A demonstrates that HuCCA cells induced platelets to aggregate in a concentration-dependent manner within the range of HuCCA cell 3.25 x 10^5 to 1.5 x 10^6 cells/ml. Figure 1B demonstrates that all three batches of HuCCA cells used in this study reveal the similar effects. Three batches of HuCCA cells were CL-2, CL-6 and CL-19 HuCCA cell lines, derived from HuCCA cells of cholangiocarcinoma patients (CL) number 2, number 6 and number 19, respectively.

2. Effects of hirudin, apyrase, EDTA and indomethacin on HuCCA cells induced platelet aggregation

Platelet aggregation induced by HuCCA cells was inhibited by hirudin, EDTA and indomethacin but not by apyrase.

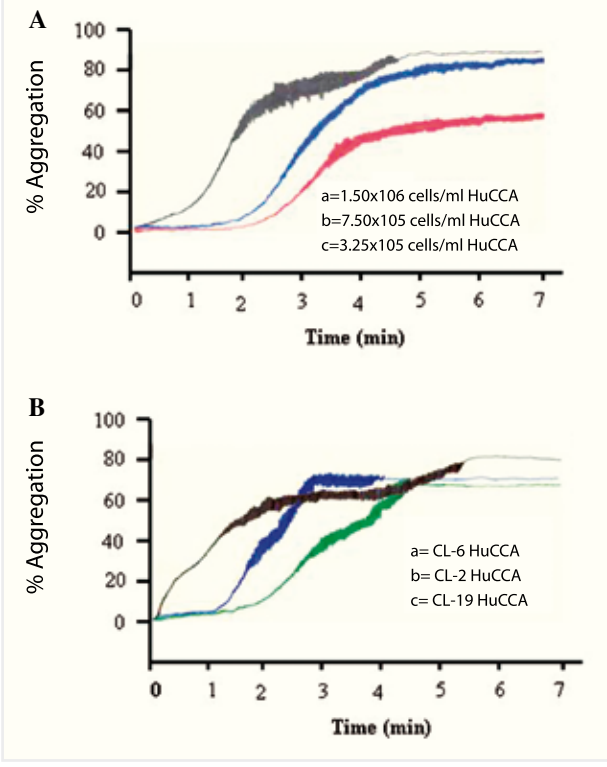

Fig 1. Effects of HuCCA cell on platelet aggregation. Panel A showed the example tracings of platelet aggregation induced by HuCCA cell in the final concentration of $3.25x10^5$ cells/ml – $1.5x10^6$ cells/ml. Panel B showed the example tracing of platelet aggregation induced by 3 batches of HuCCA cells, CL-2, CL-6 and CL-19 HuCCA cell in the final concentration of $1.5x10^6$ cells/ml. (CL-2, CL-6 and CL-19 HuCCA cell = HuCCA cell derived from cholangiocarcinoma patient number 2, 6 and 19 respectively)

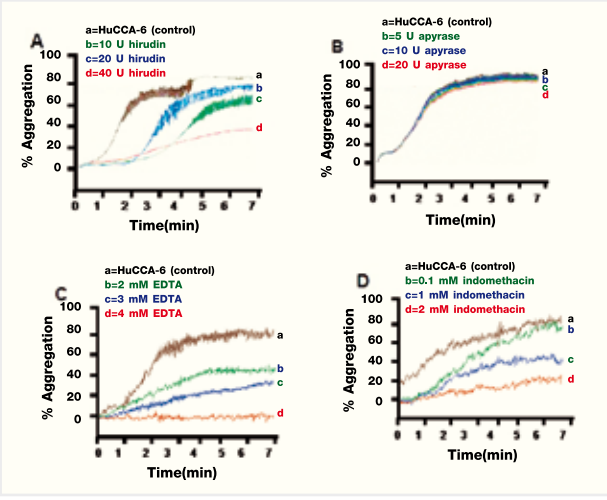

Fig 2. The example tracing of platelet aggregation induced by HuCCA cells ($1.5x10^6$ cells/ml) co-incubated with hirudin, apyrase, EDTA and indomethacin. Panel A showed hirudin effects. Panel B showed apyrase effects. Panel C showed EDTA effects. Panel D showed indomethacin effects.

2.1 Effects of hirudin on HuCCA cell induced platelet aggregation.

Hirudin inhibited HuCCA cell induced platelet aggregation in a dose dependent manner (Figure 2A). The maximum aggregation of HuCCA cell treated platelets plus hirudin (10, 20 and 40 units) were 77.27 ± 13.68%, 60.53 ± 11.14% and 34.42 ± 8.13%, respectively (n=3). The significant inhibition was found at all doses of hirudin ($p < 0.05$; Figure 3A).

2.2 Effects of apyrase on HuCCA cell induced platelet aggregation.

Apyrase did not inhibit HuCCA cell induced platelet aggregation (Figure 2B). There was no difference between the maximum aggregation of HuCCA cell treated platelets plus apyrase and of the control (HuCCA cells alone). The maximum aggregation of HuCCA cell treated platelets plus apyrase (5, 10 and 20 units) were 97.66 ± 0.38%, 97.66 ± 0.50% and 97.66 ± 0.83%, respectively (n=3, Figure 3B).

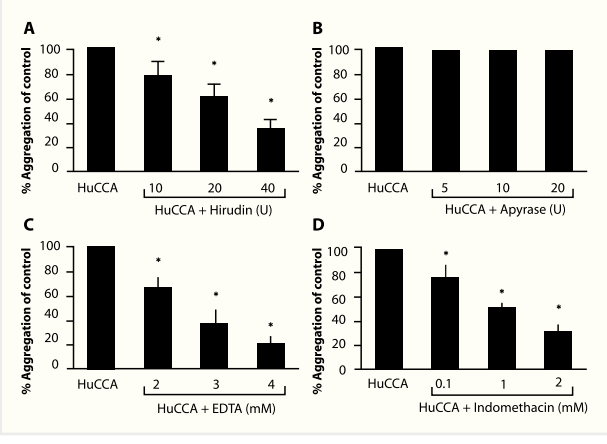

Fig 3. Effects of hirudin, apyrase, EDTA or indomethacin on HuCCA cell (1.5 x 106 cells/ml) induced platelet aggregation. Panel A showed hirudin effects. Panel B showed apyrase effects. Panel C showed EDTA effects. Panel D showed indomethacin effects. (* $p < 0.05$ when compared to control.)

2.3 Effects of EDTA on HuCCA cell induced platelet aggregation.

EDTA inhibited HuCCA cell induced platelet aggregation in a dose dependent manner (Figure 2C). The maximum of HuCCA cell treated platelets plus EDTA (2, 3 and 4 mM) were 65.70 ± 8.47%, 36.12 ± 10.65% and 4.48 ± 2.20%, respectively (n=3). The sig nificant inhibition was found at all doses of EDTA. ($p < 0.05$; Figure 3C).

2.4 Effects of indomethacin on HuCCA induced platelet aggregation.

Indomethacin inhibited HuCCA cell induced platelet aggregation in a dose dependent manner (Figure 2D). The maximum aggregation of HuCCA cell treated platelets plus indomethacin (0.1, 1 and 2 mM) were 77.15 ± 10.58%, 50.92 ± 2.80% and 31.35 ± 4.87%, respectively (n=3). The significant inhibition was found at all doses of indomethacin ($p < 0.05$; Figure 3D).

DISCUSSION

The study showed that HuCCA cells can induce platelet aggregation while their condition medium cannot (Figure 1A). The induction potency was related to the number of HuCCA cells (Figure 1A). This finding is similar to many other investigations that various tumor cells such as pancreatic cancer cell, fibrosarcoma, adenocarcinoma, lung cancer cell and brain tumors[8,10,20] can induce platelet aggregation *in vitro*. Platelets may be activated by mediators released from tumor cells, or by the direct contact with the molecules on the surface membrane of tumor cells.[21-24] Our results also revealed that the condition medium (serum free medium from HuCCA cells) did not induce platelet aggregation (data not shown). It might imply that the fresh mediators released from tumor cells are needed in the initiation of TCIPA. However, the contact of tumor cells with platelets may be another important factor because this contact may even be necessary to TCIPA in some cell systems. It may mediate via adhesion receptors on tumor cells (integrin $\alpha_2\beta_1$, integrin $\alpha IIb/\beta_3$-like recept ors, Lewis antigens, thrombospondin receptor) and on platelets (integrin $\alpha_2\beta_1$, integrin $\alpha IIb/\beta_3$, thrombospondin receptor, P-selectin).[25-29] However, some studies reported that direct contact of tumor cells with platelets may not be necessary in TCIPA. Heinmoller et al (1995) also reported that monoclonal antibodies against adhesion receptors have no effects on pancreatic cancer cell induced platelet aggregation.[8]

The mechanisms of TCIPA have been severally described. These depend on the different origins and organs of tumor cells. During TCIPA by adenocarcinoma, melanoma and neuroblastoma cell lines, ADP was released, platelet receptors were, then, stimulated and platelets were, finally, induced to aggregate.[30] Some tumors can induce platelet aggregation and subsequently activate the coagulation cascade through the generation of thrombin.[9,30] Furthermore, TCIPA is shown to associate with the production of eicosanoids that amplifies platelet aggregation such as thromboxane A_2.[31,32] Along with the production of eicosanoids, Steinert et al (1993) have shown that platelet surface glycoprotein $\alpha IIb/\beta_3$, the receptor for fibrinogen, plays an important role in platelet aggregation by tumor cells.[33] Moreover, elevation in plasma vWF levels, the common part of

an acute-phase reaction, or during endothelial cell perturbation in cancer patients, may contribute to platelet hyperreactivity.[34-35]

Hirudin, apyrase, EDTA and indomethacin are some signaling molecules that are used as pharmacological tools to understand the mechanisms underlying TCIPA. In this study, HuCCA cell induced platelet ag-gregation was inhibited by hirudin, EDTA and indo-methacin but not by apyrase. Hirudin is a specific inhibitor of thrombin and hence, the inhibition of platelet aggregation by hirudin may indicate the important pathway of thrombin in the mechanisms of HuCCA cell induced platelet aggregation. Here, we showed that hirudin inhibited HuCCA cell induced platelet aggregation in a dose dependent manner (Figure 3A). This result indicated that the signalling mechanism of HuCCA cell induced platelet aggregation is thrombin-dependent reactions.

Apyrase, is an ADP scavenger molecule. HuCCA cell induced platelet aggregation was not inhibited by apyrase which possibly indicated that the ADP pathway was not involved in this TICPA (Figure 3B). This result is in contrast to the previous works of Oleksowicz et al (1997) and Mohanty et al (1984) who reported that breast cancer and fibrosarcoma induced platelet aggregation possibly via the ADP pathway.[35,36] However, our result is similar to the study of Heinmoller et al (1995) and Katagiri et al (1991) who demonstrated that apyrase did not effect platelet aggregation induced by pancreatic cancer cells and human melanoma cells.[8,37] The different results found in these studies may be due to the various types of tumor cells used.

EDTA is the chelation of extracellular calcium. EDTA can inhibit HuCCA cell induced platelet aggregation (Figure 3C) which indicated that extracellular calcium ions are an important factor required for this TCIPA. Many studies have accordingly shown that tumor cell lines are able to induced heparinized platelets to aggregate.[3,8,10,20,38]

Indomethacin is non specific COX inhibitor. Indomethacin was used to determine whether HuCCA cell induced platelet aggregation through the cyclooxygenase pathway. The results showed that indomethacin can inhibit HuCCA cell induced platelet aggregation (Figure 3D) which indicated that this TCIPA was possibly mediated by TXA2 formation through the cyclooxygenase pathway. The result is similar to the works of Honn et al and Bastida et al (1987) who reported that platelet aggregation in response to tumor cells is dependent upon both cyclooxygenase and lipoxygenase pathways.[31,39] However, some investigators have reported that platelet aggregation is independent of the cyclooxygenase pathway.[40]

The results obtained from this study may provide an explanation for the high incidence of thrombosis in cholangiocarcinoma patients. However, thrombin and TXA2 have been accused of exerting various different effects during tumor invasion and metastasis.[41-42] It remains to be determined whether the mediators released during platelet aggregation by tumor cells affect tumor invasion and metastasis.

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REFERENCES

1. Trousseau A. Phlegmasia alba dolens. In: Clinique medicale de l'Hotel-Dieu de Paris: JB Balliere et Fils, Paris, France, 1865:654-712.
2. Goad KE, Gralnick HR. Coagulation disorders in cancer. Hematol Oncol Clin North Am 1996;10:457-85.
3. Steven M, Scates MD. Diagnosis and treatment of cancer – related thrombosis. Hematol Oncol Clin North Am 1992;6:1329-39.
4. Trikha M, Nakada MT. Platelets and cancer: implication for antiangiogenic therapy. Semin Thromb Hemost 2002;28:39-44.
5. Jurasz P, Alonso-Escolano D, Radomski MW. Platelet–cancer interactions: mechanisms and pharmacology of tumour cell-induced platelet aggregation. Br J Pharmacol 2004;143:819-26.
6. Francis JL, Biggerstaff J, Amirkhosravi A. Hemostasis and malignancy. Semin Thromb Hemost 1998;24:93-109.
7. Wojtukiewicz M, Sierko E, Kisiel W. The role of hemostatic system inhibitors in malignancy. Semin Thromb Hemost 2007;33:621-42.
8. Heinmoller E, Schropp T, Kisker O, Simon B, Seitz R, Weinel RJ. Tumor cell induced platelet aggregation in vitro by human pancreatic cancer cell lines. Scand J Gastroenterol 1995;30:1008-16.
9. Pearlstein E, Ambrogio C, Gasic G, Karpatkin S. Inhibition of the platelet aggregating activity of two human adenocarcinomas of the colon and an anaplastic murine tumor with a specific thrombin inhibitor: dansylarginine N-(3-ethyl-1,5-pentanediyl) amide. Prog Clin Biol Res 1982; 89:479-502.
10. Heinmoller E, Weinel RJ, Heidtmann HH, Salge U, Seitz R, Schmitz I, Muller KM, et al. Studies on tumor-cell-induced platelet aggregation in human lung cancer cell lines. J Cancer Res Clin Oncol 1996;122:735-44.
11. Esumi N, Todo S, Imashuku S. Human leukemia cells and mature lymphocytes induce platelet aggregation after removal of cell surface sialic acid. Nippon Ketsueki Gakkai Zasshi 1989;52:767-73.
12. Wahrenbrock M, Borsig L, Le D, Varki N, Varki A. Selectin-mucin interactions as a probable molecular explanation for the association of Trousseau syndrome with mucinous adenocarcinomas. J Clin Invest 2003;112:853-62.
13. Slattery JM, Sahani DV. What is the current state-of-the-art imaging for detection and staging of cholangiocarcinoma? Oncologist 2006;11: 913-22.
14. Schwartz DA. Helminthes in the induction of cancer: Opisthorchis viverrini, clonorchis sinensis and cholangiocarcinoma. Trop Geogr Med 1983; 32:95-100.
15. Ching CK. Trousseau's syndrome in a patient with cholangiocarcinoma. Am J Gastroenterol 1991;86:928-9.
16. Martins EB, Fleming KA, Garrido MC, Hine KR, Chapman RW. Superficial thrombophlebitis, dysplasia and cholangiocarcinoma in primary sclerosing cholangitis. Gastroenterology 1994;107:537-42.
17. Bassiri AG, Haghighi B, Doyle RL, Berry GJ, Rizk NW. Pulmonary tumor embolism. Am J Respir Crit Care Med 1997;155:2089-95.
18. Wongkajornsilp A, Sattawatthamrong Y, Huabprasert S. Primary cultures of cholangiocarcinoma established from bile fluid express insulin-like growth factor I and Fas. Thai J Pharm 2000;22:S85.
19. Born GVR. Aggregation of blood platelets. Nature 1962;194:927-9.
20. Mogi Y, Kogawa K, Takayama T, Yoshizaki N, Bannai K, Muramatsu H, et al. Platelet aggregation induced by adenosine diphosphate released from cloned murine fibrosarcoma cells is positively correlated with the experimental metastatic potential of the cells. Jpn J Cancer Res 1991; 82:192-8.
21. Nash GF, Turner LF, Scully MF, Kakkar AK. Platelets and cancer. Lancet Oncol 2002;3:425-30.
22. Nierodzik ML, Klepfish A, Karpatkin S. Role of platelets, thrombin, integrin IIb/IIIa, fibronectin and von Willebrand factor on tumor adhesion in vitro and metastasis in vivo. Thromb Haemost 1995;74:282-90.
23. Falanga A, Rickles FR. Pathophysiology of the thrombophilic state in the cancer patient. Semin Thromb Hemost 1999;25:173-82.
24. Sierko E, Wojtukiewicz MZ. Inhibition of platelet function: does it offer a chance of better cancer progression control? Semin Thromb Hemost 2007;33:712-21.
25. Chopra H, Timar J, Rong X, Grossi IM, Hatfield JS, Fligiel SE, Finch CA, et al. Is there a role for the tumor cell integrin $\alpha IIb\beta_3$ and cytoskeleton in tumor cell-platelet interaction? Clin Exp Metastasis 1992;10: 125-37.
26. Grossi IM, Hatfield JS, Fitzgerald LA, Newcombe M, Taylor JD, Honn KV. Role of tumor cell glycoproteins immunologically related to glycoproteins Ib and IIb/IIIa in tumor cell-platelet and tumor cell matrix interactions. FASEB J 1988;2:2385-95.
27. Boukerche H, Berthier-Vergnes O, Tabone E, Dore JF, Leung LK, McGregor JL. Platelet melanoma cell interaction is mediated by the glycoprotein IIb-IIIa complex. Blood 1989;74:658-63.
28. Chopra H, Hatfield JS, Chang YS, Grossi IM, Fitzgerald LA, O'Gara CY, et al. Role of tumor cytoskeleton and membrane glycoprotein IRGpIIb/IIIa in platelet adhesion to tumor cell membrane and tumor cell induced platelet aggregation. Cancer Res 1988;48:3787-800.
29. Kunita A, Kashima TG, Morishita Y, Fukayama M, Kato Y, Tsuruo T, et al. The platelet aggregation-inducing factor aggrus/podoplanin promotes pulmonary metastasis. Am J Pathol 2007;170:1337-47.
30. Bastida E, Ordinas A, Giardina SL, Jamieson GA. Differentiation of platelet-aggregating effects of human tumor cell lines based on inhibition studies with apyrase, hirudin and phospholipase. Cancer Res 1982; 42:4348-52.

31. Honn KV, Steinert BW, Moin K, Onoda JM, Taylor JD, Sloane BF. The role of platelet cyclooxygenase pathways in tumor cell induced platelet aggregation. Biochem Biophys Res Commun 1987;145:384-9.
32. Patel M, Kurek C, Dong Q. The arachidonic acid pathway and its role in prostate cancer development and progression. J Urol 2008;179:1668-75.
33. Steinert BW, Tang DG, Grossi IM, Umbarger LA, Honn KV. Studies on the role of platelet eicosanoid metabolism and integrin $\alpha IIb\beta_3$ in tumor cell induced platelet aggregation. Int J Cancer 1993;54:92-101.
34. Oleksowicz L, Mrowiec Z, Schwartz E, Khorshidi M, Dutcher JP, Puszkin E. Characterization of tumor induced platelet aggregation: the role of immunorelated GPIb and GPIIb/IIIa expression by MCF-7 breast cancer cells. Thromb Res 1995;79:261-74.
35. Oleksowicz L, Dutcher JP, Deleon-Fernandez M, Paietta E, Etkind P. Human breast carcinoma cells synthesize a protein immunorelated to platelet glycoprotein-Ib? with different functional properties. J Lab Clin Med 1997;129:337-46.
36. Mohanty D, Hilgard P. A new platelet aggregating material (PAM) in an experimentally induced rat fibrosarcoma. Thromb Haemost 1984;51: 192-5.
37. Katagiri K, Hayashi Y, Baba I, Suzuki H, Tanoue K, Yamazaki H. Characterization of platelet aggregation induced by the human melanoma cell line HMV-I: roles of heparin, plasma adhesive proteins, and tumor cell membrane proteins. Cancer Res 1991;51:1286-93.
38. Martini F, Guadagni F, Lenti L, D'Alessandro R, Aloe S, Roselli M, Gazzaniga PP, Ferroni P. CA 19-9 monosialoganglioside content of human colorectal tumor cells correlates with tumor cell-induced platelet aggregation. Anticancer Res 2000;20:1609-14.
39. Bastida E, Almirall L, Ordinas A. Tumor cell induced platelet aggregation is a glycoprotein dependent and lipoxygenase associated process. Int J Cancer 1987;39:760-3.
40. Maclouf J, Levy-Toledano S, Savariau E, Hardistry R, Caen JP. Arachidonic acid induced human platelet aggregation independent of cyclooxygenase and lipoxygenase. Prostaglandins 1984;28:383-98.
41. Nierodzik ML, Plotkin A, Kajumo F, Karpatkin S. Thrombin stimulates tumor-platelet adhesion in vitro and metastasis in vivo. J Clin Invest 1991;87:229-36.
42. Battistelli S, Stefanoni M, Lorenzi B, et al. Coagulation factor levels in non-metastatic colorectal cancer patients. Int J Biol Markers 2008; 23:36-41.