

Subcellular localization of KL-6 mucin in intraductal papillary mucinous neoplasm of the pancreas

Yoshinori Inagaki^{1,*}, Yasuji Seyama^{1,2}, Kiyoshi Hasegawa¹, Wei Tang¹, Norihiro Kokudo¹

¹ Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan;

² Department of Surgery, Tokyo Metropolitan Bokutoh Hospital, Tokyo, Japan.

Summary

This study aimed to clarify the expression profile of KL-6 mucin in intraductal papillary mucinous neoplasm (IPMN) and its relation to tumor malignancy. Expression of KL-6 mucin in 38 IPMNs (intraductal papillary mucinous adenoma (IPMA), 24 cases; minimally invasive intraductal papillary mucinous carcinoma (MI-IPMC), 8 cases; invasive carcinoma originating from IPMC (IC-IPMC), 6 cases) and 66 pancreatic ductal adenocarcinomas (PDACs) was evaluated immunohistochemically. IC-IPMCs and MI-IPMCs had positive staining of KL-6 mucin whereas 58% of IPMAs tested negative. Subcellular localization of KL-6 mucin varied among IPMNs whereas all of the PDAC had positive expression in the circumferential membrane and cytoplasm of cancer cells. IC-IPMCs and MI-IPMCs had a higher frequency of circumferential membrane and cytoplasmic localization of KL-6 mucin than did IPMAs. These results suggest that localization of KL-6 mucin could be used to predict the malignancy of IPMN.

Keywords: KL-6 mucin, intraductal papillary mucinous neoplasm, invasion

1. Introduction

A dramatic change in sialoglycoconjugate expression on the surface of cancer cells is considered to play an important role in tumor progression (1,2). In particular, overexpression of MUC1 mucin, a transmembrane mucin glycoprotein with sialo-oligosaccharides in its extracellular tandem repeat domain (3), was frequently detected in various gastrointestinal cancers and suggested an association with the invasive and metastatic capacity of those adenocarcinomas (4-7). MUC1 mucin is also frequently detected in pancreatic ductal adenocarcinoma (PDAC) and may be related to its aggressive behavior and poor outcomes (8,9). However, MUC1 comes in many forms because of the variation of its carbohydrate

components, and each form may have a different effect on cancer progression (9).

Aberrant expression or localization of KL-6 mucin, a MUC1 detected by KL-6 monoclonal antibody (mAb), was observed in various cancers of the digestive organs (10-12). Clinicopathological analyses suggested that subcellular localization of KL-6 mucin in the circumferential membrane and/or cytoplasm of cancer cells was significantly related to the malignant behavior of cancer, such as the presence of cancer cell invasion and metastasis. In pancreatic cancer tissue, positive KL-6 staining was detected in the cytoplasm and luminal surface of cancer cells in all tested cases (13). In addition, that study also noted positive KL-6 staining in 1 of 5 intraductal papillary mucinous neoplasms (IPMNs). However, IPMNs have several stages of malignancy based on the morphology and invasive characteristics of tumor cells, and subcellular localization of KL-6 mucin in IPMNs and its relation to tumor malignancy are still unclear because of the small number of patient in the previous study. In the present study, the subcellular localization of KL-6 mucin was evaluated in several histological types of IPMNs and PDACs.

Released online in J-STAGE as advance publication July 20, 2014.

*Address correspondence to:

Dr. Yoshinori Inagaki, Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.

E-mail: yinagaki-tky@umin.ac.jp

2. Patients and Methods

2.1. Patients

Tissue samples of IPMNs and PDACs were respectively collected from 38 and 66 patients who underwent pancreatoduodenectomy or distal pancreatectomy from 1994 to 2007 at the Department of Surgery of the University of Tokyo Hospital (Table 1). Patients consisted of 24 men and 14 women ages 42 to 77 years (mean \pm SD, 65.9 \pm 7.8). Pathological diagnosis of IPMN was classified as intraductal papillary mucinous adenoma (IPMA, $n = 24$), minimally invasive intraductal papillary mucinous carcinoma (MI-IPMC, $n = 8$), or invasive carcinoma originating from IPMC (IC-IPMC, $n = 6$). Pathological characteristics were evaluated according to the Japanese classification of pancreatic cancer from the Japan Pancreatic Society (14).

2.2. Immunohistochemistry

Sections (5 μ m thick) were cut from archival formalin-fixed paraffin-embedded tissue blocks, deparaffinized, and rehydrated through a graded series of ethanol. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxidase/methanol. After incubation with 5% normal goat serum for 30 min at room temperature, the sections were then incubated with KL-6 mAb (1:200 dilution; Eisai, Tokyo, Japan) for 60 min at room temperature. After the sections were incubated with biotin-conjugated secondary antibody, the biotin-streptavidin-peroxidase complex method was performed using a commercial kit per the manufacturer's instructions (Nichirei Corp., Tokyo, Japan). 3,3'-Diaminobenzidine was used as the chromogen and haematoxylin was used as a counterstain. Negative control sections were treated without the primary antibody. Staining of KL-6 mucin in 10 random microscopic fields (or in the whole section if the slide contained < 10 fields) was observed, and tumor tissues in which > 10% of the cells displayed

brown granules in subcellular structures were defined as positive. Subcellular staining patterns of KL-6 mucin were evaluated using the apical membrane, surrounding membrane, and cytoplasm, as described elsewhere (12,15).

2.3. Statistical analysis

A χ^2 test was used to evaluate the relationship between the expression of KL-6 mucin and clinicopathological parameters. Differences of $P < 0.05$ were considered significant. StatView 5.0J (Abacus Concepts, Berkeley, CA) statistical software was used for data analyses.

3. Results

3.1. Subcellular localization of KL-6 mucin in pancreatic tumor tissue

Positive staining with KL-6 mAb was observed in 24 IPMNs (63%) and 66 PDACs (100%) (Figure 1). As previously noted, the expression of KL-6 mucin was categorized on the basis of subcellular localization. This varied subcellular localization of KL-6 mucin was observed in IPMNs (Figure 2). Twenty two IPMNs (58%) had positive staining in the apical surface

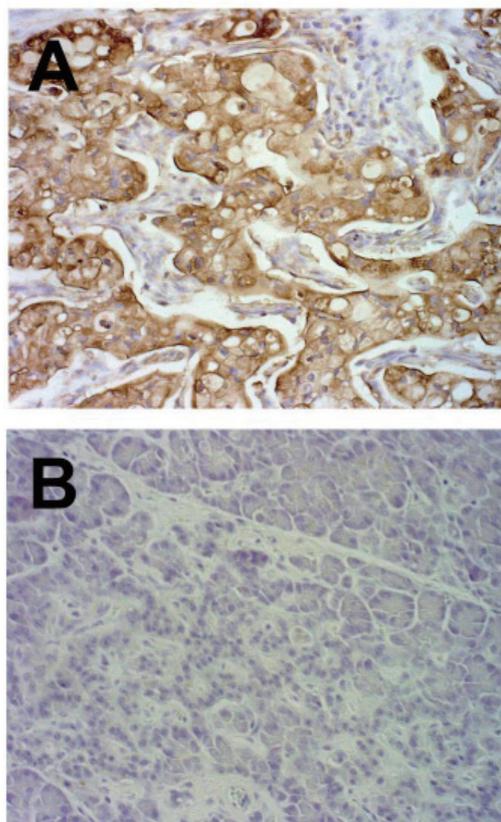


Figure 1. Typical examples of the expression profile of KL-6 mucin in PDACs (A) and non-malignant pancreatic tissue (B). Circumferential membrane and cytoplasmic localization of KL-6 mucin was frequently detected in cancer cells but not in normal pancreatic tissue. Original magnification: $\times 100$.

Table 1. Characteristics of patients with IPMNs

| Characteristics | <i>n</i> (%) |
|--|--------------|
| Age | |
| ≥ 65 | 22 (58) |
| < 65 | 16 (42) |
| Gender | |
| Male | 24 (63) |
| Female | 14 (37) |
| Location of IPMN | |
| Pancreatic head | 22 (58) |
| Pancreatic body/tail | 16 (42) |
| Pathological diagnosis of IPMN | |
| Adenoma (IPMA) | 24 (63) |
| Minimally invasive carcinoma (MI-IPMC) | 8 (21) |
| Invasive carcinoma (IC-IPMC) | 6 (16) |

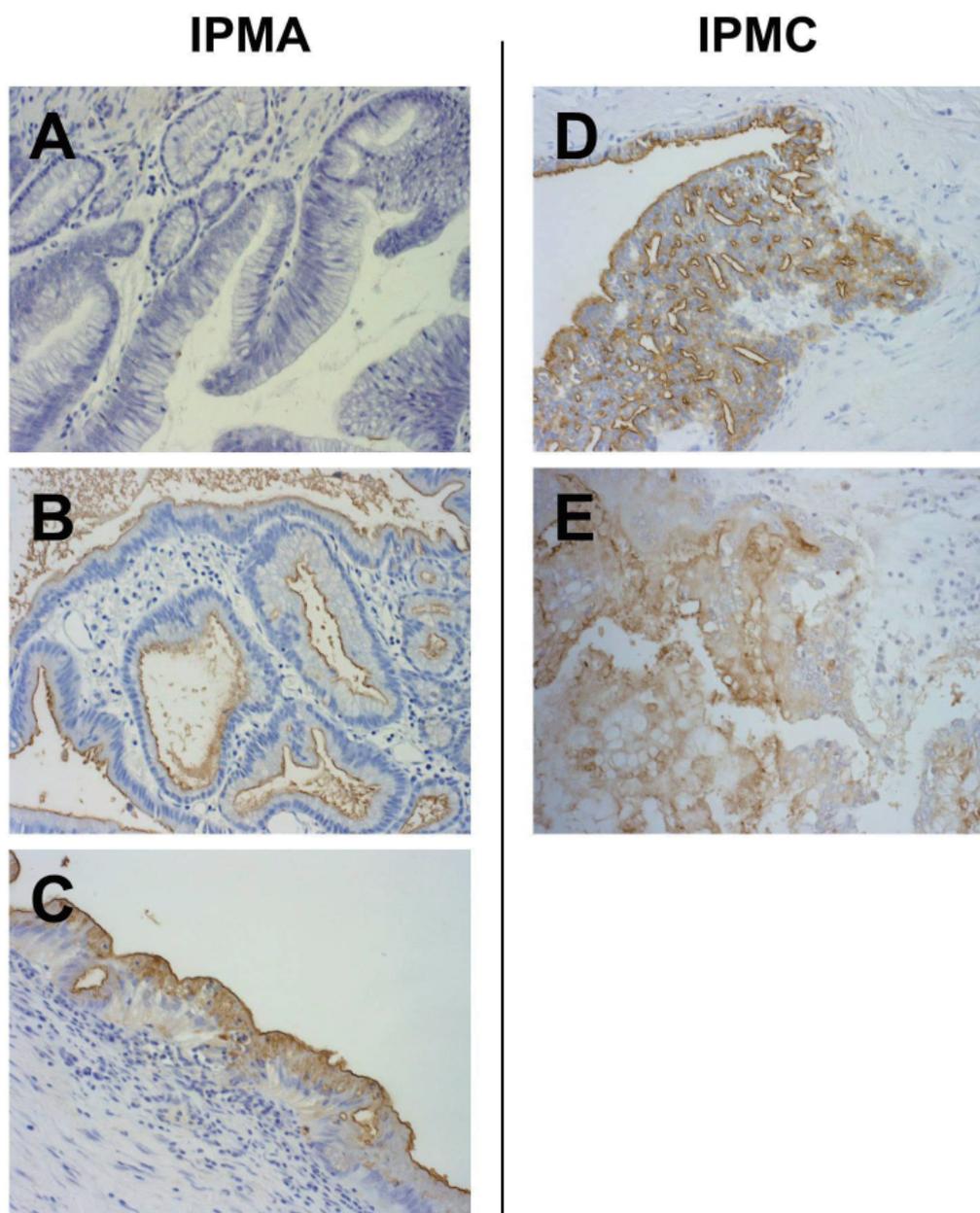


Figure 2. Typical examples of the expression profile of KL-6 mucin in non-invasive and invasive types of IPMN. IPMAs tested negative for subcellular localization of KL-6 mucin (A), the apical surface of the membrane tested positive (B), and the apical membrane and cytoplasm tested positive (C). In IPMNs in the form of carcinoma, the apical surface of the membrane, circumferential membrane and/or cytoplasm in cancer cells tested positive (D, E) but the extent varied among tissue. Original magnification: $\times 100$.

of membrane, 9 (24%) had positive staining in the circumferential membrane, and 13 (34%) had positive staining in the cytoplasm of tumor cells. All PDACs had positive staining in the circumferential membrane and cytoplasm of cancer cells (Figure 1).

3.2. Pathological significance of subcellular localization of KL-6 mucin in IPMNs

The relationship between subcellular localization of KL-6 mucin and pathological diagnosis of IPMN is summarized in Table 2. All IC-IPMCs and MI-IPMCs had positive staining of KL-6 mucin. All 6 IC-IPMCs and 7 MI-IPMCs (88%) had positive staining in the

Table 2. Subcellular localization of KL-6 mucin in IPMNs

| Subcellular localization of KL-6 mucin | IPMA (n = 24) | MI-IPMC (n = 8) | IC-IPMC (n = 6) |
|--|------------------|--------------------|--------------------|
| Negative | 14 (58%) | 0 (0%) | 0 (0%) |
| Apical surface | 9 (38%) | 7 (88%) | 6 (100%) |
| Circumferential membrane | 1 (4%) | 3 (38%) | 5 (83%) |
| Cytoplasm | 2 (8%) | 5 (63%) | 6 (100%) |

IC-IPMC, invasive carcinoma originating from intraductal papillary mucinous carcinoma; IPMA, intraductal papillary mucinous adenoma; MI-IPMC, minimally invasive intraductal papillary mucinous carcinoma.

apical surface of tumor cells. Six IPMAs (40%) had positive staining in the apical surface of tumor cells. Five

IC-IPMCs (83%) and 3 MI-IPMCs (38%) had positive staining in the circumferential membrane of tumor cells but only 1 IPMA (4%) tested positive. All 6 IC-IPMCs and 5 MI-IPMCs (63%) had positive staining in the cytoplasm of tumor cells but only 2 IPMAs (8%) tested positive. The rate of positive KL-6 mucin localization in the circumferential membrane or cytoplasm of tumor cells gradually increased in accordance with the invasive malignancy of the IPMN, and the rate of positive KL-6 mucin localization was significantly higher in IC-IPMC and MI-IPMC compared to IPMA (Table 2, $p < 0.0001$). In addition, 2 IC-IPMCs were positive for lymph node metastasis. Staining of KL-6 mucin in the circumferential membrane and cytoplasm of tumor cells was particularly intense. Age, gender, and location of IPMN were not significantly related to the subcellular localization of KL-6 mucin.

4. Discussion

This study focused on the subcellular localization of KL-6 mucin in IPMNs and PDACs and it found that MI-IPMC and IC-IPMC frequently had positive KL-6 mucin expression in the circumferential membrane or cytoplasm of tumor cells. In addition, all PDACs tested positive for KL-6 mucin expression in the circumferential membrane and/or cytoplasm of cancer cells. These results suggested that aberrant expression of KL-6 mucin is associated with the malignancy of IPMN.

KL-6 mAb was originally established from BALB/c mouse splenocytes immunized with a human pulmonary adenocarcinoma cell line, and the overexpression of KL-6 mucin has frequently been detected in this type of cancer tissue (16). Recent studies have also noted the aberrant expression of KL-6 mucin in various gastrointestinal cancer tissues and demonstrated its usefulness in clinicopathological diagnosis (12). Subcellular localization of KL-6 mucin in the circumferential membrane and/or cytoplasm of cancer cells is significantly associated with deeper invasion, lymphatic invasion, venous invasion, and lymph node metastasis (11,17). Therefore, KL-6 mucin may serve as a predictable marker of worse tumor behavior. Furthermore, overexpression of KL-6 mucin in the circumferential membrane and/or cytoplasm of cancer cells at the invasive front of gastric cancer tissue is significantly related to a higher incidence of metastasis and worse survival for patients (18). These studies suggested that aberrant localization of KL-6 mucin in the circumferential membrane and/or cytoplasm of cancer cells plays a significant role in cancer cell invasion. In the present study, localization of KL-6 mucin in the circumferential membrane and cytoplasm of tumor cells was frequently detected in MI-IPMCs or IC-IPMCs but not in IPMAs (Table 2). In addition, all PDACs that had highly invasive and metastatic characteristics had this localization of KL-6 mucin. These results suggest that

localization of KL-6 mucin in tumor cells is associated with progression of IPMN.

The relationship between the expression of MUC1 and pancreatic tumor behavior has been investigated. Expression of MUC1 was clarified to depict different profiles based on the pathology of pancreatic tumors and the glycoform of MUC1 (9). Although PDACs had frequent expression of various forms of MUC1, hyperglycosylated or sialylated MUC1 was detected in the tissue of IPMNs but hypoglycosylated MUC1 was not (19). Furthermore, MUC1 expression was detected in invasive areas of IPMNs but not in non-invasive areas (20). Expression of specific types of MUC1 may be related to the invasive characteristics of IPMN. Therefore, the detection of MUC1 is considered to be an effective way to diagnose the behavior of IPMN, but there is little evidence on IPMNs based on invasive properties and KL-6 mucin. The present study is the first to show that the rate of detection of malignant KL-6 mucin localization increased gradually in accordance with the invasive characteristics of tumor cells in IPMNs. IC-IPMCs with lymph node metastasis had particularly strong recognition by KL-6 mAb. These results suggest a relationship between the invasive properties of pancreatic tumor cells and expression of MUC1 detected by KL-6 mAb. Cell lines derived from Muc1-null tumors had diminished invasion *in vitro* (21), but the role for glycoforms of MUC1 in cancer cell behavior is still unclear. Further studies should clarify the molecular biological importance of KL-6 mucin expression in IPMN cells.

In conclusion, all MI-IPMCs and IC-IPMCs had malignant localization of KL-6 mucin while 58% of IPMAs tested negative. The frequency of positive localization of KL-6 mucin gradually increased in accordance with the invasiveness of IPMNs. The surgical outcomes for IPMNs reportedly depend on their invasive characteristics (22,23). Precise evaluation of the invasive malignancy of IPMN is therefore crucial to a better prognosis for patients and determination of surgical strategy. Although further studies should include a larger number of patient, KL-6 mucin could help to distinguish an invasive type of IPMN from a noninvasive or minimally invasive type of IPMN.

Acknowledgements

This study was supported by Grants-in-Aid from the Japan Society for the Promotion of Science (JSPS) and the Ministry of Education, Science, Sports, and Culture of Japan. The authors also wish to thank Atsuko Takeuchi for her valuable technical assistance.

References

1. Singhal A, Hakomori S. Molecular changes in carbohydrate antigens associated with cancer. *Bioessays*.

- 1990; 12:223-230.
- 2 Fogel M, Altevogt P, Schirmmacher V. Metastatic potential severely altered by changes in tumor cell adhesiveness and cell-surface sialylation. *J Exp Med.* 1983; 157:371-376.
 - 3 Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani EN, Wilson D. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J Biol Chem.* 1990; 265:15286-15293.
 - 4 Nakamori S, Ota DM, Cleary KR, Shirotani K, Irimura T. MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma. *Gastroenterology.* 1994; 106:353-361.
 - 5 Kashiwagi H, Kijima H, Dowaki S, Ohtani Y, Tobita K, Tsukui M, Tanaka Y, Matsubayashi H, Tsuchida T, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Tajima T, Makuuchi H. DF3 expression in human gallbladder carcinoma: significance for lymphatic invasion. *Int J Oncol.* 2000; 16:455-459.
 - 6 Luttgies J, Feyerabend B, Buchelt T, Pacena M, Kloppel G. The mucin profile of noninvasive and invasive mucinous cystic neoplasms of the pancreas. *Am J Surg Pathol.* 2002; 26:466-471.
 - 7 Zhang HK, Zhang QM, Zhao TH, Li YY, Yi YF. Expression of mucins and E-cadherin in gastric carcinoma and their clinical significance. *World J Gastroenterol.* 2004; 10:3044-3047.
 - 8 Osako M, Yonezawa S, Siddiki B, Huang J, Ho JJ, Kim YS, Sato E. Immunohistochemical study of mucin carbohydrates and core proteins in human pancreatic tumors. *Cancer.* 1993; 71:2191-2199.
 - 9 Yonezawa S, Higashi M, Yamada N, Yokoyama S, Goto M. Significance of mucin expression in pancreatobiliary neoplasms. *J Hepatobiliary Pancreat Sci.* 2010; 17:108-124.
 - 10 Tang W, Inagaki Y, Kokudo N, Guo Q, Seyama Y, Nakata M, Imamura H, Sano K, Sugawara Y, Makuuchi M. KL-6 mucin expression in carcinoma of the ampulla of Vater: association with cancer progression. *World J Gastroenterol.* 2005; 11:5450-5454.
 - 11 Guo Q, Tang W, Inagaki Y, Midorikawa Y, Kokudo N, Sugawara Y, Nakata M, Konishi T, Nagawa H, Makuuchi M. Clinical significance of subcellular localization of KL-6 mucin in primary colorectal adenocarcinoma and metastatic tissues. *World J Gastroenterol.* 2006; 12:54-59.
 - 12 Inagaki Y, Xu H, Nakata M, Seyama Y, Hasegawa K, Sugawara Y, Tang W, Kokudo N. Clinicopathology of sialomucin: MUC1, particularly KL-6 mucin, in gastrointestinal, hepatic and pancreatic cancers. *Biosci Trends.* 2009; 3:220-232.
 - 13 Xu H, Inagaki Y, Seyama Y, Du G, Wang F, Kokudo N, Tang W. Expression of KL-6/MUC1 in pancreatic cancer tissues and its potential involvement in tumor metastasis. *Oncol Rep.* 2011; 26:371-376.
 - 14 Japan Pancreatic Society Classification of Pancreatic Carcinoma. 1st English ed. Kanehara & Co Ltd. 1996.
 - 15 Rahn JJ, Dabbagh L, Pasdar M, Hugh JC. The importance of MUC1 cellular localization in patients with breast carcinoma: an immunohistologic study of 71 patients and review of the literature. *Cancer.* 2001; 91:1973-1982.
 - 16 Kohno N, Akiyama M, Kyoizumi S, Hakoda M, Kobuke K, Yamakido M. Detection of soluble tumor-associated antigens in sera and effusions using novel monoclonal antibodies, KL-3 and KL-6, against lung adenocarcinoma. *Jpn J Clin Oncol.* 1988; 18:203-216.
 - 17 Xu HL, Inagaki Y, Wang FS, Kokudo N, Nakata M, Tang W. Effect of benzyl-*N*-acetyl- α -galactosaminide on KL-6 mucin expression and invasive properties of a human pancreatic carcinoma cell line. *Drug Discov Ther.* 2008; 2:282-285.
 - 18 Inagaki Y, Tang W, Xu H, Nakata M, Mafune K, Konishi T, Seto Y, Kokudo N. Sustained aberrant localization of KL-6 mucin and beta-catenin at the invasion front of human gastric cancer cells. *Anticancer Res.* 2011; 31:535-542.
 - 19 Horinouchi M, Nagata K, Nakamura A, Goto M, Takao S, Sakamoto M, Fukushima N, Miwa A, Irimura T, Imai K, Sato E, Yonezawa S. Expression of different glycoforms of membrane mucin (MUC1) and secretory mucin (MUC2, MUC5AC and MUC6) in pancreatic neoplasms. *Acta Histochem Cytochem.* 2003; 36:443-453.
 - 20 Yonezawa S, Taira M, Osako M, Kubo M, Tanaka S, Sakoda K, Takao S, Aiko T, Yamamoto M, Irimura T, Kim YS, Sato E. MUC-1 mucin expression in invasive areas of intraductal papillary mucinous tumors of the pancreas. *Pathol Int.* 1998; 48:319-322.
 - 21 Besmer DM, Curry JM, Roy LD, Tinder TL, Sahraei M, Schettini J, Hwang SI, Lee YY, Gendler SJ, Mukherjee P. Pancreatic ductal adenocarcinoma mice lacking mucin 1 have a profound defect in tumor growth and metastasis. *Cancer research.* 2011; 71:4432-4442.
 - 22 Suzuki Y, Atomi Y, Sugiyama M, Isaji S, Inui K, Kimura W, Sunamura M, Furukawa T, Yanagisawa A, Ariyama J, Takada T, Watanabe H, Suda K. Cystic neoplasm of the pancreas: a Japanese multiinstitutional study of intraductal papillary mucinous tumor and mucinous cystic tumor. *Pancreas.* 2004; 28:241-246.
 - 23 Nara S, Onaya H, Hiraoka N, Shimada K, Sano T, Sakamoto Y, Esaki M, Kosuge T. Preoperative evaluation of invasive and noninvasive intraductal papillary-mucinous neoplasms of the pancreas: clinical, radiological, and pathological analysis of 123 cases. *Pancreas.* 2009; 38:8-16.

(Received June 30, 2014; Revised July 18, 2014; Accepted July 19, 2014)