Chinese-German J Clin Oncol December 2013, Vol. 12, No. 12, P581–P585
DOI 10.1007/s10330-013-1258-z

The expression and clinical significance of β-catenin and colorectal cancer stem cells marker EpCAM^{high}/CD44^{+} in colorectal cancer

Dan Liu[1], Jinghua Sun[1], Jinming Zhu[2], Huan Zhou[1], Yang Zhang[1]

[1] Department of Oncology, The Second Affiliated Hospital of DaLian Medical University, DaLian 116027, China
[2] Department of Oncology, The Affiliated Zhongshan Hospital of DaLian University, DaLian 116001, China

Received: 8 October 2013 / Revised: 17 October 2013 / Accepted: 25 December 2013

Abstract ***Objective:*** The aim of the study was to explore the role of Wnt/β-catenin signalling pathway in the maintenance, invasion and metastasis of colorectal cancer stem cells. ***Methods:*** Double immunohistochemical staining was used to detect the expression of EpCAM^{high}/CD44^{+} which is regarded as the marker of colorectal cancer stem cells in 80 cases of colorectal cancer and their corresponding liver metastases. The SP method of immunohistochemistry was used to detect the expression of the key protein β-catenin in the Wnt pathway in these tissue. The expression and correlation of β-catenin and EpCAM^{high}/CD44^{+} in colorectal cancer were analyzed and their role on the biological behavior of colorectal cancer was explored. ***Results:*** The abnormal expression of β-catenin was significantly higher in colorectal cancer than in the paraneoplastic normal intestinal mucosa [55% (44/80) vs 10% (2/20), $P < 0.05$]. The positive expression of EpCAM^{high}/CD44^{+} was significantly higher in colorectal cancer than in the paraneoplastic normal intestinal mucosa [66.25% (53/80) vs 0% (0/20), $P < 0.05$]. In the 80 cases of colorectal cancer, the abnormal expression of β-catenin has no correlation with gender ($P = 0.079$), age ($P = 0.416$) and the magnitude ($P = 0.816$) of the tumor ($P > 0.05$), but it was significantly correlated with degree of differentiation ($P = 0.001$), depth of invasion ($P = 0.001$), clinical stage ($P = 0.000$) and metastasis ($P = 0.000$). In the colorectal cancer, the expression of EpCAM^{high}/CD44^{+} cells has no correlation with gender ($P = 0.934$) and the magnitude ($P = 0.160$) of the tumor ($P > 0.05$), but was significantly correlated with age ($P = 0.021$), degree of differentiation ($P = 0.013$), depth of invasion ($P = 0.000$), clinical stage ($P = 0.000$) and metastasis ($P = 0.000$). In the corresponding liver metastases, we could also detecte EpCAM^{high}/CD44^{+} cells. In cases with abnormal expression of β-catenin, the positive expression rate of EpCAM^{high}/CD44^{+} was significantly higher than those with normal expression of β-catenin (84.1% vs 44.4%), and the difference was statistically significant ($P < 0.05$). ***Conclusion:*** The abnormal activation of Wnt/β-catenin signalling pathway may prompt the abnormal proliferation of the colorectal cancer stem cells, which leads to the recurrence and metastasis of the cancer.

Key words cancer stem cells; double immunohistochemical staining; β-catenin; EpCAM^{high}/CD44^{+}

Colorectal cancer is one of the most common gastrointestinal malignant tumor and the fifth cause of cancer-related mortality in China [1]. Recurrence and metastasis is the major problem affecting prognosis of colorectal cancer. It has been realized that a small group of cells in tumor which are named cancer stem cell is considered to be major source of recurrence and metastasis in cancer [2, 3]. Some researchers believe that cancer is a disease of stem cell, cancer stem cell is a few tumor cells with properties of stem cell such as self-renewal, differentiation potential, multi-drug resistance and tumorigenesis characteristics, which lead to the recurrence and metastasis of tumor [4]. Wnt signalling pathway plays an important role in maintaining these characteristics of cancer stem cell [5]. When the key protein β-catenin in Wnt signalling pathway is abnormally activated, the colorectal cancer stem cell has the dysfunction of self-renewal and differentiation, which leads to the genesis and development of the tumor. Thus it is significance to explore the interaction between Wnt signalling pathway and colorectal cancer stem cell that leads to recurrence and metastasis, which may provide new effect approach of therapy to target cancer stem cell and reduce recurrence and metastasis of the tumor.

Correspondence to: Yang Zhang. Email: zydl@medmail.com

Materials and methods

General materials

Make a research on 80 patients from 19 to 83 years old with resectable colorectal cancer in The 1st and 2nd Affiliated Hospital of DaLian Medical University (China)

from 2003 to 2010, including: male (38 cases), female (42 cases); average age: 60 years, > 60 years (44 cases), ≤ 60 years (36 cases); tumor size: ≤ 5 cm (50 cases), > 5 cm (30 cases); histological grade: highly differentiated (10 cases), moderate differentiated (51 cases), poorly differentiate (19 cases); TNM stage: $T_1 + T_2$ (18 cases), T_3 (28 cases), T_4 (34 cases); Dukes stage: Dukes A (16 cases), Dukes B (16 cases), Dukes C (22 cases), Dukes D (26 cases). 32 cases were without metastasis and 48 cases showed metastasis. None of patients had received either chemotherapy or radiotherapy prior to surgery and paraneoplastic intestinal mucosa samples were used as control.

Methods

Immunohistochemical staining

Paraffin-embedded sections were deparaffinized with xylene and dehydrated with a series of concentrations of ethanol, blocked with 3% H_2O_2 and goat serum, incubated with primary mouse-anti-human β-catenin monoclonal antibody (ZSGB-BIO, China) and followed by the secondary antibody (Rabbit anti-mouse, ZSGB-BIO, China). Then the sections were incubated with horseradish peroxidase and β-catenin was detected with diaminobenzidine. Positive staining was a brown-yellow precipitate.

Double immunohistochemical staining

Followed as DouSPTM double staining kit procedures. After deparaffinization and dehydration, endogenous peroxidases were blocked with 3% H_2O_2 and goat serum, sections were sequential incubated with primary antibody (mouse anti-human EpCAM monoclonal antibody, Maixin-bio, China), and then followed by secondary antibody (Goat anti-mouse/rabbit, Maixin-bio, China), incubated with alkaline phosphatase. EpCAM was detected with BCIP/NBT and positive staining was a blue-black precipitate. Then the sections were incubated with double staining enhancement solution, followed by blocked with goat serum, incubated with primary antibody (rabbit anti-human CD44 polyclonal antibody, Proteintech Group) and incubated with the secondary antibody (Goat anti-mouse/rabbit, Maixin-bio, China). CD44 was detected with AEC and positive staining was a red precipitate. Immunohistochemical of EpCAM and CD44 respectively was used as controls to prove the reliability of EpCAM^{high}/CD44^{+}.

Scoring methods

Assessment of β-catenin immunohistochemical staining

The expression of β-catenin was scored using a composite score in which the scope of stained was evaluated on a scale from 0 to 3 (0: no staining in cytomembrane, cytoplasm and nucleus; 1: staining mainly in cytomembrane; 2: staining mainly in cytoplasm; 3: staining mainly in nucleus) and the intensity of staining was categorized as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The two scores were multiplied to generate a composite score (0–9) categorized in 2 groups: 0–3 = negative; 4–9 = positive. The results were judged at Iwamoto standard [6].

Assessment of double immunohistochemical staining

EpCAM^{high}/CD44^{-} was identified as only blue-black precipitate in the cytoplasm, EpCAM^{low}/CD44^{+} was identified as only red precipitate in the cytomembrane, EpCAM^{low}/CD44^{-} as negative for the both and EpCAM^{high}/CD44^{+} as positive for the both. The results were judged at Honeth standard [7].

Statistical analysis

Adopting SPSS 17.0 software for processing, testing differences of measurement data by *t* and count data by chisquare test. Pearson correlation analysis was used for the correlation of β-catenin and EpCAM^{high}/CD44^{+}, and a *P*-value of less than 0.05 was considered as statistically significant.

Results

The expression of β-catenin in colorectal cancer and their corresponding liver metastases

The expression of β-catenin in the adjacent normal intestinal mucosa was located in the membrane, while in colorectal cancer and their corresponding liver metastases it appeared different degrees of cytoplasm and(or) nuclear expression (Fig. 1). And also in the 80 cases of colorectal cancer and their corresponding liver metastases, the abnormal expression of β-catenin had no correlation with gender, age and the magnitude of the tumor ($P > 0.05$), but it was significantly correlated with degree of differentiation, depth of invasion, clinical stage and metastasis ($P < 0.05$; Table 1). In the corresponding liver metastases, the abnormal expression of β-catenin was also higher.

The expression of EpCAM^{high}/CD44^{+} in colorectal cancer and their corresponding liver metastases

The EpCAM^{high}/CD44^{+} did not exist in the adjacent normal intestinal mucosa (Fig. 2a), while EpCAM^{high}/CD44^{+} cells were visible in colorectal cancer and their corresponding liver metastases (Fig. 2b and 2c). The expression of EpCAM^{high}/CD44^{+}-double-positive cells had no correlation with gender and the magnitude of the tumor ($P > 0.05$), but it was significantly correlated with age, degree of differentiation, depth of invasion, clinical stage and metastasis ($P < 0.05$; Table 2).

The correlation analysis between β-catenin and EpCAM^{high}/CD44^{+}

In 44 cases with abnormal expression of β-catenin, the expression rate of EpCAM^{high}/CD44^{+} is 84.1%, which was significantly higher than 44.4% in those cases with nor-

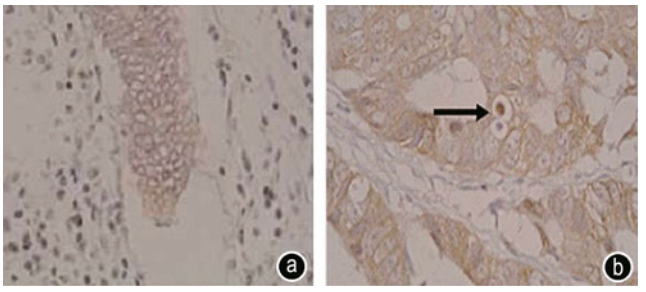

Fig. 1 The expression of β-catenin in normal intestinal mucosa and colorectal cancer (× 400). (a) β-catenin located in the membrane in normal intestinal mucosa; (b) β-catenin located in the nuclear in colorectal cancer

mal expression of β-catenin. By Pearson correlation analysis, there was significant correlation between β-catenin and EpCAM^{high}/CD44^{+} ($P = 0.000$, $r = 0.417$), and the difference was statistically significant.

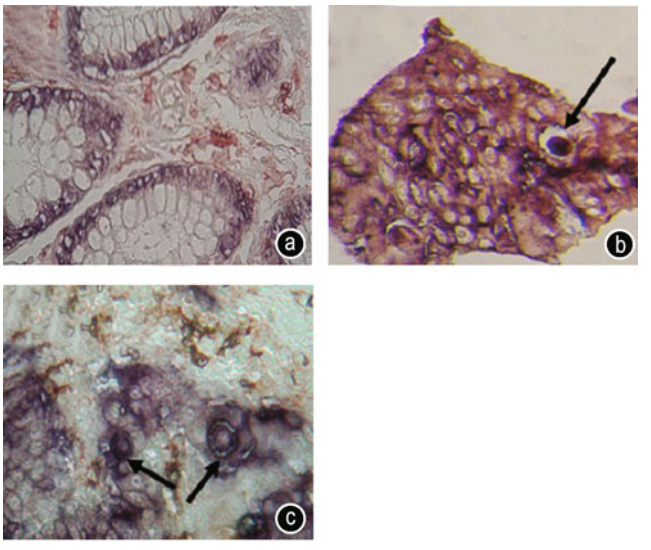

Fig. 2 The expression of EpCAM^{high}/CD44^{+} in colorectal cancer and their corresponding liver metastases (× 400). (a) No EpCAM^{high} CD44^{+} cells express in normal intestinal mucosa; (b) EpCAM^{high} CD44^{+} cells express in colorectal cancer; (c) EpCAM^{high} CD44^{+} cells express in liver metastases

Discussion

The role of β-catenin in genesis and metastasis of colorectal cancer

In the normal intestinal mucosa, β-catenin and E-cadherin binding in the cytomembrane plays a role in stabilizing the cell junction. Tyrosine phosphorylation of β-catenin in the cytoplasm make it can not enter nucleus to

Table 1 The expression of β-catenin in colorectal cancer (*n*)

Variable	*n*	β-catenin		*P*
		–	+	
Patients				
Male	38	21	17	> 0.05
Female	42	15	27	
Age (years)				
> 60	44	18	26	> 0.05
≤ 60	36	18	18	
Magnitude (cm)				
≤ 5 × 5	50	22	28	> 0.05
> 5 × 5	30	14	16	
Differentiation				
High	10	8	2	
Moderate	51	26	25	< 0.05
Low	19	2	17	
T stage				
$T_1 + T_2$	18	16	2	
T_3	28	12	16	< 0.05
T_4	34	8	26	
Dukes stage				
A	16	16	0	
B	16	11	5	< 0.05
C	22	4	18	
D	26	5	21	
Metastasis				
–	32	27	5	< 0.05
+	48	9	39	

Table 2 The expression of EpCAM^{high}/CD44^{+} in colorectal cancer (*n*)

Variable	*n*	EpCAM^{high}/CD44^{+}		*P*
		–	+	
Patients				
Male	38	13	25	> 0.05
Female	42	14	28	
Age (years)				
> 60	44	10	34	< 0.05
≤ 60	36	17	19	
Magnitude (cm)				
≤ 5 × 5	50	14	36	> 0.05
> 5 × 5	30	13	17	
Differentiation				
High	10	7	3	
Moderate	51	17	34	< 0.05
Low	19	3	16	
T stage				
$T_1 + T_2$	18	9	9	
T_3	28	15	13	< 0.05
T_4	34	3	31	
Dukes stage				
A	16	9	7	
B	16	11	5	< 0.05
C	22	5	17	
D	26	2	24	
Metastasis				
–	32	20	12	< 0.05
+	48	7	41	

regulate the expression of target gene. While in colorectal cancer, phosphorylation of β-catenin has dysregulated and then accumulate in the cytoplasm, translocate into the nucleus and activate the target gene downstream. Khramtsov AI *et al* [8] have found that increased nuclear β-catenin staining were associated with increasing degrees of dysplasia in adenomas in the 74 cases of colorectal adenoma. And also the expression of nuclear β-catenin enhanced in colorectal cancer compared with their associated adenomas. Wang *et al*[9] showed that nuclear β-catenin overexpression at the invasive front of the primary tumor in patients with liver metastasis is more evident than that in patients without liver metastasis and the expression of nuclear β-catenin in primary tumors had a positive correlation with that in the matched metastatic lesions. So the researchers point out that overexpression of nuclear β-catenin at the invasive front in colorectal cancer is strongly associated with liver metastasis and may be a promising predictor of liver metastasis.

In our study we detecte 80 cases of colorectal cancer and their corresponding liver metastases, the expression of β-catenin in the normal intestinal mucosa is located in the membrane, while in colorectal cancer and their corresponding liver metastases it appears different degree of cytoplasm and (or) nuclear expression. Moreover, β-catenin is significantly correlated with degree of differentiation, depth of invasion, clinical stage and metastasis. According to the results of this experiment we find that β-catenin is involved in the genesis and metastasis of tumors. It also can be regarded as an important reference for judging whether the colorectal cancer has the malignant potential. And from that we deduce the abnormally activated of key protein β-catenin in Wnt signalling pathway may lead to the genesis and metastasis of the tumor.

The role of EpCAM^{high}/CD44^{+} in genesis and metastasis of colorectal cancer

Recently, it has been reported that EpCAM^{high}/CD44^{+} of colorectal cancer cells have stem cell properties and have been regarded as one of the markers in colorectal cancer stem cell [10, 11]. Marhaba *et al* [12] had proposed the EpCAM^{high}/CD44^{+} cells can be identified as the marker of colorectal cancer stem cell in 2008. Dalerba *et al* [11] found that the EpCAM^{high}/CD44^{+} have stem cell-like properties in colorectal cancer. They had reported that 200-500 EpCAM^{high}/CD44^{+} cells are enough to promote tumorigenesis in NOD/SCID mice, however no tumor produced by injection 10^4 EpCAM^{low}/CD44^{-} cells.

In our study, 80 cases of colorectal cancer and their corresponding liver metastases are examined. EpCAM^{high}/CD44^{+} doesn't exist in the adjacent normal intestinal mucosa, while it is visible in the colorectal cancer and their corresponding liver metastases. The expression of EpCAM^{high}/CD44^{+}-double-positive cells has no correlation with gender and the magnitude of the tumor, but it is significantly correlated with age, degree of differentiation, depth of invasion, clinical stage and metastasis. This finding shows that EpCAM^{high}/CD44^{+} phenotype of colorectal cancer cell is significantly related to invasion and metastasis, suggesting that colorectal cancer stem cell may be one of the cause of recurrence and metastasis in colorectal cancer. Thus targeting cancer stem cell may significantly reduce the chance of recurrence and metastasis in tumor, and even elimination of tumor from the source.

The correlation and clinical significance of key protein β-catenin in Wnt signalling pathway and colorectal cancer stem cell marker EpCAM^{high}/CD44^{+} in colorectal cancer

Wnt signalling pathway is divided into classical and nonclassical pathway. The classical pathway, namely Wnt/β-catenin pathway, which can make the key protein β-catenin abnormally activate and translocate into the nucleus to regulate the expression of target genes downstream has the character of tumorigenesis and maintaining stem cell proliferation [5]. The nonclassical pathway, such as Wnt/Ca^{2+} pathway plays a role of inhibiting the growth of tumor [13]. And the canonical and non-canonical Wnt pathway are related to each other and restricted with each other [13]. In normal state, the interaction between the canonical and non-canonical reaches a state of balance to maintain the proliferation and differentiation of stem cell. And the key protein β-catenin of Wnt pathway is then located in the cytomembrane. When the interaction between the canonical and non-canonical is imbalanced, Wnt/β-catenin pathway is abnormally activated and β-catenin translocated into the nucleus to regulate the expression of target genes, which makes the self-renewal and differentiation of stem cell dysfunctioned and even lead to the genesis of tumor [13].

At present, some studies have shown that, Wnt/β-catenin pathway could regulate the proliferation of stem cell [14]. In 2010, Kanwar SS [5] had detected EpCAM and CD44 in the serum-free colono spheres that was thought to be the characteristics of cancer stem cell. Following down regulation of β-catenin by the corresponding siRNA lead to a marked reduction of EpCAM and CD44 as well as colonospheres formation, which proved that the abnormal activation of Wnt/β-catenin pathway plays a critical role in growth and maintenance of colonospheres. Recently, many researchers have already confirmed that EpCAM^{high}/CD44^{+} phenotype of colorectal cancer cell is significantly related to invasion and metastasis and also has the character of stem cell [11, 15]. So EpCAM^{high}/CD44^{+} is now used as the marker of colorectal cancer stem cell.

In summary, abnormal activation of Wnt/β-catenin pathway and EpCAM^{high}/CD44^{+} phenotype of cancer stem cells can be used as an important index to evaluate

the malignant degree of colorectal cancer, but whether there has correlation is still remains elusive. For this aim, we detecte 80 cases of colorectal cancer and their corresponding liver metastases and find that in the abnormal expression group of β-catenin, the expression of EpCAM^{high}/CD44^{+} is significantly higher than those with normal expression group of β-catenin. Furthermore, β-catenin and EpCAM^{high}/CD44^{+} are all correlated with degree of differentiation, depth of invasion, clinical stage and metastasis and also there is significantly correlated between β-catenin and EpCAM^{high}/CD44^{+}. So we can infer that the abnormal activation of Wnt/β-catenin pathway may prompt the abnormal proliferation of colon cancer stem cell, which leads to the recurrence and metastasis of colorectal cancer. Based on the above assumption, further research and development of inhibitors for Wnt/β-catenin pathway can be more effective targeted colorectal cancer stem cell, reducing the recurrence and metastasis of colorectal cancer and providing new therapies for colorectal cancer.

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