The Association of Extravasated Platelet Aggregation With Clinical Futures in Patients With Colorectal Cancer and Its Correlation With EMT Markers

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Abstract- The theory of platelet role in cancer progression was recently introduced. We investigated the association of extravasated platelets in colorectal cancer with clinicopathological features, and also the expression of epithelial-mesenchymal transition (EMT) markers. We retrospectively analyzed data from 33 patients with colorectal cancer who underwent surgery between 2013-2016. In formalin-fixed paraffin-embedded tissues, we evaluated the expression of a platelet-specific marker of CD42b and EMT markers using immunohistochemistry. The associations among the expression of the platelet‑specific marker in specimens, EMT, and clinicopathological futures were analyzed. The presence of platelets was observed in 15 out of 33 primary colorectal tumors (45%). According to multivariate analysis, CD42b expression was not correlated with clinical characteristics. Platelet-positive tumor cells did not show EMT marker expression. These data suggest that extravasated platelets may not have a central role in determining patient characteristics and clinical futures.

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Keywords: Clinical futures; Colorectal cancer; Epithelial-mesenchymal transition; Platelets

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer, with nearly 1,762,450 new cases and almost 606,880 deaths in the United States according to estimation provided in 2019 (1). Despite the decline of mortality in older age groups of CRC patients, it was increased slightly in individuals aged younger than 55 years in recent years (2). Treatment options include chemotherapy, surgery, radiotherapy, immunotherapy, or a combination of them, which increases the survival of some CRC patients (3). However, most of the time, there is the chance of metastasis and subsequent cancer recurrence (4). It has been proved that reciprocal interactions between tumor cells and cellular and non-cellular components of the tumor microenvironment control most aspects of tumorigenesis (5). Recently, platelets, as one of the cellular components of the tumor microenvironment, have been recognized to play a crucial role in the regulation of tumor metastasis. Theoretical and experimental evidence indicates that platelets can aggravate cancer metastasis in a number of ways: enhancing tumor cells survival in the circulatory system, encouraging tumor cell arrest in the vasculature, promoting extravasation, and stimulating tumor proliferation and angiogenesis in the secondary tumor site (6). Platelets can modulate tumor cell survival and growth via secreting several types of growth factors (7). To clarify the presence of elements in the cancer microenvironment that affect tumor progress, we focused on platelets in resected specimens from primary tumor of colorectal cancer patients. We examined the association between the presence of extravasated platelets and the clinicopathologic features in CRC patients.

Moreover, one potential mechanism which is important for cancer metastasis is the process of epithelial-mesenchymal transition (EMT) (8). During EMT, epithelial cells loss their adhesion junctions and cell polarity, simultaneously acquire migratory properties to become invasive mesenchymal cells. One of the main

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characteristics of EMT is the downregulation of E-cadherin, leading to the destabilization of adherens junctions (9). Loss of E-cadherin expression is associated with disease recurrence and shorter survival in CRC (10,11). Moreover, the association of vimentin expression with EMT and tumor malignancy has been proved in CRC. It functions as an organizer of a number of critical proteins involved in attachment, migration, and cell signaling during EMT (12). Furthermore, EMT is usually associated with the nuclear accumulation of β-catenin, which may activate the transcription of downstream target genes to promote tumor invasion and metastasis (13). It has been confirmed that the aberrant expression of MMP-9 is also associated with EMT and invasion property in a variety of cancer types (14,15).

Although the role of EMT in cancer progression has been proved, however, the presence of platelets in primary tumor site and its association with EMT markers have not been understood well yet. Accordingly, in this study, we aimed to find whether extravasated platelets could be detected in primary tumor sites and could be associated with any of clinicopathological futures and also an expression of EMT markers in patients with colorectal cancer.

Materials and Methods

Tissue specimens

Formalin-fixed paraffin-embedded tissues from patients with CRC (n=33) who underwent surgery during the period from 2013-2016 were retrieved from the archive of the Imam Hossein Hospital, Shahroud University of Medical Sciences, Iran. Patients did not receive preoperative radiotherapy or chemotherapy.

Immunohistochemistry

Paraffin-embedded tissue samples of 33 patients with CRC were obtained from the Imam Hossein Hospital, Shahroud University of Medical Sciences. The specimens were previously fixed in 10% formalin and embedded in paraffin. Several 5-mm-thick sections were cut from each paraffin block; one was stained with hematoxylin and eosin, and five were subjected to immunohistochemical staining for CD42b, E-cadherin, β-catenin, vimentin, and MMP9. For immunohistochemistry, the paraffin sections were first incubated at 60° C for 30 min, then de-paraffinized in xylene and rehydrated in decreasing concentrations of ethanol. For antigen retrieval, the slides were placed in 10 mM citrate buffer (pH=6.0) and maintained at a sub-boiling temperature for 10 min. Endogenous peroxidase activity was blocked by placing the slides in 3% hydrogen peroxide for 10 min at room temperature. This was followed by blocking each section with 1% BSA for one h at room temperature. The tissue sections were placed in a 4º C humidified chamber with primary antibody. The rabbit monoclonal E-cadherin, vimentin, β-catenin, and MMP9 antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse monoclonal MMP9, and CD42b (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:100. After washing with washing buffer, the sections were incubated with a secondary antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) diluted 1:2000 for one h and washed three times for five min with wash buffer. Finally, the color reaction was developed using 3,3′-diaminobenzidine (DAB) reagent, then sections were faintly counterstained with hematoxylin, dehydrated, and sealed. For the negative control, a washing buffer was used as the primary antibody.

Evaluation of immunostaining

Two independent researchers examined the sections. Protein expression was scored for CD42b (16), E-cadherin (17), vimentin (18), β-catenin (19), and MMP9 (20) as previously described. Cases in which >10% of cancer cells were stained were defined as positive. Measurements were made on five independent regions of each slide.

Statistical analysis

Differences were analyzed for significance using the chi-square test. Data management and statistical analysis were performed using Prism version 6 software. Data values were considered statistically significant when the P was <0.05.

Results

Patient and clinicopathological characteristics

Patient characteristics, including age, gender, tumor location and size, histologic grade, depth of invasion, number of lymph nodes involved, lymphovascular invasion, liver and lung metastasis, and tumor stage are summarized in Table 1. Patients included 19 men and 14 women with an average age of 62 years (range 32-86 years). Six patients had poorly differentiated or mucinous adenocarcinomas, whereas 27 patients had well or moderately differentiated colorectal tumors. Ten patients were diagnosed with liver metastasis. The tumor stages were as follows: stage I, n=14; stage Ⅱ, n=4; stage Ⅲ, n=4, and stage IV, n=11.

Table 1. Patient characteristics and the correlation between CD42b and each factor.

Variables		CD42 positive (≥10%)	CD42 negative (<10%)
Age (y)	<65	9	10
	>65	6	8
Gender	Female	7	7
	Male	8	11
Location	Colon	11	12
	Rectum	4	6
Histological grade	Well-differentiated	9	10
	Moderately differentiated	5	3
	Poorly differentiated	0	4
	Mucinous adenocarcinoma	1	1
Depth of invasion	mp:muscularis propria	8	6
	ss: subserosa	7	12
Tumor size	<40mm (small)	7	9
	≥40mm (large)	8	9
Lymph node metastasis	Absent	8	10
	Present	7	8
No of lymph nodes involved	0	8	10
	1-3	5	5
	≥4	2	3
Lymphovascular invasion	Absent	9	12
	Present	6	6
Liver metastasis	Absent	10	13
	Present	5	5
Lung metastasis	Absent	13	16
	Present	2	2
T classification	T1-T2	10	8
	T3-T4	5	10
N classification	N0-N1	13	15
	N2-N3	2	3
M classification	M0	9	13
	M1	6	5
Clinical stage	I-II	8	10
	III-IV	7	8

CD42b expression

All tumors were evaluated for CD42b expression, a platelet‑specific marker. CD42b was mainly expressed in the pericellular spaces of cancer cells. Positive CD42b expression was found in 15 of 33 primary colon tumors (45%) (Figure 1).

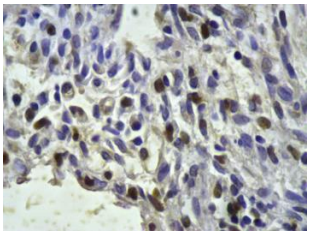

Figure 1. Representative photomicrograph of the specimen from colon cancer lesion. Immunohistological images of CD42b positive platelets (brown color), (original magnification, x100)

Relationship between extravasated platelets and clinicopathological features

The relationships between CD42b expression and clinicopathological features, including age, gender, tumor location and size, histologic grade, depth of invasion, number of lymph nodes involved, lymphovascular invasion, liver and lung metastasis, and tumor stage are summarized in Table 1. No statistically significant association was noted between CD42b expression and clinicopathological characteristics.

E-cadherin, vimentin, β-catenin, and MMP9 expressions

The expression of E-cadherin, vimentin, β-catenin, and MMP9 were also examined in studies samples (Figure 2).

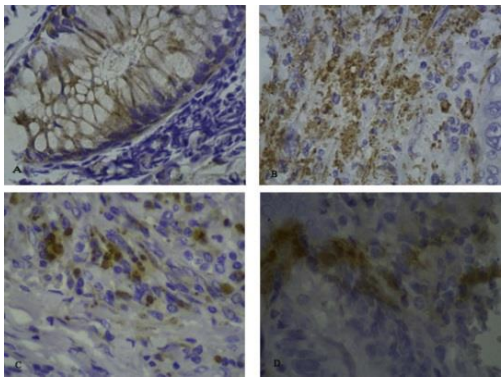

Figure 2. Representative photomicrographs from Immunohistochemical analysis of E-cadherin, vimentin, β-catenin, and MMP9 expression in specimens from patients with colorectal cancer. (A) Few cells were positive for E-cadherin. (B) Vimentin expression was detected around primary tumor cells. (C) nuclear staining of β-catenin in CD42b positive primary tumor cells, (D) MMP9 was stained in tumor cytoplasm, (original magnification, x100)

Correlation between CD42b and expression of E-cadherin, vimentin, β-catenin, and MMP9

There was no correlation between CD42b and expression of E-cadherin, vimentin, β-catenin, and MMP9.

Discussion

It has been suggested that platelets are one of the factors promoting cancer migration, infiltration, and metastasis (21). Although intravasated platelet aggregation has focused attention on cancer progression and metastasis, extravasated platelets have been less noticeable. Qi *et al*., reported that platelet aggregation within colorectal cancers is associated with tumor stage and lymph node metastasis (22). Mikami *et al*., showed that interactions between platelets and gastric cancer cells increase tumor proliferation (23). Moreover, the association between expression of CD42b as the specific marker of platelets, EMT, neo-adjuvant chemotherapy, and survival in breast cancer biopsy specimens were analyzed by immunohistochemistry. The results showed that platelet-positive tumor cells have an EMT-like morphology and express EMT markers. There was a significant association between the presence of platelets around tumor cells and the chemotherapeutic response in breast cancer (16). In pancreatic cancer specimens, extravasated platelet aggregation was associated with the first step in the formation of the EMT (21).

In this study, we demonstrated that platelets aggregated around primary tumor cells in 45% of the colorectal cancer specimens. Moreover, we examined the association between the surrounding platelets and clinical characteristics. We could not find any correlation between the presence of platelets and histologic grade, depth of invasion and tumor size, lymph node and liver metastasis, and also clinical stage. Additionally, in the present study, we were unable to demonstrate a significant relationship between CD42b and expression of EMT markers vimentin, β-catenin, MMP9, and reduction and/or loss of E-cadherin expression in tumor cells.

This discrepancy with other studies may be due to sampling size, potential selection bias, heterogeneity of tumor characters, and follow uptime.

Because our study consisted of different subtypes of colorectal tumors, there is a necessity to evaluate a greater number of samples. Moreover, we examined the specimens at the time of diagnosis, and it seems that the duration of follow-up limited the ability to evaluate the recurrence and metastasis; increased follow-up time is required.

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