Ki-*ras* Point Mutation and p53 Expression in Human Pancreatic Cancer: A Comparative Study among Chinese, Japanese, and Western Patients

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Abstract

The aim of this study was to clarify features of Ki-*ras* point mutation (PM) and p53 expression in Chinese pancreatic cancer and to compare those with that in other countries. Dot blot hybridization and immunohistochemical methods were performed in 59 Chinese patients. The results showed that Ki-*ras* PMs at codon 12 and p53 expression were frequent in this group. No relationships were found between Ki-*ras* PM alone and p53 expression alone, and clinicopathological parameters, including age, gender, clinical stage, and histological grade and classification in Chinese patients. However, their cooperation was significantly associated with a poor prognosis in this group. Comparison showed that there were significant differences in the overall frequency and substitution of Ki-*ras* PM and in the ratio of transition:transversion in pancreatic cancer among various countries. In addition, the effect of Ki-*ras* PM and p53 expression on a poor prognosis of pancreatic cancer may be different among various countries. These findings suggested that not only Ki-*ras* PM and p53 expression are frequent in Chinese pancreatic cancer, but also a gene component to pancreatic cancer may be different between Asian and Western pancreatic cancer. In addition, it seems that cooperation of Ki-*ras* PM and p53 expression may predict a poor prognosis in Chinese patients with pancreatic cancer.

Introduction

Pancreatic cancer is a major contributor to cancer-related death both in developed Western countries (1) and in the developing country, China (2). The frequencies of Ki-*ras* PM[2] have been found in the range of 75–95% of this cancer (3) and implicated as an early event in pancreatic carcinogenesis in both nitrosamine-induced hamster models (4) and humans (3, 5, 6). On the other hand, it is also known that the p53 tumor suppressor gene plays an important role in restriction of abnormal cell proliferation and that loss of this safeguard function induced by its mutation may be a key factor in carcinogenesis. Mutations of the p53 tumor suppressor gene are found in up to 70% of this cancer and have been involved in an entire progression of pancreatic carcinogenesis, especially in a late stage (7, 8). Moreover, the cooperation of the *ras* oncogene with other cancer-related genes in tumorigenic transformation has been extensively studied both *in vitro* (9) and *in vivo* (10). These findings indicated not only that mutations in these two genes were the most common gene abnormalities in pancreatic cancer, but that the malignant progression of pancreatic cancer was accompanied by the progressive accumulation of multiple genetic abnormalities. Thus, the relationships of these two genes with pancreatic carcinogenesis have widely attracted the interests of oncologists.

On the other hand, geographical differences in the Ki-*ras* PM pattern had been reported in developed countries (1, 11, 12). p53 expression and its clinicopathological implications also were significantly seen among the Westerns, Japanese, and Chinese (13). However, the effects of Ki-*ras* PM (5–7, 14–16) and p53 expression (8, 12, 17) on the biological characters of pancreatic cancer had been mainly published in advanced countries. No clinical paper on Ki-*ras* PM and its cooperation with p53 expression in Chinese pancreatic cancer was published and similarly, no comparative study of Ki-*ras* PM and p53 expression between Asian and Western pancreatic cancer has been published to date.

It is known that Ki-*ras* PM was relatively easily detected because it is generally limited to one codon, but the detection of p53 tumor suppressor gene mutations is more difficult because of its multiple sites of mutations. Many comparative studies, however, have suggested that p53 expression was an approximate indicator to the real mutation rate with >90% specificity between immunohistochemistry and gene analysis (18). These findings make us easily assess the statues of the p53 gene by means of immunohistochemistry. The aim of this study was to clarify the biological characteristics of Ki-*ras* PM and p53 expression and the possible clinicopathological significance in Chinese pancreatic cancer and to compare those with that in other countries. Our study may provide a valuable clue for the epidemiological study of pancreatic cancer.

Patients and Methods

Clinical Data. From December 1989 to November 1997, a total of 59 Chinese patients underwent pancreatectomy for the purpose of treating pancreatic cancer and were verified to have primary invasive ductal carcinoma of the pancreas histologically at the second Department of Surgery, the First Affiliated Hospital of China Medical University (Shenyang, China). During this period, the main method for treating pancreatic cancer was pancreatectomy, although the diagnostic procedure has

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[2] The abbreviation used is: PM, point mutation.

relatively improved. In addition, four patients who died from surgery-related causes such as bleeding and acute renal failure within 1 month after surgery were excluded from this study. The mean age was 55.5 ± 10.4 years (range, 30–73 years), and there were 38 men and 21 women. According to TNM stage classification by the International Union Against Cancer (19), six patients were classified in stage I, 8 in stage II, 40 in stage III, and 5 in stage IV. None of them received any type of treatment before surgical procedures. All specimens were formalin-fixed and paraffin-embedded. Postoperative survival was defined as the time that elapsed from surgery to cancer-related death. Twelve percent (7 of 59) of the patients received adjuvant chemotherapy after surgery, but none of them received radiotherapy.

DNA Extraction and Amplification. Genomic DNA was extracted from both samples and cell line by treatment with SDS and proteinase K and was followed by extraction with phenol/chloroform and ethanol precipitation (16). Genomic DNA from a cell line of SW 480 colon cancer (Japanese Cancer Research Resource Bank, Tokyo, Japan) was used as a positive control. This cell line has a Ki-*ras* PM at codon 12 that changes wild-type glycine (GGT) to valine (GTT). Genomic DNA from the normal pancreas of the organ donor program serves as the GGT control to eliminate possible false positive results. The DNA fragment, including codon 12 of the Ki-*ras* gene, was amplified by PCR. The primers used were 5′-ATGACTGAATATAAACTTGTGG-3′ and 3′-GCTTATACTAGGTTGTTATC-5′, respectively (TaKaRa Corp., Kyoto, Japan). Genomic DNA (0.5 μg) was subjected to PCR in a total of 100 μl of reaction mixture containing 2.5 units of *Taq* polymerase, 10 μl of 10× PCR buffer, 8 μl of dNTP mixture (2.5 mM each; TakaRa Corp., Kyoto, Japan), and 75 pmol of each primer. PCR was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer) for 35 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 2 min. After the last cycle of amplification, the extension was continued for an additional 7 min at 72°C.

Dot Blot Hybridization. Ki-*ras* PM was assessed using a method as described previously (16). Briefly, 50 μl of adjusted PCR product were spotted and fixed onto Hybond-N+ nylon membranes (Amersham International plc, Buckinghamshire, England). Prehybridization was appealed with hybridization buffer (0.1% hybridization buffer component, 0.02% SDS, 0.5% blocking agent) at 56°C for 30 min. Ki-*ras* codon 12 wild or mutant oligonucleotide probes were labeled by the enhanced chemiluminescence 3′-oligolabeling kit (Amersham International plc). Hybridization was performed at 56°C for 90 min. The membranes were washed with buffer 1 [0.15 M NaCl, 0.1 M Tris base (pH 7.5)], blocked with 0.5% (w/v) blocking reagent for 30 min, and then incubated in the antifluorescein horseradish peroxidase conjugate [diluted 1:1000 in 0.4 M NaCl, 0.1 M Tris base (pH 7.5) containing 0.5% BSA] for 30 min. After they were washed with buffer 2 [0.4 M NaCl, 0.1 M Tris base (pH 7.5)], the filters were incubated in an equal volume of chemiluminescence detection 1 and 2 (Amersham International plc) for 1 min. Finally, the filters were autoradiographed with Hyperfilm (Amersham International plc) for 5–10 min.

Immunohistochemistry. Immunostaining was performed according to the method as described previously (13). Briefly, 5-μm sections were deparaffinized in xylene and rehydrated in graded ethanol, and endogenous peroxidase was blocked with 0.3% hydrogen peroxidase in methanol. After antigen retrieval by microwave oven and preventing nonspecific binding by 10% normal rabbit, p53 monoclonal antibody (mouse monoclonal antibody, DO-1, Oncogene Sciences, Inc., Cambridge, MA) was used for immunohistochemical analysis. The sections were incubated in biotinylated second antibody, the streptavidin-biotinylated horseradish peroxidase, and diaminobenzidine (Nichirei Corp., Tokyo, Japan), respectively. Sections of p53-positive colon carcinoma were performed in every staining batch as positive controls. PBS was used as a negative control medium. Only the nuclear staining of the tumor cells was considered as positive for p53 expression. Twenty percent of the positive cells were considered as the threshold for p53 positivity (17).

Statistics. The tumors with a double mutation were scored twice for substitution with the corresponding amino acid. The effects of adjuvant chemotherapy on a slightly improved prognosis in Chinese pancreatic cancer were excluded from this study because they are too few in number. The data were analyzed with the χ^2 test (or Yates' correction test) and the post-hoc test (by Bonferroni method) using StatView 5.0 software (SAS institute Inc.). The survival curves were calculated according to the Kaplan-Meier method and compared using the generalized Wilcoxon test. Significant differences were accepted at $P < 0.05$.

Results

Ki-*ras* PMs at codon 12 were detected in 76.3% (45 of 59) of Chinese patients, including a single mutation in 38 cases and a double mutation in 7 cases (Fig. 1). The pattern of Ki-*ras* PMs at codon 12 was listed in Table 1. The seven specimens with a double mutation involved GAT plus GTT in three, GTT plus AGT in two, GTT plus TGT in one, and GAT plus GCT in one case. A total of 45 Ki-*ras* PM specimens included 52 mutated amino acids because of seven cases with a double mutation. Of 52 mutated amino acids, 19% (10 of 52) of the mutations were at the first base, and the remaining 81% (42 of 52) were at the second base of the codon 12 Ki-*ras* gene.

Compared to previous reports, the differences in the overall frequency of Ki-*ras* PMs were found between Asians and between Asian and Western patients ($P < 0.05$–0.001; Table 1; Refs. 20–27). Multiple comparisons showed that there were significant differences in all six substitutions of Ki-*ras* PMs at codon 12 among these countries ($P < 0.01$). Furthermore, the differences between Chinese patients and patients from other countries were found in the following substitution: aspartic acid, China *versus* Austria ($P = 0.012$), China *versus* England ($P < 0.001$), China *versus* America ($P = 0.043$), China *versus* Spain ($P = 0.043$); valine, China *versus* England ($P = 0.027$), China *versus* Italy ($P = 0.042$), China *versus* Spain ($P = 0.048$); arginine, China *versus* Austria ($P = 0.019$). However, no significant differences in substitutions of Ki-*ras* PMs at codon 12 were found between Chinese and Japanese patients with pancreatic cancer.

p53 expression was seen in 69.5% (36 of 59) of Chinese patients. Strong and moderate intensity of the nuclear p53 staining was found in up to 90% tumor cells in the positive-stain slide. The pattern of nuclear p53 staining was usually diffuse throughout stained slides (Fig. 2).

In addition, 53% (31 of 59) of cases harbored simultaneously both Ki-*ras* PM and p53 expression, but no correlation was found between Ki-*ras* PMs at codon 12 and p53 expression ($P = 0.26$). No significant relationships were found between Ki-*ras* PMs at codon 12 alone, as well as p53 expression alone, and clinicopathological parameters, including age, gender, clinical stage, and histological grade and classification in Chinese

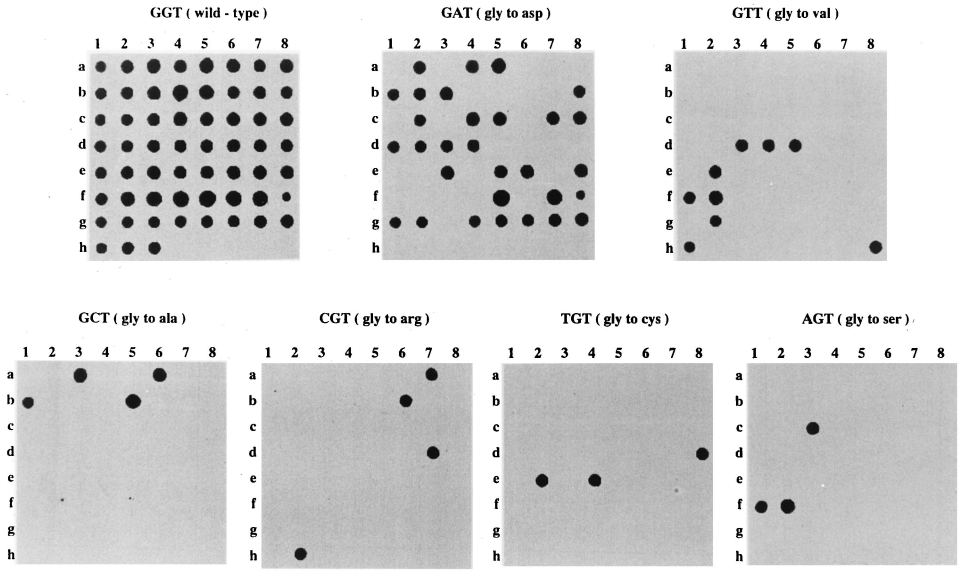

Fig. 1. The results of dot blot hybridization. A total of seven blots, each containing the same PCR product at the same position, have been hybridized using wild or mutant oligonucleotide probes. h8 is a positive control for GTT.

Table 1 Frequency and pattern of Ki-*ras* point mutation at codon 12 in pancreatic cancer in various countries

Country	Total frequency % (positive/samples)	Double mutation % (positive/samples)	Pattern of Ki-*ras* PM (%)[a]						Transition/ transversions (second base)	Reference No.
			GAT (aspartic acid)	GTT (valine)	GCT (alanine)	CGT (arginine)	TGT (cysteine)	AGT (serine)		
China	76 (45/59)	16 (7/45)	30 (58)	8 (15)	4 (8)	4 (8)	3 (6)	3 (6)	2.5	This study
Japan	91 (166/182)[b]	5 (10/166)	108 (60)	39 (22)	3 (2)	18 (10)	4 (2)	7 (4)	2.6	12, 14, 15, 16, 20, 21
American	79 (99/125)	0	44 (44)	32 (32)	1 (1)	17 (17)	5 (5)	0	1.3	1, 7, 22, 23
Austria	75 (47/63)	4 (2/47)	18 (37)	15 (31)	1 (2)	15 (31)	0	0	1.1	5
Dutch	93 (28/30)	0	9 (32)	8 (29)	0	1 (4)	10 (36)	0	1.1	27
England	78 (29/37)	3 (1/29)	4 (13)	12 (40)	0	3 (10)	6 (20)	5 (17)	0.3	24, 25
Italy	73 (40/55)	13 (5/40)	21 (51)	16 (39)	0	2 (5)	1 (2)	1 (2)	1.3	3, 11
Spain	78 (62/80)[c]	3 (2/58)	27 (47)	22 (38)	0	6 (10)	3 (5)	0	1.2	11, 26

[a] The tumors with a double mutation were scored more times for substitution with the corresponding amino acid.
[b] An adenosquamous carcinoma of the pancreas (in Ref. 12) was excluded from this analysis.
[c] Six cases with *ras* mutation in Scarpa's paper (11) were excluded from analysis because using different primers and restriction enzymes gave no clear results.

pancreatic cancer. The median survival months according to Ki-*ras* PM, p53 expression, and their cooperation were summarized in Table 2. Ki-*ras* PM alone or p53 expression alone was not associated with the prognosis of the Chinese patients. However, their cooperation showed a significant relationship with a poor prognosis of the patients (Fig. 3).

Discussion

In the present study, we studied Ki-*ras* PMs at codon 12 and p53 expression in Chinese patients with pancreatic cancer and compared their differences between Asian (Chinese and Japanese) and Western pancreatic cancer.

Previous reports indicated that the frequency, the pattern, and the ratio of transition:transversion at the second base of codon 12 Ki-*ras* PMs of pancreatic cancer are different in various countries (1, 11, 12). In this study, the frequency of Ki-*ras* PMs in the Chinese patients was significant lower than that in the Japanese, but similar to that in the developed Western patients. On the other hand, the frequency of Ki-*ras* double mutations was highest in the Chinese patients. Previous reports indicated that a GGT-to-GAT transition is 38% in European and 44% in American patients. But it was 58% in Chinese and 55% in Japanese. Previous studies and additional reviews also demonstrated that the majority of Ki-*ras* PMs were at the second base of its codon 12, with the range from 53 to 90% in developed countries compared to 81% in developing China. For this reason, we analyzed the ratio of transition:transversion at this site and found that the ratio is lower in both European and American patients (range, from 0.3 to 1.3) than that in Asian patients, with 2.5 for the Chinese and 2.6 for the Japanese patients (Table 1). Multiple comparisons showed that there were significant differences in the substitution of Ki-*ras* PMs at codon 12 in various countries. Furthermore, the significant differences in aspartic acid, valine, and arginine substitution of Ki-*ras* PMs at codon 12 were found between Chinese and Western patients, but not between Chinese and Japanese patients. These findings suggested that the pattern of Ki-*ras* PM at 12 codon was quite different between Asians and Europeans and/or Americans. Although Japan is also a well-developed country, and Japanese life-styles have been affected by Western countries during the last half-century, their life-styles are still different from those of Europeans or Americans (13). These differences in life-styles may generate different carcinogens and may be one of the reasons responsible for the similar pattern of Ki-*ras* PMs between Asian patients and for the different pattern of Ki-*ras* PMs between Asian and Western

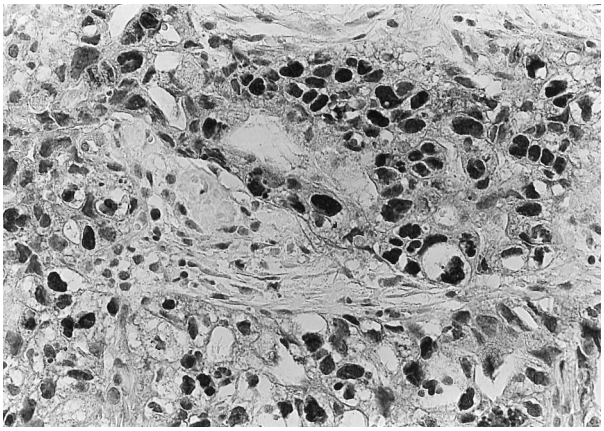

Fig. 2. p53 expression in invasive ductal carcinoma of the pancreas. Diffuse nuclear staining is found in the majority of the tumor cell (original magnification, ×200).

Table 2 Association of Ki-*ras* PM at codon 12 and p53 expression with prognosis in pancreatic cancer

Group	No. (%)	Median survival (mo)	
Overall	59 (100)	9.9	
p53 (−)	18 (30.5)	11.0	
p53 (+)	41 (69.5)	10.2	
Ki-*ras* PM (−)	14 (23.7)	11.7	
Ki-*ras* PM (+)	45 (76.3)	9.7	
Single mutation	38 (38/45, 84.4)	10.6	
Double mutation	7 (7/45, 15.6)	6.7	
p53 (−) Ki-*ras* PM (+)	14 (23.7)	8.0	
p53 (+) Ki-*ras* PM (+)	31 (52.5)	10.2[a]	9.7[b]
p53 (+) Ki-*ras* PM (−)	10 (16.9)	9.1	
p53 (−) Ki-*ras* PM (−)	4 (6.8)	18.2[a,b]	

[a] $P = 0.048$.
[b] $P = 0.027$ (9.7, median of three groups).

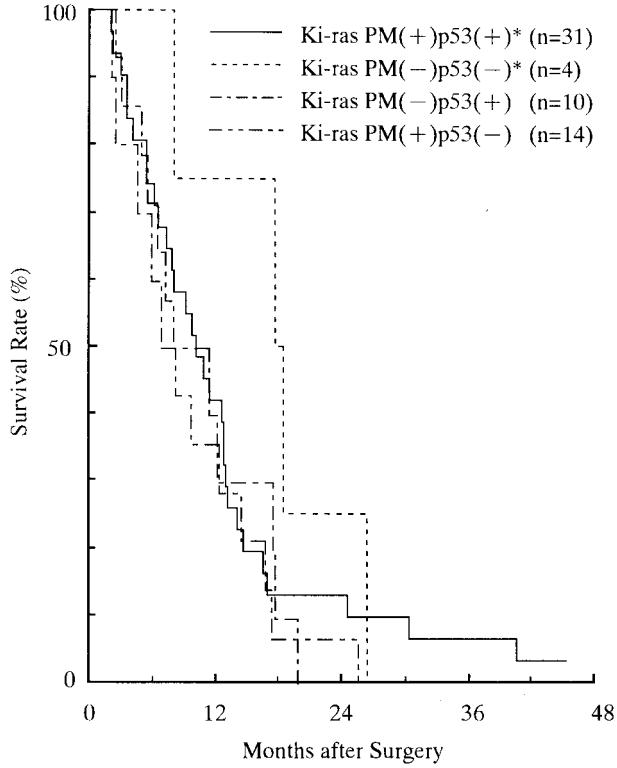

Fig. 3. Survival curves (Kaplan-Meier method) after surgery according to the cooperation of p53 expression and Ki-*ras* PMs at codon 12. *$P = 0.048$. Furthermore, the group with Ki-*ras* PM(−)p53(−) had a significantly higher survival curve compared with the other three groups ($P = 0.027$).

patients. Epidemiological studies have suggested the influences of life-styles on development and progression of pancreatic cancer. For example, the morbidity of pancreatic cancer in the Chinese who settled in China is significant lower than that in the Chinese who settled in American. The mortality of the patients with this cancer is positively correlated with the consumption of oils and fats, milk and dairy product, sugar, eggs, and coffee, but negatively with that of pulse, although these relationships remain controversial (1, 15, 28). These findings suggested that not only the ethnic or racial factors, but also life-style might be closely associated with the status of the cancer-related gene.

A recent study indicated that Ki-*ras* PM was significantly associated with TNM tumor stage and a poor prognosis in Spanish pancreatic cancer (29). Another study indicated that a double mutation compared to a single mutation of the Ki-*ras* gene was significantly associated with a poor prognosis in Japanese pancreatic cancer (16). These situations, however, were not seen in Chinese patients, although the substitution of Ki-*ras* PMs in Japanese patients was similar to that in Chinese patients. A previous report also suggested that the TGT and AGT mutation of the codon 12 Ki-*ras* gene might mean a low potential for malignant transformation in pancreatic tissues (6). In the present study, however, the patients with these two patterns of mutation did not show statistically better survivals than those with the other pattern of mutation. These findings suggested that the biological significance of Ki-*ras* PMs in pancreatic cancer might be different with various populations.

We have reported that the frequency of p53 expression and its biological features in pancreatic cancer were different in various countries (13). The frequency of p53 expression in pancreatic cancer was 70% in the Chinese, ranged from 15 to 49% in the Japanese, and ranged from 40 to 63% in the Westerns. Moreover, p53 expression showed a significant relationship with advanced clinical stage and poorly histological grade in Western, but not in Asian pancreatic cancer. However, the effect of p53 expression on a poor prognosis in pancreatic cancer remains controversial. We were aware of Japanese investigators whose data showing a significant relationship between positive p53 expression and a poor prognosis in pancreatic cancer at the level of either p53 immunostaining or p53 tumor suppressor gene mutation were not consistent with the majority of studies from advanced countries, as well as the developing country, China (13). Besides the differences in life-styles stated above, one of the reasons responsible for these controversial conclusions might be that p53 expression does not entirely reflect the status of p53 mutation at the gene level. That is, when p53 is not stained immunohistochemically, p53 can be deleted, carry a nonsense mutation, or be epigenetically suppressed, resulting in an immunohistochemically p53-negative, clinically malignant phenotype. WAF-1 or MDM2 overexpression may also affect the real function of the p53 gene (8, 30). If p53 gene mutation is truly related to a poor prognosis,

absence of p53 expression may include a different distribution of normal and abnormal p53 status in various populations (13). Recently, coexpression of the p53 and MDM2 protein phenotype, as well as WAF-1 has been confirmed to be a useful prognostic indicator for human cancer (17, 30). In addition, some reports suggested that the adjuvant chemotherapy might mask the true effect of p53 expression on a poor prognosis of pancreatic cancer (13, 17).

Although a separate analysis of Ki-*ras* PM and p53 expression did not significantly indicate any clinicopathological implications in the present study, their cooperation showed an association with a poor prognosis of pancreatic cancer (Fig. 3; Table 2), suggesting that the status of the Ki-*ras* or p53 gene alone might have only a weak influence on the biological characteristics of pancreatic cancer. However, it is very difficult to explain this phenomenon. This may explain why the median survival between the groups with and without abnormality of these two genes differs only by a month or two (Table 2). A study suggested that an activated *ras* gene alone, if there was no cooperation with the inactivation of the p53 tumor suppressor gene, was not sufficient to transform cells *in vitro* (31). Similarly, inactivation of the p53 tumor suppressor gene alone was also not sufficient to cause pancreatic carcinogenesis in transgenic mice (11). The mutant p53 protein has been involved in maintaining the transformed phenotype in cells transformed with p53 plus *ras* in culture (9). A recent study indicated that pancreatic carcinogenesis was also associated with multiple cancer-related genes, including Ki-*ras*, p53, p16, and DPC4 genes. Alterations in three or four of these genes were present in 76% of this tumor (32). In addition, 53% (31 of 59) of Chinese patients harbored simultaneously both Ki-*ras* PM and p53 expression in the present study. These findings suggested that *ras* and p53 genes have been involved in pancreatic carcinogenesis, and their cooperation may affect biological features of pancreatic cancer.

In summary, the present study indicated that Ki-*ras* PMs at codon 12 and p53 expression were frequently seen in Chinese pancreatic cancer. A gene component to pancreatic cancer might be different between Asian and Western pancreatic cancer. In addition, it seems that the cooperation of Ki-*ras* PM and p53 expression may be associated with a poor prognosis of pancreatic cancer patients. However, the cases in the subgroup with negative p53 staining and the wild-type *ras* gene were too small to draw a definitive conclusion. The cooperation of Ki-*ras* PM and p53 expression in the development and progression of pancreatic cancer needs to be clarified in the future.

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