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Clinicopathological and prognostic significance of α5β1-integrin and MMP-14 expressions in colorectal cancer

B. YANG[1], J. GAO[1], Z. RAO[1], Q. SHEN[2,*]

[1]*Department of Oncology, Wuhan General Hospital of Guangzhou Command, People's Liberation Army, Wuhan, Hubei Province, 430070, China;*
[2]*Medical Research Center, Zhongnan Hospital of Wuhan University, Wuhan, Hubei Province, 430071, China*

**Correspondence: qinglinshen2011@163.com*

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The purpose of this study was to evaluate the association of expression level of α5β1-integrin and MMP-14 with clinicopathologic features and prognosis in colorectal cancer (CRC). The expressions of α5β1-integrin and MMP-14 in normal colorectal mucosa and CRC tissue were detected with immunohistochemistry. We estimated the five-year survival rate by the Kaplan-Meier method. The positive expressions rates of α5β1-integrin and MMP-14 in CRC tissue were 60.6% and 63.3% respectively, and there were significant differences on their positive expression rates between in CRC tissue and in normal colorectal mucosa($P<0.05$). The expression rates of α5β1-integrin and MMP-14 in patients with poor histological differentiation, lymph node metastasis and high clinical staging were heightened. There was a significant difference ($P<0.05$) on the five-year survival rate for α5β1-integrin expression, which was 44.6% in positive groups and 75.5% in negative groups. And there was a significant difference ($P<0.05$) on the five-year survival rate for MMP-14 expression, which was 48.2% in positive group and 73.1% in negative group. The expression of α5β1-integrin and MMP-14 is correlated with the progression and metastasis of CRC, and α5β1-integrin and MMP-14 may be used as prognostic markers in CRC.

Key words: CRC, immunohistochemistry, α5β1-integrin, MMP-14, prognosis, survival

CRC is the third most common cancer worldwide [1]. CRC is a cause of significant morbidity and cancer-related mortality in China both men and women. More than 17 million new cases of CRC were reported every year in China mainland. Despite recent treatment options and prognosis for patients with advanced CRC have improved through the development of novel drugs, progress in the treatment of CRC has been limited [2,3]. Most newly diagnosed patients will present with incurable disease, and have a median survival of less than 1 year. If metastasis has occurred, patient five-year survival rate after surgery falls dramatically from 90% to less than 10% [4]. It is, therefore, important to increase our understanding of the molecular changes leading to development, spread and metastasis of CRC and to identify potentially prognostic and predictive biomarkers for the disease.

The conspicuous characteristic of malignant tumor is invasion and metastasis. To date, it is now clear that adhesive interaction play a critical role in the process of metastatic tumor dissemination [5].Cell adhesion molecules (CAMs) are involved in cell-cell and cell-extracellular matrix(ECM) binding, a highly complex process [6]. Integrins are the major adhesive molecules in cells and have been associated with metastasis of cancer cells. The integrins , a superfamily of heterodimer cell surface glycoprotein receptors composed of distinct α and β subunits [7], were originally described as cell adhesion receptors, but their functions in cell behavior including motility and invasion and their interactions with classical growth factor receptor signaling pathways have been increasingly recognized in the past few years [8]. Integrins have a modulating effect on several signalling pathways involved in the regulation of cell survival, proliferation, differentiation and apoptosis, and their functions and expression levels are altered in many types of cancer [9]. The majority of integrins demonstrate multiple ligand binding owing to which different integrins may interact with the same matrix protein and produce identical signals [10]. One of the key mechanisms by which integrins modulate tumor progression is the transduction of signals regulating the expression of matrix-specific metalloproteinases (MMP) [11,12]. The role of these enzymes in invasion and metastasis have been dem-

onstrated in a number of reports [13]. The classical fibronectin receptor (FnR), the α5β1-integrin, binds to fibronectin (FN) and has a well-defined role in cell adhesion, migration, matrix formation and angiogenesis. Down-regulation of α5β1-integrin subunit expression level or redistribution in the surface of cancer cells may alter cell adhesion ability to ECM. Within this matrix are a host of matricellular proteins that regulate the expression and function of a myriad of proteins that regulate tumorigenic processes [14]. MMP-14 mainly functions as proteolytic enzymes, and it can break down ECM, and participate in cancer infiltration, metastasis and angiogenesis [15]. A variety of studies demonstrated that expressions of α5β1-integrin and MMP-14 are closely related to occurrence, development and prognosis of CRC [16-20]. Up to now, however, the correlations between expressions of α5β1-integrin and MMP-14 and survival or prognosis in CRC is still inconclusive. Here,we investigated retrospectively the expressions of α5β1-integrin and MMP-14 are correlated to tumor metastasis and poor prognosis in CRC patients.

Materials and methods

Ethics statement. The study was approved by the Ethics Committee of Wuhan General Hospital, Guangzhou Command of the People's Liberation Army. Written informed consent was obtained from all participants.

Patients and tissue samples. The patient cohort comprised 259 consecutive CRC cases from 2000 through 2006 documenting pathologic and clinical factors and clinical outcome at the Department of Oncology of Wuhan General Hospital, Guangzhou Command of People's Liberation Army. None of the patients underwent radiotherapy or chemotherapy before surgery. There were 153 men and 106 women with a mean age of 64.3 (range 26-89). The Dukes' stage was used to classify the tumors, and stage A, B, C, and D lesions were present in 26, 86, 113 and 34 patients, respectively. This study included adenocarcinoma of Non Other Specified type, usually gland-forming carcinomas and classified as well, moderately or poorly differentiated adenocarcinomas, without mucinous differentiation. The grade was based on the percentage of the gland-like structures as follows : well differentiated lesions exhibited glandular structures in > 95 % of the tumor; moderately differentiated lesions had 95-50 % glands; poorly differentiated adenocarcinomas had 5-50% glands and undifferentiated carcinomas had < 5 % glands [21], and 87 tumors were graded as well differentiated, 132 as moderately differentiated, and 40 as poorly differentiated adenocarcinoma. Infiltrative depth classified as mucosa and submucous membrane layer, muscular layer and Serosa layer, and 19 in mucosa and submucous membrane layer, 113 in muscular layer and 127 in serosa layer. Lymph node metastasis happened in 112 patients, and other 147 patients had no metastatic lymph node. The non-tumor part was taken from the grossly normal colorectal mucosa more than 5 cm away from the tumor in resected colorectal specimen.Sixteen patients were excluded from this study because of absence of follow-up. During the follow-up period from the date of surgery until December 31, 2011, 96 patients died and 147 were alive (median follow-up time, 58 mo, range, 2-99 mo).

Immunohistochemistry analysis. The paraffin embedded CRC tissues and distal normal mucosa tissues were cut at 4 μm and mounted on glass slides. Then the slides were dewaxed in xylene and rehydrated in ethanol, and treated with a solution of peroxidase-blocking reagent (Dako, Glostrup, Denmark) to exhaust endogenous peroxidase activity. They were put in 0.01 mol/L citrate buffer at pH 6.0 for 15 minutes in an 800W microwave oven and then left at room temperature for 20 minutes to expose antigen hidden inside the tissue due to formalin fixation. To inhibit non-specific antigen–antibody reactions possible in immunohistochemical staining, protein blocker (Research Genetics, Huntsville, AL, USA) was used for 5 minutes and the slides were washed thoroughly with PBS buffer. Then the slides were incubated overnight with the primary antibodies against α5β1-integrin (1:100, MAB1969, Chemicon (Millipore), USA) and MMP-14 (1:150, MAB3317, Chemicon (Millipore), USA)at 4 centigrade. Biotinylated goat anti-rabbit secondary antibody (1:200; AP307R, Chemicon (Millipore), USA)was applied for 20 minutes at room temperature, followed by further washing with buffer to remove unbound antibody. A complex of avidin with horseradish peroxidase was then applied for 20 minutes at room temperature. For color development, the slides were stained with 3,3'diaminobenzidine (DAB, Sigma–Aldrich, St Louis, MO, USA) then were counterstained with hematoxylin. A reddish brown precipitate in the cytoplasm of cells indicated a positive reaction. In each immunohistochemistry run, the positive section provided by reagent company served as positive control and omission of the primary antibody served as negative control.

Evaluation of score. Immunohistochemistry stained slides were reviewed by two investigators independently blinded to all clinical data. Staining was graded (0, negative; 1, weak; 2, moderate; 3, strong) and percentage of positive staining cells was counted (0, < 5%; 1, 6%-25%; 2, 26%-50%; 3, > 50%). The final score was determined by the combined staining score (intensity + extent). Score 0 was defined as negative expression (-), 1-2 as weak staining pattern(+), 3-4 as moderate staining pattern(++), and 5-6 as strong expression(+++).

Statistical analysis. Positive rates of α5β1-integrin and MMP-14 expressions in CRC and normal mucosa were compared by χ^2 test. The χ^2 test was used to examine the various clinicopathological characteristics and α5β1-integrin and MMP-14 expression. The relation between α5β1-integrin and MMP-14 was assessed by the Spearman correlation test. Cumulative survival curves were drawn by the Kaplan-Meier method. The prognostic value of the 9 variables was tested by univariate analysis using the log-rank test. Multivariate Cox proportional hazard models were used to define the potential prognostic significance of individual parameter. A p value <0.05 was considered statistically significant. All statistical analyses were performed with *SPSS* 15.0 (SPSS Inc., Chicago, IL, USA).

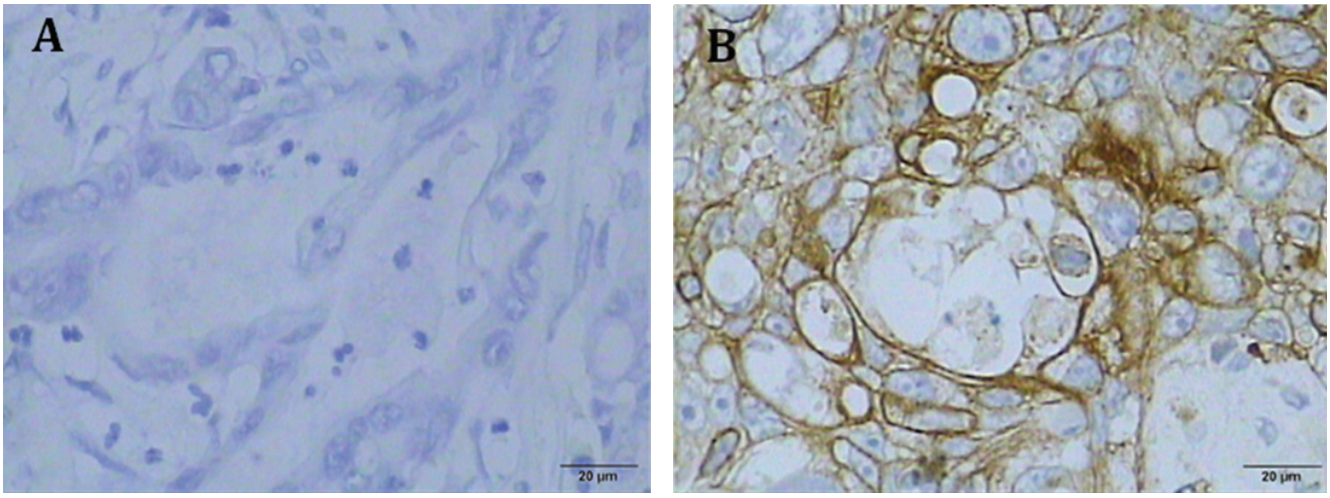

Figure 1. Expression of α5β1-integrin by immunohistochemistry in CRC (IHC, × 400).The negative(A) and positive(B). Bar indicates 20 μ m.

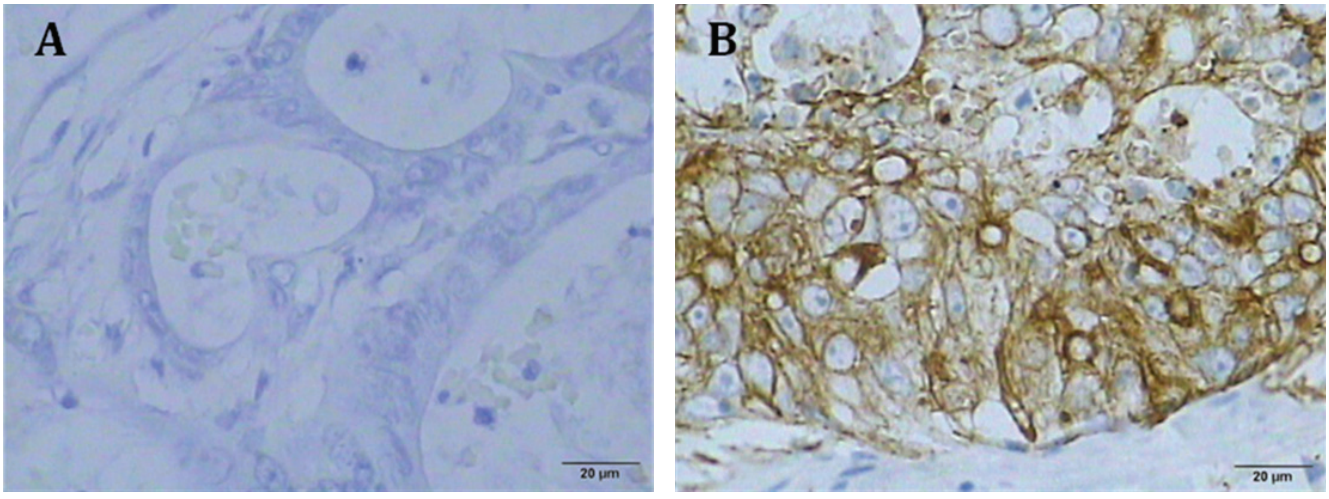

Figure 2. Expression of MMP-14 by immunohistochemistry in CRC (IHC, × 400).The negative(A) and positive(B). Bar indicates 20 μ m.

Results

Pattern of α5β1-integrin and MMP-14 expressions in CRC and normal mucosa. The α5β1-integrin and MMP-14 staining were as performed in 259 CRC patients and 76 cases of normal tissues by immunohistochemistry. The α5β1-integrin expression was detected in 60.6% (157/259) CRC, and in 15.8% (12/76) distal normal mucosa (Figure 1). The MMP-14 expression was detected in 63.3% (164/259) CRC, and in 11.8% (9/76) distal normal mucosa (Figure 2) The expression of α5β1-integrin and MMP-14 protein was found in cytoplasm only. The difference of α5β1-integrin and MMP-14 expression between CRC and normal mucosa was statistically significant (χ^2 = 47.235, P <0.001, χ^2 = 62.351, P < 0.001 respectively).

Correlation of α5β1-integrin and MMP-14 expressions and clinicopathological features in CRC. When comparing the α5β1-integrin and MMP-14 status with clinicopathological variables, we found significant positive correlations between α5β1-integrin and MMP-14 expressions and degree of differentiation (P = 0.004, P = 0.008 respectively), depth of infiltration (P = 0.024, P = 0.002 respectively), lymph node metastasis (P = 0.011, P = 0.039 respectively) .The level of α5β1-integrin and MMP-14 in cases of low Dukes stage (A + B)was lower than that of high stage (C + D) (P = 0.006, P = 0.042 respectively) (Table1).

Correlation between α5β1-integrin and MMP-14 expressions in CRC patients. The positive expression rate of α5β1-integrin and MMP-14 in CRC were 60.6% (157/259) and 63.3% (164/259) respectively. Both for positive expression were

Table 1. The relationship of expression of α5β1-integrin and MMP-14 in CRC and clinicopathological feature

Items	Cases	α5β1-integrin			MMP-14		
		Positive cases	x^2	*P*	Positive cases	x^2	*P*
Gender							
Male	153	98	1.847	0.174	95	0.243	0.622
Female	106	59			69		
Age							
<60years	67	38	0.576	0.448	41	0.176	0.675
≥60years	192	119			123		
Tumor size							
<5cm	108	69	1.013	0.314	73	1.456	0.228
≥5cm	151	88			91		
Differentiated degree							
Well differentiation	87	45			53		
Moderately differentiation	132	79	10.938	0.004**	77	9.724	0.008**
Poor and Undifferentiation	40	33			34		
Infiltrative depth							
Mucosa and submucous	19	11			10		
Muscular	113	79	7.436	0.024*	85	12.244	0.002**
Serosa	127	67			69		
Metastasis of lymph node							
Negative	112	58	6.448	0.011*	63	4.247	0.039*
Positive	147	99			101		
Dukes' stage							
A	26	13			11		
B	86	45	12.461	0.006**	52	8.229	0.042*
C	113	70			75		
D	34	29			26		

* Statistically significant $p < 0.05$, ** $p < 0.01$

134 cases .The Spearman correlation test revealed a significant positive relation between α5β1-integrin and MMP-14 (r = 0.487, $P < 0.001$) (Table 2).

Relationship between α5β1-integrin and MMP-14 expressions and five-year survival rate of CRC patients. There were 16 patients absence of follow-up. The 5-year survival rate for 243 CRC cases was 56.8%. The five-year survival rate of the α5β1-integrin negative patients (75.5%) was higher than that of the α5β1-integrin positive group (44.6%)(Figure 3**A**). The α5β1-integrin expressions appeared as a significant independent prognostic factors (log-rank test , P=0.003). The five-year survival rate for MMP-14-negative and MMP-14-positive cases were 73.1% and 48.2%, respectively. The MMP-14 expressions appeared as a significant independent prognostic factors (log-rank test ,P = 0.011) (Figure 3**B**). The results also showed that both α5β1-integrin and MMP-14 overexpression are two independent alterations that produce the same survival outcome(Figure 3**C**).

Kaplan-Meier analysis showed that the differences of survival in α5β1-integrin and MMP-14 expression groups, tumor differentiation groups, infiltrative depth groups, metastasis of lymph node groups and Dukes' stage groups were highly statistically significant. Importantly, there was no sig-

Table 2. The correlation of expression of α5β1-integrin and MMP-14 in CRC

α5β1-integrin	MMP-14			
	-	+	++	+++
-	72	7	14	9
+	13	11	15	12
++	6	14	23	16
+++	4	10	13	20

Table 3. Univariate Cox regression analysis of overall survival

Factor	Log-rank	*p*
α5β1-integrin expression	8.911	0.003**
MMP-14 expression	6.439	0.011*
Gender	0.016	0.899
Age	0.129	0.719
Tumor size	0.180	0.671
Tumor differentiation	12.264	0.002**
Infiltrative depth	35.011	0.000**
Metastasis of lymph node	5.168	0.023*
Dukes' stage	20.060	0.000**

* Statistically significant $p < 0.05$, ** $p < 0.01$

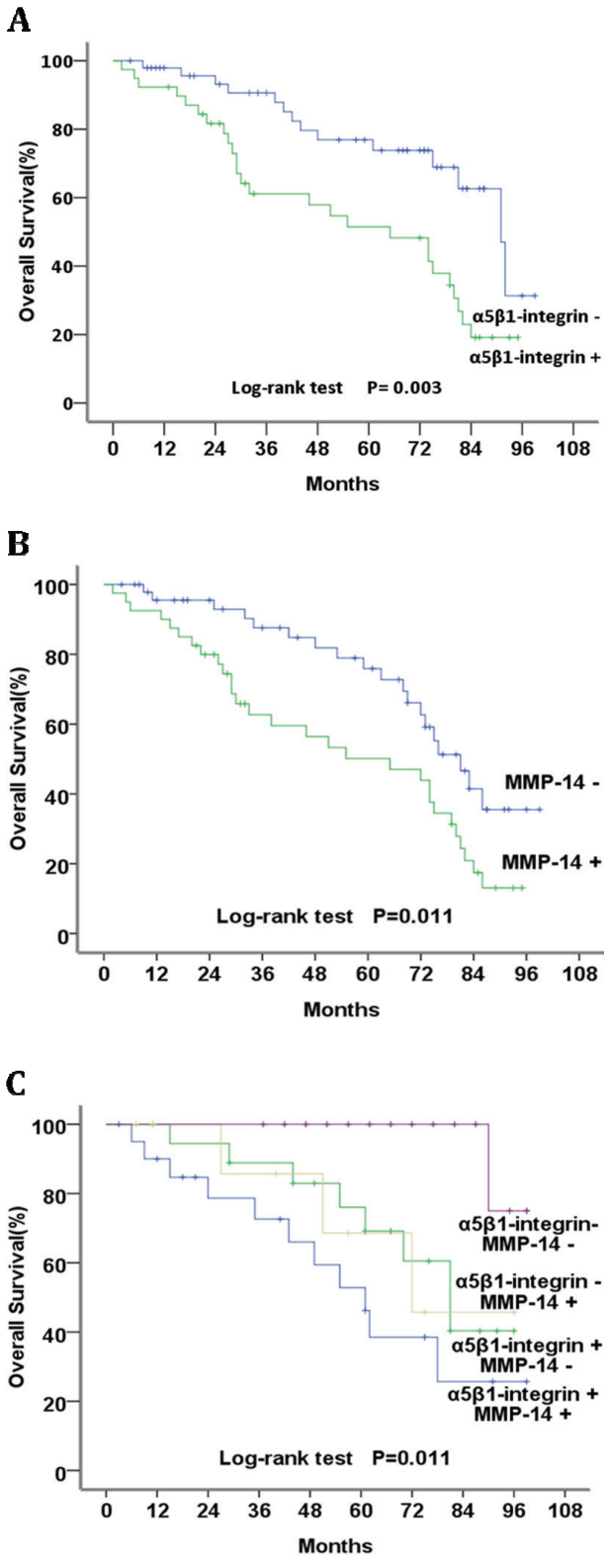

Figure 3. Kaplan-Meier survival curves. Expression of α5β1-integrin, MMP-14, or a combination of both was correlated to Overall Survival (A, B, and C).For some variables, the summed number of cases is lower than the total size of sample because of missing data.

nificant difference in terms of patient age,gender and tumor size(Table 3). The inα5β1-integrin and MMP-14 expression appeared as significantly independent prognostic factors with a relative risk of 1.337 (95%confidence interval, 1.060-1.686) and 1.358 (95%confidence interval, 1.146-1.609) respectively in Cox multivariate analysis, which were done with the following variables for each case: α5β1-integrin expression, MMP-14 expression, infiltrative depth, metastasis of lymph node, and Dukes' stage (Table 4).

Discussion

In previous studies, strong associations between α5β1-integrin expression and poor prognostic factors were revealed in human breast cancer and chondrosarcoma [22,23] .CRC cell mainly express α5β1-integrin and αvβ3-integrin, but α5β1-integrin was more closely correlated to CRC cells biological behavior. Stoeltzing reported [24] that they reduced CRC metastases in mice and enhanced its overall survival by inhibiting α5β1-integrin expression. This study found that α5β1-integrin expression in CRC tissues (157/259,60.6%) is significantly higher than in the corresponding normal mucosa (12/76,15.8%), and there was statistically significant (χ^2=47.235, P < 0.001) . Moreover, the expression of α5β1-integrin in patients with poorly histological differentiation , deep infiltration, lymph node metastasis and high Dukes' staging were heightened, and there were statistically significant (Table 1). Our data suggest that α5β1-integrin play an important role in occurrence, development and metastasis process of CRC.

Despite demonstrating a strong correlation between α5β1-integrin expression and tumor occurrence, development and metastasis, the possible mechanism responsible for this association is that α5β1-integrin stimulate tumor angiogenesis [25].In addition to mediating cell adhesion, integrins make transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways [26,27].The α5β1-integrin/fibronectin interactions may influence also cell survival by modulating apoptosis. In addition, α5β1-integrin signalling can promote also cell survival by up-regulation of anti-apoptotic proteins [28,29], or suppression of apoptotic mediators [30].

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that play a vital role in the proteolysis of structure

Table 4. Multivariate Cox regression analysis of overall survival

Factor	Risk ratio	95% CI	*p* value
α5β1-integrin expression	1.337	1.060-1.686	0.014*
MMP-14 expression	1.358	1.146-1.609	0.036*
Tumor differentiation	1.197	0.891-1.607	0.232
Infiltrative depth	1.604	1.119-2.297	0.010*
Metastasis of lymph node	2.183	0.978-4.873	0.003**
Dukes' stage	2.547	1.997-3.250	0.000**

* Statistically significant p <0.05, ** p <0.01

and signaling components of ECM and in the influence on differentiation, migration, invasion, and proliferation of cells [31]. MMPs play roles within various areas of cancer pathology, including tumor growth, metastasis, and angiogenesis, and MMP activation is increased in nearly all human cancers when compared with normal tissue [32]. Invasion and metastasis of CRC have a close relationship with basement membrane adhesion and ECM degradation. Interestingly, others have reported that MMP-14 acts either directly by degrading ECM components such as type III collagen or indirectly by activating pro-MMP-2 and also by inducing highly vascularized tumors through vascular endothelial growth factor (VEGF) up-regulation [33-35]. In this study, we found MMP-14 that expression in CRC tissues (164/259, 63.3%) is significantly higher than the corresponding normal mucosa (9/76, 11.8%), and there was statistically significant (χ^2=62.351, P<0.001) . Furthermore, high levels of MMP-14 in cancer cells correlated with poorly histological differentiation, deep infiltration, lymph node metastasis and high Dukes' staging (Table 1). Therefore this finding suggests that MMP-14 is likely to play a role in promoting tumor invasion and metastasis.

Our data suggest that α5β1-integrin and MMP-14 may serve as markers for poor prognoses. Roman et al. reported that α5β1-integrin expression have a correlation with the poor prognosis of lung cancer [36]. Fabienne et al. showed that α5β1- integrin engagement could regulate specific pro-survival functions through the activation of glycogen synthase kinase 3β(GSK3β) in Leukemic Cells [37]. Although the detailed molecular mechanism involved in this process is inconclusive, our study have potentially clinical benefits. The α5β1-integrin and MMP-14 expressions that could be detected by IHC might be two useful molecular markers to predict the prognosis in CRC patients.

Moreover, we found that there was a positive correlation between the expression of α5β1-integrin and MMP-14.The Spearman correlation test revealed a significant positive relation between α5β1-integrin and MMP-14 ($r = 0.487$, $P < 0.001$) (Table 2). Hartney et al. [38] showed that elevated integrin-mediated expression of MMPs by mutant macrophages likely contributes to lung pathology. This indicated that integrin and MMPs had mutual effect .These findings suggested that α5β1-integrin plays a functional role in the invasion of studied cells, perhaps by triggering a signaling pathway controlling the activity of MMP-14 gene. Most importance was that α5β1-integrin and MMP-14 also have the common functions of activating MMP-2 [39-42] and stimulating tumor angiogenesis [25,43]. The α5β1-integrin and MMP-14 could promote each other in tumor with invasion and metastasis process and act synergistically.

Taken together, our present study has highlighted the importance of α5β1-integrin and MMP-14 in tumor initiation, progression and metastasis process. We have demonstrated the synergistic effect between α5β1-integrin and MMP-14. Although further work is required to define the molecular mechanisms underlying this relationship, and the expressions of α5β1-integrin and MMP-14 may serve as a valuable tool of clinical assessment of tumour biological behaviour and prognosis in patients with CRC.

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