Int Surg 2017;**102:**272–277
DOI: 10.9738/INTSURG-D-17-00072.1

Prediction of Postoperative Early Recurrence and Prognosis in Pancreatic Cancer Patients

Hiroshi Kurahara[1], Kosei Maemura[1], Yuko Mataki[1], Masahiko Sakoda[1], Satoshi Iino[1], Yota Kawasaki[1], Takaaki Arigami[1], Shinichiro Mori[1], Hiroyuki Shinchi[2], Shoji Natsugoe[1]

[1]*Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, and*

[2]*Health Sciences, Kagoshima University, Kagoshima, Japan*

Background: Stratification of pancreatic cancer patients based on early recurrence and prognosis is essential for selecting optimal therapeutic strategies. The aim of this study was to investigate whether serum carbohydrate antigen (CA) 19-9 levels can be better used to predict early recurrence and prognosis.

Methods: Patients whose CA 19-9 levels obtained in association with total bilirubin >2.0 mg/dL and whose CA 19-9 levels <5.0 U/mL were excluded to avoid the influence of obstructive jaundice and Lewis phenotype Le^{a-b-}, respectively. A total of 125 pancreatic cancer patients who underwent surgical resection were enrolled.

Results: Larger tumor size, higher serum CA 19-9 level, higher C-reactive protein (CRP) level, more advanced T status, and lymph node metastasis were significantly associated with recurrence within 1 year after surgery (early recurrence). The rate of early recurrence in patients with CA 19-9 levels >100 U/mL (76.9%) was significantly higher than that of patients with CA 19-9 levels ≤100 U/mL (27.9%). We performed combination analysis of 2 additional risk factors, CRP level (cutoff: 1.0 mg/dL) and tumor size (cutoff: 3.0 cm), with serum CA 19-9 level (cutoff: 100 U/mL). Patients were divided into 3 groups according to their number of risk factors. Rates of early recurrence in patients with 0, 1, and 2 or 3 risk factors were 22.0%, 45.2%, and 91.7%, respectively. Moreover, overall survival was significantly different in each group.

Conclusion: Serum CA 19-9 level, in addition to CRP level and tumor size, improve patient stratification in early recurrence as well as prognosis.

Key words: CA 19-9 – CRP – Recurrence – Metastasis – Prognosis

Corresponding author: Hiroshi Kurahara, MD, Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, Kagoshima University, Kagoshima, Japan.
Tel.: +81 99 275 5361; Fax: +81 99 265 7426; E-mail: h-krhr@m3.kufm.kagoshima-u.ac.jp

Pancreatic cancer is one of the most lethal malignancies due to its rapid growth, aggressive invasiveness, and early systemic metastasis.[1] Despite recent development in diagnosis and management, the 5-year survival rate of pancreatic cancer patients is only 4%.[2] Most cases of pancreatic cancer are already advanced by the time of diagnosis; more than 50% are metastatic.[3] Even after potentially curative resection, more than 50% of patients develop local or distant metastasis despite adjuvant therapy.[4] The most common site of distant metastasis is the liver, and calculations based on estimates of tumor doubling times indicate that occult liver metastases may exist in many patients diagnosed as having localized tumor at the time of surgical resection.[5] Intensive neoadjuvant chemotherapies for both borderline and fully resectable pancreatic cancers have recently been adopted to reduce postoperative recurrence and achieve a more favorable prognosis.[6–9] However, it remains unclear which patient subpopulation would benefit from intensive preoperative therapy. Prediction of early recurrence after surgery is critical for selecting the optimal therapeutic strategy for this formidable disease.

To this end, serum carbohydrate antigen (CA) 19-9 has been investigated in pancreatic cancer, and its roles in diagnosis and therapeutic management have been increasingly elucidated.[10–12] In patients who underwent surgical resection of pancreatic cancer, serum CA 19-9 level has been reported to correlate with tumor resectability, postoperative recurrence, and prognosis.[13,14] Furthermore, several studies reported that changes in serum CA 19-9 levels over the course of chemotherapy or chemoradiotherapy are indicative of the responses of advanced pancreatic tumors to treatment.[15–17] However, serum CA 19-9 can be affected by obstructive jaundice and Lewis blood group antigen. Specifically, biliary obstruction has been reported to increase serum CA 19-9 levels,[18] whereas patients belonging to the Lewis phenotype Le^{a-b-}, which occurs in 5%–10% of the population, are not able to produce CA 19-9.[19] Therefore, exclusion of patients with obstructive jaundice and Lewis phenotype Le^{a-b-} is essential to properly investigate the efficacy of serum CA 19-9 level as a prognostic or metastatic predictor in pancreatic cancer patients.

Many previous studies have demonstrated that inflammatory status is associated with prognosis in patients with various malignant tumors.[20] Several recent studies demonstrated that pancreatic cancer patients with high CRP levels had poor prognoses.[21,22] However, the cutoff values of for CRP level varied by study, and its significance as a predictor of early recurrence after surgical resection is therefore unclear. Additionally, previous large cohort studies revealed several other prognostic clinicopathologic factors such as lymph node metastasis, pathologic T stage, histologic grade, tumor size, and adjuvant chemotherapy.[23,24] Among these factors, tumor size is potentially evaluable before surgery.

The primary aim of this study was to investigate the feasibility of employing CA 19-9 level, in combination with CRP level and tumor size, for better stratification of pancreatic cancer patients based on their prognosis and the risk of early postoperative recurrence.

Materials and Methods

Patient enrollment

Pancreatic cancer patients who underwent surgical resection at our department between January 2000 and December 2014 were enrolled in this study. Patients whose serum CA 19-9 values were not examined before treatment were excluded. This study was approved by the institutional ethics review board of Kagoshima University Hospital, and written informed consent for data analysis regarding the association between clinicopathologic factors and prognosis was obtained. All resected specimens were examined histologically according to the 7th TNM classification system.[25] Imaging examinations that included computed tomography (CT) and magnetic resonance imaging (MRI) were performed every 4 months for the first 2 years and every 6 months for the following 3 years. The median follow-up duration was 23.0 months (mean: 30.2 months).

Clinical examination

Before treatment, all patients underwent a clinical evaluation, routine laboratory tests, and imaging studies [CT plus magnetic resonance imaging and or endoscopic ultrasonography (EUS)]. Serum CA 19-9 levels obtained in association with total bilirubin $\leq$2.0 mg/dL were considered evaluable. Patients in whom total bilirubin was >2.0 mg/dL at the time baseline CA 19-9 levels were measured were excluded from the study, as biliary obstruction may lead to an artificial increase in serum CA 19-9 levels.[15,16] Furthermore, CA 19-9 levels in patients with Lewis phenotype Le^{a-b-} may not reflect accurate disease statuses. In a previous genetic study of the association between Lewis phenotype and CA 19-9 levels, all

patients with true Le^{a-b-} exhibited <1.0 U/mL CA 19-9.[19] Because genotyping was not performed in all patients, those with Le^{a-b-} were not identified. Therefore, patients with CA 19-9 levels <5 U/mL were excluded from this study to constrain the analysis to only those who could produce CA 19-9.[15] Laboratory data including CA 19-9 and CRP levels were obtained within 7 days before surgery.

Statistical analysis

Continuous variables, including CA 19-9 levels, were presented as the median (interquartile range), and were compared using the Mann–Whitney *U* test. Associations between different categoric variables were assessed using the χ^2 test or Fisher's exact test. *P* values <0.05 were considered statistically significant. Overall survival (OS) curves were plotted using the Kaplan–Meier method and analyzed using the log-rank test. OS periods were calculated starting from immediately after surgery. Statistical evaluation was performed using SigmaPlot version 12.5 for Windows (Hulinks Inc., Tokyo, Japan).

Results

Patient characteristics

Of 143 patients who underwent surgical resection for pancreatic cancer, 6 were excluded because of total bilirubin levels >2.0 mg/dL. Of the remaining 137 patients, 125 (91.2%) with serum CA 19-9 levels >5 U/mL were enrolled. Microscopically curative resection (R0) was achieved in 101 patients (80.8%). Forty-one patients (32.8%) underwent preoperative chemotherapy or chemoradiotherapy, and 81 (64.8%) received postoperative adjuvant chemotherapy. The mean size of the 125 tumors was 31 mm (median: 27 mm). Eighty-five (68.0%) of the 125 patients had postoperative recurrence; these were detected within 1 year after surgery in 54 patients. We regarded recurrence within 1 year after surgery as early recurrence in this study. Of these 54 early recurrence patients, 31 (57.4%) had hematogenous metastases; the most common site of the metastasis was the liver (28 cases).

Association between early recurrence and clinicopathologic factors

As shown in Table 1, larger tumor size, higher CA 19-9 level, higher CRP level, more advanced T factor, and lymph node metastasis were significantly associated with early recurrence after surgery.

Table 1 Association between recurrence within 1 year after surgery and clinicopathologic factors in patients with pancreatic cancer (n = 125)

Factor (n)	Recurrence (−) (n = 71)	Recurrence (+) (n = 54)	*P* value
Sex			
Male (73)	37	36	0.146
Female (52)	34	18	
Age (years)	69.0 (61.0–75.0)	67.5 (62.8–74.0)	0.962
Tumor size (mm)	25.0 (15.0–30.0)	33.5 (25.0–50.0)	<0.001
Tumor position			
Head (80)	46	34	0.982
Body-tail (45)	25	20	
CA 19-9 (U/mL)	20.4 (11.1–63.7)	135.2 (44.0–431.4)	<0.001
Albumin (g/dL)	4.0 (3.8–4.2)	4.1 (3.8–4.3)	0.507
CRP (mg/dL)	0.08 (0.03–0.20)	0.16 (0.08–0.61)	<0.001
Histology			
Grade 1 (60)	36	24	0.608
Grade 2/3 (65)	35	30	
T factor			
1/2 (15)	13	2	0.027
3/4 (110)	58	52	
N factor			
0 (52)	41	11	<0.001
1 (73)	30	43	
Postoperative adjuvant therapy			
No (45)	20	25	0.057
Yes (80)	51	29	

CA 19-9, carbohydrate antigen 19-9; CRP, C-reactive protein.

Relationship between preoperative serum CA 19-9 level, CRP level, tumor size, and early recurrence

We analyzed the serum CA 19-9 level while testing several cutoff values to predict early recurrence after surgery (Table 2). A cutoff value of 100 U/mL was the most reliable for predicting early recurrence (sensitivity: 55.6%, specificity: 87.3%, and accuracy: 73.6%). The rate of early recurrence in patients with serum CA 19-9 levels >100 U/mL (76.9%) was

Table 2 Association between each prognostic predictor and recurrence within 1 year after surgery in patients with pancreatic cancer (n = 125)

Cut-off value	Sensitivity	Specificity	PPV	NPV	Accuracy
CA 19-9 level					
50 U/mL	72.2%	73.2%	67.2%	77.6%	72.8%
100 U/mL	55.6%	87.3%	76.9%	72.1%	73.6%
200 U/mL	40.7%	94.4%	84.6%	67.7%	71.2%
CRP level					
0.2 mg/mL	48.1%	74.6%	59.1%	65.4%	63.2%
0.5 mg/mL	25.9%	91.5%	70%	61.9%	63.2%
1.0 mg/dL	20.4%	100%	100%	62.3%	65.6%
Tumor size					
2 cm	88.9%	42.3%	88.9%	42.3%	62.4%
3 cm	55.6%	74.6%	62.5%	68.8%	66.4%
4 cm	37.0%	87.3%	69.0%	64.6%	65.6%

CA 19-9, carbohydrate antigen 19-9; CRP, C-reactive protein; NPV, negative predictive value; PPV, positive predictive value.

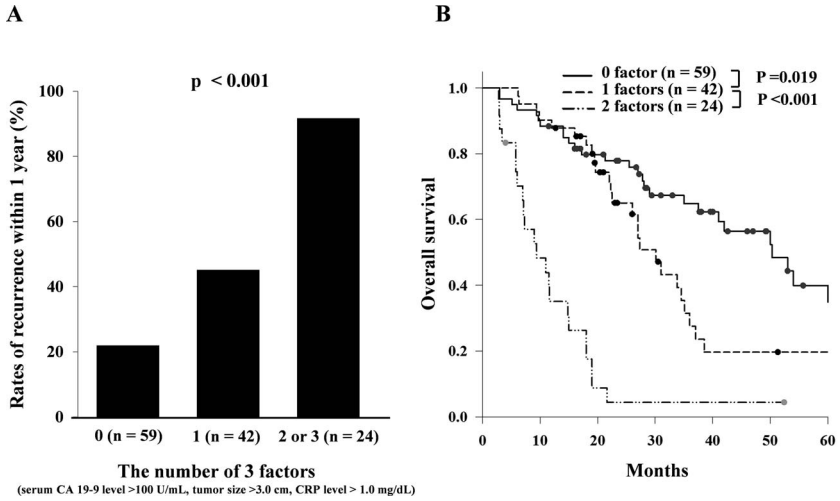

Fig. 1 (A) Rates of pancreatic cancer recurrence within 1 year after surgery according to the containing the number of the 3 risk factors (serum CA 19-9 level >100 U/mL; CRP level >1.0 mg/dL; and tumor size >3.0 cm). (B) Kaplan–Meier survival curves according to the number of these risk factors present.

significantly higher than in those with serum CA 19-9 levels ≤100 U/mL (27.9%). Next, we analyzed tumor size and CRP level using several cutoff values as predictors of early recurrence after surgery (Table 2). The accuracies of CRP level and tumor size were lower than that of serum CA 19-9 level (60%-plus range). Cut-off values with highest accuracy were 1.0 mg/dL and 3.0 cm for CRP and tumor size, respectively. We assessed the diagnostic significance of tumor size in 81 patients who underwent all 3 imaging modalities (CT, MRI, and EUS). When categorizing tumor sizes into ≤3 cm versus and >3 cm, 72 patients (88.9%) were accurately diagnosed using this parameter.

Prediction of early recurrence and prognosis using serum CA 19-9 level, tumor size, and CRP level

We performed combination analysis using the 3 risk factors (serum CA 19-9 level, CRP level, and tumor size) to achieve better stratification based on the risk of early recurrence after surgery (Fig. 1A). We divided all patients into 3 groups according to their number of positive risk factors. Rates of early recurrence after surgery in patients with 0, 1, and 2 or 3 risk factors were 22.0%, 45.2%, and 91.7%, respectively. Using this method, patients could clearly be stratified by the risk of early recurrence after surgery. Next, we performed survival analysis based on these 3 risk factors (Fig. 1B). There was a significant difference in OS between each patient group, indicating that patients could be readily stratified according to their postoperative prognoses. Median OS in patients with 0, 1, and 2 or 3 risk factors were 50.3 months, 27.3 months, and 9.4 months, respectively.

Discussion

Pancreatic cancer produces distant metastases at an early stage; the liver is the most common site of such metastases.[5] *In vivo* lineage tracing revealed that entry of pancreatic cancer cells into the bloodstream and seeding into the liver occurs before detectable primary tumor formation.[26] In the present study, 54 of the 125 pancreatic cancer patients (43.2%) had postoperative recurrence within 1 year; hepatic recurrence occurred most frequently. Better patient stratification based on the risk of early recurrence and prognosis is critical for devising optimal therapeutic strategies for pancreatic cancer patients. CA 19-9 has become the most important tumor marker for pancreatic cancer since first reported by Koprowski *et al* in 1979.[27] Patients who developed early recurrence after surgery showed higher serum CA 19-9 levels, larger tumor sizes, higher CRP levels, and positive nodal statuses. With regard to prediction of early recurrence, the serum CA 19-9 cut-off value of 100 U/mL demonstrated 55.6% sensitivity, 87.3% specificity, and 73.6% accuracy. Patients with serum CA 19-9 levels >100 U/mL showed significantly higher early recurrence rates compared with those with serum CA 19-9 levels ≤100 U/mL.

Many previous studies reported the clinical utility of serum CA 19-9 in predicting recurrence and prognosis in pancreatic cancer patients after adopting various cutoff values.[10,15,16,28] Optimal cutoff values may be different depending on the type of tumor (resectable, borderline resectable, or unresectable pancreatic cancer) or the purpose (screening, resectability, recurrence, or response to chemotherapies). As mentioned already, the value of

CA 19-9 can also be affected by obstructive jaundice and Lewis antigen expression. In this study, the ratio of patients excluded because of CA 19-9 levels <5.0 U/mL (8.8%) is similar to that of the reported percentage of Lewis phenotype Le^{a-b-} patients (5%–10%).[19]

We incorporated 2 additional risk factors, CRP level and tumor size, to achieve better stratification of patients based on the risk of early recurrence after surgery. CRP level and tumor size were previously reported to be prognostic factors in pancreatic cancer patients;[21–24,29] this was reaffirmed in our study. With regard to prediction of early recurrence after surgery, cutoff values of 1.0 mg/dL for CRP and 3.0 cm for tumor size showed highest accuracy. By using the 3 risk factors, serum CA 19-9 level (cutoff value: 100 U/mL), CRP level (cutoff value: 1.0 mg/dL), and tumor size (cutoff value: 3.0 cm), we divided the patients into 3 groups based on the number of the containing risk factors. This classification could stratify the pancreatic cancer patients more clearly based on not only early postoperative recurrence but also the OS. Microenvironments with dense, desmoplastic stroma rich in inflammatory cells, fibroblasts, and extracellular matrix proteins are traits of pancreatic cancer.[30] The inflammatory response to tumor cells, reflected by an elevated serum CRP level, may produce proinflammatory cytokines, angiogenic and lymphogenic factors, and chemokines that lead to tumor growth, angiogenesis, and metastasis.[21]

There were several limitations in this study. First, because of its retrospective nature, the treatment strategy was not uniform. Patients not subjected to adjuvant therapy tended to experience more frequent early recurrence. Second, it is difficult to judge the tumor size accurately before surgery. However, by using EUS in addition to CT and MRI, whether the tumor was larger or smaller than 3 cm was judged accurately in a majority of patients (88.9%). Despite these limitations, the present study demonstrated the ability to stratify patients based on the risk of early recurrence after surgery. Such reliable stratification may be useful to determine the treatment strategy, including intensive neoadjuvant therapies, for pancreatic cancer patients. Patients with a high risk of early recurrence ought to be administered intensive systemic chemotherapy before surgery, even those with resectable tumors.

In conclusion, our findings demonstrated that serum CA 19-9 level, together with CRP level and tumor size, improved the ability to stratify patients based not only on early recurrence, but prognosis as well.

Acknowledgments

The authors state that they have no conflicts of interest and that they received no financial or material support.

References

1. Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. *Nat Rev Clin Oncol* 2010;**7**(3):163–172
2. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet 2011;**378**(9791):607–620
3. Ying JE, Zhu LM, Liu BX. Developments in metastatic pancreatic cancer: is gemcitabine still the standard? *World J Gastroenterol* 2012;18(8):736–745
4. Cameron JL, Riall TS, Coleman J, Belcher KA. One thousand consecutive pancreaticoduodenectomies. *Ann Surg* 2006;244(1):10–15
5. Yachida S, Iacobuzio-Donahue CA. The pathology and genetics of metastatic pancreatic cancer. *Arch Pathol Lab Med* 2009;**133**(3):413–422
6. Rose JB, Rocha FG, Alseidi A, Biehl T, Moonka R, Ryan JA *et al*. Extended neoadjuvant chemotherapy for borderline resectable pancreatic cancer demonstrates promising postoperative outcomes and survival. *Ann Surg Oncol* 2014;**21**(5):1530–1537
7. Ferrone CR, Marchegiani G, Hong TS, Ryan DP, Deshpande V, McDonnell El *et al*. Radiological and surgical implications of neoadjuvant treatment with FOLFIRINOX for locally advanced and borderline resectable pancreatic cancer. *Ann Surg* 2015;**261**(1):12–17
8. Christians KK, Heimler JW, George B, Ritch PS, Erickson BA, Johnston F *et al*. Survival of patients with resectable pancreatic cancer who received neoadjuvant therapy. *Surgery* 2016;**159**(3):893–900
9. Ielpo B, Duran H, Diaz E, Fabral I, Caruso R, Ferri V *et al*. Preoperative treatment with gemcitabine plus nab-paclitaxel is a safe and effective chemotherapy for pancreatic adenocarcinoma. *Eur J Surg Oncol* 2016;**42**(9):1394–1400
10. Katz MH, Varadhachary GR, Fleming JB, Wolff RA, Lee JE, Pisters PW *et al*. Serum CA 19-9 as a marker of resectability and survival in patients with potentially resectable pancreatic cancer treated with neoadjuvant chemoradiation. *Ann Surg Oncol* 2010;**17**(7):1794–1801
11. Ballehaninna UK, Chamberlain RS. The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: an evidence based appraisal. *J Gastrointest Oncol* 2012;**3**(2):105–119

12. Choe JW, Kim JS, Kim HJ, Hwang SY, Joo MK, Lee BJ *et al*. Value of early check-up of carbohydrate antigen 19-9 levels for pancreatic cancer screening in asymptomatic new-onset diabetic patients. *Pancreas* 2016;**45**(5):730–734
13. Schlieman MG, Ho HS, Bold RJ. Utility of tumor markers in determining resectability of pancreatic cancer. *Arch Surg* 2003; **138**(9):951–955; discussion 955–956
14. Ferrone CR, Finkelstein DM, Thayer SP, Muzikansky A, Fernandez-del Castillo C, Warshaw AL. Perioperative CA19-9 levels can predict stage and survival in patients with resectable pancreatic adenocarcinoma. *J Clin Oncol* 2006; **24**(18):2897–2902
15. Park JK, Paik WH, Ryu JK, Kim YT, Kim YJ, Kim J *et al*. Clinical significance and revisiting the meaning of CA 19-9 blood level before and after the treatment of pancreatic ductal adenocarcinoma: analysis of 1, 446 patients from the pancreatic cancer cohort in a single institution. *PloS One* 2013;**8**(11):e78977.
16. Tzeng CW, Balachandran A, Ahmad M, Lee JE, Krishnan S, Wang H *et al*. Serum carbohydrate antigen 19-9 represents a marker of response to neoadjuvant therapy in patients with borderline resectable pancreatic cancer. *HPB (Oxford)* 2014; **16**(5):430–438
17. Chung KH, Ryu JK, Lee BS, Jang DK, Lee SH, Kim YT. Early decrement of serum carbohydrate antigen 19-9 predicts favorable outcome in advanced pancreatic cancer. *J Gastroenterol Hepatol* 2016;**31**(2):506–512
18. Kang CM, Kim JY, Choi GH, Kim KS, Choi JS, Lee WJ *et al*. The use of adjusted preoperative CA 19-9 to predict the recurrence of resectable pancreatic cancer. *J Surg Res* 2007;**140**(1):31–35
19. Narimatsu H, Iwasaki H, Nakayama F, Ikehara Y, Kudo T, Nishihara S *et al*. Lewis and secretor gene dosages affect CA19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. *Cancer Res* 1998;**58**(3):512–518
20. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; **420**(6917):860–867
21. Szkandera J, Stotz M, Absenger G, Stojakovic T, Samonigg H, Kornprat P *et al*. Validation of C-reactive protein levels as a prognostic indicator for survival in a large cohort of pancreatic cancer patients. *Br J Cancer* 2014;**110**(1):183–188
22. Mitsunaga S, Ikeda M, Shimizu S, Ohno I, Takahashi H, Okuyama H *et al*. C-reactive protein level is an indicator of the aggressiveness of advanced pancreatic cancer. *Pancreas* 2016; **45**(1):110–116
23. Qiu M, Qiu H, Jin Y, Wei X, Zhou Y, Wang Z *et al*. Pathologic diagnosis of pancreatic adenocarcinoma in the United States: Its status and prognostic value. *J Cancer* 2016;**7**(6):694–701
24. Paniccia A, Hosokawa P, Henderson W, Achulick RD, Edil BH, McCarter MD *et al*. Characteristics of 10-year survivors of pancreatic ductal adenocarcinoma. *JAMA Surg* 2015;**150**(8): 701–710
25. Sobin LH, Gospodarowicz MK, Wittekind C, International Union against Cancer. TNM Classification of Malignant Tumours, 7th ed. Chichester, West Sussex, UK; Hoboken, NJ: Wiley-Blackwell, 2010
26. Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F *et al*. EMT and dissemination precede pancreatic tumor formation. *Cell* 2012;**148**(1–2):349–361
27. Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somatic Cell Genet* 1979;**5**(6):957–971
28. Berger AC, Garcia M Jr., Hoffman JP, Reqine WF, Abrams RA, Safran H *et al*. Postresection CA 19-9 predicts overall survival in patients with pancreatic cancer treated with adjuvant chemoradiation: a prospective validation by RTOG 9704. *J Clin Oncol* 2008;**26**(36):5918–5922
29. Miura T, Hirano S, Nakamura T, Tanaka E, Shichinohe T, Tsuchikawa T *et al*. A new preoperative prognostic scoring system to predict prognosis in patients with locally advanced pancreatic body cancer who undergo distal pancreatectomy with en bloc celiac axis resection: a retrospective cohort study. *Surgery* 2014;**155**(3):457–467
30. Kurahara H, Takao S, Kuwahata T, Nagai T, Ding Q, Maeda K *et al*. Clinical significance of folate receptor beta-expressing tumor-associated macrophages in pancreatic cancer. *Ann Surg Oncol* 2012;**19**(7):2264–2271