Optimize CA19-9 in detecting pancreatic cancer by Lewis and Secretor genotyping

Guopei Luo^{†,a,b,c}, Meng Guo^{†,a,b,c}, Kaizhou Jin^{†,a,b,c}, Zuqiang Liu^{a,b,c}, Chen Liu^{a,b,c}, He Cheng^{a,b,c}, Yu Lu^{a,b,c}, Jiang Long^{a,b,c}, Liang Liu^{a,b,c}, Jin Xu^{a,b,c}, Quanxing Ni^{a,b,c},
Xianjun Yu^{*,a,b,c}

^aDepartment of Pancreas Surgery, Fudan University Shanghai Cancer Center;

^bDepartment of Oncology, Shanghai Medical College, Fudan University;

^cPancreatic Cancer Institute, Fudan University.

[†]Equal contribution

*Correspondence to: Xianjun Yu, MD, PhD, No. 270, Dong'An Road, Xuhui District, Shanghai, 200032, China, Tel: 86-21-64175590-1308; Fax: 86-21-64031446; Email: yuxianjun@fudanpci.org

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ABSTRACT

Background: Carbohydrate antigen 19-9 (CA19-9) is currently the most widely used biomarker for pancreatic cancer. It is well-known that Lewis and Secretor status can affect CA19-9 biosynthesis. This study was performed to optimize CA19-9 in detecting pancreatic cancer using Lewis and Secretor dependent cut-off values.

Methods: Lewis and Secretor genotypes were determined by Sanger sequencing in a large cohort of subjects (578 cases with pancreatic cancer, 210 cases with benign pancreatic disease, 315 normal subjects). The effectiveness of CA19-9 for detecting pancreatic cancer using Lewis and Secretor group dependent cut-off values was evaluated.

Results: The Lewis (-), Mixed, and Secretor (-) groups had low, medium, and high CA19-9 biosynthesis, respectively. In Lewis (-) pancreatic cancer (all stages), CA19-9 had a sensitivity of 48.6% and a specificity of 95.9% when 1.8 U/mL was used as the cut-off value. The sensitivity of CA19-9 in detecting all stages of pancreatic cancer improved from 80.1% to 88.0% and the negative predictive value increased from 81.2% to 87.1% without compromising other values when using group dependent cut-off values. The sensitivity of CA19-9 for the detection of stage I, II pancreatic cancer increased from 76.1% to 87.2%.

Conclusions: The value of CA19-9 in detecting pancreatic cancer was optimized by using group dependent cut-off values based on Lewis and Secretor genotypes. CA19-9 can be applied as an early detector of pancreatic cancer using group dependent cut-off values.

Introduction

Pancreatic cancer is a dismal malignancy with a mortality rate close to its incidence [1, 2]. Surgical resection is the only potentially curative treatment for patients with pancreatic cancer [2]. However, approximately 20% of patients are diagnosed at the localized stage and have the opportunity to undergo curative resection [1-3]. Therefore, there is an urgent need to develop or optimize potential biomarkers that improve pancreatic cancer management.

CA19-9 is currently the most important and widely used biomarker for pancreatic cancer [2, 4-6]. The recommended cut-off point for CA19-9 in detecting pancreatic cancer is 37.0 U/mL [6]. The sensitivity of CA19-9 is approximately 80%, which limits its use in screening pancreatic cancer [6]. It is well known that the Lewis blood group (also called fucosyltransferase 3, or FUT3) has a great impact on the biosynthesis of CA19-9, and subjects with Lewis inactivity do not secrete or scarcely secrete CA19-9 [7-9]. Secretor (also called fucosyltransferase 2, or FUT2) has also been reported to affect the biosynthesis of CA19-9, and subjects with Secretor inactivity can increase secretion levels of CA19-9 [7-9]. Therefore, the detection of pancreatic cancer using CA19-9 should be based on Lewis and Secretor genotypes and activities.

In the study, we divided subjects into three groups according to Lewis and Secretor genotypes and activities (Lewis (-), Mixed, and Secretor (-)). The value of CA19-9 in detecting pancreatic cancer was optimized by using group dependent cut-off values.

Materials and methods

Patients

Patients with pancreatic adenocarcinoma or benign pancreatic disease (chronic pancreatitis, solid pseudopapillary neoplasm, intraductal papillary mucinous neoplasm, serous cystadenoma, mucinous cystadenoma, pancreatic intraepithelial neoplasm, and others) who had been treated at the Fudan University Shanghai Cancer Center were retrieved from a prospectively maintained database. Pathological or cytological evidence was obtained from all patients with pancreatic diseases (pancreatic adenocarcinoma, benign pancreatic tumor, and pancreatitis). CA19-9 was examined as previously described [5]. The exclusion criteria for patients were as follows: no genomic DNA available to perform genotyping; unsuccessful genotyping by sequencing; and no available CA19-9. Patients with pancreatic neuroendocrine tumors were also excluded. Normal subjects with no evidence of any malignancy were used as controls. Demographic characteristics, including gender and age, were obtained for normal subjects. The value of CA19-9 in detecting pancreatic cancer patients with normal total bilirubin was also evaluated taking a cut off value of serum total bilirubin of <2 mg/d. The study was approved by the local ethics committee. Written informed consent was acquired from all of the subjects.

Lewis and Secretor genotyping

Lewis and Secretor genotyping was performed using first-generation sequencing (Sanger). Lewis genotypes were determined based on the following variants: T59G, T202C, C314T, G508A, and T1067A, as previously described [7, 10]. Secretor genotypes were determined based on the following variants: G428A, A385T, and

T357C, as previously described [11, 12]. Secretor homozygous loss-of-function mutations are mainly caused by G428A homozygous mutations in Western populations and by A385T homozygous mutations in Asian populations [11, 12]. Before initiating major treatment, blood specimens were obtained by the Tissue Bank from patients with pancreatic diseases. Genomic DNA was extracted from the blood samples based on standard protocols. PCR amplification was performed using the following primers: (Lewis, 358-F, GGGTGCAGCCAAGCCACAA, 358R, AGGTGGGAGGCGTGACTTAGG; Lewis, P1F, ACTTGGAGCCACCCCCTAACTGCCA, 508R, CGGCCTCTCAGGTGAACCAAGCCGCT; Secretor, 21F, CTAACGTGTCCCGTTTTCCTC, 21R, CCACTCCGGCAGGAAGGC). The PCR products were sequenced using a 3730XL DNA Sequencer (Applied Biosystems, USA).

Group Definition

The definition of groups was based on the activities and genotypes of Lewis and Secretor (Fig. 1) [8, 9]. The Lewis (-) group was defined as subjects with no Lewis activity regardless of Secretor activity. The genotypes of Lewis (-) included homozygous mutated (T202C or C314T or G508A or T1067A), compound heterozygous (T59G/G508A and T202C/C314T, T59G/T1067A and T202C/C314T, T59G and T202C/C314T, T59G/G508A/T1067A and T202C/C314T) and homozygous mutated for T59G and heterozygous mutated for T202C, C314T, G508A or T1067A in the Lewis gene. The Secretor (-) group was defined as subjects who had

no Secretor activity but did have Lewis activity, including all subjects who had Lewis activity but were classified as homozygous mutated for Secretor. The Mixed group was composed of all combinations other than the genotypes in the Lewis (-) and the Secretor (-) groups.

Statistical Analysis

The optimal CA19-9 cut-off values for detecting all stages of pancreatic cancer or stage I, II pancreatic cancer in the three groups were determined by receiver operating characteristic (ROC) analysis. Wilcoxon rank-sum tests were used to compare the CA19-9 values between the three groups and between patients with pancreatic cancer, patients with benign pancreatic diseases, and normal subjects. The area under the ROC curve was calculated for each group. The point closest to the top-left part of the ROC curve was selected as the optimal cut-off point according to the following criterion: $\min((1 - \text{sensitivities})^2 + (1 - \text{specificities})^2)$ [13]. The positive predictive value and negative predictive value were examined using a contingency table. Dichotomous variables were examined using the χ^2 test. Statistical analysis was performed using the STATA 12.0 statistical software package (StataCorp LP, College Station, TX, USA). P values less than 0.05 were considered statistically significant.

Results

Subjects characteristics

The study included 578 cases of pancreatic cancer, with a mean age of 61.3 years and a male-to-female ratio of 1.5. More than half of patients had stage I, II pancreatic cancer (309, 53.4%) and 73.7% (426) patients never had jaundice and they had serum

total bilirubin less than 2 mg/dL. Patients with benign pancreatic disease (210 cases) had a mean age of 49.4 years and a male-to-female ratio of 0.6. Benign pancreatic diseases consisted of chronic pancreatitis (36), solid pseudopapillary neoplasm (42), intraductal papillary mucinous neoplasm (32), serous cystadenoma (63), mucinous cystadenoma (23), and others (14). Normal subjects (315 cases) had a mean age of 36.3 years and a male-to-female ratio of 2.8. No statistically significant difference in the Lewis or Secretor genotype distribution was found between patients with pancreatic cancer, patients with benign pancreatic disease, and normal subjects (patients with pancreatic cancer, Lewis (-), 6.4%, Mixed, 72.0%, Secretor (-), 21.6%; patients with benign pancreatic disease, Lewis (-), 8.6%, Mixed, 73.3%, Secretor (-), 18.1%; Normal, Lewis (-), 9.8%, Mixed, 71.4%, Secretor (-), 18.7%, $P = 0.315$). No deviations in genotype analyses were observed from the Hardy-Weinberg equilibrium. *Lewis (-), Mixed, Secretor (-) groups had low, intermediate, and high CA19-9 biosynthesis potential, respectively*

In normal subjects, the Lewis (-) group had a low, the Mixed group had a medium, and the Secretor (-) group had a high ability to biosynthesize CA19-9 (median and 95% confidence interval (95% CI), Lewis (-), 0.60 U/mL (95CI, 0.60-0.60); Mixed, 7.49 U/mL (95CI, 6.83-8.31); Secretor (-), 18.72 U/mL (95CI, 16.22-20.73), $P < 0.001$, Fig. 2). This trend was also observed in patients with benign pancreatic disease (Lewis (-), 0.87 U/mL (95CI, 0.60-1.10); Mixed, 9.27 U/mL (95CI, 8.34-9.76); Secretor (-), 25.16 U/mL (95CI, 19.06-30.92), $P < 0.001$, Fig. 2), patients with pancreatic cancer (Lewis (-), 1.73 U/mL (95CI, 0.99-10.11); Mixed, 239.35 U/mL

(95CI, 194.00-289.50); Secretor (-), 570.80 U/mL (95CI, 386.53-1000.00), $P < 0.001$, Fig. 2), and patients with pancreatic cancer who had normal total bilirubin (Supplemental Fig. 1).

Grouping cut-off values greatly enhanced the sensitivity and negative predictive value without compromising the specificity and positive predictive value

A ROC curve was used to analyze CA19-9 to detect all of the stages of pancreatic cancer or all of the stages of Lewis (-), Mixed, and Secretor (-) pancreatic cancers (Fig. 3). The area under the ROC curve was 0.90, with a sensitivity of 80.1%, a specificity of 94.7%, a positive predictive value of 94.3%, and a negative predictive value of 81.2% when using 37.0 U/mL as the cut-off value (Table 1). For the Lewis (-) group, the sensitivity of CA19-9 in detecting pancreatic cancer was only 18.9% when using 37.0 U/mL as the cut-off value (specificity, 100.0%; positive predictive value, 100.0%; negative predictive value, 62.0%). However, the area under ROC curve was 0.82, with a sensitivity of 48.6%, a specificity of 95.9%, a positive predictive value of 90.0%, and a negative predictive value of 71.2% when using 1.8 U/mL as the cut-off value. CA19-9 showed promising results for detecting pancreatic cancer in the Mixed group (cut-off value, 22.0 U/mL; sensitivity, 87.5%; area under ROC curve, 0.94) and the Secretor (-) group (cut-off value, 49.4 U/mL; sensitivity, 89.6%; area under ROC curve, 0.94). The diagnostic value was greatly enhanced when using a group dependent cut-off value in the Mixed and Secretor (-) groups (sensitivity, 88.0%; specificity, 92.0%; positive predictive value, 92.6%; negative predictive value, 97.1%). These results were also demonstrated in pancreatic cancer patients with

normal total bilirubin (Supplemental Fig. 2, Supplemental Table 1).

Grouping cut-off values in detecting early stage pancreatic cancer

For stage I, II pancreatic cancer, the area under the ROC curve was 0.89, with a sensitivity of 76.1%, a specificity of 94.7%, a positive predictive value of 89.4%, and a negative predictive value of 87.0% when using 37.0 U/mL as the cut-off value (Fig. 4, Table 1). For Lewis (-) group, the area under the ROC curve was 0.86, with a sensitivity of 50.0%, a specificity of 95.9 %, a positive predictive value of 83.3%, and a negative predictive value of 82.5% when using 1.8 U/mL as the cut-off value. The diagnostic value was greatly enhanced when using the group dependent cut-off value in the Mixed and Secretor (-) groups (sensitivity, 87.2%; specificity, 92.2%; positive predictive value, 87.2%; negative predictive value, 92.2%). These results were also shown in pancreatic cancer patients with normal total bilirubin (Supplemental Fig. 3, Supplemental Table 1).

Discussion

Narimatsu *et al.* [8] reported that individuals who had homozygous inactive Secretor alleles and homozygous active Lewis alleles exhibited the highest mean CA19-9 value, and all Lewis negative individuals had CA19-9 values under 1.0 U/mL. The authors suggested that revised Lewis and Secretor genotype-dependent cutoff values for CA19-9 be used for more accurate cancer detection. In another study, Wannhoff *et al.* [9] aimed to improve the diagnostic accuracy of CA19-9 for biliary malignancy in primary sclerosing cholangitis by assessing the impact of the FUT2/3 genotypes. Their results indicated low, intermediate, and high CA19-9 biosynthesis in

A, no FUT3 activity regardless of FUT2 activity; B, all other FUT2/FUT3 combinations; and C, no FUT2 activity, respectively [9]. They further demonstrated improved sensitivity and reduced the number of false positive results by applying group-dependent cut-off values [9]. Our study confirmed low, medium, and high CA19-9 biosynthesis levels in the Lewis (-), Mixed, and Secretor (-) groups, respectively. By using group-dependent cut-off values, the value of CA19-9 in detecting pancreatic cancer was optimized by improved sensitivity and a negative predictive value without compromising specificity and positive predictive value.

Mounting evidence has demonstrated that Lewis negative individuals have no or limited CA19-9 secretion and that CA19-9 has limited value in detecting cancer for Lewis negative patients [7-9, 14]. Narimatsu *et al.* [8] reported that all 40 Lewis negative normal individuals and all 15 Lewis negative patients with colorectal cancer had CA19-9 values under 1.0 U/mL. Tempero *et al.* [14] reported a finding that Le^{a-b-} pancreatic cancer patients had normal or low serum levels of CA 19-9 (under 37.0 U/mL). However, in the present study, we found that the area under the ROC curve was 0.82 for CA19-9 in cases of Lewis negative pancreatic cancer (cut-off value, 1.8 U/mL; sensitivity, 48.6%; specificity, 95.9%), indicating that CA19-9 can be used to detect Lewis negative pancreatic cancer by using a group-dependent cut-off value. However, more studies are needed to explore potential biomarkers in Lewis negative patients with pancreatic cancer.

Although viewed as the most important biomarker for pancreatic cancer, CA19-9 is inadequate for the early detection of pancreatic cancer [6, 15]. In the present study,

the sensitivity of CA19-9 for the detection of stage I, II pancreatic cancer was 76.1%. However, the sensitivity was greatly improved when using the group dependent cut-off values in the Mixed and Secretor (-) groups, which accounted for 93.6% of patients with pancreatic cancer (sensitivity, 87.2%; positive predictive value, 87.2%). These findings suggest that CA19-9 can be applied in the screening or early detection of pancreatic cancer using Lewis and Secretor group-dependent cut-off values.

Biliary tract obstruction or inflammation is another important factor affecting the secretion of CA19-9 [4, 5]. In the present study, the sensitivity of CA19-9 for detecting all stages of pancreatic cancer improved from 77.7% to 85.3% and the negative predictive value increased from 84.0% to 88.1% when using group dependent cut-off values; this occurred without compromising the other values. Additionally, the sensitivity of CA19-9 for detecting stage I, II pancreatic cancer increased from 71.6% to 83.2%. These findings suggest that the value of CA19-9 for detecting pancreatic cancer was optimized by using group dependent cut-off values in patients with normal total bilirubin.

Several techniques have been used to determine the Lewis and Secretor status. For example, modified enzyme-linked immunosorbent assays in saliva and hemagglutination assays in red blood cells have been applied for testing Lewis and Secretor phenotypes [14]. However, these techniques have a high proportion of false-negatives because Lewis positive individuals can become Lewis negative under certain circumstances [16]. Therefore, our technique of Sanger sequencing is more reliable than the previously reported methods.

This study has several limitations. First, CA19-9 is not an ideal biomarker for detecting Lewis negative pancreatic cancer, even when using group dependent cut-off values. More studies are needed to explore potential biomarkers in these patients. Second, additional testing by sequencing will be needed in optimizing CA19-9 performance for pancreatic cancer. Moreover, the optimal group dependent cut-off values for CA19-9 in detecting pancreatic cancer should be based on large-scale studies.

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Author contribution

Guopei Luo, Chen Liu, Quanxing Ni, Xianjun Yu designed study. All authors collected and analyzed data. Zuqiang Liu wrote the draft. Guopei Luo and Xianjun Yu revised it critically. All the authors approved the version to be published.

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Figure Legends

Fig. 1. Grouping algorithm based on Lewis and Secretor genotypes and activities.

Fig. 2. CA19-9 levels in normal control patients (A), patients with benign pancreatic disease (B), and patients with pancreatic cancer (C). A comparison was made between Lewis (-), Mixed, and Secretor (-) groups. Significant differences were observed between groups in normal control patients (A), patients with benign pancreatic disease (B), and patients with pancreatic cancer (C). For all subjects, the Lewis (-) group had lower and the Secretor (-) group had higher CA19-9 levels than the Mixed group. * $P < 0.001$. CA19-9 is presented logarithmically.

Fig. 3. ROC curves and area under the ROC curve for CA19-9 detection of all stages of pancreatic cancer. The groups included all pancreatic cancers (A), Lewis (-) (B), Mixed (C), and Secretor (-) (D). The CA19-9 levels was more effective for detecting cancer in the Mixed group (0.94) and Secretor (-) group (0.94) than in the Lewis (-) group (0.82).

Fig. 4. ROC curves and area under ROC curve for CA19-9 detection of stage I or II pancreatic cancer. Groups included all pancreatic cancers (A), Lewis (-) (B), Mixed (C), and Secretor (-) (D). CA19-9 was more effective for detecting cancer in the Mixed group (0.94) and Secretor (-) group (0.92) than that in the Lewis (-) group (0.86).

Table 1 The diagnostic value of CA19-9 in different groups of patients with pancreatic cancer (all, Lewis (-), Mixed, Secretor (-), Mixed plus Secretor (-)), as determined by ROC analysis

Subjects	Group	Cut-off ¹	Sens.	Spec.	PPV	NPV	ROC
All stages pancreatic cancer + control ²	All	37.0	80.1%	94.7%	94.3%	81.2%	0.90
	Lewis (-)	1.8	48.6%	95.9%	90.0%	71.2%	0.82
	Mixed	22.0	87.5%	91.8%	92.2%	87.0%	0.94
	Secretor (-)	49.4	89.6%	92.8%	94.1%	87.4%	0.94
	Mixed plus Secretor (-)	/	88.0%	92.0%	92.6%	87.1%	/
Stage I, II pancreatic cancer + control ²	All	37.0	76.1%	94.7%	89.4%	87.0%	0.89
	Lewis (-)	1.8	50.0%	95.9%	83.3%	82.5%	0.86
	Mixed	22.2	87.3%	91.8%	86.1%	92.6%	0.94
	Secretor (-)	70.7	87.0%	93.8%	90.9%	91.0%	0.92
	Mixed plus Secretor (-)	/	87.2%	92.2%	87.2%	92.2%	/

¹ U/mL. ² The control includes normal subjects and subjects with benign pancreatic disease. Sens., sensitivity; Spec., specificity; PPV, positive predictive value; NPV, negative predictive value; ROC, receiver operating characteristic.

Figure 1

Figure 2

Figure 3

Figure 4