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Ⅱ. 過 去 の 研 究 歴

1992年～1995年6月　中国医科大学 医学修士課程　膵腫瘍のK-ras変異の研究

1995年6月～1996年3月　東京大学第1外科 客員研究員　膵胆管腫瘍の分子生物学変化の研究

1996年4月～現在　東京大学医学系大学院 博士課程　膵腫瘍の分子生物学変化の研究

Ⅲ. 過 去 の 研 究 実 績

1. 膵癌におけるK-ras遺伝子点突然変異の研究
2. 12指腸乳頭部癌におけるp53, p21/Waf1およびK-rasの意義
3. 剖検膵癌のK-ras遺伝子変異とそのheterogeneity

Ⅳ. 本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

1. 第97回 日本外科学会総会 "乳頭部癌におけるp53蛋白overexpressionの意義" (poster)
2. Fourth Congress of the Asian Society of Hepato-Biliary-Pancreatic Surgery "p53 and p21/Waf1 expression in carcinoma of the papilla of Vater" (poster)
3. 第98回 日本外科学会総会 "乳頭部癌におけるp53, p21/waf1およびK-ras変異の意義" (口演予定)

(2) 学会誌等に発表した論文　無 ・ (有)　（雑誌名・論文名）

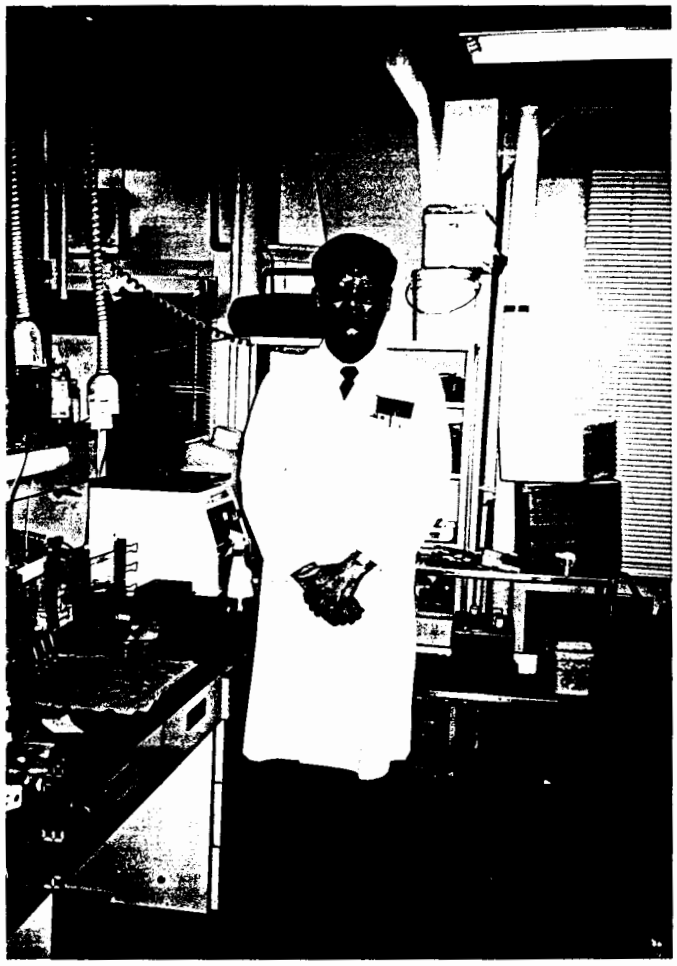
1. Japanese Journal of Clinical Oncology 1997; 27: 58~61 Renal Cell Carcinoma of the spindle type with metastasis to the pancreas: a case report
2. 臨床雑誌[外科] 1997年 第59巻 第1号 "膵液K-ras点突然変異は膵癌診断に有用か?"
3. 胆と膵 1997; 18: 251~257 "先天性嚢胞の概念と病態"

Ⅴ. 今後の研究計画及び希望

膵癌は悪性度が高く、その発生が増えつつある一方、発見時にはほとんどのものが助からないのは現状であ早期診断と加療がその予後改善の肝要である。遺伝子の研究によって膵癌の発癌機序の解明、そして早期診断に貢献できることは期待される。今後、膵癌によく見られるhyperplasiaとdysplasiaなどいわゆる癌前病変における遺伝子変異の検索で、膵癌がこれらの病変から発生するものであるか否かを解明したい。

Ⅵ. 研　究　報　告（日本語、又は英語で書いて下さい。 4,000字以上で記載して下さい。別紙可）

別紙



Ⅶ. 指導教官の意見

趙斌は東京大学医学系博士課程外科専攻の大学院生で膵臓癌・胆道癌などの研究をしています．研究業績，日本語力を始めとする周囲とのコミュニケーション能力において卓越したものを持っており，中国からの留学生達のリーダー的存在となっています．研究に取り組む姿勢は大変真摯で，時間と手間のかかる研究に対しても積極的に取り組み，着実な成果を上げ，既に英文論文１本を完成し，現在も英文原著論文を執筆中です．国際学会でも，日本語で日本の学会でも演者として発表するなど業績を上げています．日本語，英語ともに堪能で日本語検定試験１級に合格し，現在は日常生活はもちろん，研究過程におけるディスカッションも日本語ですべてこなしています．性格は温厚でとても思いやりがあり，周囲の人皆から慕われております．将来素晴らしい研究成果を携えて日中友好の架け橋になってくれるものと確信しております．

研 究 報 告

Theme: p53 and p21/Waf1 Protein Expression and K-*ras* Codon 12 Mutation in Carcinoma of the Papilla of Vater

I. Aim and Background

Carcinoma of the papilla of Vater accounts for nearly 40% of all surgically operable pancreatoduodenal tumors, and is second only pancreatic carcinoma in the periampullary region. Carcinoma of the papilla of Vater originates mainly in three anatomical regions, i.e., the common channel, the intraduodenal portion of the common bile duct, or the intraduodenal portion of the pancreatic duct. Thus, carcinoma of the papilla of Vater may have a different biological behavior depending on its origin. However, there have been few reports on the molecular changes in carcinoma of the papilla of Vater.

Human malignancies are associated with the activation of oncogenes and inactivation of tumor suppressor genes. Among these molecular changes, K-*ras* and p53 have frequently been reported to be related to the development and progression of many malignancies. p53 is also related to apoptosis and progression of the cell cycle through regulation of some downstream factors, e.g., p21. The p21/Waf1 gene encodes a cyclin-dependent kinase inhibitor (CDI) which inhibits multiple complexes of cyclin and cyclin-dependent kinase in initiating the progression of cells from G1 to S phase.

In this study, p53, p21/Waf1 immunohistochemical expression and K-*ras* codon 12 mutation in carcinoma of the papilla of Vater were investigated.

II. Materials and Methods

Thirty seven cases of carcinoma of the papilla of Vater were studied. Formalin-fixed, paraffin-embedded samples were available for all cases. The mean age of the patients was 65 years (range 41 to 80). There were 24 men and 13 women. The average size of the tumors was 25 mm in diameter. The TNM stage was classified according to the staging manual of AJCC. Macroscopically, the carcinoma was ulcerative type in 15 cases, and non-ulcerative type in 22 cases. Histologically, the tumors were divided into intestinal and pancreaticobiliary types as described previously. There were nine intestinal type, 27 pancreaticobiliary type, and one undifferentiated. Five micrometer-thick serial sections were cut and one piece was used for hematoxylin and eosin (HE) staining, two for immunohistochemical staining of p53 and p21/Waf1, and another for DNA extraction to detect K-*ras* codon 12 mutation.

The correlations among p53 overexpression, p21/Waf1 expression, K-*ras*

codon 12 mutation and the clinicopathological parameters described above were studied. The significance of these molecular changes in determining the patient's prognosis was analyzed. The relationship between p53 and p21/Waf1 expression was also studied.

Immunohistochemical staining

1. After deparaffinization, antigen retrieval with citrate buffer was performed by heating in a microwave oven and boiling for p53 and p21/Waf1, respectively.
2. Samples were incubated at 4°C overnight with monoclonal anti-p53 antibody Do7 and monoclonal anti-p21/Waf1 antibody EA10, respectively.
3. Endogenous peroxidase activity was blocked in methanol with H_2O_2.
4. Samples were then incubated with biotinylated antibody.
5. After incubation with Avidin-Biotin Complex, samples were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB).
6. Finally, samples were counterstained with hematoxylin and mounted.
7. Staining was evaluated using an Image Cytometer CAS 200R. Briefly, 20 fields were randomly selected in each stained section, and the percentage of stained cells in each field was counted. For p53 and p21/Waf1, percentages above 10% and above 5% were defined as positive, respectively.

Detection of K-*ras* codon 12 mutation

1.DNA extraction. Both normal duodenal mucosa and tumor tissues were scraped from paraffin-embedded slices under microscopic investigation. The scraped samples were dried overnight at 37°C. After deparaffinization, samples were incubated in digestion buffer for 24 h at 48°C. DNA was extracted by the phenol-chloroform method.

2. Two-step PCR-RFLP. K-*ras* codon 12 was amplified using the two-step PCR-RFLP method. PCR was carried out in a total volume of 50 μl containing genomic DNA, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.25 U of Taq Polymerase, and 12.5 pM of each primer. The system was subjected to 40 cycles of PCR (95°C for 2 min, 55°C for 3 min, and 72°C for 1.5 min in each cycle) using an automatic thermal cycler. The following primers were used:

A(sense): 5' ACTGAATATAAACTTGTGGTAGTTGGACCT 3'
B(antisense): 5' GTCCTGCACCAGTAATATGC 3'
C(antisense): 5' CTATTGTTGGATCATATTCG 3'

Primer A is a mutant in which the underlined base represents a mismatch to the K-*ras* gene sequence and provides an artificial restriction site for *Mva*I if K-*ras* codon 12 is wild-type. The first PCR was performed with primers A and B, and generated a 147-bp fragment. Eight μl of the product was digested with 10 units of *Mva*I and

0.6 μl of the digested product was subjected to a second PCR under the same conditions as the first PCR with primers A and C, and generated a 106-bp fragment. Eight μl of the product of the second PCR was then digested with 10 units of *Mva*I followed by electrophoresis on an 8% acrylamide gel.

3. Direct sequencing. The dye-terminator cycle sequencing method was used to analyze the mutation on codon 12 if a mutant band was detected on electrophoresis. Briefly, the band was removed from the gel and incubated in elution buffer overnight at 37°C. DNA was precipitated with ethanol and sodium acetate. PCR and purification of the PCR products were performed according to the manufacturer's protocol. The reaction was performed in a volume of 20 μl containing 8.8 μl of the template DNA, 8.0 μl of the terminator premix, and 3.2 μl (1pM/μl) of primer C, which was used in the PCR-RFLP method. The samples were subjected to 25 cycles of PCR (96°C for 10 sec, 55°C for 5 sec, 60°C for 4 min). The PCR products were purified with a Centri-Sep spin column to discriminate the extra ddNTP. Electrophoresis was performed using an ABI PRISM™ 90 Genetic Analyzer. Data were analyzed with sequencing analysis software.

Statistical Analysis

A statistical analysis was performed using the chi-square test. Survival was analyzed using the Kaplan-Meier method.

III. Results

p53 overexpression was found in 45% (17/37) of the cases. The staining was confined to the nuclei of tumor cells. No normal epithelium was positive for p53. p53 overexpression was seen in 67% (10/15) of the ulcerative cases, which was significantly higher than the incidence in non-ulcerative cases (32%; 7/22, $p<0.05$). There was no significant difference in p53 overexpression between the intestinal and pancreaticobiliary types. No correlation was found between p53 overexpression and tumor size, lymph node metastasis or tumor stage.

p21/Waf1 staining was found in 41% (15/37) of the cases. Again, staining was confined to the nuclei of tumor cells, but was scattered among tumor cells. Staining was seen in both primary lesions and infiltrating parts. Normal duodenal mucosa was slightly stained near the intestinal lumen. Tumors larger than 3 cm in diameter more frequently exhibited p21/Waf1 expression than those smaller than 3 cm. However, p21/Waf1 expression did not correlate with any other clinicopathological parameters.

There was no correlation between p53 and p21/Waf1 expression. In 18 of 37 (49%) cases, the staining pattern was either positive for p53 while negative for p21, or negative for p53 while positive for p21. In the other 19 (51%) cases, p53 and

p21/Waf1 were either both positive or both negative. We further investigated their relationship in 33 cases by comparing the staining in 20 pairs of samples taken from the same site in one tumor on adjacent slices stained for p53 and p21/Waf1, respectively. Among the 660 pairs of sites studied, 421 sites were negative for both p53 and p21/Waf1, and 21 sites were positive for both. One hundred fifty five sites were positive for p53 but negative for p21/Waf1, while 63 sites were positive for p21/Waf1 but negative for p53. No significant correlation was found by this analysis. Moreover, even within the same case, staining showed heterogeneity regarding the relationship between p53 and p21/Waf1.

The frequency of K-*ras* codon 12 mutation was 38% (14/37). The mutation was significantly more frequent in carcinoma of the intestinal type (67%, 6/9) than in that of the pancreaticobiliary type (30%, 8/27, $p<0.05$). No significant difference in the frequency of K-*ras* codon 12 mutation was found between ulcerative and non-ulcerative types. The mutation did not correlate with any other clinicopathological parameters including tumor size, lymph node metastasis, or tumor stage.

On direct sequencing, the mutation exclusively involved the second base of codon 12, and the base-pair changes were GGT to GAT (9/14), GGT to GTT (4/14), and GGT to GCT (1/14). GGT to GAT was also the main type in tumors of the pancreaticobiliary type (6/8). GGT to GAT and GGT to GTT were detected equally in the intestinal type. There was no significant difference in the type of mutation between the two histological types.

In a survival analysis, neither p53 nor p21/Waf1 expression or K-*ras* codon 12 mutation was correlated with postoperative survival.

IV. Summary

1. p53 overexpression was significantly more frequent in the ulcerative type than the non-ulcerative type. This indicated that p53 overexpression may play a role in ulcer formation in carcinoma of the papilla of Vater. ulceration was more frequently seen in the histologically pancreaticobiliary type, which had a much higher incidence and malignant potential in carcinoma of the papilla of Vater. The non-ulcerative type was more associated with the histologically intestinal type, which has a relatively lower malignant potential than the pancreaticobiliary type. Thus, it was assumed that p53 overexpression was related to highly malignant tumors although a significant correlation was not found between p53 overexpression and this histological classification into intestinal and pancreaticobiliary types in carcinoma of the papilla of Vater.

2. With regard to the relationship between p53 and p21/Waf1, in the present study, in addition to areas that were positive for p53 but negative for p21/Waf1 and *vice versa*, there were also areas that were positive for both p53 and p21/Waf1 and areas that were negative for both p53 and p21/Waf1. Furthermore, even in the same

case, heterogeneity was seen with regard to the relationship between p53 and p21/Waf1 expression. These results indicate that p21/Waf1 protein induction also follows a p53-independent pathway in carcinoma of the papilla of Vater.

3.The two histological types, i.e., intestinal type and pancreaticobiliary type, varied regarding the incidence of K-*ras* codon 12 mutation, with the higher mutation rate in the intestinal type. In colorectal carcinoma, it has been clarified that there is an adenoma-adenocarcinoma sequence and K-*ras* mutation is closely associated with the progression from small to relatively larger adenomas in this sequence. Moreover, K-*ras* mutation is mainly associated with the development of carcinomas from polypoid adenomas, while carcinomas that have developed from flat adenomas rarely involve this genetic alteration. In carcinomas of the papilla of Vater, some authors have claimed that there is an adenoma-adenocarcinoma sequence similar to that in colorectal carcinoma. Although it is possible that carcinoma of the papilla of Vater and that of the colon and rectum are quite different with regard to development, carcinoma of the papilla of Vater of the intestinal type closely resembles tubular adenocarcinoma of the colon and rectum, histologically. Therefore, it can be assumed that carcinoma of the papilla of Vater of the intestinal type may develop in the same way as that of the colon and rectum, and may originate from a polypoid adenoma. On sequencing, it seems that mutation itself rather than the type of mutation is more important in carcinogenesis in this malignancy.