

## ABO Blood Group IgM Isoagglutinins Interact with Tumor-Associated O-Glycan Structures in Pancreatic Cancer

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### Abstract

**Purpose:** The *ABO* gene locus is associated with the risk of developing pancreatic ductal adenocarcinoma (PDAC) resulting in an increased incidence in individuals with non-O blood groups. Up to 90% of PDAC specimens display alterations in mucin type O-GalNAc glycosylation. Because aberrant O-GalNAc glycans (Tn and T antigen) are structurally related to blood group A and B glycans, we investigated the role of IgM isoagglutinins in PDAC.

**Experimental Design:** Binding studies of IgM isoagglutinins toward blood group A, B, Tn antigen, and T antigen glycoconjugates from patients with PDAC and healthy individuals were conducted. Isoagglutinin titers and total IgM were compared between patients with PDAC and control group. An anti-A antibody was used for immunoprecipitation of aberrant O-glycosylated tumor proteins and subsequent mass spectrometric analysis.

**Results:** We found that IgM isoagglutinins bind blood group antigens, Tn and T glycoconjugates as well as tumor-derived glycoproteins. Blood group A isoagglutinins exhibited a strong binding toward blood group B antigen and T antigen, whereas blood group B showed binding to blood group A antigen and Tn antigen. Furthermore, we confirmed a decreased frequency in individuals with blood group O and observed a significant decrease of IgM isoagglutinin titers in PDAC sera compared with control sera, whereas total IgM levels were unaltered. We identified new PDAC-derived O-GalNAc glycoproteins by mass spectrometry using a blood group A-specific antibody.

**Conclusion:** Our data elucidated a novel interaction of blood group IgM isoagglutinins and PDAC O-GalNAc glycoproteins that may contribute to the pathogenesis and progression of pancreatic cancer. *Clin Cancer Res*; 20(23); 6117–26. ©2014 AACR.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Introduction

Pancreatic ductal adenocarcinoma (PDAC) is amongst the four most deadly cancers in Western societies with a mortality rate that exceeds 95% (1). ABO blood groups are associated with pancreatic cancer (2–4). Individuals with blood group non-O (A, B, AB) are significantly more likely to develop PDAC compared with blood group O. A genome-wide association study already showed the relationship between *ABO* gene locus and pancreatic cancer (5), but the underlying mechanism remains obscure.

Landsteiner first described the ABO antigens in 1900 as red cell antigens (6) and chemical characterization of the determinants defining the histo-blood groups showed that these antigens were oligosaccharides (7, 8). The human ABO blood groups are based on a single gene *ABO* encoding a glycosyltransferase displaying different alleles for A and B defined by seven nucleotide substitutions (9). The O allele differs from the A allele by a

### Translational Relevance

The relationship between ABO blood groups and the risk of developing pancreatic cancer has been known for over 50 years, but the underlying mechanism remains obscure. We provide evidence for the interaction of ABO blood group IgM isoagglutinins and tumor-associated O-glycans (Tn and T antigen) on secreted and cellular pancreatic ductal adenocarcinoma (PDAC)-derived proteins. Our study suggests a role for IgM isoagglutinins during tumorigenesis of PDAC.

deletion of guanine at position 261. The deletion causes a frame shift resulting in expression of a truncated protein lacking glycosyltransferase activity (10).

ABO antibodies, also termed isoagglutinins, are of major clinical significance because they are naturally occurring in individuals who do not possess the corresponding antigen and are highly reactive. The appearance can be explained by inapparent stimulations particularly from intestinal bacteria because blood group substances are widely distributed in the environment (11). Anti-A titers are usually higher than anti-B titers and titers from O individuals tend to be higher than blood group A or B individuals (12). Anti-A and anti-B antibodies are usually IgM antibodies, whereas blood group O individuals provide IgG isoagglutinins additionally (11). All isoagglutinins are able to activate the complement cascade (11).

Cell surfaces display a diverse array of carbohydrates which are involved in virtually every aspect of biology, like immune response (13), protein folding (14), host-pathogen interactions (15), embryonic development (16), and disease (17). The mucin type O-GalNAc glycosylation (hereafter called O-glycosylation) is a common posttranslational modification of serine, threonine and likely tyrosine residues of mammalian glycoproteins. O-glycosylation is initiated by the family of polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts) that utilize UDP-GalNAc as the nucleotide donor substrate to modify protein substrates (18). The resulting O-GalNAc precursor structure is named Tn antigen (Tn; refs. 19, 20). It is further elaborated by downstream *C1GALT1* (T-synthase) to generate the core 1 structure, also known as T antigen (T; ref. 21).

The presence of Tn and T antigens is a hallmark of cancer and contributes to the phenotype and biology. The molecular basis for Tn and T expression in cancer are mutations in the T-synthase chaperone *C1GALT1C1* (COSMC; ref. 22) and dysregulated ppGalNAc-Ts (23).

Because Tn and T antigen are structurally related to blood group A and B glycoforms, we investigated a possible interaction of isoagglutinins with PDAC-associated O-GalNAc glycans and the influence on tumorigenesis and PDAC progression.

## Materials and Methods

### Patients

All patients had a pathologically proven PDAC and were patients of the General, Visceral, and Thoracic Surgery Hamburg-Eppendorf. The control group involved blood donors from the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). Most of the serum and tumor samples were previously recruited during the years 2010 until 2012. Sera and tissues were collected with a standard protocol to ensure high quality of samples. Studies on human tissue samples and sera were approved by the local ethical review board (Ärztchamber Hamburg, Germany, PV3548).

### Isoagglutinin titers

PDAC sera were taken preoperatively at the day of surgery. Serum samples of the control group were taken on blood donation day. Because the average age of patients with was 62.1 years compared with average age of blood donors, which was 29.3 years, PDAC and control sera were matched regarding age of the participants. PDAC sera were also selected as accurately as possible regarding histopathological tumor stadium (T1-T4) and their distribution among different blood groups corresponding to the total PDAC cohort. Serum was prepared in Serum-Gel Monovettes (Sarstedt) according to the manufacturer's instructions. Isoagglutinin titers were determined by incubating 50  $\mu$ L of 5% diluted test erythrocyte solution in saline with 100  $\mu$ L of serially diluted serum for 15 minutes at room temperature (RT) in round bottom glass tubes. Agglutination was measured after centrifugation for 5 minutes at 1,000  $\times$  g. Quality control was conducted using standards of blood group A, B, or O sera.

### Quantification of overall IgM

IgM quantification was performed with Dimension Vista System and Flex reagent cartridge (Siemens) according to the manufacturer's instructions. The Dimension Vista System automatically performed sampling, reagent delivery, mixing, and processing. Obtained group values were compared using the *t* test. PDAC and control sera were matched regarding age of participants.

### Tumor and normal tissue preparation

Tumor samples and corresponding normal tissue were collected intraoperatively and PDACs were pathologically characterized according to tumor-node-metastasis (TNM) classification of PDAC of the classification of malignant tumors, UICC 7th edition. Samples were stored at  $-80^{\circ}\text{C}$ . Tissue was grounded in mortars in liquid nitrogen and further processed for immunoprecipitation and Western blot analysis.

### Western blot analysis

Grounded tissues were treated with Complete Lysis-M (Roche) according to the manufacturer. Proteins (30  $\mu$ g) were separated by SDS-PAGE in 4% to 15% Mini-PROTEAN

TGX gels (Bio-Rad) and blotted on a nitrocellulose membrane (Thermo Fischer Scientific). Membrane was blocked with 1 × Carbo-Free Blocking Solution (Biozol) and subsequently incubated with serum (1:20) in TBS-T overnight. Tn antigen antibody (MA1-80055; Thermo Fischer Scientific), T antigen antibody (MA1-34862; Thermo Fischer Scientific), and sTn antigen antibody (ABIN356328; antibodies-online) were diluted 1:250 in TBS-T. Washing was performed five times with TBS-T. The membrane was incubated with HRP-mouse anti-human IgM Antibody (1:1250; Invitrogen) for 1 hour at RT. After five times washing with TBS-T buffer, immunodetection was performed using enhanced chemiluminescence (GE Healthcare). The experiment was repeated three times with three different blood group O PDAC specimens and adjacent healthy control tissues.

### Glycan ELISA

Neoglycoprotein conjugates of blood group A and B, Tn and T antigen were purchased from Dextra composed of a human serum albumin (HSA) backbone to which carbohydrates were chemically cross-linked. Glycan ELISA was performed as previously described (24). Briefly, individual sera were diluted 1:200 in PBS and binding was performed for 4 hours with subsequent washing, anti-human IgM-HRP incubation, and final washing. Absorbance was measured at 405 nm on a microplate reader. As a quality control of unspecific binding toward HSA, blank values were individually subtracted and absorption was calculated to percent normalized to anti-A from blood group B. Significance was evaluated using *t* test.

### Immunoprecipitation and mass spectrometry

Immunoprecipitation was performed as previously described using 60 μg of protein and incubation with anti-blood group A antibody (Novus; HE-193) overnight (25). Proteins were separated by PAGE and followed by

Coomassie staining (Carl Roth). ESI-MS was performed as previously described (26).

### Statistical analyses

Participant characteristics were examined for cases and controls, and by blood group among controls. Distribution of blood groups in patients with PDAC was compared with healthy controls. We used logistic regression to calculate OR and 95% confidence intervals (CI) for PDAC by ABO blood group. Fisher exact test was applied to analyze relationship between blood groups and patient group. Survival curves were plotted using Kaplan–Meier method and analyzed using log-rank test. Univariate analyses were performed for prognostic factors for overall survival using the Cox regression model. Titer values were logarithmized. Antibodies against A and B were considered separately. ANOVA models were used to compare values between patients and controls. Spearman correlation coefficient was applied to investigate the relationship between titer values and UICC. In addition, logistic regression analysis was performed to analyze relationship between titer values and UICC cancer classification. The level of significance was set to  $P < 0.05$ , two sided. Calculations were performed using SPSS (Version 20.0.0).

### Results

#### Decreased frequency of PDACs accompanied by more often well-differentiated tumors in blood group O

Seven hundred and seventy-five patients with PDAC and 31,316 healthy controls were available for analysis. Blood group distribution among patients with PDAC and control group was evaluated (Table 1). Blood group distribution of PDAC individuals was: 31.9% O, 47.7% A, 14.6% B, and 5.8% AB, whereas blood group distribution of the control group was: 39.4% O, 41.8% A,

**Table 1.** Blood group distribution among patients with PDAC and a healthy control group

		PDAC	Control	Total	OR (95% CI)
Blood group O	Absolute number	247	12,349	12,596	
	Percentage	31.9%	39.4%	39.3%	
A	Absolute number	370	13,091	13,461	1.413 (1.201–1.663)
	Percentage	47.7%	41.8%	41.9%	
B	Absolute number	113	4,057	4,170	1.393 (1.112–1.745)
	Percentage	14.6%	13.0%	13.0%	
AB	Absolute number	45	1,819	1,864	1.237 (0.897–1.706)
	Percentage	5.8%	5.8%	5.8%	
Total	Absolute number	775	31,316	32,091	
	Percentage	100.0%	100.0%	100.0%	

NOTE: The ABO blood group distribution of 775 patients with PDAC and 31,316 healthy persons was compared. The cohort ( $n = 32,091$ ) is composed of 12,596 individuals with blood group O, 13,461 blood group A, 4,170 blood group B, and 1,864 blood group AB.

13.0% B, and 5.8% AB. The mean age of patients with PDAC with blood group O, A, B, and AB was 62.5 (range 30–90), 63.3 (range 33–87), 61.9 (range 33–87), and 61.5 years (range 28–78), respectively. This indicates that the age of the patients is an independent factor for blood group distribution among patients with PDAC.

Associations between ABO blood group, frequency of PDACs, and other risk factors were estimated by unconditional logistic regression analysis. Individuals of blood group A and B compared with blood group O had a significantly higher frequency for PDACs ( $P < 0.0001$ ). The unadjusted ORs (95% CI) were 1.413 (1.201–1.663) and 1.393 (1.112–1.745). On the other hand, blood group AB did not show higher frequency in patients with PDAC with the OR of 1.237 (0.897–1.706). Clinicopathologic data were collected retrospectively and 602 PDAC patient data were available for analysis (Table 2). Tumor size (T;  $P = 0.259$ ), lymph node metastasis (N;  $P = 0.854$ ), infiltration of resection margin (R;  $P = 0.186$ ), and distant metastasis (M;  $P = 0.629$ ) did not differ significantly between different blood groups. Blood group O patients had more often well-differentiated PDACs compared with blood group non-O, whereas blood group AB patients displayed more often poorly differentiated tumors ( $P = 0.035$ ; Table 2).

Survival data of 645 of 775 patients were available during the study period, including 206 patients with blood group O, 310 blood group A, 96 blood group B, and 33 blood group AB. Kaplan–Meier survival analysis (log-rank test) did not show significant difference in relative survival rate of patients with PDAC in different blood groups ( $P = 0.2$ ; Supplementary Fig. S1). The overall survival rate was 41% at 1 year, 8% at 3 years, and 3% at 5 years. The median survival rate for blood group O, A, B, and AB was 14.6, 16, 13.4, and 10.8 months, respectively.

### Serum IgM isoagglutinins bind PDAC-associated glycostructures

Forty-one PDAC sera and 43 control sera were age matched and used for analysis of IgM isoagglutinin glycan-binding. Eleven PDAC and 11 control sera were available for blood group O. Ten PDAC sera and 10 control sera were analyzed for blood group A and B and blood group AB included 10 PDAC and 12 control sera. Glycan ELISAs were performed for each blood group and isoagglutinin binding to A, B, Tn, and T glycoconjugates was measured.

Blood group O sera showed binding to A, B, Tn, and T glycostructures. PDAC blood group O sera showed a significant decrease of isoagglutinin binding compared with control sera ( $P < 0.001$ ; Fig. 1A).

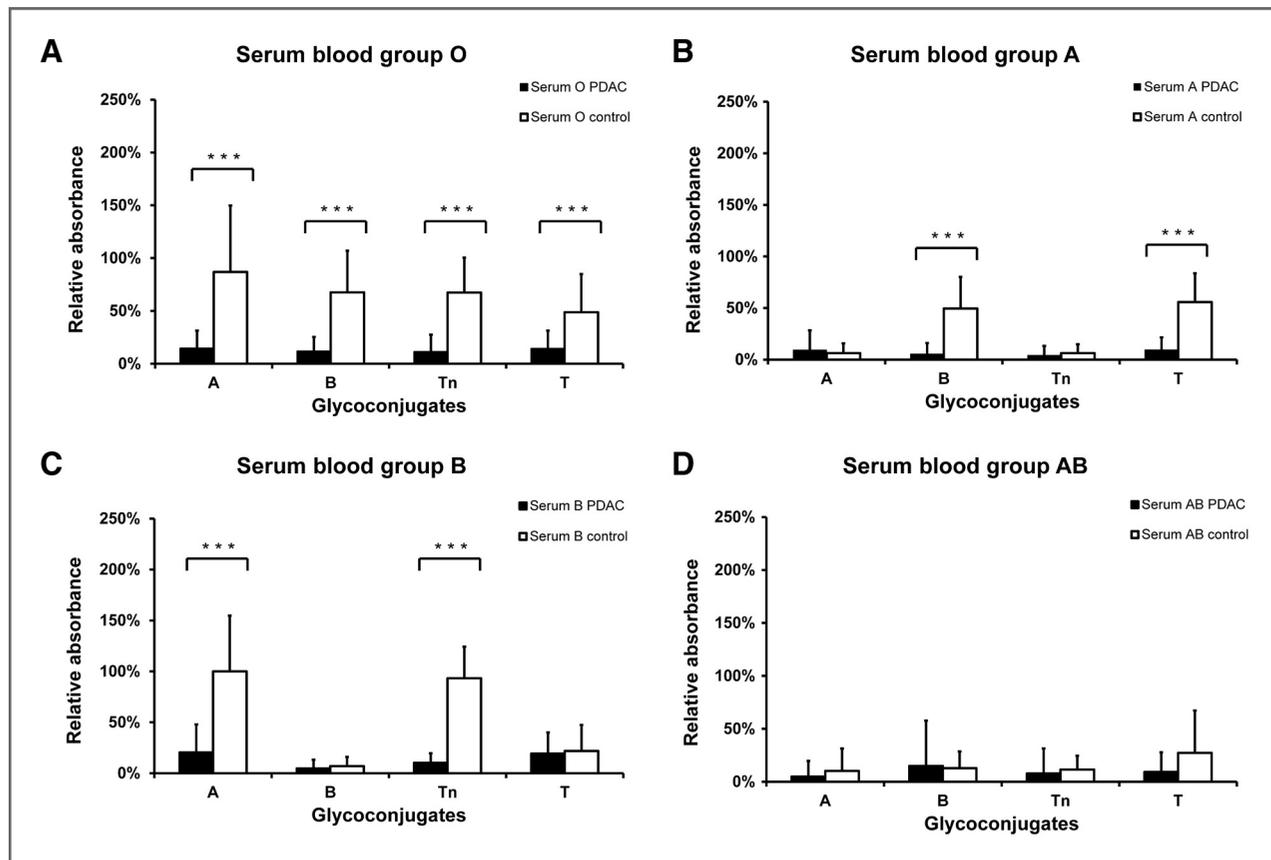
Blood group A sera exhibited binding to B and T glycoconjugates and were significantly decreased in PDAC sera compared with control sera ( $P < 0.001$ ). Isoagglutinins of blood group A sera did not bind A and Tn glycoconjugates (Fig. 1B).

Comparable results were obtained analyzing sera of blood group B. Blood group B isoagglutinin had a strong

Table 2. Clinicopathologic parameters of patients with PDAC

TNM classification	Blood group				Total	P
	O	A	B	AB		
T	14	15	7	0	35	0.259
	41	50	11	4	106	
	95	160	53	21	329	
	41	65	15	10	131	
N	73	110	35	10	228	
	122	172	51	25	370	
	2	2	0	0	4	
M	132	177	55	21	385	
	70	99	38	10	217	
R	126	166	55	15	362	
	21	41	9	7	77	
R2	46	75	31	9	162	
G	140	185	55	19	399	
	51	100	35	17	203	

NOTE: TNM classification of PDAC was used according to the classification of malignant tumors, UICC 7th edition and assorted by blood group. T1–4 describes the tumor size or direct extends of the primary tumor, N0–1 involved lymph nodes, M0–1 distant metastasis, R0–2 resection margin, and G1–4 describes pathologic grading of tumor cells.



**Figure 1.** Evaluation of serum IgM isoagglutinin-binding properties toward A, B, Tn, and T glycoconjugates in PDAC and control sera (\*\*\*,  $P < 0.001$ ). ELISAs with A, B, Tn, and T glycoconjugates were used and OD was measured at 405 nm. A, blood group O sera of PDAC and control group showed binding activity toward A, B, Tn, and T glycoconjugates. Binding activity of PDAC O sera were significantly decreased compared with control. B, blood group A sera showed decreased binding capacity of PDAC sera compared with control sera for B and T glycoconjugates. C, blood group B sera showed decreased binding activity of PDAC sera compared with control sera for A and Tn glycoconjugates. D, blood group AB serum showed no differences of PDAC sera compared with control sera.

binding toward A and Tn glycoconjugates and showed decreased binding of PDAC sera compared with control sera ( $P < 0.001$ ). Isoagglutinins of PDAC sera and control sera did not bind B and T glycoconjugates (Fig. 1C).

Because blood group AB sera do not contain isoagglutinins, glycan ELISAs were used to display possible binding of IgM antibodies beside isoagglutinins. PDAC and control sera with blood group AB displayed very weak binding of IgM antibodies to A, B, Tn, and T glycoconjugates and showed no significant differences in binding between PDAC and control sera ( $P = 0.45$ ,  $P = 0.25$ ,  $P = 0.33$ ,  $P = 0.17$ , Fig. 1D).

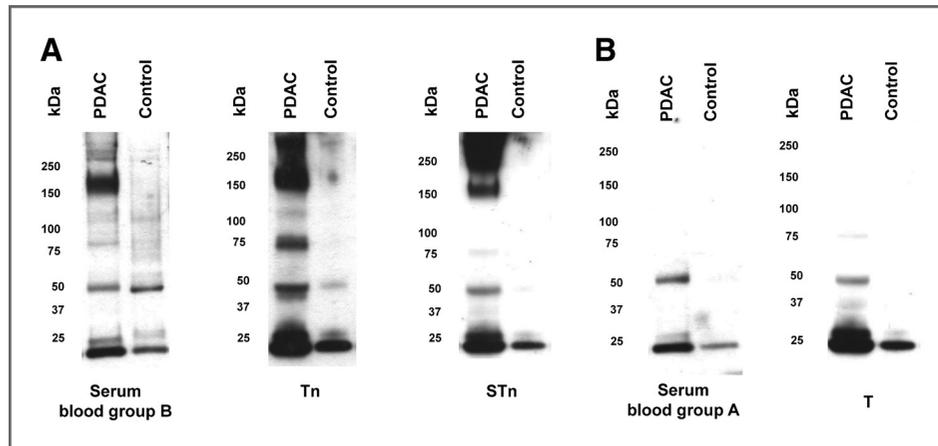
### Serum IgM isoagglutinins bind differentially glycosylated PDAC proteins

PDAC and adjacent healthy pancreatic tissues were obtained from 3 different patients with blood group O and TNM classification for these tumors was T3 N1 M0 G2, T3 N2 M0 G2, and T3 N0 M0 G3. Western blot analysis of tissue protein lysates showed distinct staining patterns using pooled serum of healthy blood group B individuals as well as Tn antigen (Tn) and sialyl-Tn (sTn)

antigen antibodies. PDAC proteins showed stronger staining compared with corresponding controls and staining patterns were highly similar between blood group B sera (anti-A isoagglutinins), Tn and sialyl-Tn antibodies, indicating similar binding of differentially glycosylated pancreatic cancer proteins (Fig. 2A). Western blot analysis were also performed with pooled serum of healthy blood group A sera and T antigen (T) antibody. PDAC proteins showed a stronger staining compared with control protein staining. Furthermore, serum blood group A isoagglutinins (anti-B isoagglutinins) showed similar staining pattern compared with T antigen antibody, highlighting preferentially binding of glycosylated PDAC proteins (Fig. 2B).

### Mass spectrometry identified O-GalNAc proteins precipitated with anti-A antibody

After immunoprecipitation of PDAC tumor proteins using an anti-A antibody, mass spectrometry identified eight O-GalNAc proteins: lithostathine-1- $\alpha$ , myosin-9, myosin-11, histone H2B, serotransferrin, carbonic anhydrase 1, Ig- $\gamma$ -2 chain-C-region, and serine/threonine-



**Figure 2.** Analysis of PDAC lysates from blood group O patients with sera and glycan-specific antibodies. A, PDAC and adjacent healthy pancreatic tissue were obtained from the same patient. Western blot analysis was performed with pooled blood group B sera, Tn antigen (Tn), and sialyl-Tn (sTn) antigen antibodies. B, Western blot analysis of pooled blood group A sera and T antigen (T) antibody.

protein kinase mTOR (Table 3). Lithostathine-1- $\alpha$ , serotransferrin, and carbonic anhydrase 1 were previously identified as O-GalNAc glycoproteins (29–33). Using the O-GalNAc prediction tool (NetOGlyc 4.0), identified proteins were evaluated for possible O-glycosylation sites. All identified proteins have a high possibility for O-GalNAc glycosylation (Table 3).

#### Decreased IgM isoagglutinin titers in PDAC sera

Isoagglutinin serum titers derived from 50 patients with PDAC and 43 healthy, age-matched controls were examined for blood groups O, A, and B (Fig. 3A). Blood group AB was not included because A and B antigens are expressed and therefore blood group AB lacks isoagglutinins. We observed significantly decreased isoagglutinin titers in patients with PDAC (mean titer 1:8) compared with the control group (mean titer 1:58; Fig. 3B).

PDAC sera did not contain an anti-A and anti-B isoagglutinin titer greater than 1:128 and 1:32, respectively. Titers

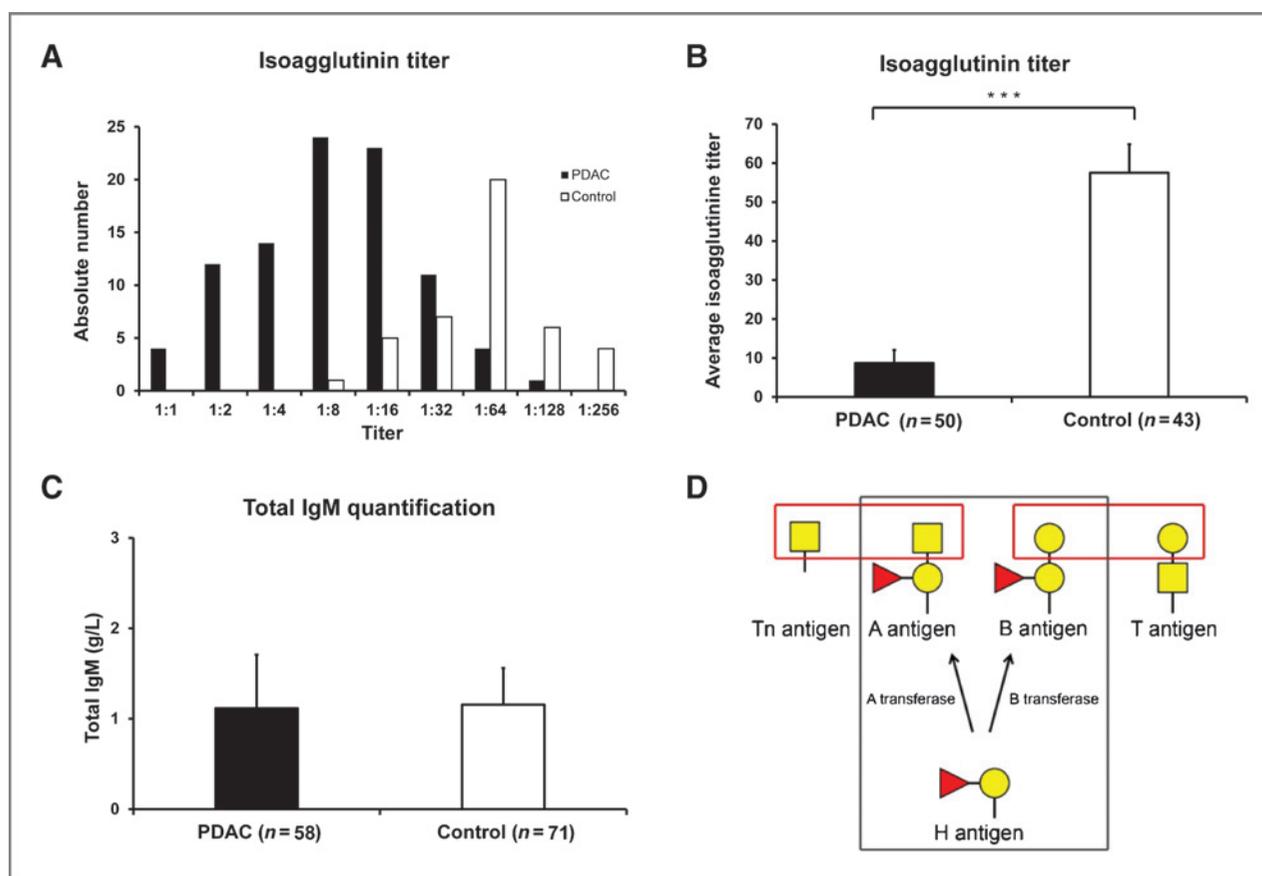
of control sera containing anti-A and anti-B isoagglutinins did not exceed 1:256. Control sera had significantly higher titers compared with PDAC sera ( $P < 0.001$ ). Median anti-A and anti-B titers in blood group O were 1:64 (range 48–80) and 1:64 (range 32–96) in control sera, whereas median isoagglutinin titer of blood group O derived from PDAC sera was 1:16 (range 15–17) and 1:8 (range 6–10; Supplementary Fig. S2A and S2B). For blood group A, healthy control's median anti-B titers were 1:64 (range 48–80) and median titers of PDAC sera were 1:8 (range 7–9; Supplementary Fig. S2C). For blood group B, median control titer was 1:64 (range 56–72) and median titer of PDAC sera was 1:4 (range 2–6; Supplementary Fig. S2C). Isoagglutinin titers of PDAC and control sera differed significantly for anti-A ( $P < 0.0001$ ) and anti-B titers ( $P < 0.0001$ ).

Low anti-A and anti-B titers did not significantly correlate with UICC tumor stage ( $P = 0.127$  and  $P = 0.173$ ) or presence of distant metastasis ( $P = 0.942$  and  $P = 0.301$ ).

**Table 3.** Mass spectrometry-based protein identification

Protein	Symbol	Uniprot acc. number	NetOGlyc 4.0	References
Lithostathine-1- $\alpha$	REG1A	P05451	+	(39)
Myosin-9	MYH9	P35579	+++	NA
Myosin-11	MYH11	P35749	+++	NA
Histone H2B	H2B1C	P62807	+++	NA
Serotransferrin	TRFE	P02787	+	(33, 40, 41)
Carbonic anhydrase 1	CAH1	P00915	++	(33)
Ig $\gamma$ -2 chain C region	IGHG2	P01859	+	NA
Serine/threonine-protein kinase mTOR	MTOR	P42345	+++	NA

NOTE: Proteins identified from human PDAC by immunoprecipitation using an anti-A antibody accompanied by mass spectrometry. The O-GalNAc prediction tool (NetOGlyc 4.0; ref. 42) was used to assess possibility of O-glycosylation. Score indicates percentage of possibly O-GalNAc modified Serin/Threonin residues (+, <10%; ++, <20%; +++, >20%). References are presented for previously identified O-GalNAc glycans.



**Figure 3.** Quantification of isoagglutinin titers in PDAC sera and control sera. Isoagglutination was determined in comparison with test erythrocyte solutions. A, isoagglutinin titers with absolute numbers of PDAC sera or control sera. Titers range from 1:1 until 1:256. B, average isoagglutinin titers of 50 PDAC sera and 43 control sera (\*\*\*,  $P < 0.001$ ). C, average of total IgM was measured for 58 PDAC sera and 71 sera of healthy controls. Comparison of the two groups failed to reach statistical significance ( $P = 0.653$ ). D, blood group antigen synthesis and structural relationship between A antigen and Tn antigen as well as B antigen and T antigen. H antigen (blood group O) is the basis for A and B antigen synthesis. The A synthase ( $\alpha$ 1,3-N-acetylgalactosaminyltransferase) transfers N-acetylgalactosamine to form the A antigen, whereas B synthase ( $\alpha$ 1,3-galactosyltransferase) transfers galactose for B antigen synthesis (grey frame). Structural-related glycan-moieties of A and Tn antigen (terminal N-acetylgalactosamine), B and T antigen (terminal  $\alpha$ 1,3-galactose) are highlighted by red frames.

### No difference between total IgM levels in PDAC sera and healthy control

Total IgM levels were quantified for 58 PDAC sera and 71 healthy controls sera and showed no significant differences between both groups ( $P = 0.653$ ; Fig. 3C). Blood group O had average IgM levels of 0.99 g/l in 19 PDAC sera and 1.03 g/l ( $P = 0.809$ ) in 21 control sera. Blood group A, with 19 PDAC sera and 21 control sera, showed an average level of 1.14 g/l and 1.27 g/l IgM ( $P = 0.4151$ ). Blood group B had 13 PDAC sera and 18 control sera with average IgM levels of 1.12 g/l and 1.25 g/l ( $P = 0.470$ ) and in blood group AB were 7 PDAC and 11 control sera with IgM levels of 1.29 g/l and 1.10 g/l ( $P = 0.499$ ) analyzed (Supplementary Fig. S3).

### Discussion

It has long been recognized that ABO blood groups are associated with pancreatic cancer risk (3, 4) and a recent genome-wide association study discovered the relationship

between the ABO locus and pancreatic cancer (5). Because the underlying mechanism is still enigmatic, we decided to correlate substantiated clinical data with glyco-biological and molecular biologic experiments, focusing on glycan-specific IgM isoagglutinins and its interaction with PDAC O-glycan tumor antigens.

Analyzing 775 patients with PDACs after potential curative resection, the frequency of PDACs was significantly decreased in blood group O compared with blood group A and B ( $P < 0.0001$ ). Blood group AB did not show a change in frequency, but this is probably due to the small sample size of patients with PDAC. In general, our findings are in accordance to previous studies, which as well showed a lower frequency of PDAC in blood group O (3, 27, 28).

TNM status of the tumors did not correlate significantly between blood groups. These findings are also in consistency with a previous study, which failed to show significant differences in clinicopathologic characteristics among patients with different ABO blood groups (28). In contrast, tumor grading (G) showed significant differences within

blood groups ( $P = 0.035$ ). Blood group O patients showed more often well-differentiated PDACs, whereas patients with blood group other than O had a higher frequency of poorly differentiated PDACs.

However, this result does not promote prolonged survival of blood group O PDAC patients. There were no significant differences in the median overall survival between blood groups ( $P = 0.2$ ). Data of previous studies about blood group-dependent survival of patients with PDAC are conflicting. In one study based on a Han Chinese population who underwent potentially curative resection, patients with blood group O had a favourable prognosis compared with non-O patients. Interestingly, in this study, patients who did not undergo potentially curative resection had no survival differences amongst different blood groups (27). A study with mainly Caucasian population revealed a favorable and independent impact of blood group O on survival using multivariate analysis (3). However, univariate analysis of ABO blood group status did not correlate with survival (3, 28).

A frequently occurring and poorly understood characteristic of PDAC is its atypical glycosylation. Aberrant glycosylation is a typical characteristic of malignant transformation in epithelial cells (20). The Tn and T antigens are tumor-associated aberrant O-glycans and detectable in approximately 75% to 90% of PDACs (29–31). Tn and T antigen share structural-related glycan-moieties with A and B blood group antigens. A antigen and Tn antigen have a terminal N-acetylgalactosamine, whereas B antigen and T antigen share a terminal  $\alpha$ 1,3-galactose (Fig. 3D). For this reason we hypothesized, that blood group isoagglutinins interact with tumor-associated T or Tn antigens and may affect thereby the blood group-related frequency and survival as afore mentioned.

Because blood group O naturally contains isoagglutinins against A and B antigen, we analyzed blood group O isoagglutinin binding toward A and B glycoconjugates as well as structurally related T and Tn glycoconjugates using a glycan ELISA. Interestingly, blood group O isoagglutinins did not only bind A and B glycoconjugates, but also Tn and T glycoconjugates. Similar to blood group O, blood group B sera displayed binding to A and Tn glycoconjugates, whereas blood group A sera showed preferential binding to B and T glycoconjugates.

The isoagglutinin binding of PDAC sera toward glycoconjugates was significantly decreased compared with control sera. Low isoagglutinin binding in PDAC sera suggest an enhanced isoagglutinin depletion possibly due to the presentation of antigens on the tumor surface followed by an increased binding of isoagglutinins or a depletion by binding of soluble tumor proteins by isoagglutinins and their subsequent decay. Antibody binding of tumor surface glycoproteins and tumor-associated soluble glycoproteins including T and Tn antigen has been described previously (32, 33) and may cause the low isoagglutinin titers in patients with PDAC.

Blood group AB showed very low binding toward A, B, Tn, and T glycoconjugates and showed no significant differ-

ences between PDAC and healthy control sera. The observed binding is probably not isoagglutinin related, because blood group AB sera has no naturally occurring isoagglutinins and observed binding might be due to other IgM antibodies, besides isoagglutinins. We therefore assume that low binding of blood group A sera toward A and Tn conjugates as well as blood group B sera toward B and T conjugates are not isoagglutinin related.

Altogether, our results indicate a specific binding of isoagglutinins to the structurally related glycan-moieties in each particular blood group.

Moreover, we investigated whether isoagglutinins are also capable of binding glycoproteins derived from primary PDAC tissue. We detected an increased binding of isoagglutinins of blood group A and B to PDAC proteins, compared with proteins of healthy pancreatic tissue. We used an anti-human IgM secondary antibody to exclude binding by IgG immunoglobulins. Blood group B serum had similar staining pattern as Tn and sTn antibodies and blood group A serum was highly similar to T antibody staining pattern. These results suggest binding of IgM isoagglutinins to proteins detectable with T or Tn antigen. We used pooled sera of healthy controls and thereby excluded specific antibodies toward aberrant O-glycosylated PDAC proteins. Moreover, O-glycans usually induce weak immune response.

Blood group antigens are expressed in PDAC (34, 35) as well as in normal pancreatic acinar cells (35, 36). Staining of blood group antigens were ruled out using protein lysates and sera of PDAC and healthy controls with identical blood groups. Furthermore, the Tn, sTn, and T antibodies do not bind blood group antigens. Altogether, isoagglutinins bind very likely PDAC glycoproteins, which in turn can be detected with Tn, sTn, and T antibodies.

Because we found decreased binding of isoagglutinins in PDAC patient sera using glycan ELISA, we hypothesized whether the reduced binding was due to decreased level of isoagglutinins. Thus, we examined PDAC and healthy controls sera and compared isoagglutinin titers. We found a blood group independent and significant decrease of isoagglutinin titers of PDAC sera compared with healthy controls. The median isoagglutinin titer of PDAC sera was 1:8, whereas median isoagglutinin titer of healthy control sera was 1:58. The decreased level of isoagglutinins in PDAC sera might be due to an enhanced binding of isoagglutinins to PDAC and cancer-derived O-GalNAc serum glycoproteins, reduced isoagglutinin production, or a general immunosuppression resulting in total decrease of IgM levels. Therefore, we examined total IgM levels in PDAC sera and control sera. Overall IgM levels showed no difference between both groups ( $P = 0.653$ ) and suggest a distinct reduction of isoagglutinins rather than global immunosuppression. Whether this reduction is due to reduced production or an enhanced binding is unclear. A literature review failed to display any reduced production of isoagglutinins even under immunosuppressive therapy (37).

Whether the decreased titer of isoagglutinins in patients with pancreatic cancer is causally related to tumorigenesis

is unclear at present. Binding of isoagglutinins may cause an antibody-dependent cell-mediated cytotoxicity reaction. Suppression of this reaction would facilitate tumor growth. In the case of anti-A isoagglutinins, antibody binding to Tn antigen may interfere with the interaction of Tn glycans on tumor cells with the Tn-binding glyco-receptor CLEC10A, which is expressed on dendritic cells and macrophages (38).

In summary, healthy controls display significantly higher isoagglutinin titers compared with patients with PDAC and higher binding to Tn and T antigen. Because we failed to detect a correlation of isoagglutinin titers and clinical parameters like overall survival or tumor stage, we assume that isoagglutinins are important during tumorigenesis but not during tumor growth once it is present.

Finally, we examined possible isoagglutinin target proteins and identified already known as well as previously unknown PDAC-derived glycoproteins. We used an anti-A antibody for immunoprecipitation because glycan-ELISAs confirmed an interaction between blood group B isoagglutinins with A and Tn antigen. Therefore, the identified PDAC proteins express Tn antigen, which was supposed to interact with the anti-A antibody.

Careful review of the literature revealed that lithostathine-1- $\alpha$ , serotransferrin, and carbonic anhydrase 1 were previously described as O-glycosylated proteins, further supporting an excellent agreement of our results (33, 39–41). To the best of our knowledge, there is no report of O-glycosylated Myosin-9, myosin-11, histone H2B, and mTOR in the published literature. Noteworthy, the glycosylation prediction database NetOGlyc 4.0 predicted all identified proteins as potentially O-glycosylated. Certainly, additional investigations are of pivotal interest to determine the influence of isoagglutinins for tumorigenesis at all which may include other GI tumors besides PDAC as well. Future prospective studies may also dissect serum isoagglutinin levels as a possible diagnostic

marker of PDAC tumor growth as well as their potential therapeutic application.

In conclusion, our results suggest that isoagglutinins are able to bind differentially expressed O-glycan-derived PDAC proteins and may play an important role in the genesis of pancreatic cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Clinical Cancer Research

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