Tumor-associated CD75s- and iso-CD75s-gangliosides are potential targets for adjuvant therapy in pancreatic cancer

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Abstract

Pancreatic adenocarcinoma confers one of the highest mortality rates in malignant human tumors with very poor prognosis. Because as yet no treatments are available that produce a substantial survival benefit for this fatal neoplasia, new therapeutic concepts are urgently required to support cancer standard treatment. In search of tumor-associated gangliosides with therapeutic background, we probed a random collection of cancerous and adjacent normal postoperative tissue samples from 38 patients for the expression of CD75s- and iso-CD75s-gangliosides. We exhaustively analyzed the expression of CD75s-1-ganglioside (IV^{6}Neu5Ac-nLc4Cer) and structurally closely related iso-CD75s-1-ganglioside (IV^{3}Neu5Ac-nLc4Cer) by means of immunohistology of cryosections and semiquantitative TLC of tissue lipid extracts combined with mass spectrometry. CD75s-1- and iso-CD75s-1-ganglioside showed an elevated expression in 42% and 66% of the tumors, respectively, indicating a significant association with neoplastic transformation (P = 0.001). Thus, increased expression of CD75s-1- and iso-CD75s-1-gangliosides renders these cell surface molecules promising candidates for oncologic applications. Further statistical analysis revealed a significant enhancement of CD75s-1-ganglioside in the group of less differentiated tumors (grade >2) suggesting this ganglioside as a potential marker for poor differentiation. The CD75s-specific antitumor drug rViscumin, which represents the recombinant counterpart of the ribosome-inactivating lectin viscumin, has successfully passed clinical phase I trials and provides an opportunity for treating pancreatic cancer. Consequently, if an enhanced expression is existent in malignant tissues, we propose the targeting of CD75s-gangliosides with rViscumin as a novel potential strategy in adjuvant treatment of pancreatic malignancies. [Mol Cancer Ther 2008;7(8):2464–75]

Introduction

Pancreatic carcinoma confers one of the highest mortality rates in malignant human tumors and is ranked fourth among cancer-related death in the United States, responsible for estimated 33,370 deaths in 2007 (1). The prognosis of patients with pancreatic cancer is very poor with a median survival period of ~6 months and 5-year survival rates of <5%. Despite advances in the past decade, the most lethal of the common cancers continues to pose an enormous challenge to clinicians and cancer scientists to diagnose and treat this formidable disease. The nucleoside gemcitabine, a widely used drug that targets the proliferative potential of tumor cells, is the only substance that results in a short gain of patients' survival time alone (2) or when combined with other established anticancer drugs and radiotherapy (3). Several new agents have been developed from the molecular understanding of pancreatic tumors, which are now being assessed in large clinical trials in advanced pancreatic cancer (4). Removal of the residual disease after surgical resection could be achieved by the development of new therapeutic strategies based on, for example, genetically engineered monoclonal antibodies for specific targeting of cell surface–exposed growth factor receptors, shown to be overexpressed in pancreatic cancer (4).

Glycosphingolipids (GSLs) are amphipathic molecules, which are composed of a hydrophilic oligosaccharide chain and a hydrophobic ceramide moiety (5). They play pivotal roles in myriad biological communication events, including cell differentiation and cell surface recognition (6). GSLs are located primarily in the outer leaflet of the plasma membrane of animal cells and are organized in microdomains (7, 8). Changes in the composition of cell surface GSLs occur during neoplastic transformation in essentially all types of human cancers (9, 10). Clinically important, the

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enhanced expression of tumor-associated gangliosides (=sialylated GSLs) makes them promising candidates as potential targets for oncologic applications (11). The detailed structural analysis of gangliosides from liver metastases of a human pancreatic adenocarcinoma revealed, besides sialyl Lewis^a- and sialyl Lewis^x-gangliosides, the expression of the type II ganglioside Neu5Acα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer (12), now renamed as CD75s-ganglioside (13). Along the lines of the newly defined carbohydrate CD categories, we introduced the term ''iso-CD75s-ganglioside'' for terminally α2-3-sialylated type II ganglioside with Neu5Acα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer structure (14).

In search of cancer-associated GSLs with therapeutic background, preliminary data of random samples (n = 3) taken from different types of gastrointestinal tumors (14, 15) revealed evidence for an enhanced expression of CD75s- and iso-CD75s-gangliosides in pancreatic tumors. CD75s-gangliosides are specific receptors of the ribosome-inactivating protein viscumin and its recombinant counterpart rViscumin (16, 17). As a potential anticancer drug, rViscumin is currently under clinical development and has successfully passed clinical phase I trials (18, 19). In this study, we report about the elevated expression of CD75s-gangliosides in a cohort of 38 clinically characterized pancreatic cancer cases. Moreover, the enhanced expression of iso-CD75s-gangliosides in the majority of pancreatic tumors suggests a further potential target structure that could help oncologists tailor treatments to individual patients suffering from pancreatic cancer.

Materials and Methods

Surgical Specimens and Serum Samples

The study was carried out with institutional review board consent using tissue samples from 38 patients that have undergone surgery for their primary tumors under an approved protocol of the local ethic committees of the University Hospital Dresden (20) and the University Hospital Münster (15). Tumor histology was determined according to the criteria of the WHO. The stage of tumors was assessed according to Union Internationale Contra Cancrum (21). All cases were ductal adenocarcinomas. The clinicopathologic characteristics of the tumor cohort are described in Supplementary Table S1.[8] Tumor specimens were snap frozen in liquid nitrogen immediately after removal and stored at −80°C until use. Corresponding control specimens were obtained from the same patient at organ sites without macroscopic tumor involvement and a minimal distance of 2 cm to the tumor. Tissue wet weights of normal tissues ranged from 8.5 to 197.5 mg (median, 34.4 mg) and cancerous tissues from 19.0 to 153.4 mg (median, 56.9 mg; see Supplementary Table S1).[8] Serum samples were taken from patients before surgery and stored at −80°C until analyzed.

Preparation of Lipid Extracts from Surgical Specimens

Tissues were homogenized and extracted each with 4 mL chloroform/methanol (1:2, v/v), 4 mL chloroform/methanol (1:1, v/v), and 4 mL chloroform/methanol (2:1, v/v). The combined supernatants of each tissue sample (12 mL) were dried by rotary evaporation and phospholipids were saponified by incubation with 4 mL aqueous 1 N NaOH for 1 h at 37°C. After neutralization with 400 μL of 10 N HCl, the samples were dialyzed against deionized water and dried by rotary evaporation. The extracts were adjusted to defined volumes of chloroform/methanol (2:1, v/v) corresponding to 0.1 mg wet weight/μL.

Reference Gangliosides

The CD75s-1- and CD75s-2-gangliosides IV^{6}Neu5Ac-nLc4Cer and VI^{6}Neu5Ac-nLc6Cer, respectively, and the isomeric iso-CD75s-1- and iso-CD75s-2-gangliosides IV^{3}Neu5Ac-nLc4Cer and VI^{3}Neu5Ac-nLc6Cer, respectively, from human granulocytes served as reference gangliosides (ref. 22; see Supplementary Table S2).[8] The nomenclature of GSLs follows the IUPAC-IUBM recommendations 1997 (23).

Anti-ganglioside Antibodies, Viscumin, and rViscumin

The preparation and specificities of chicken polyclonal AB2-3 antibody, which recognize the iso-CD75s-1-ganglioside IV^{3}Neu5Ac-nLc4Cer and iso-CD75s-2-ganglioside VI^{3}Neu5Ac-nLc6Cer both with Neu5Acα3Galβ4GlcNAc-terminus, and chicken polyclonal AB2-6 antibody, which specifically binds to the CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer and CD75s-2-ganglioside VI^{6}Neu5Ac-nLc6Cer both with Neu5Acα6Galβ4GlcNAc-residue, have been described previously (14, 15, 24). The CD75s binding specificity of heterodimeric viscumin (also called mistletoe lectin I or *Viscum album* agglutinin) and its recombinant counterpart rViscumin has been described in previous publications (refs. 16, 17 and references therein). Binding specificities of antibodies and lectins are summarized in Supplementary Table S3.[8]

High-Performance TLC

Reference gangliosides and tissue lipid extracts were applied to glass-backed silica gel 60 precoated high-performance TLC plates (Merck) with an automatic applicator (Linomat IV; CAMAG). GSLs were separated in chloroform/methanol/water (120:85:20, each by volume) supplemented with 2 mmol/L $CaCl_2$ and visualized with orcinol.

TLC Overlay Assay

The TLC immunodetection procedure using primary chicken anti-GSL antibodies in conjunction with secondary alkaline phosphatase–labeled anti-chicken IgY antibodies was employed as described previously (14, 16, 17, 24). Binding of viscumin and rViscumin toward CD75s-gangliosides was detected with the monoclonal mouse IgG1 antibody TA5, which recognizes the A-chain of both lectins, and secondary alkaline phosphatase–labeled anti-mouse IgG antibodies (16, 17).

Binding of CD75s-1-gangliosides by serum antibodies from cancer patients was investigated as follows: Aliquots

[8] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

of 20 μL patients' sera were diluted 1:2.5 in 1% bovine serum albumin, 0.02% NaN_3 in PBS (=solution A). Resulting 50 μL volumes were sprinkled on silica-fixed chromatograms of reference gangliosides (see Reference Gangliosides). This ''microscale method'' (because only microliter volumes of primary anti-GSL antibodies containing sera are applied in the first step of the overlay assay to strips of 1.5 × 10 cm of size) has been described previously by Müthing et al. (25). Binding of serum antibodies was detected with secondary alkaline phosphatase–labeled anti-human IgG plus IgM antibodies.

Bound secondary antibodies were visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Roth) in glycine buffer [0.1 mol/L glycine, 1 mmol/L $ZnCl_2$, 1 mmol/L $MgCl_2$ (pH 10.4)]. The immunostained chromatograms were washed with glycine buffer and stored at −20°C. Deep blue-colored overlay assay detected immunopositive ganglioside bands were analyzed with a CD60 scanner (Desaga, software Pro-Quant^R, version 1.06.000) and their amounts determined semiquantitatively as relative expression compared with reference gangliosides. Bands were scanned in reflectance mode at 630 nm with a light beam slit of 0.02 × 3 mm.

Statistical Analysis

The TLC overlay data of immunostained CD75s-1- and iso-CD75s-1-gangliosides were compiled with the software package SPSS version 14.0.2 (SPSS) for nonparametric statistical analysis of primary densitometric data of normal and tumor tissues and secondary difference values calculated by subtraction of normal from related tumor tissue values. The sign test was used for the set of primary densitometric data to examine the significance of relationship between ganglioside expression and neoplastic transformation. The median test was done for primary and secondary densitometric data to test whether ganglioside expression was significantly different among the tumors comprising various clinicopathologic categories. Correlation coefficients were calculated for ranked values of the primary densitometric data of normal and tumor tissues, respectively, and the ranked difference values. Kendall's τ was used to assess the degree of association between ganglioside expression and histopathologic grading. Kendall's τ was also calculated to measure the correlation between CD75s- und iso-CD75s-ganglioside expression. All tests used were two tailed and the level of significance was set to $P = 0.05$.

Immunohistochemistry

Frozen tissues of cancer and healthy controls were cut into 4 μm sections with a cryomicrotome (Microm) and mounted on microscope glass slides (SuperFrost, R. Langenbrinck). Air-dried cryosections (48 h) were fixed for 10 min in 3.7% formaldehyde (Fischar) and unspecific binding of antibodies was blocked with 3% bovine serum albumin, 0.02% NaN_3 in PBS for 30 min. Sections were incubated overnight at 4°C with 50 μL of an anti-ganglioside, anti-CD14 (mouse IgG1, clone 18D11; Immunotools), and anti-CD45 antibody (mouse IgG1, clone MEM-28, Immunotools), diluted 1:50 (AB2-6), 1:25 (AB2-3), and 1:200 (anti-CD14 and anti-CD45, respectively) in solution A. Sections were stained for 1 h with dichlorotriazinylamino fluorescein and Alexa Fluor 568–labeled secondary antibodies, diluted 1:40 and 1:250 in solution A, respectively. Dichlorotriazinylamino fluorescein–conjugated affinity chromatography-purified rabbit anti-chicken IgY was from Dianova and Alexa Fluor 568 goat anti-mouse IgG was from Invitrogen. Control slides were stained with mouse IgG1 isotype control (Immunotools) and chicken preimmune serum instead of primary antibodies as well as only with the secondary antibodies. Nuclear DNA of the cells was stained with 4′,6-diamidine-2-phenylindole (DAPI; Sigma-Aldrich). Fifty microliters of a 1:1,000 diluted stock solution (1 mg/mL DAPI in H_2O) were used for staining per section followed by embedding with fluorescent mounting medium (DAKOCytomation).

Fluorochrome-labeled antibodies and DAPI-stained nuclei were evaluated under a fluorescence microscope (Axioskop; Zeiss), original magnification ×40 (objective lense Plan-Neofluar, numerical aperture 0.75), following published protocols (26). To confirm the lipid nature of the positive staining with anti-ganglioside antibodies, lipids were extracted from air-dried sections with methanol and chloroform/methanol (1:1, v/v) for 10 min each and stained by the same procedure as described above.

Infrared Matrix-Assisted Laser Desorption/Ionization Orthogonal Time-of-Flight Mass Spectrometry

The specifications of the infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometer (IR-MALDI-o-TOF-MS; MDS Sciex, Concord) have been described in two recent publications (14, 27). Direct TLC-IR-MALDI-o-TOF-MS analysis was done *in situ* from immunopositive bands (see TLC Overlay Assay) as described in detail by Distler et al. (14). The immunostained TLC plates were soaked for 2 h in 10 mmol/L ammonium acetate buffer (pH 3.6) and the silica gel fixative (Plexigum P28, Röhm) was removed by chloroform extraction. The TLC plates were cut into pieces of appropriate size and the gangliosides were analyzed in the positive ion mode using glycerol as a matrix.

Results

In search of tumor-associated gangliosides (gangliosides showing enhanced expression in cancerous tissue), we probed samples of 38 patients for the expression of CD75s- and iso-CD75s-gangliosides in malignant compared with normal postoperative pancreas tissue samples. These structures differ in terminal sialylation of the type II gangliosides, which results in α2-6-sialylated CD75s-gangliosides and α2-3-sialylated iso-CD75s-gangliosides. The pathologic characteristics of the pancreatic adenocarcinomas and tissue wet weights of the malignant and unaffected tissue samples are listed in Supplementary Table S1.[8] Probing the expression of CD75s- and iso-CD75s-gangliosides, we employed a recently developed strategy, which avoids laborious GSL isolation but allows the analysis of GSLs in crude lipid extracts from small-sized tissue samples (14).

TLC Immunodetection of Tumor-Associated CD75s-Gangliosides

Identical aliquots of GSL extracts from malignant and corresponding unaffected tissues, equivalent to 2 mg wet weight and harboring the full repertoire of tissue-specific GSLs, were separated simultaneously by TLC. CD75s-gangliosides were detected with the TLC overlay assay using the anti-CD75s-ganglioside antibody AB2-6. According to the different expression, the 38 tumors were ranked from 1 to 38 whereby rank 1 indicates highest, rank 2 the second highest, etc., and rank 38 the lowest expression of CD75s-gangliosides (see Table 1). Based on the ranking data, tumors were grouped into categories I to IV corresponding to high (I) and moderate (II) overexpression and equal (III) and lowered (IV) expression. Four representative TLC immunostains of tumor extracts with the AB2-6 antibody are shown for each tumor category along with the related healthy tissue samples in Fig. 1A. In total, 42.1% of pancreatic tumors showed a moderate to high overexpression of CD75s-1-gangliosides, whereas 55.3% exhibited an equal expression with undetectable quantities of CD75s-1-gangliosides in the majority of the tumor and related normal tissue

Table 1. Expression of CD75s-1- and iso-CD75s-1-gangliosides in pancreatic carcinoma tissues

CD75s-1					iso-CD75s-1				
Category*	%	Rank†	Grade‡	Patient	Category*	% Age	Rank†	Grade‡	Patient
I	10.5	1	2	38	I	7.9	1	2	38
		2	3	24			2	4	18
		3	4	18			3	3	24
		4	3	2	II	57.9	4	2	1
II	31.6	5	2	6			5	2	3
		6	2	3			6	2-3	8
		7	2	11			7	3	14
		8	3	34			8	2	27
		9	2	1			9	3	30
		10	3	21			10	2	13
		11	3	35			11	2	19
		12	3	28			12	3	22
		13	X	4			13	2	5
		14	3	15			14	2	32
		15	3	17			15	3	29
		16	2	32			16	2	25
III	55.3	17	3	29			17	3	21
		18	3	22			18	3	15
		19	2	13			19	2	36
		20	2	19			20	3	35
		21	3	14			21	2	6
		22	2	31			22	3	28
		23	2	36			23	2	10
		24	3	37			24	3	37
		25	3	30			25	2	16
		29	2	5	III	21.1	26	2	9
		29	2-3	8			27	2	31
		29	2	10			28	2	33
		29	2	16			29	2	11
		29	2	23			30	3	17
		29	3	26			31	3	34
		29	2	27			32	X	4
		33	2	9			33	3	2
		34	2	25	IV	13.2	34	2	12
		35	2	12			35	3	26
		36	2	7			36	2	20
		37	2	33			37	2	7
IV	2.6	38	2	20			38	2	23

*Based on the ranking data, tumors were grouped into categories I to IV corresponding to high (I) and moderate (II) overexpression and equal (III) and lowered (IV) expression.

†The 38 tumors were ranked from 1 to 38, whereby rank 1 indicates the highest, rank 2 the second highest, etc., and rank 38 the lowest expression of CD75s-gangliosides.

‡Histopathologic grading: G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; G4, undifferentiated; X, histopathologic grading cannot be assessed.

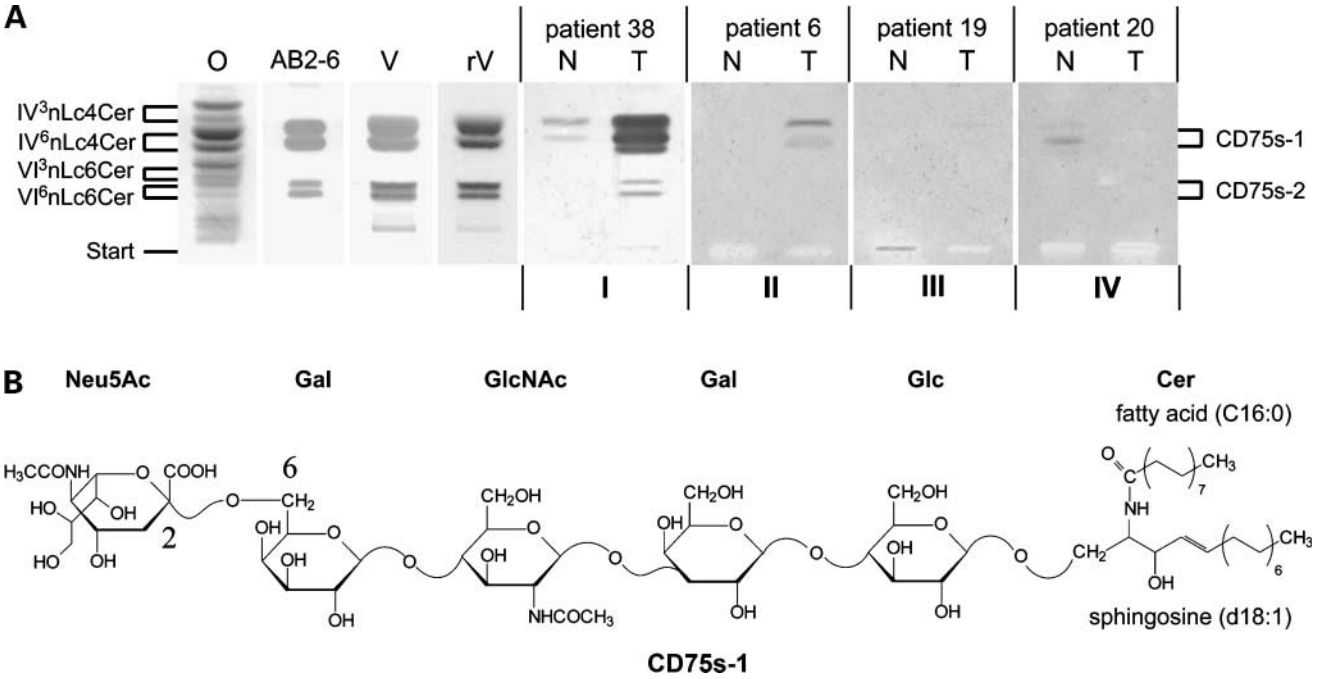

Figure 1. **A,** TLC immunostain of tumor-associated CD75s-gangliosides in pancreatic cancer. Aliquots from crude lipid extracts corresponding to 2 mg wet weight of normal (*N*) and tumor tissue (*T*) were separated simultaneously by TLC and immunostained with the anti-CD75s-ganglioside antibody AB2-6. The different expression of CD75s-gangliosides in malignant versus healthy tissue is shown for patients 38, 6, 19, and 20, corresponding to the four tumor categories: I, high overexpression; II, moderate overexpression; III, equal expression (mostly undetectable in malignant and adjacent normal tissue); and IV, lowered expression (enhanced expression in healthy tissue). The synopsis of CD75s-ganglioside expression of all 38 patients investigated is provided in Table 1 and the pathologic data of pancreatic tumors are listed in Supplementary Table S1. Comparative TLC runs were done employing 10 μg reference gangliosides for orcinol stain (*O*), overlay detection of CD75s-gangliosides using viscumin (*V*) and rViscumin (*rV*), respectively, and 1.5 μg for TLC immunostain of CD75s-gangliosides with anti-CD75s-ganglioside antibody (AB2-6). **B,** structure of CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer (d18:1, C16:0). Terminally α2-6-sialylated CD75s-gangliosides represent the receptors for viscumin and its recombinant counterpart rViscumin, whereas isomeric α2-3-sialylated iso-CD75s-gangliosides do not bind. CD75s-2-ganglioside VI^{6}Neu5Ac-nLc6Cer with elongated nLc4Cer core structure by one Galβ4GlcNAc repeat retains antibody and lectin binding activity. All of those gangliosides appear in human tissues preferentially with C24 or C16 fatty acids but constant sphingosine (d18:1) in their ceramide parts, leading to typical double bands in TLC. This is shown in **A** of the figure for reference CD75s-gangliosides, whereby the upper band represents the gangliosides with the long-chain fatty acids and the lower band represents the gangliosides with the short-chain fatty acids (for structures of reference gangliosides, see Supplementary Table S2).

samples as exemplarily shown for patient 19 in Fig. 1A. Only 2.6% (=1 patient) showed a diminished expression (see synopsis of the CD75s-ganglioside expression of all 38 patients in Table 1). Most of the normal tissues exhibited only low expression (e.g., patient 38 of category I) or no expression (e.g., patients 6 and 19 of category II and III, respectively) of CD75s-1-ganglioside compared with the related malignant tissues. The identical CD75s specificity of antibody AB2-6, viscumin, and rViscumin is shown in Fig. 1A using reference gangliosides. Those controls indicate that antibody AB2-6, native, and recombinant viscumin all recognize both CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer and CD75s-2-ganglioside VI^{6}Neu5Ac-nLc6Cer but do not bind to isomeric iso-CD75s-1-ganglioside IV^{3}Neu5Ac-nLc4Cer and iso-CD75s-2-ganglioside VI^{3}Neu5Ac-nLc6Cer.

Immunohistochemical Detection of Tumor-Associated CD75s-Gangliosides

To determine the histologic distribution of CD75s epitopes, immunofluorescence microscopy was done with cryosections from tumor and normal samples. Sections were stained with the antibody AB2-6 in parallel with antibodies against CD14 (specific for granulocytes and macrophages/monocytes) and CD45 (expressed on all leukocytes) and DAPI (nuclear stain). As exemplarily shown for the tumor of patient 24 (tumor category I), the anti-CD75s-ganglioside antibody AB2-6 binds to certain areas of the tumor tissue, whereas the unaffected tissue of the same patient revealed an extremely low content of CD75s-gangliosides (Fig. 2A). CD14/CD45-positive leukocytes were found in low numbers and to be singularly distributed in the tumor tissue. The AB2-6 antibody coimmunostains only to minor extent single leukocytes compared with malignant tissue. Thus, the overall fluorescence intensity of CD75s-positive tumor areas exceeded by far that of leukocytes (most likely polymorphonuclear leukocytes; that is, neutrophilic granulocytes; ref. 28). In all cases, sections incubated with mouse IgG1 isotype control, chicken preimmune serum as well as the secondary dichlorotriazinylamino fluorescein–labeled antibodies only, did not stain. After section treatment with methanol and chloroform/methanol (1:1, v/v), CD75s antigens were undetectable indicating the lipid nature of CD75s epitopes (the extraction of CD75s-gangliosides; data not shown).

Structural Characterization of Tumor-Associated CD75s-Gangliosides

For unequivocal structural characterization of tumor-associated CD75s-gangliosides, the AB2-6 antibody-positive bands of TLC-separated lipid extracts of tumors were subjected to *in situ* TLC-IR-MALDI-MS according to Distler et al. (14). As an example for an immunohistologically anti-CD75s-positive tumor (patient 24; see Fig. 2A), mass spectra obtained from tumor-associated CD75s-1-gangliosides of the lipid extract of the same patient are shown in Fig. 2B. The $[M_1 + 2Na\text{-}H]^+$ and $[M_1 + Na]^+$ ions of the AB2-6-immunopositive upper band at *m/z* 1672.93/1674.95 and 1650.94/1652.98, respectively, were assigned to the CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer (d18:1, C24:1/C24:0) and the $[M_2 + 2Na\text{-}H]^+$ ions at *m/z* 1646.92 to CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer (d18:1, C22:0). The $[M_3 + 2Na\text{-}H]^+$ and $[M_3 + Na]^+$ ions of the AB2-6 immunopositive lower band at *m/z* 1562.81 and 1540.82, respectively, evidenced the CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer (d18:1, C16:0). The structural assignment of *m/z* values was deduced from *m/z* values of CD75s-1-gangliosides of reference gangliosides (see Supplementary Table S2)[8] according to Distler et al. (14). Thus, our data indicate total agreement of immunohistologically

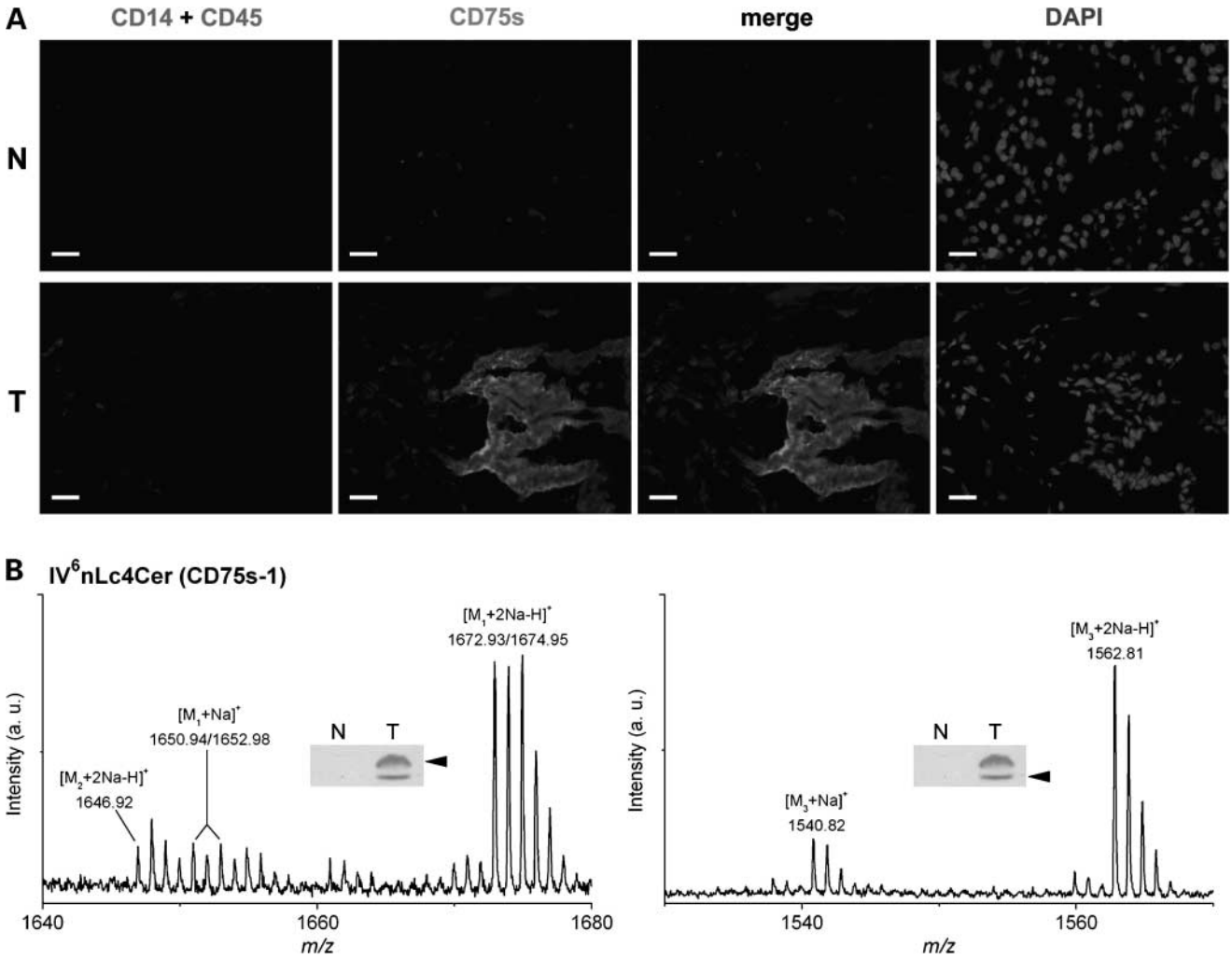

Figure 2. Immunofluorescence microscopic detection (**A**) and structural characterization by TLC-IR-MALDI-MS (**B**) of tumor-associated CD75s-gangliosides in pancreatic cancer. Cryosections and lipid extracts of normal and tumor tissue of patient 24 (tumor category I; see Table 1) were investigated with the anti-CD75s antibody AB2-6. Cryosections (**A**) were coimmunostained with CD14- and CD45-specific antibodies, and cell nuclei were detected with DAPI. Bars, 20 μm. Lipid extracts (**B**) equivalent to 2 mg normal and tumor wet weight tissue, respectively, were separated simultaneously by TLC and immunostained. *Arrowhead,* gangliosides analyzed by TLC-IR-MALDI-MS. The $[M_1 + 2Na\text{-}H]^+$ and $[M_1 + Na]^+$ ions of the immunopositive upper band of the tumor tissue with *m/z* values of 1672.93/1674.95 and 1650.94/1652.98, respectively, correspond to the CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer (d18:1, C24:1/C24:0) and the $[M_2 + 2Na\text{-}H]^+$ ions with *m/z* values of 1646.92 correspond to CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer (d18:1, C22:0). The $[M_3 + 2Na\text{-}H]^+$ and $[M_3 + Na]^+$ ions of the immunopositive lower band with *m/z* values of 1562.81 and 1540.82, respectively, are indicative for the CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer (d18:1, C16:0). (For comparison, see *m/z* values of CD75s-gangliosides of reference gangliosides listed in Supplementary Table S2).

detected overexpression of CD75s epitopes in cryosections with (*a*) the TLC overlay assay detected overexpression and (*b*) the structural fine characterization of tumor-associated CD75s-1-gangliosides by TLC-IR-MALDI-MS.

TLC Immunodetection of Tumor-Associated iso-CD75s-Gangliosides

Identical aliquots of GSL extracts from cancerous and corresponding healthy tissues, equivalent to 2 mg wet weight, were separated simultaneously by TLC. Iso-CD75s-gangliosides were detected with the TLC overlay assay using the anti-iso-CD75s-ganglioside antibody AB2-3. Tumors were ranked from 1 to 38 and grouped in categories I to IV as described above for CD75s-gangliosides (see Table 1). Four representative TLC immunostains of tumor extracts with the AB2-3 antibody are shown for each tumor category along with the related healthy tissue samples in Fig. 3A. In total, 65.8% of pancreatic tumors showed a moderate to high overexpression of iso-CD75s-1-gangliosides, whereas 21.1% exhibited an equal and only 13.2% a diminished expression (see synopsis of iso-CD75s-ganglioside expression of all 38 patients in Table 1). The normal tissues of all patients exhibited notable expression of iso-CD75s-1-ganglioside that differed between patients, indicating some natural biological variation in the unaffected tissues. The antibody AB2-3 recognizes both the iso-CD75s-1-ganglioside IV^{3}Neu5Ac-nLc4Cer and the iso-CD75s-2-ganglioside VI^{3}Neu5Ac-nLc6Cer but does not bind to CD75s-1-ganglioside IV^{6}Neu5Ac-nLc4Cer and CD75s-2-ganglioside VI^{6}Neu5Ac-nLc6Cer using reference gangliosides as shown in Fig. 3A.

Immunohistochemical Detection of Tumor-Associated iso-CD75s-Gangliosides

The histologic distribution of iso-CD75s epitopes was determined by immunofluorescence microscopy as described above. Sections were stained with the antibody AB2-3 together with anti-CD14 and anti-CD45 antibodies and DAPI. The tumor of patient 18 (tumor category I) was chosen to show binding of the anti-iso-CD75s-ganglioside antibody AB2-3 to certain areas of the tumor tissue, whereas the healthy tissue of the same patient was found being less intensively stained (Fig. 4A). Single CD14/CD45-positive cells were detected in the tumor tissue indicating a low number of weakly stained leukocytes when compared with the considerably higher overall fluorescence intensity

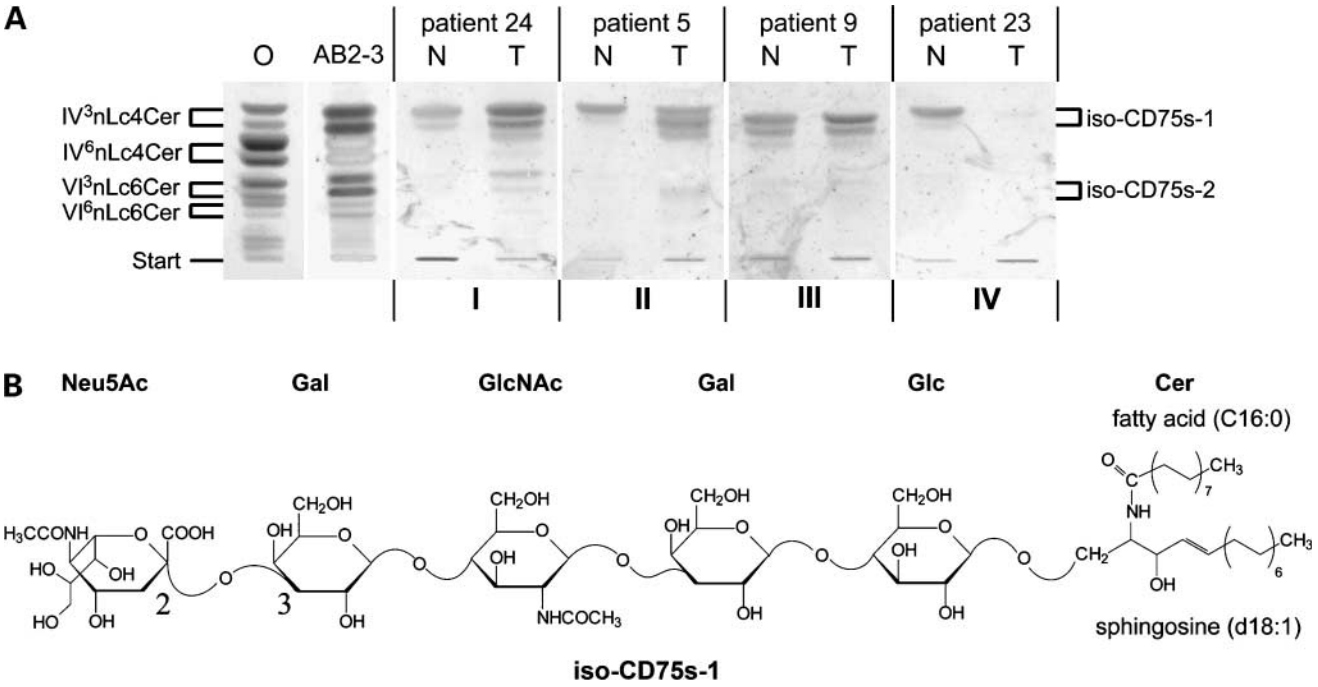

Figure 3. **A,** TLC immunostain of tumor-associated iso-CD75s-gangliosides in pancreatic cancer. Aliquots from crude lipid extracts corresponding to 2 mg wet weight of normal and tumor tissue were separated simultaneously by TLC and immunostained with the anti-iso-CD75s-ganglioside antibody AB2-3. The different expression of iso-CD75s-gangliosides in malignant versus healthy tissue is shown for patients 24, 5, 9, and 23, corresponding to the four tumor categories: I, high overexpression; II, moderate overexpression; III, equal expression; and IV, lowered expression (enhanced expression in healthy tissue). The synopsis of iso-CD75s-ganglioside expression of all 38 patients investigated is provided in Table 1 and the pathologic data of pancreatic tumors are listed in Supplementary Table S1. The orcinol stain and the TLC immunostain of iso-CD75s-gangliosides with anti-iso-CD75s-ganglioside antibody (AB2-3) were done with 10 and 1.5 μg reference gangliosides, respectively. **B,** structure of iso-CD75s-1-ganglioside IV^{3}Neu5Ac-nLc4Cer (d18:1, C16:0). Elongation of nLc4Cer core structure by one Galβ4GlcNAc repeat resulting in iso-CD75s-2-ganglioside VI^{3}Neu5Ac-nLc6Cer retains antibody binding activity. All of those gangliosides appear in human tissues preferentially with C24 or C16 fatty acids but constant sphingosine (d18:1) in their ceramide parts leading to typical double bands in TLC. This is shown for reference iso-CD75s-gangliosides, whereby the upper band represents the gangliosides with the long-chain fatty acids and the lower band represents the gangliosides with the short-chain fatty acids (for structures of reference gangliosides, see Supplementary Table S2).

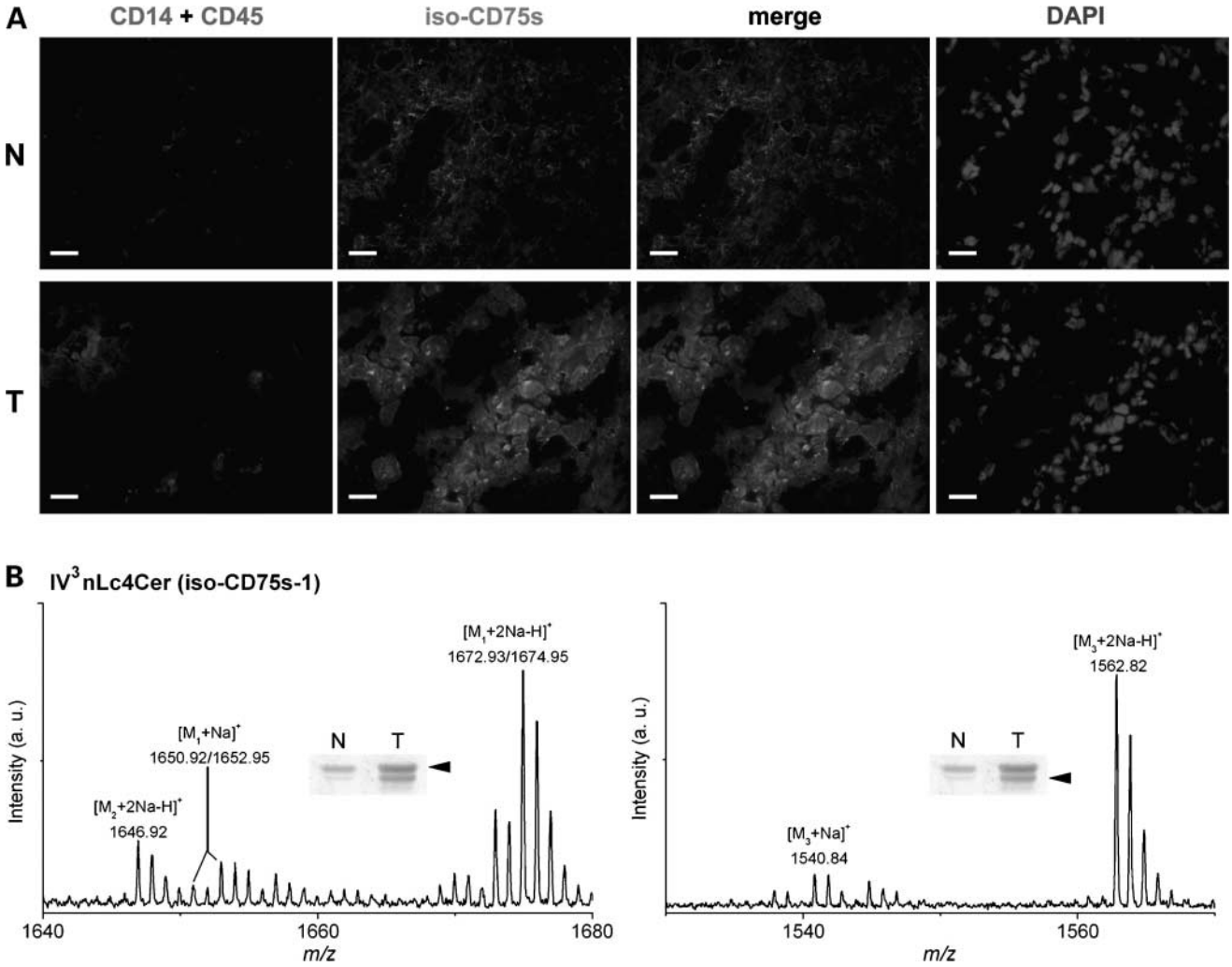

Figure 4. Immunofluorescence microscopic detection (**A**) and structural characterization by TLC-IR-MALDI-MS (**B**) of tumor-associated iso-CD75s-gangliosides in pancreatic cancer. Cryosections and lipid extracts of normal and tumor tissue of patient 18 (tumor category I, see Table 1) were investigated with the anti-iso-CD75s antibody AB2-3. Cryosections (**A**) were coimmunostained with CD14- and CD45-specific antibodies, and cell nuclei were detected with DAPI. Bars, 20 μm. Lipid extracts (**B**) equivalent to 2 mg normal and tumor wet weight tissue, respectively, were separated simultaneously by TLC and immunostained. *Arrowhead,* gangliosides analyzed by TLC-IR-MALDI-MS. The $[M_1 + 2Na\text{-}H]^+$ and $[M_1 + Na]^+$ ions of the immunopositive upper band of the tumor tissue with *m/z* values of 1672.93/1674.95 and 1650.92/1652.95, respectively, correspond to the iso-CD75s-1-gangliosides IV^3Neu5Ac-nLc4Cer (d18:1, C24:1/C24:0) and the $[M_2 + 2Na\text{-}H]^+$ ions with *m/z* values of 1646.92 correspond to iso-CD75s-1-ganglioside IV^3Neu5Ac-nLc4Cer (d18:1, C22:0). The $[M_3 + 2Na\text{-}H]^+$ and $[M_3 + Na]^+$ ions of the immunopositive lower band with *m/z* values of 1562.82 and 1540.84, respectively, are indicative for the iso-CD75s-1-ganglioside IV^3Neu5Ac-nLc4Cer (d18:1, C16:0) (for comparison, see *m/z* values of iso-CD75s-gangliosides of reference gangliosides listed in Supplementary Table S2).

of iso-CD75s-positive tumor areas. All control stains (outlined above) were negative and treatment of the sections with organic solvents revealed the lipid nature of iso-CD75s epitopes (the elimination of iso-CD75s-gangliosides; data not shown).

Structural Characterization of Tumor-Associated iso-CD75s-Gangliosides

Tumor-associated iso-CD75s-gangliosides were structurally characterized by *in situ* TLC-IR-MALDI-MS as described above. The tumor of patient 18 (category I) served as an example for an immunohistologically anti-iso-CD75s-positive tumor (see Fig. 4A) and mass spectra obtained from tumor-associated iso-CD75s-1-gangliosides of the same patient are shown in Fig. 4B. The $[M_1 + 2Na\text{-}H]^+$ and $[M_1 + Na]^+$ ions of the AB2-3 immunopositive upper band with *m/z* 1672.93/1674.95 and 1650.92/1652.95, respectively, indicate the iso-CD75s-1-ganglioside IV^3Neu5Ac-nLc4Cer (d18:1, C24:1/C24:0) and the $[M_2 + 2Na\text{-}H]^+$ ions with *m/z* 1646.92 indicate the iso-CD75s-ganglioside IV^3Neu5Ac-nLc4Cer (d18:1, C22:0). The $[M_3 + 2Na\text{-}H]^+$ and $[M_3 + Na]^+$ ions of the AB2-3 immunopositive lower band with *m/z* 1562.82 and 1540.84, respectively, were assigned to the iso-CD75s-1-ganglioside IV^3Neu5Ac-nLc4Cer (d18:1, C16:0). The structural assignment of *m/z*

values was deduced from *m/z* values of iso-CD75s-1-gangliosides of reference gangliosides (see Supplementary Table S2)[8] according to Distler et al. (14). Thus, compliance was determined between immunohistologically detected overexpression of iso-CD75s epitopes and (*a*) the TLC overlay assay detected overexpression and (*b*) the identification of tumor-associated iso-CD75s-1-gangliosides by TLC-IR-MALDI-MS.

Statistical Analysis

Box plots of the densitometrically determined CD75s- and iso-CD75s-gangliosides in pancreatic tissues of 38 patients investigated in this study are shown in Fig. 5A and B, respectively. The expression of both types of gangliosides was significantly increased in the tumors in comparison with the adjacent normal tissues ($P = 0.001$). Thus, our data suggest a statistically significant association between neoplastic transformation and CD75s-1- and iso-CD75s-1-ganglioside expression. The calculation of correlation coefficients from ranked primary densitometric values of the tumor samples and from the difference values (calculated by subtraction of normal from related tumor tissue values) revealed a significant correlation of CD75s-1- and iso-CD75s-1-ganglioside expression in malignant pancreatic tissues ($P = 0.002$; $\tau = 0.364$ and $P = 0.023$; $\tau = 0.260$, respectively).

The statistical analysis of the CD75s-1-ganglioside expression did not show any correlation with extent of the primary tumor (pT), regional lymph node metastasis (pN), presence of distant metastasis (pM), or stage grouping (Union Internationale Contra Cancrum stage). However, the comparison of tumors assigned as histopathologic grade 2 ($n = 21$ patients) and those with a histopathologic grade >2 ($n = 16$) revealed a statistically significant enhancement of CD75s-1-ganglioside expression in the group of poorly differentiated and undifferentiated tumors (grade >2). This association between CD75s-1-ganglioside expression and tumor differentiation was found using both primary densitometric values of the tumor samples and the difference values ($P = 0.049$ for both types of values) and is shown in the box plots of Fig. 5C. In addition, primary CD75s-1-ganglioside values of tumors and difference values of CD75s-1-ganglioside expression correlated with the grade of tumor differentiation ($P = 0.028$; $\tau = 0.305$ and $P = 0.011$; $\tau = 0.346$, respectively). In conclusion, the expression of CD75s-1-gangliosides is prone to increase in less differentiated malignant tissue suggesting the CD75s-1-ganglioside as a potential marker for poor differentiation of pancreatic tumors.

Finally, it should be mentioned that no significant association between the expression of iso-CD75s-1-gangliosides and any of the clinicopathologic variables of tumors could be observed.

Anti-CD75s-1-Ganglioside Antibodies in Patients' Sera

TLC overlay assay detection of anti-CD75s-1-ganglioside antibodies in sera from patients with pancreatic carcinoma was done with 19 available serum samples (50% of the total cohort of 38 patients), which have been taken before

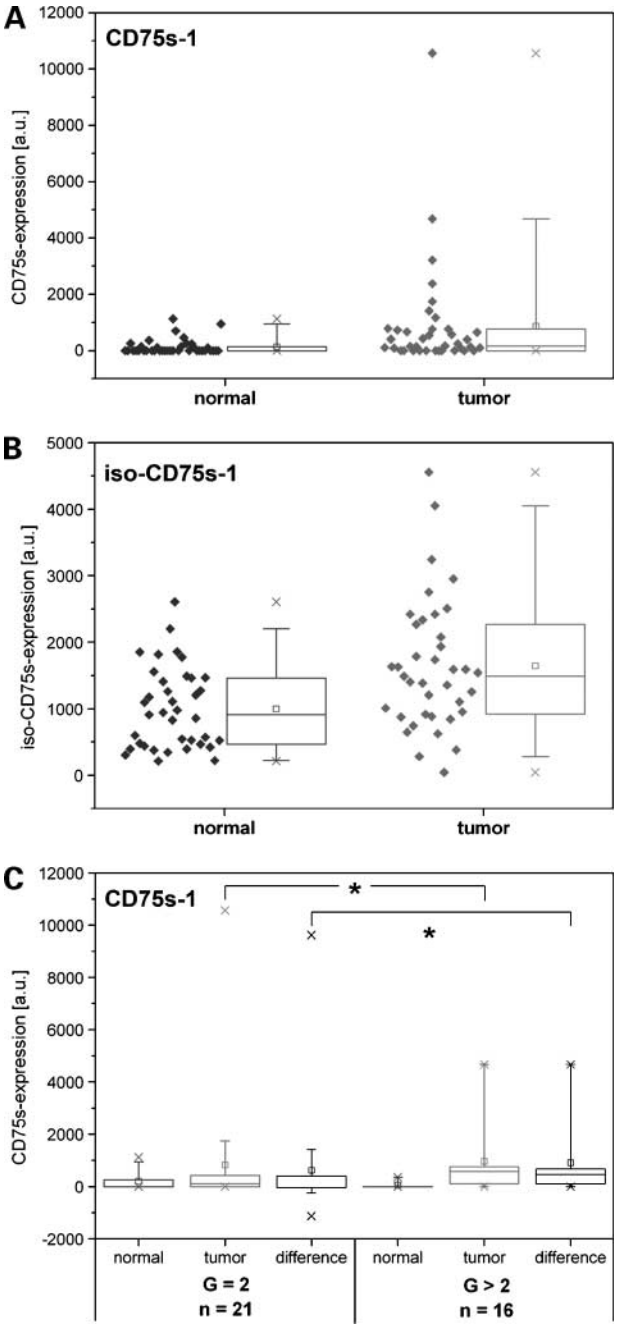

Figure 5. Box plots of CD75s-1- (**A**) and iso-CD75s-1-ganglioside expression (**B**) in pancreatic normal and tumor tissues ($n = 38$ patients). The boxes are bounded above and below by the 25% and 75% percentiles, respectively. *Lines in the boxes* and *little squares,* median and mean, respectively; *two crosses,* minimum and maximum values, respectively. CD75s-1- and iso-CD75s-1-ganglioside expression in tumor tissues was significantly enhanced compared with that in adjacent normal tissues indicating an association of both types of gangliosides with neoplastic transformation ($P = 0.001$). **C,** correlation between CD75s-1-ganglioside expression and tumor differentiation ($n = 37$ patients, because one tumor could not be assigned; see Table 1). Tumors were divided into two groups according to histopathologic grading: grade 2 ($n = 21$ patients) and grade >2 ($n = 16$ patients). Both values of the tumors and difference values (*square brackets with asterisks*) point to a statistically significant higher CD75s-1-ganglioside expression in tumors graded as >2 ($P = 0.049$).

surgery. The TLC immunostained CD75s-1-ganglioside bands were categorized according to their intensities as ++ (moderate positive), + (faint positive), and - (negative). The majority of sera showed a moderate or weak positive reaction toward CD75s-1-gangliosides (57.9%), whereas 42.1% were negative (for synopsis see Supplementary Table S4).[8] No correlation could be detected at all neither with tumor staging nor with histopathologic grading. Thus, the biological and/or clinical significance of serum antibodies against CD75s-1-gangliosides remains obscure at this stage of research. Three examples of sera from patients 32, 31, and 33, corresponding to moderate, weak, and negative anti-CD75s-1 binding, respectively, are shown in Supplementary Fig. S1.[8] The structural interpretation of positive gangliosides that chromatograph beneath CD75s-1-gangliosides (marked with question marks in Supplementary Fig. S1)[8] remains elusive.

Discussion

Gangliosides have aroused exceedingly interest in tumor diagnosis and as potential targets for anticancer therapy (11, 29, 30). Their multivalent presentation in microdomains of the plasma membrane is important to acquire strong affinity of an oligosaccharide toward its ligand in carbohydrate-protein interactions, often called the cluster glycoside effect (31). Thus, the clustered assembly in microdomains renders gangliosides excellent candidates for tumor targeting employing lectins, toxins, or monoclonal antibodies directed toward tumor-associated gangliosides. As an example, a novel anti-ganglioside monoclonal antibody has been recently produced (32), which binds to a ganglioside and directly induces apoptosis of targeted tumor cells without the need for immune effector cells or complement, suggesting a new therapeutic approach for this class of antibodies. Interestingly, the tumor-associated target ganglioside has been thus far preliminarily characterized as a sialyltetraosylceramide, thereby excluding GM1 or sialyl-Lewis^x antigens as binding structures. However, this anti-ganglioside antibody seems to be a promising anticancer drug and is supposed to increase efficacy of treatment of colorectal cancer.

When analyzing GSL extracts of human tumors, the possibility must be considered that these components may be derived at least to some extent from nonneoplastic host infiltrating cells. Tumor-infiltrating macrophages (33) and granulocytes (28) can significantly contribute to the total cell population of certain tumors and thus to the composition of GSL extracts obtained from solid tumors. The fact that granulocytes and, to a minor extent, macrophages do express type II gangliosides (22, 34) indicates the necessity of a thorough microscopic evaluation of the tumor tissue for the presence of neutrophils and macrophages. CD75s-1- and CD75s-2-gangliosides as well as iso-CD75s-1- and iso-CD75s-2-gangliosides are common in human granulocytes but very minor constituents of monocytes/macrophages (22, 34). Based on (*a*) careful immunohistologic analysis of tissue sections and (*b*) failure of detecting relevant quantities of CD75s-2- and iso-CD75s-2-gangliosides (=granulocyte markers) in the tumor extracts, we could show that the detected CD75s-1- and iso-CD75s-1-gangliosides in the GSL extracts from normal and malignant tissues are indeed intrinsic compounds of pancreatic tissues and do not derive from leukocytes.

An accumulation of sialyl(α2-6)paragloboside (=CD75s-1-ganglioside) has been reported previously for human hepatoma (35), suggesting that the biosynthetic pathway of gangliosides containing Neu5Acα6Galβ4GlcNAc structure is activated in hepatoma cells. This early report is in line with our previous studies suggesting that an enhanced expression of CD75s-gangliosides may not be unique to malignant pancreatic tissue but a widespread feature of other tumor entities derived from the gastrointestinal tract (14, 15). As an alternative to gain information about the sialylation status of tumor tissues, a body of data has been produced by several clinical groups employing the genetic approach to identify the expression of sialyltransferases being responsible for the specific transfer of Neu5Ac in α2-6- or α2-3-configuration. However, frequent inconsistency regarding the quantitative relationship between sialyltransferase transcripts, the enzyme activity determined on the protein level, and the appearance of related sialylglycoconjugates suggested that the enzyme/product relationship does not appear to be direct and the expression of α2-6-sialylated oligosaccharides is controlled at multiple levels (36). Therefore, we favored in our study the reliable structural proof of CD75s- and iso-CD75s-gangliosides and provided objective evidence for the existence of those isomeric type II gangliosides representing potential targets for antitumor therapies of pancreatic cancer.

We could detect a significant relationship between neoplastic transformation and CD75s-1-ganglioside as well as iso-CD75s-1-ganglioside expression, whereby both gangliosides were found to correlate in ductal adenocarcinoma of the pancreas. Furthermore, the expression of CD75s-1-gangliosides was found to correlate with the degree of histologic differentiation of the tumors. A significant trend of elevated expression of CD75s-1-gangliosides was observed in poorly differentiated and undifferentiated tumors (grade >2). Generally speaking, less differentiated pancreatic tumors tend to express increased amounts of CD75s-1-gangliosides carrying terminally α2-6-linked sialic acid. The first *in vivo* evidence for a functional role of α2-6-linked sialic acids in the development of mammary cancer was shown in an elegant study done by Hedlund et al. (37) supporting the hypothesis that α2-6-sialylation modulates carcinoma differentiation causing a less differentiated phenotype. This study in turn substantiates our results obtained from primary pancreatic tumors that underline a correlation between enhanced expression of α2-6-sialylated gangliosides and poor differentiation. Because poor differentiation is known to correlate with poor prognosis, the analysis of CD75s-1-gangliosides could provide useful prognostic information in patients suffering from one of the most aggressive cancers.

Conclusion

Increased expression of CD75s- and iso-CD75s-gangliosides in pancreatic tumors renders these cell surface molecules promising candidates for oncologic applications. The CD75s-ganglioside specific viscumin and its recombinant counterpart rViscumin, both being under clinical investigation as potential antitumor drugs, are of particular interest due to their cancer-destructive potential. Besides their primordial antitumor activity on tumor cell lines including multidrug-resistant human cancer cell lines (38), synergistic antitumoral effects of the native and recombinant lectin have been documented in numerous *in vitro* studies employing conventional antitumor drugs (39) and ionizing radiation treatment (40). Furthermore, rViscumin has been successfully probed in treating cancer cells in animal models *in vivo* (41, 42). Surgery, chemotherapy, radiotherapy, and hormone therapy have proven their cancer-destructive potencies and curative feasibilities and have emerged as the gold standard in the treatment of carcinoma. However, as yet no standard therapy has produced a substantial survival benefit of patients suffering from pancreatic cancer. Therefore, new therapeutic concepts are urgently required to support the cancer standard treatments. Thus, the first goal should be to match rViscumin to the CD75s-ganglioside profiles of individual pancreatic tumors. In case of enhanced expression, those patients should be suitable candidates for an adjuvant rViscumin therapy. In conclusion, rViscumin, which has successfully passed clinical phase I trials (18, 19), provides an opportunity for treating patients afflicted by this devastating malignancy with very poor prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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