

β -catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: Relationships with carcinogenesis and metastasis

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Abstract

AIM: To investigate whether abnormal expression of β -catenin in conjunction with overexpression of cyclinD1, c-myc and matrix metalloproteinase-7 (MMP-7) correlated with the carcinogenesis, metastasis and prognosis of pancreatic cancer, and to analyze the relationship of β -catenin expression with cyclinD1, c-myc and MMP-7 expression.

METHODS: Using immunohistochemistry, we examined the expression of β -catenin, cyclinD1, c-myc and MMP-7 in 47 pancreatic adenocarcinoma tissues, 12 pancreatic intraepithelial neoplasia (PanIN) and 10 normal pancreases, respectively. Proliferation cell nuclear antigen was also tested as the index of proliferative activity of pancreatic cancer cells.

RESULTS: In 10 cases of normal pancreatic tissues, epithelial cells showed equally strong membranous expression of β -catenin protein at the cell-cell boundaries, but the expression of cyclinD1, c-myc and MMP-7 was negative. The expression of β -catenin, cyclinD1, c-myc and MMP-7 in PanIN and pancreatic adenocarcinoma tissues had no significant difference [6/12 and 32/47 (68.1%), 6/12 and 35/47 (74.5%), 5/12 and 33/47 (70.2%), 7/12 and 30/47 (63.8%), respectively]. The abnormal expression of β -catenin was significantly correlated to metastasis and one-year survival rate of pancreatic cancer, but had no relation with size, differentiation and cell proliferation. The expression of cyclinD1 was correlated with cell proliferation and extent of differentiation, but not with size, metastasis and one-year survival rate of the pancreatic cancer. The expression of c-myc was not correlated with size, extent of differentiation, metastasis and 1-year survival rate, but closely with cell proliferation of pancreatic cancer. The overexpression of MMP-7 was significantly associated with metastasis and 1-year survival rate of

pancreatic cancer, but not with size, extent of differentiation and cell proliferation. There was a highly significant positive association between abnormal expression of β -catenin and overexpression of cyclinD1, c-myc and MMP-7 not only in PanIN ($r = 1.000, 0.845, 0.845$), but also in pancreatic cancer ($r = 0.437, 0.452, 0.435$).

CONCLUSION: The abnormal expression of β -catenin plays a key role in the carcinogenesis and progression of human pancreatic carcinoma by up-regulating the expression of cyclinD1, c-myc and MMP-7, resulting in the degradation of extracellular matrix and uncontrolled cell proliferation and differentiation. β -catenin abnormal expression and MMP-7 overexpression may be considered as two useful markers for determining metastasis and prognosis of human pancreatic cancer.

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Key words: cyclinD1; c-myc; MMP-7

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INTRODUCTION

β -catenin is a multifunctional cytoplasmic protein. It links E-cadherin and α -catenin to cytoskeleton constituting E-cadherin-catenin complex to maintain normal epithelial polarity and intercellular adhesion, and regulate cellular differentiation and proliferation^[1-4]. Reduction and loss of β -catenin or other molecules might disrupt stability and integrity of the E-cadherin-catenin complex and disturb cellular adhesive junction, resulting in cell proliferation, invasion and metastasis of tumor^[5-9]. Our previous study has found that abnormal expression of E-cadherin and α , β -catenin significantly correlated with differentiation, lymph node and liver metastasis of pancreatic cancer^[10]. Recently, β -catenin has been found as an important member in the Wnt signaling pathway, which is conserved in various organisms from worms to mammals, and plays important roles in cellular development, proliferation and differentiation^[11-14]. In the absence of Wnt signals, β -catenin is sequestered in a complex with the adenomatous polyposis coli tumor suppressor, AXIN, and a serine threonine glycogen synthetase kinase-

3 β (GSK-3 β), enabling phosphorylation and degradation of free β -catenin by the ubiquitin-proteasome system^[11-14]. Thus, in normal epithelial cells, levels of free cytosolic β -catenin are low. During embryogenesis and activation of the Wnt signaling pathway, phosphorylation by GSK-3 β is inhibited, and β -catenin accumulates in the cytosol at high levels, binds to cytosolic T cell-factor/lymphoid-enhancer-factor (Tcf/Lef) transcription factors, and the resulting complex is shuttled to the nucleus, leading to activation of target genes expression^[14-16]. Recent studies have demonstrated that cyclinD1, c-myc and MMP-7 were important target genes of Wnt signaling pathway and overexpression of them was highly associated with accumulation of β -catenin and mutational defects of the Wnt signaling pathway in numerous tumor types^[17-23], but there were only few reports in pancreatic cancer, and the results were conflicting^[7,23-28].

To investigate the role of the Wnt signaling pathway in carcinogenesis and progression of pancreatic cancer, we detected expression of β -catenin, cyclinD1, c-myc and MMP-7 in normal pancreas, pancreatic intraepithelial neoplasia (PanIN) and pancreatic adenocarcinoma by immunohistochemistry.

MATERIALS AND METHODS

Patients and specimens

Forty-seven specimens of pancreatic carcinomas were collected from the Department of Pathology, Affiliated Hospital of Medical College, Qingdao University from 1995 to 1999. These included 29 male and 18 female patients with a mean age of 56.7 years (range 27-75 years). No patient received radiotherapy or chemotherapy before surgery. The tumor size was divided into three groups: smaller than 3 cm in diameter in 9 patients, 3-5 cm in 15 patients and larger than 5 cm in 23. Histologically, all cases were infiltrative ductal adenocarcinoma. According to the Modified Kloppel Histological Grading System^[29], 10 cases were in grade I (well differentiated), 16 in grade II (moderately differentiated) and 21 in grade III (poorly differentiated). Twenty-five cases were with local and distant lymph node metastases, and 22 without metastasis. All patients were followed up for more than 1 year; 13 patients survived more than 12 mo and 34 patients died within 12 mo. The one-year survival rate was 27.6%. Twelve of 47 cases of pancreatic carcinoma tissues had regions containing identifiable PanIN lesions (all cases were PanIN-2). Ten normal adult pancreatic tissues were obtained from healthy young men who died of traffic accidents.

Immunohistochemistry

All specimens were fixed in 100 mL/L neutral formalin for 24-48 h, embedded by paraffin, and cut into 4- μ m thick sections for immunohistochemical staining. PicTureTM method was used (Zymed, USA) as well as the following antibodies: β -catenin monoclonal antibody (diluted 1:100, Sigma, USA); cyclinD1, c-myc and MMP-7 monoclonal antibodies (ready for use, Zymed, USA); proliferation cell nuclear antigen (PCNA) monoclonal antibody (ready for use, Dako, USA) respectively. Before staining, the sections were pretreated with microwave (4 min \times 4 min at 900 W) in 0.1 mol/L

citrate buffer for antigen retrieval. DAB was used for chromogen. PBS was substituted for primary antibodies as negative control.

Evaluation of immunohistochemical staining

The slides were reviewed by two independent observers who had no knowledge of patients' outcome. The positive staining of β -catenin appeared in brown, and was located in the cell membrane. Staining grade was based on the method described by Jawhari *et al*^[30]: 0 score: no expression; 1 score: expression in cytoplasm; 2 scores: decreased expression; 3 scores: expression in cell membrane, namely normal expression. A score of 0-2 was abnormal expression. The expression of cyclinD1 and c-myc was stained in nucleus. A semi-quantitative evaluation was used to determine positive expression of cells by viewing 10 high power fields (\times 400)^[31]: negative (-), cells were stained less than 10%; mildly positive, cells were stained at 11-25%; moderately positive, cells were stained at 26-50%; strongly positive, cells were stained over 50%. And we regarded the last three grades as positive (+, or overexpression). Positive immunostaining of MMP-7 was predominantly localized in cytoplasm. The slides were regarded as negative (-), and positive (+, or overexpression) if the count of positive cells was less than 20% and exceeded 20%. The positive staining of PCNA was located in the nuclei of pancreatic cancer cells with brown-yellow granules. The expression of PCNA was classified into four grades according to the count of positive cells, as follows^[32]: 1, less than 25%; 2, between 26 and 50%; 3, between 51 and 75%; 4, more than 76%. Grades 1 and 2 were defined as low proliferative activity, and grades 3 and 4 were defined as high proliferative activity.

Statistical analysis

Statistical analysis was performed using the χ^2 test or Fisher's exact test and Spearman rank correlation coefficient analysis. $P < 0.05$ was considered significant.

RESULTS

Expression of β -catenin, cyclinD1, c-myc and MMP-7 in normal pancreas, PanIN and pancreatic carcinoma tissues

The immunoreactivity of β -catenin was expressed by normal ductal and acinar cells with strong membranous staining at the intercellular border in 10 cases of adult normal pancreases. The rates of abnormal expression of β -catenin in pancreatic carcinoma and PanIN tissues were 68.1% (32/47) and 6/12 ($\chi^2 = 0.689$, $P > 0.05$). The expression of β -catenin in most cases was in cytoplasm, whereas membranous staining reduced or disappeared (Figures 1A-C). The positive staining of cyclinD1 and c-myc was located in the nucleus (Figures 2A-D), and was not detected in 10 normal tissues of the pancreas. The positive rates of cyclinD1 and c-myc in pancreatic carcinoma and PanIN tissues were 74.5% (35/47) and 6/12 ($\chi^2 = 0.213$, $P > 0.05$), 70.2% (33/47) and 5/12 ($P = 1.000$, $P > 0.05$), respectively. Positive immunostaining of MMP-7 was predominantly localized in cytoplasm (Figures 3A-C), and it was detected in 30 (63.8%) of the 47 tumor tissues and 7 of the 12 PanIN tissues ($\chi^2 = 0.124$, $P > 0.05$), but not in any of the normal pancreases.

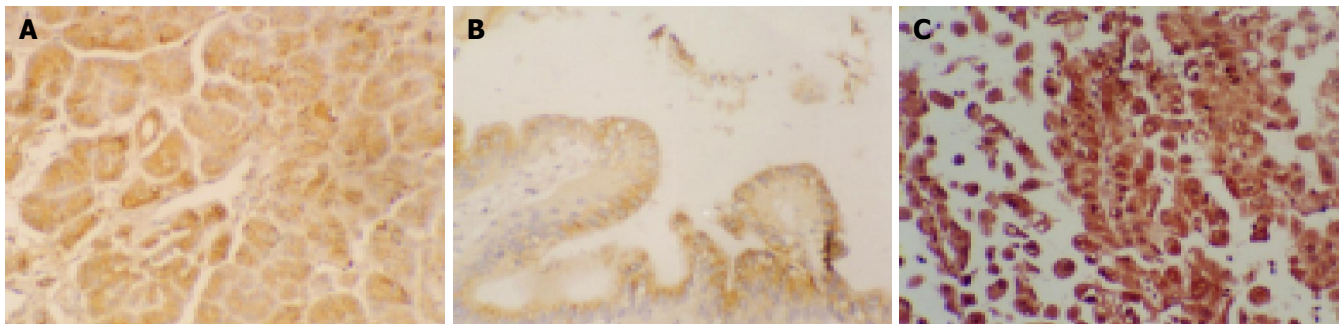


Figure 1 Immunohistochemical PicTure™ staining. A: Immunoreactivity of β -catenin was expressed by normal ductal and acinar cells with strong membranous staining at intercellular border, $\times 100$; B and C: Immunoreactivity of β -catenin was mainly located in cytoplasm of PanIN and pancreatic cancer cells, $\times 200$.

Expression of PCNA in pancreatic carcinoma tissues

The positive staining of PCNA was located in the nuclei of pancreatic cancer cells with brown-yellow granules (Figures 3A-C). There were 6 cases of grade 1, 13 cases of grade 2, 16 cases of grade 3 and 12 cases of grade 4 in 47 cases of pancreatic cancer. Low proliferative activity was in 19 cases (grades 1 and 2) and high proliferative activity in 28 cases (grades 3 and 4).

Relationships between expression of β -catenin, cyclinD1, c-myc and MMP-7 and clinicopathologic characteristics of pancreatic carcinoma

The abnormal expression of β -catenin was significantly correlated to metastasis and 1-year survival rate of pancreatic cancer ($\chi^2 = 6.23, 5.50$; $P < 0.05$), but had no relation with tumor size, histological grade and cell proliferation ($P = 0.981, \chi^2 = 3.51, 1.17$; all $P > 0.05$). The overexpression of cyclinD1 was correlated with cell proliferation and histological grade ($P = 0.006, \chi^2 = 6.19$; $P < 0.05$), but not with size, metastasis and one-year survival

rate of the pancreatic cancer ($\chi^2 = 0.01, 0.86, 0.78$; all $P > 0.05$). The overexpression of c-myc was not correlated with size, histological grade, metastasis and survival rate ($P = 1.000, 0.208$; $\chi^2 = 0.90, 1.35$; all $P > 0.05$), but closely related with cell proliferation of pancreatic cancer ($\chi^2 = 4.17$; $P < 0.05$). The overexpression of MMP-7 was significantly associated with metastasis and survival rate of pancreatic cancer ($\chi^2 = 6.05, 6.61$; both $P < 0.05$), but not with size, histological grade and cell proliferation ($\chi^2 = 0.40, 0.74, 1.34$; all $P > 0.05$) (Table 1).

Relationships between abnormal expression of β -catenin and overexpression of cyclinD1, c-myc and MMP-7 in PanIN

In PanIN, β -catenin abnormal expression rates in tumor tissues of cyclinD1, c-myc and MMP-7 overexpression group were 6/6, 5/6, 6/6, and β -catenin normal expression rates in tumor tissues of cyclinD1, c-myc and MMP-7 negative expression group were 6/6, 6/6 and 5/6, respectively. There was a highly significant positive association between abnormal expression of β -catenin and overexpression of

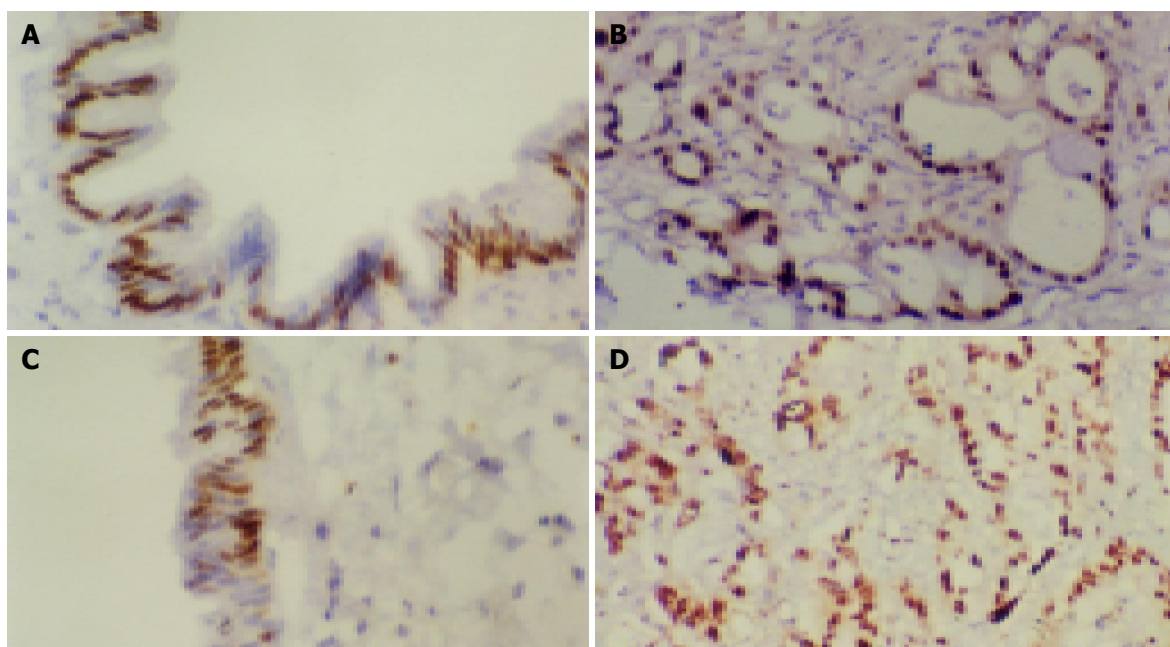


Figure 2 Immunohistochemical PicTure™ staining. A and B: CyclinD1 expression was in the nuclei of PanIN and pancreatic cancer cells, $\times 200$; C and D: c-myc expression located in the nuclei of PanIN and pancreatic cancer cells, $\times 200$.

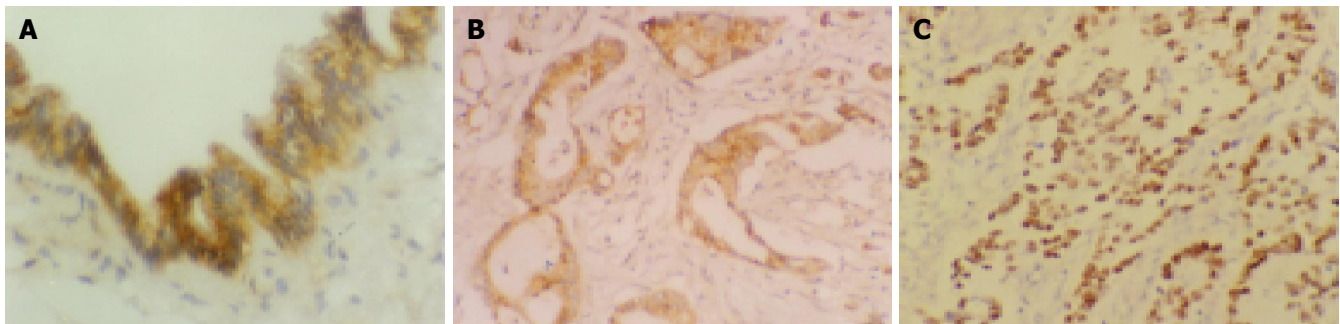


Figure 3 Immunohistochemical PicTure™ staining. **A and B:** The expression of MMP-7 was mainly located in cytoplasm of PanIN and pancreatic cancer cells, $\times 200$. **C:** Expression of PCNA protein was located in the nuclei of pancreatic cancer cells with brown-yellow granules, $\times 200$.

cyclinD1, c-myc and MMP-7 in PanIN ($P = 0.002, 0.015, 0.015$; $r = 1.000, 0.845, 0.845$) (Table 2).

Relationships between abnormal expression of β -catenin and overexpression of cyclinD1, c-myc and MMP-7 in pancreatic carcinomas

As summarized in Table 3, β -catenin abnormal expression rates in cancer tissues of cyclinD1, c-myc and MMP-7 overexpression group were 87.5%, 84.7%, 78.1%, and β -catenin normal expression rates in cancer tissues of cyclinD1, c-myc and MMP-7 negative expression group were 8/15, 9/15 and 10/15, respectively. There was a close positive and significant association between abnormal expression of β -catenin and overexpression of cyclinD1, c-myc and MMP-7 in pancreatic cancer ($\chi^2 = 6.94, 7.61, 8.87$; $r = 0.437, 0.452, 0.435$; all $P < 0.05$).

DISCUSSION

In addition to its role in regulating intercellular adhesion, β -catenin has a critical role in the highly conserved Wnt signaling pathway. Mutations of β -catenin and aberrant

expression of its protein have been identified in a number of different types of human cancers, and implicated in tumor invasion and metastasis^[33-35]. However, reports regarding the expression of β -catenin in human pancreatic cancer are very few, and the results are controversial. Gerdes *et al*^[27], reported that intracellular accumulation of β -catenin was not observed in any of the 40 pancreatic adenocarcinomas, and neither 78 pancreatic adenocarcinomas nor 14 pancreatic cancer cell lines had mutations in exon 3 of the β -catenin gene. Thus, Wnt- β -catenin pathways did not appear to be common targets of genetic alterations in pancreatic carcinogenesis. But most researchers^[17,24,25] found that alterations in β -catenin expression were common in pancreatic cancer and correlated with loss of tumor differentiation, metastasis and prognosis. In our study, we found that the expression of β -catenin was located in normal ductal and acinar cells with strong membranous staining in adult normal pancreases, but in most cases of pancreatic carcinomas was in cytoplasm and membranous staining reduced or disappeared, and the abnormal expression rates of β -catenin in pancreatic carcinoma and PanIN tissues were 68.1% and 6/12 ($P > 0.05$), and abnormal β -catenin was correlated with metastasis and survival time of pancreatic cancer. These data suggest that the abnormal expression of β -catenin plays an important role in the process of pancreatic tumorigenesis and metastasis, and may be considered as a valuable marker for early diagnosis and prognosis of pancreatic cancer.

CyclinD1 and c-myc are critical genes involved in cell proliferation and differentiation. As two target genes of Wnt signaling pathway, the amplification and/or overexpression of cyclinD1 and c-myc in tumor cells are extremely common, indicating that their activation may be essential during carcinogenesis^[36-40]. To date, only few data on the biological significance and prognostic value of cyclinD1 and c-myc overexpression have been reported in pancreatic cancer. Several investigators^[41-44] have reported that cyclinD1 overexpression was detected in pancreatic carcinoma and PanIN, whereas normal human pancreas stained negative, and its overexpression was associated with tumor stage, cell proliferating activity, differentiation, perineural invasion, lymph node metastasis and poor prognosis of pancreatic adenocarcinoma. Schlegel *et al*^[45], recently have shown that c-myc activation was detected in some high-grade PanIN lesions, and its overexpression was common in pancreatic cancer, and significantly associated with poor tumor differentiation, but not with tumor stage or metastasis. So

Table 1 Relationships between expression of β -catenin, cyclinD1, c-myc and MMP-7 and clinicopathologic characteristics of pancreatic cancer

Parameter	n	β -catenin		c-myc		cyclinD1		MMP-7	
		Normal	Abnormal	+	-	+	-	+	-
Mass size (cm)									
<3	9	3	6	6	3	6	3	6	3
3-5	15	4	11	11	4	10	5	10	5
>5	23	8	15	16	7	19	4	14	9
Histological grade									
I	10	4	6	5	5	4	6	7	3
II	16	6	10	11	5	12	4	11	5
III	21	5	16	17	4	19 ^a	2	12	9
Proliferation degree									
High	28	6	22	23	5	25	3	16	12
Low	19	9	10	10 ^c	9	10 ^c	9	14	5
Metastasis									
Positive	25	4	21	19	6	20	5	20	5
Negative	22	11 ^e	11	14	8	15	7	10 ^e	12
Survival time (yr)									
>1	13	8	5	8	5	9	4	4	9
≤ 1	34	7 ^s	27	25	9	26	8	26 ^s	8

^a $P < 0.05$ vs grade I; ^c $P < 0.05$ vs high proliferation degree; ^e $P < 0.05$ vs positive metastases. ^s $P < 0.05$ vs > 1 year.

Table 2 Relationships between expression of β -catenin and cyclinD1, c-myc and MMP-7 in PanIN

β -catenin	<i>n</i>	cyclinD1		c-myc		MMP-7	
		+	-	+	-	+	-
Normal	6	0	6	0	6	1	5
Abnormal	6	6	0	5	1	6	0

$P = 0.002, 0.015, 0.015; r = 1.000, 0.845, 0.845.$

they considered that c-myc activation was involved in early stages of development and progression of pancreatic neoplasia, rather than in late stages of local spread and lymph node metastasis of invasive adenocarcinoma. In the present study, we found that cyclinD1 and c-myc overexpression was detected in 35 and 33 of 47 cases of pancreatic carcinoma, 6 and 5 of 12 PanIN tissues, respectively, but not in 10 normal pancreases. Both proteins' overexpression was significantly correlated with cell proliferation activity and degree of differentiation, but not with size, metastasis or one-year survival rate of the pancreatic cancer. These results suggest that cyclinD1 and c-myc overexpression plays an important role in pancreatic carcinogenesis, but does not affect tumor progression independently. Masuda *et al*^[46], have also reported that overexpression of cyclinD1 was not associated with the presence of nodal metastasis and clinical stage in head and neck squamous cell carcinoma.

Degradation of the extracellular matrix mediated by matrix metalloproteinases (MMPs) is a crucial mechanism during tumor invasion and metastasis. They are necessary to create a microenvironment supporting the growth of primary tumor and metastasis^[47]. Unlike the majority of MMPs family members, MMP-7 mRNA and protein are expressed not only in the epithelium of premalignant lesions in multiple glandular tissues^[48] but also in the tumor cells, and associated with tumor progression of colorectal^[49], gastric^[50] and hepatic carcinomas^[51]. In contrast to these gastrointestinal cancers, there has been little information on the significance of MMP-7 expression in pancreatic carcinoma. Some researchers^[52-54] have found that expression of MMP-7 mRNA and protein was significantly correlated with infiltrating growth pattern, lymph node and liver metastasis, TNM stage, postoperative recurrence and survival time of pancreatic carcinoma. Crawford *et al*^[55], have shown that MMP-7 was expressed in the majority of human pancreatic adenocarcinomas and PanIN, but was never detected in the normal pancreas. In our study, we also found that 10 normal pancreases did not express MMP-7, but 63.8% pancreatic adenocarcinoma tissues and 7/12 PanIN lesions showed MMP-7 positive expression. Moreover, overexpression of MMP-7 was significantly associated with metastasis and survival time of pancreatic cancer, but not with tumor size, cell proliferation activity and degree of differentiation. These results suggest that MMP-7 plays a key role not only in the earlier stages of pancreatic tumorigenesis, but also in the progression of pancreatic carcinoma, and thereby contributes to a poor prognosis.

As an effector of the Wnt signaling pathway, β -catenin is able to bind to Tcf/Lef transcription factors, and subsequently activate target genes^[14-16]. Recently studies demonstrated that

Table 3 Relationships between expression of β -catenin and cyclinD1, c-myc and MMP-7 in pancreatic carcinomas

β -catenin	<i>n</i>	CyclinD1 (%)		c-myc (%)		MMP-7 (%)	
		+	-	+	-	+	-
Normal	15	7	8	6	9	5	10
Abnormal	32	28 (87.5)	4	27 (84.5)	5	25 (78.1)	7

$\chi^2 = 6.94, 7.61, 8.87; r = 0.437, 0.452, 0.435; P < 0.05.$

cyclinD1, c-myc and MMP-7 were important target genes of Wnt signaling pathway^[16-18]. Immunohistochemical studies confirmed that overexpression of cyclinD1, c-myc and MMP-7 was highly associated with accumulation of β -catenin and mutational defects of the Wnt signaling pathway in numerous tumor types^[17,19-22]. For instance, a strong correlation was reported between β -catenin deregulation and cyclinD1 and MMP-7 expression in primary colorectal tumors^[56,57]. Brabletz *et al*^[20], also found a tight correlation between nuclear β -catenin accumulation and c-myc expression in colorectal adenomas, although neither feature correlated with grade of dysplasia or proliferative activity. Zhai *et al*^[17], found that nuclear accumulation of β -catenin was highly associated with overexpression of cyclinD1 and MMP-7, but not with c-myc overexpression in ovarian endometrioid adenocarcinomas. In pancreatic carcinomas, only one literature^[26] reported that β -catenin might be involved in the tumorigenesis of pancreatic cancer and exhibited its effects mainly by the transactivation of cyclinD1. In this study, we examined the expression of β -catenin, cyclinD1, c-myc and MMP-7 in pancreatic carcinomas and PanIN, and confirmed that there was a highly significant positive association between abnormal expression of β -catenin and overexpression of cyclinD1, c-myc and MMP-7 not only in PanIN ($r = 1.000, 0.845, 0.845$), but also in pancreatic cancer ($r = 0.437, 0.452, 0.435$). It can be inferred from our findings that the abnormal expression of β -catenin plays a key role in the carcinogenesis and progression of human pancreatic carcinoma by up-regulating the expression of cyclinD1, c-myc and MMP-7, resulting in the degradation of extracellular matrix and uncontrolled cell proliferation and differentiation.

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