



Observational Study

## Integrin $\alpha v \beta 6$ and matrix metalloproteinase 9 correlate with survival in gastric cancer

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### Abstract

**AIM:** To investigate the expression of integrin  $\alpha v \beta 6$  and matrix metalloproteinase 9 (MMP-9), their association with prognostic factors and to assess their predictive role in gastric cancer patients.

**METHODS:** Immunohistochemistry was used to determine the expressions of integrin  $\alpha v \beta 6$  and MMP-9 in 126 specimens from patients with primary gastric carcinoma. Associations between immunohistochemical staining and various clinic pathologic variables of tissue specimens were evaluated by the  $\chi^2$  test and Fisher's exact test. Expression correlation of  $\alpha v \beta 6$  and MMP-9 was assessed using bivariate correlation analysis. The patients were followed-up every 3 mo in the first two years and at least every 6 mo afterwards, with a median follow-up of 56 mo (ranging from 2 mo to 94 mo).

Four different combinations of  $\alpha v\beta 6$  and MMP-9 levels (that is, both markers positive, both markers negative,  $\alpha v\beta 6$  positive with MMP-9 negative, and  $\alpha v\beta 6$  negative with MMP-9 positive) were evaluated for their relative effect on survival. The difference in survival curves was evaluated with a log-rank test. Survival analysis was conducted using the Kaplan-Meier survival and Cox proportional hazards model analysis.

**RESULTS:** The expressions of integrin  $\alpha v\beta 6$  and MMP-9 were investigated in 126 cases, among which 34.92% were positive for  $\alpha v\beta 6$  expression, and 42.06% for MMP-9 expression. The expression of  $\alpha v\beta 6$  was associated with Lauren type, differentiation, N stage, and TNM stage (the *P* values were 0.006, 0.038, 0.016, and 0.002, respectively). While MMP-9 expression was associated with differentiation, T stage, N stage, and TNM stage (the *P* values were 0.039, 0.014, 0.033, and 0.008, respectively). The positive correlation between  $\alpha v\beta 6$  and MMP-9 in gastric cancer was confirmed by a correlation analysis. The Kaplan-Meier survival analysis showed that patients with expression of  $\alpha v\beta 6$  or MMP-9 alone died earlier than those with negative expression and that patients who were both  $\alpha v\beta 6$  and MMP-9 positive had a shorter overall survival than those with the opposite pattern (both  $\alpha v\beta 6$  and MMP-9 negative) (*P* = 0.000). A Cox model indicated that positive expression of  $\alpha v\beta 6$  and MMP-9, diffuse Lauren type, as well as a senior grade of N stage, M stage, and TNM stage were predictors of a poor prognosis in univariate analysis. Only  $\alpha v\beta 6$  and MMP-9 retained their significance when adjustments were made for other known prognostic factors in multivariate analysis (RR = 2.632, *P* = 0.003 and RR = 1.813, *P* = 0.007).

**CONCLUSION:** The expression of  $\alpha v\beta 6$  and MMP-9 are closely correlated, and the combinational pattern of  $\alpha v\beta 6$  and MMP-9 can serve as a more effective prognostic index for gastric cancer patients.

**Key words:** Gastric cancer; Integrin  $\alpha v\beta 6$ ; Prognosis; Matrix metalloproteinase 9; Survival

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**Core tip:** Both integrin  $\alpha v\beta 6$  and matrix metalloproteinase 9 (MMP-9) play an important role in the development and progression of cancer. However, the combined effect of the two proteins in gastric cancer is not yet clear. We would like to draw attention to this aspect. In the present study, we demonstrated that the expression of  $\alpha v\beta 6$  and MMP-9 are closely correlated, and the combinational pattern of  $\alpha v\beta 6$  and MMP-9 can serve as a more effective prognostic index for gastric cancer patients.

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## INTRODUCTION

Gastric cancer is the fourth most common cancer world-wide and accounts for approximately 989600 cases annually, with more than half of these cases occurring in East Asia. Gastric cancer also ranks as the second leading cause of cancer-related death<sup>[1]</sup>. Clinical management of gastric cancer is, to some extent, based on the availability of markers that are predictive of prognosis and alterations commonly present in gastric cancer have the potential to serve such a purpose.

Integrin  $\alpha v\beta 6$  is a member of the integrin family and is expressed in epithelial cells only, with fibronectin (FN) as its major ligand. The expression of  $\alpha v\beta 6$  is rare and can hardly be detected in normal epithelial cells<sup>[2]</sup>, but increases substantially in response to injury and/or inflammation and in epithelial tumors<sup>[3]</sup>. Previous studies have shown that the *de novo* expression of integrin  $\alpha v\beta 6$  is involved in the pathogenic processes of gastrointestinal malignancies, including cell adhesion, proliferation, apoptosis, and matrix metalloproteinase (MMPs) secretion<sup>[4-6]</sup>.

Similar to the integrin family, members of the MMP family, including MMP-9, have been implicated in tumor invasion and metastasis, which are characterized by a zinc atom at the active site and classified according to homologies in sequence and substrate affinity<sup>[7]</sup>. MMP-9 has been confirmed to be associated with the malignant behavior of tumors or the eventual clinical outcome of patients afflicted with the disease and has a unique ability to degrade type IV collagen, which is a major component of the basement membrane<sup>[8]</sup>.

Our prior research has suggested that  $\alpha v\beta 6$  integrin is an independent prognostic indicator of gastric cancer<sup>[9]</sup>. Since that initial report, limited work has been conducted to highlight the predictive value of  $\alpha v\beta 6$  in gastrointestinal cancers, until the research of Peng *et al*<sup>[10]</sup> on colorectal cancer. The fact that no index alone can be simply explicable in terms of its effect on cancer prognosis prompted us to focus on the combined analysis of both  $\alpha v\beta 6$  and MMP-9. In the present study, we undertook an immunohistochemical analysis of  $\alpha v\beta 6$  and MMP-9 expression in gastric cancer. Their correlation with clinical characteristics and prognosis was assessed to determine whether the combinational pattern of  $\alpha v\beta 6$  and MMP-9 expression could be used as a more effective prognostic index for gastric cancer patients after curative surgery.

## MATERIALS AND METHODS

### Antibodies and reagents

The mouse monoclonal integrin  $\alpha v\beta 6$  antibody 6.2A1 (1:500, Biogen, Cambridge, MA, United States)<sup>[9]</sup> and

rabbit polyclonal MMP-9 antibody ab38898 (1:100, Abcam, Cambridge, MA, United States)<sup>[11]</sup> were used for integrin  $\alpha v \beta 6$  and MMP-9 immunohistochemical staining, respectively. Specificity of the two staining patterns was confirmed by the use of irrelevant control antibodies.

### Immunohistochemistry

Immunohistochemistry was performed on 5 micrometer sections of the formalin-fixed, paraffin-embedded routine sections, and tissue microarray (TMA) analysis using antibodies against  $\alpha v \beta 6$  (6.2A1) and MMP-9 (ab38898) was applied. The sections were deparaffinized and hydrated, and the heat induced epitope retrieval was performed with Borg decloaking high pH buffer in the Biocare decloaking chamber. Subsequently, endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and then the slides were incubated first with an avidin-biotin kit, followed by incubations with the  $\alpha v \beta 6$  primary antibody (1:500) and the MMP-9 primary antibody (1:100) overnight at 4 °C, streptavidin-horseradish peroxidase, and betazoid diaminobenzidine for color development. Negative controls were prepared by using the identical concentration of mouse immunoglobulin IgG1 (Dako). The next day, biotinylated anti-mouse IgG and anti-rabbit IgG (1:200) were applied to the slides, which were subsequently treated using horseradish peroxidase (HRP)-labelled streptoantibiotin (Dako) for 15 min. Finally, all the slides were counterstained with Dako hematoxylin, rinsed with water, and then dehydrated with alcohol and xylene, and cover-slipped. Appropriate controls were included. All incubations were conducted at room temperature.

### Assessment of $\alpha v \beta 6$ and MMP-9 scoring in the tissue sections

Assessment of the two proteins' staining was evaluated independently by two pathologists who were blinded to the patients' clinical outcome. Disagreements were resolved by discussion in a meeting to obtain the final results. The staining stratification was established based on two scores: (1) the proportion score representing the fraction of positive staining cells (0, 0%; 1, < 20%; 2, 20%-50%; 3, 51%-75%; and 4, > 75%, respectively); and (2) the intensity of the staining (0, no staining; 1, weak staining, pale brown; 2, moderate staining, brown; and 3, strong staining, dark brown, respectively). Such assessment allows for a semi-quantitative estimate of the expression levels of protein in the tissue section. The two scores were added, and the final definition of every section was obtained. Finally, tumors with a final score of < 2 were designated as negative, and > 2 as positive, in both  $\alpha v \beta 6$  and MMP-9 expression.

### Patients and follow-up

A series of consecutive specimens from tumors of 126

primary gastric cancer patients, with curative resection, were obtained from the Department of Pathology, Qilu Hospital, Shandong University (Jinan, China), from January 2007 to June 2010 and were formalin-fixed and paraffin-embedded. All of the specimens were procured before any adjuvant treatment and after the informed consent from the patients, with the approval of the Ethics Committee of Qilu Hospital.

According to our protocol, all patients were followed up every 3 mo in the first two years and at least every 6 mo afterwards, until June 2015, with a median follow-up of 56 mo (ranging from 2 mo to 94 mo). They were examined by regular abdomen ultrasonography or computed tomography (CT), monitoring the relapse of the disease. Observations were censored at either the last follow-up date or the terminal date of the follow-up if death had not occurred.

### Statistical analysis

Statistical analyses were performed with SPSS 13.0. Associations between immunohistochemical staining and clinicopathologic variables of tissue specimens were evaluated by the  $\chi^2$  test and Fisher's exact test. Expression correlation of  $\alpha v \beta 6$  and MMP-9 was assessed using bivariate correlation analysis. The difference in survival curves was evaluated with a log-rank test. Survival analysis was conducted using the Kaplan-Meier survival and Cox proportional hazards model analysis. A value of  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Expression of $\alpha v \beta 6$ and MMP-9 and the association with clinical factors

We characterized the expression of  $\alpha v \beta 6$  and MMP-9 by immunohistochemical staining in gastric cancer from 126 patients (Figure 1), aged from 27 to 81. Of these, 44 (34.92%) were positive for  $\alpha v \beta 6$  expression, with 82 (65.08%) being negative. For MMP-9, 53 (42.06%) patients were positive for expression, and 73 (57.93%) were negative.

As in our clinical correlation studies, the immunohistochemical analysis was conducted without knowledge of any clinical information regarding prognostic outcome. Levels of the two proteins' expression were compared with tumor characteristics and risk factors (Table 1). The following analysis showed that Lauren type, differentiation, N stage, and TNM stage were associated with  $\alpha v \beta 6$  expression (the  $P$  values were 0.006, 0.038, 0.016, and 0.002, respectively). Regarding MMP-9 expression, statistically significant differences existed in differentiation, T stage, N stage, and TNM stage (the  $P$  values were 0.039, 0.014, 0.033, and 0.008, respectively). No evidence of a significant association was observed between alteration of  $\alpha v \beta 6$  or MMP-9 expression and other characteristics or risk factors.

**Table 1** Expression of integrin  $\alpha v \beta 6$  and MMP-9 and patient characteristics

Characteristic	No.	$\alpha v \beta 6$		P value	MMP-9		P value
		Negative	Positive		Negative	Positive	
Age (yr)							
< 60	51	33	18	0.942	31	20	0.593
> 60	75	49	26		42	33	
Gender							
Male	78	50	28	0.769	43	35	0.416
Female	48	32	16		30	18	
Location							
Upper	27	20	7	0.136	16	11	0.847
Middle	51	28	23		28	23	
Lower	48	34	14		29	19	
Lauren type							
Intestinal	83	47	36	0.006	46	37	0.427
Diffuse	43	35	8		27	16	
Differentiation							
Well	19	15	4	0.038	6	13	0.039
Moderate	30	14	16		18	12	
Poor	77	53	24		49	28	
T stage							
T1	14	10	4	0.578	3	11	0.014
T2	13	7	6		8	5	
T3	18	10	8		14	4	
T4	81	55	26		48	33	
N stage							
N0	37	26	11	0.016	18	19	0.033
N1	17	12	5		10	7	
N2	26	10	16		11	15	
N3	46	34	12		34	12	
M stage							
M0	124	81	43	0.652	73	51	0.094
M1	2	1	1		0	2	
TNM stage							
I	12	4	8	0.002	3	9	0.008
II	42	36	6		22	20	
III	70	41	29		48	22	
IV	2	1	1		0	2	

MMP-9: Matrix metalloproteinase 9.

**Table 2** Expression correlation of integrin  $\alpha v \beta 6$  and MMP-9

$\alpha v \beta 6$ expression	MMP-9 expression		Total
	Negative	Positive	
Negative	56	26	82
Positive	17	27	44
Total	73	53	126

MMP-9: Matrix metalloproteinase 9.

**Role of both  $\alpha v \beta 6$  and MMP-9 in cancer-related survival**

The Kaplan-Meier plot (Figure 2A and B) showed that patients with positive  $\alpha v \beta 6$  expression had a much shorter survival time than the negative cases ( $P = 0.000$ ), consistent with our previous study<sup>[9]</sup>. The same tendency was present in MMP-9 positive expression cases ( $P = 0.000$ ).

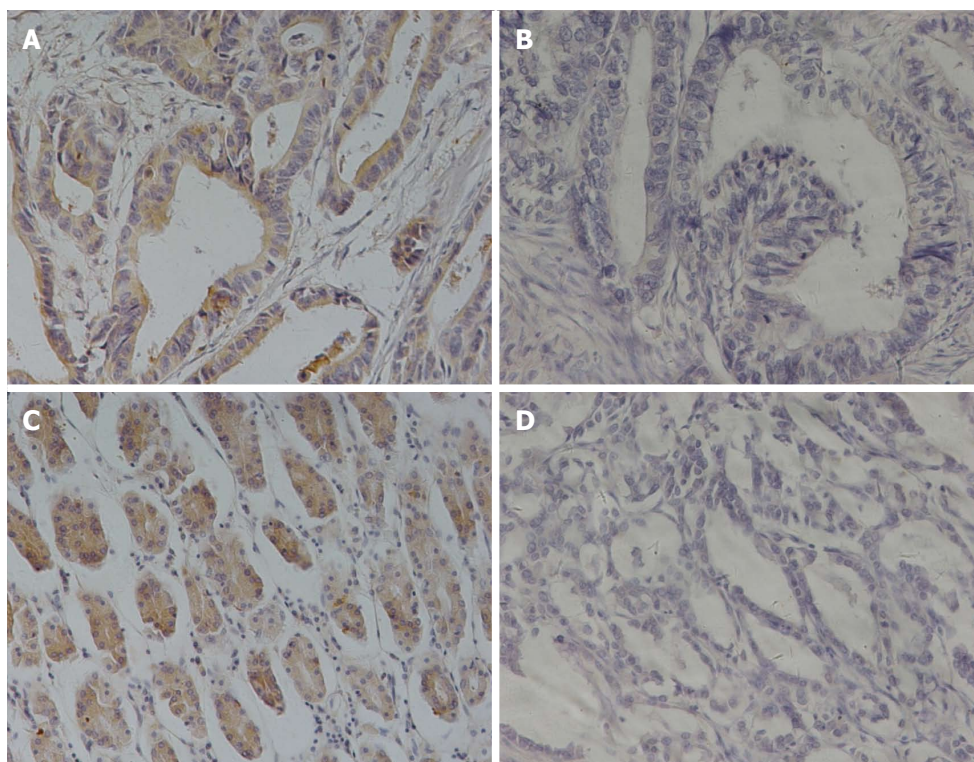
The expression of  $\alpha v \beta 6$  and MMP-9 were closely correlated in gastric cancers ( $r = 0.286$ ,  $P = 0.001$ ) (Table 2), which indicated that the combined analysis of the two proteins may be more helpful in predicting the survival for this disease. A striking stratification

of mortality risk was observed when four different combinations of  $\alpha v \beta 6$  and MMP-9 levels (that is, both markers positive, both markers negative,  $\alpha v \beta 6$  positive with MMP-9 negative, and  $\alpha v \beta 6$  negative with MMP-9 positive) were evaluated for their relative effect on survival. Kaplan-Meier plots showed that patients who were both  $\alpha v \beta 6$  and MMP-9 positive died earlier ( $26.45 \pm 5.54$ ) than the other three groups ( $53.40 \pm 5.90$ ,  $51.54 \pm 6.86$ , and  $68.86 \pm 4.87$ , respectively;  $P = 0.000$ ). The greatest difference in survival was found between patients with both markers being positive and those with the opposite pattern (both markers negative) (Figure 2C).

**Univariate and multivariate analysis for prognosis of patients with gastric cancer**

Sufficient clinical follow-up data were obtained from all 126 patients, allowing for evaluation of the association between protein alteration and prognostic outcome. Of these, 69 (54.8%) cases were confirmed as cancer-related deaths within 5 years of prognosis and 57 (45.2%) were censored, as the follow-up survey was





**Figure 1** Comparison of immunohistochemical staining of  $\alpha v\beta 6$  and MMP-9 in the same gastric cancer specimen evaluated with formalin-fixed, paraffin-embedded tissue (streptavidin-peroxidase method; original magnification  $\times 400$ ). Positive  $\alpha v\beta 6$  expression was detected predominantly in the cell membrane, and matrix metalloproteinase 9 (MMP-9) mainly exhibited a cytoplasmic immunostaining in tumor cells. A: Positive  $\alpha v\beta 6$  expression; B: Negative  $\alpha v\beta 6$  expression; C: Positive MMP-9 expression; D: Negative MMP-9 expression.

discontinued or patients were alive beyond 60 mo or died of reasons other than this disease.

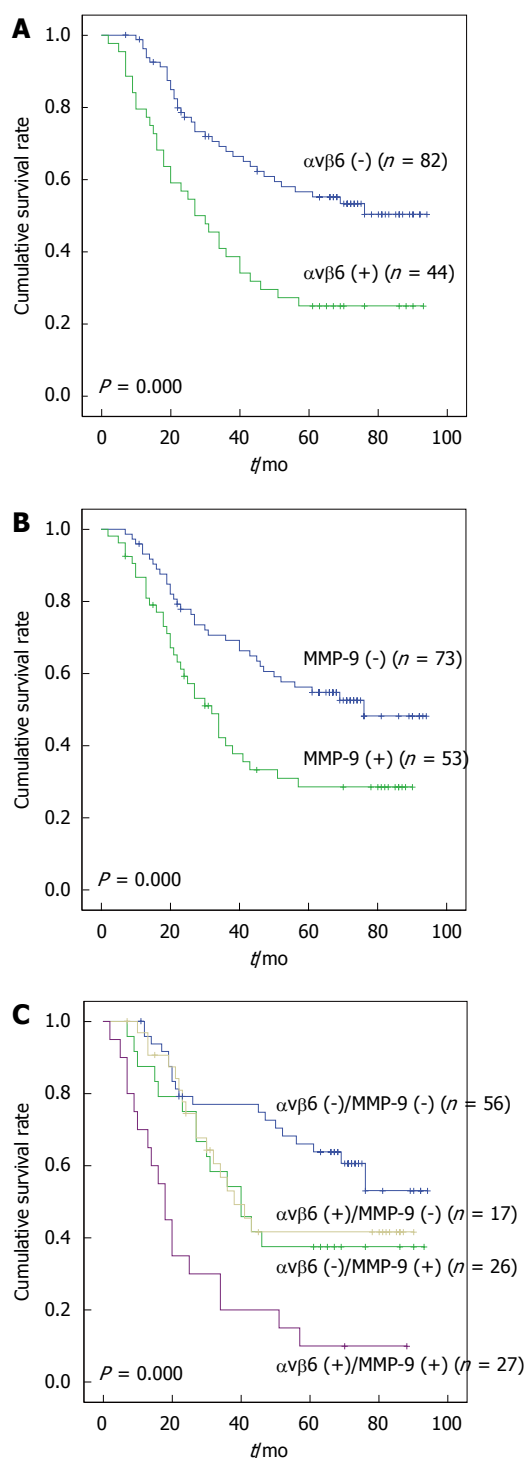
We estimated the relative risks (RR) (both univariate and multivariate) of dying and 95%CI, using the Cox proportional hazard model (Table 3). The Cox model indicated that positive expressions of  $\alpha v\beta 6$  and MMP-9, diffuse Lauren as well as high grades of N, M, and TNM stages were predictors of a poor prognosis in the univariate analysis. Then, the variables with statistical values of  $P < 0.05$  were chosen for multivariate analysis, and only  $\alpha v\beta 6$  and MMP-9 retained their significance after adjustments for other known prognostic factors, which revealed that positive expression of  $\alpha v\beta 6$  and MMP-9 were unfavorable independent prognostic factors ( $P = 0.003$  and  $P = 0.007$ , respectively) and superior to all other known prognostic factors.

## DISCUSSION

Molecular markers associated with distinct clinical outcomes have been identified in gastric cancer, laying the groundwork for improved clinical management through more personalized medicine. Despite improvements during the past years, the prognostic outcome of gastric cancer remains unsatisfactory. Molecular investigation of cancerous pathogenesis would contribute to the treatment of patients with gastric cancer<sup>[12]</sup>, which suggests that the current

molecular research is far from sufficient.

Considerable evidence now exists that alteration in the expression of  $\alpha v\beta 6$  or MMP-9 may serve as an important pathogenic event for some human malignancies. We have previously shown that  $\alpha v\beta 6$  is an independent prognostic indicator in gastric cancer<sup>[9]</sup> and simultaneously extended its pathogenic role to mediating the potential for colon cancer cells to colonize in and metastasize to the liver<sup>[13]</sup>. When it came to MMP-9, a number of studies have been published on its prognostic value in human cancers. High expression of MMP-9 was found to be associated with lymph node metastasis and a higher tumor stage in breast cancer<sup>[14,15]</sup>. Moreover, MMP-9 is also implicated in the progression of esophageal squamous cell carcinoma<sup>[16]</sup>. Meanwhile, MMP-9 status represents a novel prognostic factor in evaluation of colorectal cancer<sup>[17]</sup>, especially in combination with the membrane-anchored MMP regulator RECK<sup>[18]</sup>. As to the prognostic role of MMP-9 in gastric cancer, a consensus has not been reached in previous reports<sup>[19-23]</sup>. Given the variable natural history and heterogeneity of gastric cancer, conflicting studies published to date suffer from a similar problem: the interference from neoadjuvant chemotherapy, which was recently confirmed<sup>[24,25]</sup>. To exclude the possibility of chemotherapy interfering with the two proteins' predictive role in gastric cancer, only patients who had not received neoadjuvant chemotherapy were enrolled.



**Figure 2** Kaplan-Meier plots show the association of survival and expression of integrin  $\alpha v\beta 6$  and MMP-9 in all gastric cancer patients tested. A: Positive expression of  $\alpha v\beta 6$  was associated with decreased survival ( $P = 0.000$ ); B: The same tendency was present in matrix metalloproteinase 9 (MMP-9) positive expression cases ( $P = 0.000$ ); C: When  $\alpha v\beta 6$  and MMP-9 alterations were analyzed together, patients with both  $\alpha v\beta 6$  and MMP-9 positive expression experienced the highest mortality of the four groups ( $P = 0.000$ ).

In the present study, the expression of integrin  $\alpha v\beta 6$  and MMP-9 in 126 gastric cancer specimens was investigated, which showed a 34.92% incidence of  $\alpha v\beta 6$  expression, concordant with previously published positive rates of 36.7% in gastric cancer<sup>[9]</sup>, as well as a

**Table 3** Univariate and multivariate analyses comparing overall survival to prognostic factors in 126 gastric cancer patients

Factors	Univariate analysis		Multivariate analysis	
	Relative risk (95%CI)	P value	Relative risk (95%CI)	P value
Age (yr)				
< 60	1			
> 60	1.800 (1.090-2.973)	0.258		
Gender				
Female	1			
Male	1.450 (0.882-2.381)	0.143		
Location				
Upper	1			
Middle/Lower	1.171 (0.856-1.602)	0.323		
Lauren type				
Intestinal	1			
Diffuse	1.836 (1.061-3.177)	0.03	1.431 (0.805-2.545)	0.222
Differentiation				
Well	1			
Moderate/Poor	1.045 (0.764-1.429)	0.784		
T stage				
T1	1		1	
T2-4	1.295 (1.006-1.668)	0.050	1.248 (0.813-1.916)	0.311
N stage				
N0	1		1	
N1-3	1.343 (1.105-1.633)	0.003	1.249 (0.818-1.909)	0.303
M stage				
M0	1		1	
M1	26.25 (2.887-238.81)	0.004	8.555 (0.735-99.62)	0.087
TNM stage				
I	1		1	
II-IV	2.314 (1.502-3.565)	< 0.001	1.654 (0.643-4.257)	0.296
$\alpha v\beta 6$				
Negative	1		1	
Positive	2.862 (1.452-3.747)	< 0.001	2.632 (1.569-4.416)	0.003
MMP-9				
Negative	1		1	
Positive	2.193 (1.110-2.787)	0.005	1.813 (1.398-2.351)	0.007

MMP-9: Matrix metalloproteinase 9.

42.06% incidence of MMP-9 expression. Subsequently, a positive correlation between the two proteins was confirmed, and each of them was associated with a poor prognosis in gastric cancer. When comparing different combinational expression patterns in the Kaplan-Meier survival analysis, patients who were both  $\alpha v\beta 6$  and MMP-9 positive suffered a higher mortality rate than the other three expression states, especially in comparison to the opposite pattern. Furthermore, a Cox model indicated that positive expression of  $\alpha v\beta 6$  and MMP-9, diffuse Lauren type, as well as a higher grade of N, M, and TNM stage were

predictors of a poor prognosis in univariate analysis. Only  $\alpha v \beta 6$  and MMP-9 retained their significance when adjustments were made for other known prognostic factors in multivariate analysis. Based on these data, we proposed that the concomitant expression of  $\alpha v \beta 6$  and MMP-9 in gastric cancer could serve as a more effective independent predictor to determine early relapse, metastases and prognosis in gastric cancer patients.

As an exploratory attempt, this paper intends to provide some enlightenment for the clinical management of gastric cancer patients in the future. We have previously confirmed a direct linkage between ERK2 and the cytoplasmic domain of  $\beta 6$ , as well as the binding domain for ERK2 within the cytoplasmic tail of the  $\beta 6$ -integrin subunit<sup>[26,27]</sup>, through which integrin  $\alpha v \beta 6$  transmitted a growth enhancing signal to cancer cells. Previous studies have shown that the expression of MMP-9 has a direct correlation to integrin  $\alpha v \beta 6$ <sup>[5,28]</sup>. Thus, a signaling pathway mediated by ERK2 between  $\alpha v \beta 6$  and MMP-9 was theoretically supposed to exist to play a key role in the biological behavior and/or clinical outcome of human gastric cancer.

In summary, we concur with Zhang *et al.*<sup>[22]</sup> that  $\alpha v \beta 6$  should be a focus of attention for diagnosis and therapy in human gastric cancers; our current findings extend this idea to the combination of  $\alpha v \beta 6$  and MMP-9. The expression of  $\alpha v \beta 6$  and MMP-9 are closely correlated, and the combinational pattern of  $\alpha v \beta 6$  and MMP-9 can serve as a more effective prognostic index for gastric cancer patients. However, further research is necessary to identify the signaling pathway between  $\alpha v \beta 6$  and MMP-9 as an important target for gastric cancer therapy.

## COMMENTS

### Background

Both integrin  $\alpha v \beta 6$  and matrix metalloproteinase 9 (MMP-9) play an important role in the development and progression of cancer. However, the combined effect of the two proteins in gastric cancer is not yet clear. The fact that no index alone can be simply explicable in terms of its effect on cancer prognosis prompted us to focus on the combined analysis of both  $\alpha v \beta 6$  and MMP-9.

### Research frontiers

The prior research by the authors suggested that  $\alpha v \beta 6$  integrin is an independent prognostic indicator of gastric cancer. Since that initial report, limited work has been conducted to highlight the predictive value of  $\alpha v \beta 6$  in gastrointestinal cancers, until the research of Peng *et al.* on colorectal cancer.

### Innovations and breakthroughs

The combinational pattern of  $\alpha v \beta 6$  and MMP-9 expression was demonstrated to be more effective than  $\alpha v \beta 6$  alone in prognosis of gastric cancer.

### Applications

The combinational detection of  $\alpha v \beta 6$  and MMP-9 expression could be used as a more effective prognostic index for gastric cancer patients after curative surgery.

### Terminology

Integrin  $\alpha v \beta 6$  is a member of the integrin family and is expressed in epithelial

cells only, but increases substantially in response to injury and/or inflammation and in epithelial tumors.

## Peer-review

In the present study, the authors undertook an immunohistochemical analysis of  $\alpha v \beta 6$  and MMP-9 expression in gastric cancer. The correlation with clinical characteristics and prognosis was assessed to determine whether the combinational pattern of  $\alpha v \beta 6$  and MMP-9 expression could be used as a more effective prognostic index for gastric cancer patients after curative surgery. Over all, this is an interesting manuscript about the expression of integrin  $\alpha v \beta 6$  and MMP-9 in gastric cancer patients.

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