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Original Article

Overexpression of CO-029 in pancreatic ductal adenocarcinoma is associated with poor prognosis

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is a high lethal disease with a poor prognosis. Identifying prognostic markers that promote progression of PDAC is important for the treatment of pancreatic cancer. CO-029 appears to be involved in multiple processes during tumor development and metastasis. Its activity is increased in malignant cells compared to noncancerous cells. However, the clinical and significance of CO-029 expression has not been characterized previously in PDAC. The aim of this study was to investigate the CO-029 expression and to explore its contribution to PDAC. The mRNA and protein expression levels of CO-029 in 80 cases of PDAC tissues were determined using qualitative Real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry staining, respectively, and the relationship between the CO-029 expression level and clinicopathological parameters was analyzed in 80 cases of PDAC. Kaplan-Meier analysis and Cox regression analysis were used to investigate the correlation between CO-029 expression and prognosis of PDAC patients. CO-029 expression was significantly higher in PDAC tissues compared to normal tissues ($P < 0.001$). In univariate analysis, CO-029 was found to be associated with lymph node metastasis ($P < 0.001$). Kaplan-Meier analysis and COX regression analysis showed that CO-029 was significantly correlated with poor survival in PDAC patients ($P < 0.001$). Furthermore, Cox regression analyses showed that CO-029 expression was an independent predictor of overall survival. Taken together, our study reveals that high CO-029 expression and its association with lymph node metastasis in PDAC. It also provided the first evidence that CO-029 expression in PDAC was an independent prognostic factor of patients, which might be a potential diagnostic and therapeutic target of PDAC.

Keywords: CO-029, prognosis, pancreatic ductal adenocarcinoma, tissue microarray

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal types of human cancer worldwide for its early invasion and metastasis [1]. The long-term prognosis remains poor with a 5-year survival rate of less than 5% after diagnosis [2]. Surgical resection and chemotherapy are important part of management and can acquired the goals of diagnosis and are associated with improved survival of PDAC patients. However, despite recent advances in multimodal therapeutic approaches, the fatal prognosis of pancreatic cancer has remained unchanged over the last few decades. Many efforts have been undertaken to find new molecular markers and targeted therapies to achieve earlier diagnosis and better treatment, and ultimately to identify a subset of patients who are more likely to benefit from surgery, chemoradiotherapy and molecular targeted therapy based on their particular profile. Although various molecular changes have been revealed in this malignancy, mechanisms underlying the aggressiveness of this neoplasm remain largely unclear.

CO-029, also known as TSPAN8, is one member of tetraspanins, has been found to be expressed on gastric, colon, rectal, pancreatic and hepatocellular carcinomas [3, 4]. Tetraspanins are known to contribute to the tumor metastasis. For instance, CD82 and CD9 have been described as the tumor metastasis suppressor gene and related with favorable prognosis by inhibiting cell motility and invasiveness [5, 6]. By contrast, CO-029 and CD151 are associated with tumor progression [7]. Previous studies have shown that CO-029 is

involved in development of hepatocellular carcinoma, Barrett esophagus and the migration of Isreco colon cancer cells in humans [8, 9]. In the Isreco colon cancer, p-120-catenin and E-cadherin prevent CO-029 migrating of cancer cells. Furthermore, CO-029 is associated with other tetraspanin members, such as CD151, promotes tumor dissemination. Recently, the latest study reported that CO-029 deregulates cell-matrix and cell-cell adhesions to promote cancer cell movement in colorectal cancer [10]. They found that the loss of CO-029 significantly attenuated cell motility and altered the balance of cell-cell and cell-matrix adhesions, leading to the decreased metastatic potential of tumor cells. Gesierich *et al*. reported that CO-C029 is associated with CD151 and α6β4 correlates with increased tumor cell motility in pancreatic cancer [7]. These results suggest that CO-029 might play an important role in promoting tumorigenesis.

However, little is known about the clinical significance of CO-029 in PDAC. In this study, we explored the expression of CO-029 in 80 cases with pancreatic ductal adenocarcinoma (PDAC) and evaluate its correlation with clinicopathologic characteristics and patient prognosis. We demonstrated the prognostic significance of CO-029 expression in PDAC and the potential value of this marker as a prognostic indicator in patients with PDAC.

Materials and methods

Tissue specimens

Eighty PDAC tissues specimens were obtained from patients with PDAC who underwent surgical treatment at Department of Hepatobiliary Surgery, Shanghai Tenth People's Hospital, during the period from September 2009 and December 2013. PDAC was diagnosed according to AJCC Cancer Staging Atlas [11]. Patient consent was obtained for the collection of specimens, and all study protocols were approved by Ethics Committee for Clinical Research of Shanghai Tenth People's Hospital. The selection criteria were as follows: 1) the subject had a diagnosis of PDAC and no history of other tumors, 2) the subject had complete clinical data, such age, gender, clinical stage, histological differentiation, lymph node metastasis and tumor diameter. Tissue samples for immunohistochemistry were fixed in 5% formalin.

Tissue microarray and immunohistochemistry

After tumor verification with hematoxylin and eosin (H&E) staining, we constructed tissue microarray slides (Outdo Co., Shanghai, China). One core punch sample was taken from each specimen, measuring 1 mm in the greatest dimension from the center of tumor foci. Immunohistochemical staining using an anti-CO-029 antibody (No. sc-292058, Santa Cruze, Dallas, Texas, USA) was performed using an avidin-biotin complex (ABC) method (vector laboratories Burlingame, CA, USA). Two pathologists who were blinded with respect to the clinical and histopathologic features independently evaluated Immunostained results. Briefly, staining intensity in the cytoplasma was graded according to the method of Fromowitz semi-quantitative grading, a scale from 0 to 3 (0 for no immunostaining, 1 for light-brown color, 2 for medium-brown color, and 3 for dark-brown color). The percentage of positively stained cells was scored as follows: 0, no staining; 1, < 25% of the entire malignant cell population; 2, 25%-75% of entire malignant cell population; 3, > 75% of the entire malignant cell population. The final composite score was the product of the intensity and percentage scores, being classified as: strong (+++, final score > 6), moderate (++, final score = 4-6), weak (+, final score = 1-3), or null (-, final score = 0). For analysis, CO-029 expression was divided into "high" (++ and +++) versus "low" (+ and -). Discrepancies in scoring were resolved through discussion.

Qualitative Real-time PCR

Qualitative Real-time PCR was then employed to determine the change of CO-029 mRNA in pancreatic cancer tissues. Total RNA was isolated from tumor and normal pericarcinous tissue using the RNeasy Plus Universal Mini Kit (73404; Qiagen) following the manufacturer's guidelines. mRNA was subsequently transcribed into cDNA using the Reverse Transcription System kit (Promega, USA) as described previously [12]. PCR were performed with the SYBR Green method in an ABI 7500 sequence detection system (Applied Biosystems, USA) as described previously [13]. All oligonucleotides were designed using Primer Express 2.0 (Applied Biosystems, USA) computer software, and synthesized by Invitrogen Life Technology (USA). The sequence spanning nucleotides 130-842 of tetraspanin CO-029

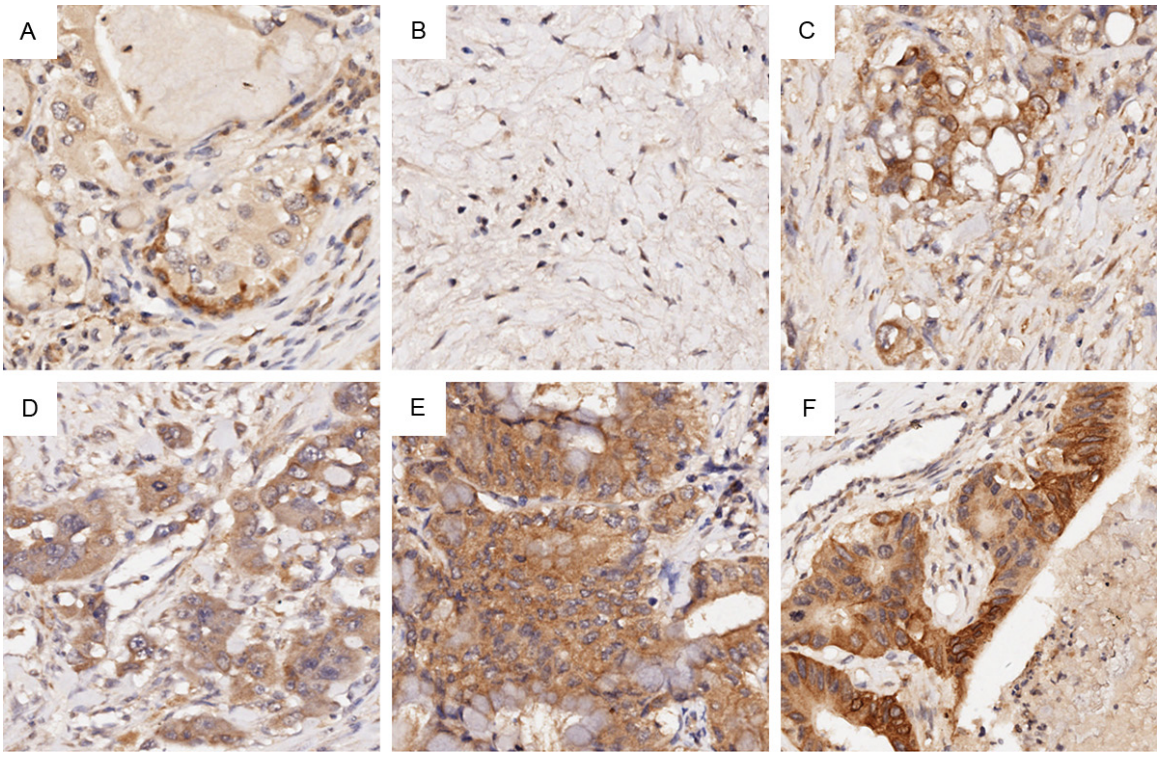

Figure 1. Immunohistochemical analysis of CO-029 expression in human pancreas specimens. A: Representative image of immunohistochemical staining of CO-0C29 in PDAC tissues (×400 magnification). B: Representative images were negative control (×400 magnification). C-F: The four images showed different levels of CO-029 in the PDAC cells (×400 magnification).

were amplified by PCR using the forward primer: 5'-TCGAATTCTTTCCGAAATGGCAGGTGT-GAG-3', and the reverse primer: 5'-ATGTCG-ACTGCATCCACAGATTCATTTGTTC-3'. The reactions were carried out as follows: 2 min at 50°C, followed by 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Data are expressed as relative mRNA abundance obtained from the CT values from the target versus the endogenous reference gene GAPDH.

Follow-up

Follow-up was carried out in all patients, with survival time being censored on 31th December 2014. Follow-up was conducted every 6 months by telephone. Other treatment options, including adjuvant chemotherapy and radiotherapy, were fully discussed with the patients.

Statistical analysis

Values were expressed as mean ± SD. Correlations between categorical variables were analyzed using Pearson's chi-square tests. For survival analysis, overall survival was defined as the time interval between the date of surgery to the date of death or the last follow-up. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Seven possible prognostic factors were analyzed by univariate analysis and the results of $P < 0.2$ were included in multivariate analysis, using COX regression with stepwise backward method. Cox's proportional hazards model was used to identify the fators that had a significant influence on survival. All statistics were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

Results

Correlation between CO-029 expression and clinicopathological factors in PDAC

In all 80 PDAC patients, tissue microarray analysis reveals high CO-029 expression in 29 patients (29/80) and low CO-29 expression in

Table 1. The associations of CO-029 expression with clinicopathological characteristics in 80 PDAC patients

Characteristics	Value	CO-029 expression		*p*-Value
		High	Low	
Gender	80	29	51	
Male	53	20	33	0.699
Female	27	9	18	
Age (years)				
≤ 60	49	18	31	0.910
> 60	31	11	20	
TMN-stage				
I-II	42	15	27	0.235
III-IV	28	14	14	
Histological grade				
I-II	37	15	22	0.873
III-IV	33	14	19	
Lymph node metastasis				
Positive	64	24	40	< 0.001
Negative	16	5	11	
Tumor size (cm)				
< 2	51	19	32	0.804
≥ 2	29	10	19	
Survival outcomes				
Alive	45	1	44	< 0.001
Dead	35	28	7	

P-Values were two-tailed and based on the Pearson chi-square test.

51 patients (51/80) (**Figure 1**). The clinicopathologic characteristics stratified by high CO-029 and low CO-029 were presented in **Table 1**. There was no significant correlation between CO-029 expression and gender, age, tumor differentiation, histological grade or tumor size, as determined by the χ^2 test ($P > 0.05$). However, CO-029 expression was associated with lymph node metastasis ($P < 0.01$) and survival outcomes ($P < 0.01$). 24 of 29 (82.76%) patients with CO-029 high expression had lymph node metastasis. In contrast, 40 of 51 (78.43%) patients with CO-029 low expression had lymph node metastasis. Twenty-eight patients (96.55%) with high level of CO-029 had a much higher mortality than those with low CO-029 expression of patients (13.73%). The clinicopathological data indicate that decreased expression of CO-029 is associated with lymph node metastasis and survival outcomes, which are involved in tumor progression of PDAC.

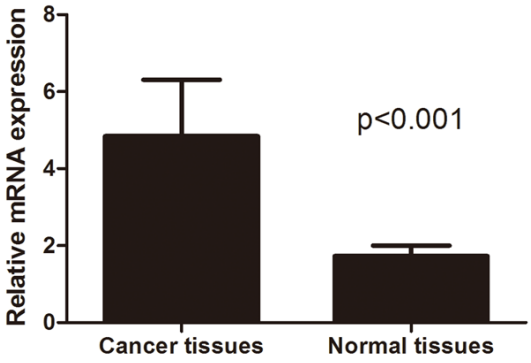

Figure 2. Different expression of CO-029 mRNA in pancreatic cancer tissues and pericaicinous tissues. RT-PCR analysis of revealed that CO-029 mRNA expression was significantly higher in cancer tissues than that in normal tissues ($P = 0.001$).

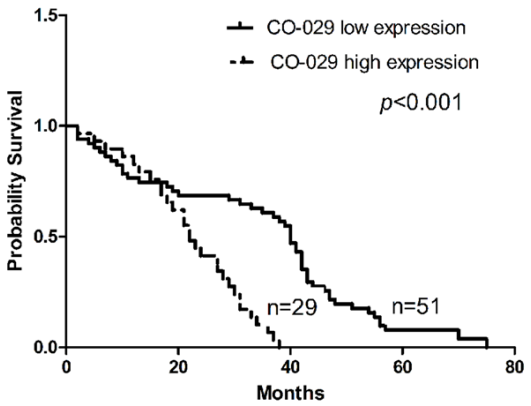

Figure 3. Kaplan-Meier survival curves in PDAC patients according to CO-029 expression level. The median overall survival of patients with high expression CO-029 was 13.5 months, and that of patients with low expression CO-029 was 65 months. Kaplan-Meier survival analysis demonstrated that patients with high expression CO-029 had a significantly shorter overall survival than patients with lower levels of CO-029 expression ($P < 0.001$).

Overexpression of CO-029 mRNA in pancreatic cancer tissues

To examine the expression of CO-029 mRNA in PDAC tissues, Real-time PCR was performed. The RT-PCR analysis showed that the levels of CO-029 mRNA were significantly higher in PDAC tissues than in pericarcinous tissues ($P < 0.001$) (**Figure 2**).

Survival analysis in patients with PDAC

To investigate the prognostic value of CO-029 for PDAC, we assessed the association between

Table 2. Multivariate analyses of prognostic factors PDAC patients

Independent factor	*B*	*P*-value	Risk ratio
Age	0.008	0.663	1.008
Gender	0.441	0.249	0.644
CO-029	2.660	< 0.001	5.150
Histological grade	0.382	0.390	0.683
TNM stage	0.400	0.410	0.510
Lymph node metastasis	1.179	0.069	0.308
Tumor size	1.450	0.078	0.401

CO-029 expression and survival duration using a Kaplan-Meier analysis with a log-rank test. After median follow-up of 5 years (ranging from 1 to 5 years), 35 of 80 (43.75%) patients with PDAC have died. The median overall survival for the entire cohort was 45 months. The median overall survival of patients with high expression CO-029 was 13.5 months, and that of patients with low expression CO-029 was 65 months. Furthermore, Kaplan-Meier survival analysis demonstrated that patients with high expression CO-029 had a significantly shorter overall survival than patients with lower levels of CO-029 expression ($P < 0.001$) (**Figure 3**). These observations indicated that overexpression of CO-029 is associated with PDAC clinical progression.

Prognostic values of CO-029 in patients with PDAC

Meanwhile, to evaluate the possibility of CO-029 used as an independent risk factor for poor prognosis, conventional clinicopathological factors and CO-029 levels were assessed by Cox's multivariate hazard regression model (**Table 2**). The multivariate Cox regression analysis shows that the CO-029 was an independent prognostic factor in pancreatic cancer patients ($P < 0.001$). The results showed that low expression of CO-029 may serve as a prognostic indicator for patients with PDAC.

Discussion

The incidence of pancreatic cancer, particularly ductal adenocarcinoma, is increasing in the world. Thus, it is critical to evaluate novel prognostic marker for this disease. Tetraspanins regulate a kind of pathological and physiological processes, and some tetraspanins can modulate the tumor progression and metastasis by modulating cell adhesion, migration, fusion and proliferation. Early reports show that some tetraspanins form multi-molecular complexes to modulate cells adhesion, migration and fusion. The tumor metastatic partially depends on the motility and adhesiveness of tumor cells. Therefore, some tetraspanins could promote the massive metastatic spread of tumor by modulating the abilities of tumor cells to adhere and move [14-16].

Recent studies suggested that CO-029 could serve as an oncogene in several cancer types [17]. The possible clinical significance role of CO-029 was poorly known in the PDAC, although prior studies have shown that CO-029 is overexpressed in PDAC tissues compared with normal pancreas tissues [7]. This finding promoted us to speculate that CO-029 could be used as a prognostic marker for PDAC. In this study, we found that CO-029 was overexpressed in PDAC tissues compared with normal pancreas tissues by qRT-PCR and immunohistochemistry. CO-029 was primary expressed on pancreatic ductal cells in human pancreas. A prior study has also shown evidence of CO-029 expression in pancreatitis, implying that CO-029 is involved in the early progression of PDAC and may function as an oncogene in PDAC. Moreover, in this study, we further demonstrated that high expression CO-029 was positively correlated with lymph node metastases ($P < 0.001$) and survival outcomes ($P < 0.001$), which might be predominantly caused by CO-029 associating with CD151 to enhance tumor cell motility and stimulate the expression of several integrins and tetraspanins. Indeed, survival analysis indicated that the expression of CO-029 was correlated with median survival time, and lower CO-029 expression was associated with longer survival time. Moreover, multivariate analysis demonstrated that CO-029 expression is an independent risk factor in prognosis of PDAC patients. Our clinical evidence obviously suggests that CO-029 may be an important mediator of PDAC progression.

Emerging evidence shows that CO-029 plays an important role in cancer. For instance, CO-029 is upregulated among the progression of liver, esophageal, pancreatic and colorectal cancer and is associated with poor prognosis of these cancers [18]. High expression of

CO-029 could promote the lung, lymph and liver metastasis [19]. It is also noteworthy that CO-029 also promotes tumor cells movement by altering cell-adhesions, and tumor cell migration and invasion are necessary for tumor metastasis. In colorectal cancer, CO-029 expression was silenced. It was found that the CO-029 significantly reduced cell migratory ability and likely inhibited cell adhesion on laminar and the diminished cell migration was accompanied by the upregulation of both integrin-dependent cell-matrix adhesion on laminar and calcium-dependent cell-cell adhesion [10]. Decreased cell adhesion at early stage of metastasis helps releasing tumor cells from the primary tumor. It is well established that CO-029 can regulate cell migration. Similarly, Gesierich *et al*. reported that the association of α6β4 integrins with CD151 and CO-029 correlates with increased tumor cell motility [7]. Thus, it is reasonable to speculate that high expression of CO-029 could modulate cell-cell adhesion, promote cell-cell movement, ultimately contributes to cancer development. Moreover, CO-029 perturbs other molecules, such as CD44 and MelCAM, which are important for metastasis [20, 21]. CD44 could directly engage cell-matrix and -cell adhesions and accelerate cancer progression [22]. In agreement with this notion, we showed that high CO-029 expression was associated with poorer outcomes in PDAC patients. In this study, we found that CO-029 mRNA was significantly increased in PDAC tissues compared with normal tissues. Cox regression analysis unraveled that CO-029 expression was regarded as an independent prognostic factor for PDAC patient overall survival time. Moreover, CO-029 expression was strongly correlated with lymph node metastasis, consistent with CO-029-dependent regulation of cell adhesive structures. Taken together, our data not only imply a potentially promising application of CO-029 as a valuable prognostic marker, but suggest a possible relationship between the molecular functions of CO-029 and carcinogenesis of pancreas cancer. However, further studies are needed to elucidate the mechanism underlying CO-029 in the development and metastatic process of PDAC, and to clarify whether CO-029 could be used as a novel therapeutic target for PDAC.

In summary, high CO-C029 expression in PDAC is an independent predictor for survival of patients and high CO-029 expression is able to enhance lymph node metastasis. In combination, these results indicate that CO-029 plays an important role in PDAC progression and may represent a potential therapeutic target in the treatment of PDAC.

Disclosure of conflict of interest

None.

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References

[1] Ducreux M, Boige V, Goere D, Deutsch E, Ezra P, Elias D, Malka D. The multidisciplinary management of gastrointestinal cancer. Pancreatic cancer: from pathogenesis to cure. Best Pract Res Clin Gastroenterol 2007; 21: 997-1014.

[2] Rhim AD, Stanger BZ. Molecular biology of pancreatic ductal adenocarcinoma progression: aberrant activation of developmental pathways. Prog Mol Biol Transl Sci 2010; 97: 41-78.

[3] Sela BA, Steplewski Z, Koprowski H. Colon carcinoma-associated glycoproteins recognized by monoclonal antibodies CO-029 and GA22-2. Hybridoma 1989; 8: 481-491.

[4] Kanetaka K, Sakamoto M, Yamamoto Y, Yamasaki S, Lanza F, Kanematsu T, Hirohashi S. Overexpression of tetraspanin CO-029 in hepatocellular carcinoma. J Hepatol 2001; 35: 637-642.

[5] He B, Liu L, Cook GA, Grgurevich S, Jennings LK, Zhang XA. Tetraspanin CD82 attenuates cellular morphogenesis through down-regulating integrin alpha6-mediated cell adhesion. J Biol Chem 2005; 280: 3346-3354.

[6] Nakazawa Y, Sato S, Naito M, Kato Y, Mishima K, Arai H, Tsuruo T, Fujita N. Tetraspanin family member CD9 inhibits Aggrus/podoplanin-induced platelet aggregation and suppresses pulmonary metastasis. Blood 2008; 112: 1730-1739.

[7] Gesierich S, Paret C, Hildebrand D, Weitz J, Zgraggen K, Schmitz-Winnenthal FH, Horejsi V, Yoshie O, Herlyn D, Ashman LK, Zoller M. Colocalization of the tetraspanins, CO-029 and CD151, with integrins in human pancreatic adenocarcinoma: impact on cell motility. Clin Cancer Res 2005; 11: 2840-2852.

[8] Greco C, Bralet MP, Ailane N, Dubart-Kupperschmitt A, Rubinstein E, Le Naour F, Boucheix C. E-cadherin/p120-catenin and tetraspanin Co-029 cooperate for cell motility control in human colon carcinoma. Cancer Res 2010; 70: 7674-7683.

[9] Botelho NK, Schneiders FI, Lord SJ, Freeman AK, Tyagi S, Nancarrow DJ, Hayward NK, Whiteman DC, Lord RV. Gene expression alterations in formalin-fixed, paraffin-embedded Barrett esophagus and esophageal adenocarcinoma tissues. Cancer Biol Ther 2010; 10: 172-179.

[10] Guo Q, Xia B, Zhang F, Richardson MM, Li M, Zhang JS, Chen F, Zhang XA. Tetraspanin CO-029 inhibits colorectal cancer cell movement by deregulating cell-matrix and cell-cell adhesions. PLoS One 2012; 7: e38464.

[11] Compton CC, Byrd DR, Aguilar JG, Kurtzman SH, Olawaiye A, MK. W. AJCC cancer staging atlas. 2nd edition. New York: Springer; 2012. pp. 297-308.

[12] García-Vallejo JJ, van Liempt E, da Costa Martins P, Beckers C, van het Hof B, Gringhuis SI, Zwaginga JJ, van Dijk W, Geijtenbeek TB, van Kooyk Y, van Die I. DC-SIGN mediates adhesion and rolling of dendritic cells on primary human umbilical vein endothelial cells through LewisY antigen expressed on ICAM-2. Mol Immunol 2008; 45: 2359-2369.

[13] Garcia-Vallejo JJ, Gringhuis SI, van Dijk W, van Die I. Gene expression analysis of glycosylation-related genes by real-time polymerase chain reaction. Methods Mol Biol 2006; 347: 187-209.

[14] Jones PH, Bishop LA, Watt FM. Functional significance of CD9 association with beta 1 integrins in human epidermal keratinocytes. Cell Adhes Commun 1996; 4: 297-305.

[15] Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. FASEB J 1997; 11: 428-442.

[16] Claas C, Zöller M. Association between the rat homologue of CO-029, a metastasis-associated tetraspanin molecule and consumption coagulopathy. J Cell Biol 1998; 141: 267-280.

[17] Richardson MM, Jennings LK, Zhang XA. Tetraspanins and tumor progression. Clin Exp Metastasis 2011; 28: 261-270.

[18] Huerta S, Harris DM, Jazirehi A, Bonavida B, Elashoff D, Livingston EH, Heber D. Gene expression profile of metastatic colon cancer cells resistant to cisplatin-induced apoptosis. Int J Oncol 2003; 22: 663-670.

[19] Boucheix C, Soria C, Mirshahi M, Soria J, Perrot JY, Fournier N, Billard M, Rosenfeld C. Characteristics of platelet aggregation induced by the monoclonal antibody ALB6 (acute lymphoblastic leukemia antigen p 24). Inhibition of aggregation by ALB6Fab. FEBS Lett 1983; 161: 289-295.

[20] Marhaba R, Zoller M. CD44 in cancer progression: adhesion, migration and growth regulation. J Mol Histol 2004; 35: 211-231.

[21] Sales KM, Winslet MC, Seifalian AM. Stem cells and cancer: an overview. Stem Cell Rev 2007; 3: 249-255.

[22] Zou GM. Cancer stem cells in leukemia, recent advances. J Cell Physiol 2007; 213: 440-444.