Analysis of sialyl-Lewis x on MUC5AC and MUC1 mucins in pancreatic cancer tissues.

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Abstract

Pancreatic adenocarcinoma (PDAC) lacks efficient biomarkers. Mucins are glycoproteins that can carry aberrant glycosylation in cancer. Our objective was to identify cancer-related glycan epitopes on MUC1 and MUC5AC mucins in PDAC as potential biomarkers. We have analysed the tumour-associated carbohydrate antigens sialyl-Lewis x (SLe^x) and sialyl-Tn (STn) on MUC1 and MUC5AC in PDAC tissues. The selected cohort for this study consisted of twenty-one PDAC tissues positive for SLe^x antigen and three normal pancreas specimens as controls. STn expression was shown in 76% of the PDAC tissues. MUC1 and MUC5AC were detected in 90% of PDAC tissues. We performed *in situ* proximity ligation assay combining antibodies against mucins and glycan epitopes to identify specific mucin glycoforms. MUC1-SLe^x and MUC5AC-SLe^x were found in 68% and 84% respectively, of the mucin expressing PDAC tissues, while STn hardly colocalized with any of the evaluated mucins. Further analysis by Western blot of MUC5AC and SLe^x in eight PDAC tissue lysates showed that six out of eight cases were positive for both markers. Moreover, immunoprecipitation of MUC5AC from positive PDAC tissues and subsequent SLe^x immunodetection confirmed the presence of SLe^x on MUC5AC. Altogether, MUC5AC-SLe^x glycoform is present in PDAC and can be regarded as potential biomarker.

Keywords: mucins; pancreatic cancer; sialyl-Lewis x.

Abbreviations: PDAC, pancreatic cancer; PLA, *in situ* proximity ligation assay; SLe^x, sialyl-Lewis x; STn, sialyl-Tn

1. Introduction

Pancreatic adenocarcinoma (PDAC) is estimated to be the third leading cause of cancer death in the United States [1] and is projected to become the second cause of cancer-related death by 2030 [2].

PDAC is one of the cancers with the lowest survival rate (7% in 5 years), due to a late diagnosis and the intrinsic aggressiveness of this tumour [3]. Unfortunately, and in contrast to the declining trends for most cancers, PDAC is one of the few neoplasms with increasing incidence and mortality in both genders [1, 4].

Despite the efforts made in the last decades to discover new biomarkers, there is still a lack of biomarkers with enough specificity and sensitivity. This reduces the chances of detection of PDAC in early stages, when curative procedures are most effective [5]. For the majority of patients the only available treatment is the administration of gemcitabine, a chemotherapeutic agent that has shown beneficial effects in only 20-30% of patients [6, 7].

Mucins are high molecular weight glycoproteins expressed on epithelial cells. A multitude of oligosaccharides are attached to the polypeptide backbone of mucins which can be bound to the cell membrane or secreted into the organ lumen. Mucins constitute a selective barrier and play an important role in cell adhesion, immune response, cell signalling and in the renewal and differentiation of the epithelium [8-10]. Mucin expression and their glycosylation are altered in many tumours [11, 12], including breast [13], colon [14, 15], gastric [16-18], lung [19], and PDAC [20, 21]. These alterations influence cellular growth, differentiation, malignant transformation, adhesion, invasion and immune surveillance [8, 11, 22, 23].

MUC1 is a membrane-bound mucin that contains a variable number of tandem repeats (VNTR), between 20 and 125, which are abundantly *O*-glycosylated. Its molecular mass is therefore variable ranging from 120-225 kDa (protein backbone) and significantly larger when glycosylated, up to 500 kDa. MUC1 can also be secreted after cleavage of its extracellular domain. It is expressed at the apical surface of ductal epithelia of tissues, including breast, pancreas, bronchus and the gastrointestinal tract [17, 24, 25]. In normal pancreas, the common staining of MUC1 is at the cytoplasmic level in acinar cells and in the apical area of ductal cells [26], and it is well-established that MUC1 is overexpressed in malignant situation [26, 27]. MUC5AC is a secreted mucin of a polypeptide chain of approximately

586 kDa, but with a much higher molecular weight especially due to the large number of *O*-glycosylated chains attached to Ser and Thr residues in the VNTR regions. It is only expressed in 2-4% of healthy pancreas [6]. Regarding PDAC, MUC5AC has been shown to be expressed in the cytoplasm and is *de novo* expressed in around 75-92% of the PDAC cases [27-29] and is also detected in 70% of PanIN1A cases, which is the earliest stage of the most common precursor lesions of PDAC [27].

Aberrant glycosylation of cell surface glycolipids, membrane associated glycoproteins and secreted glycoproteins is a universal feature of malignant transformation [23, 30-32]. For instance, in malignant pancreatic tissues the carbohydrate antigens sialyl-Lewis x (SLe^x) and sialyl Tn (STn) have been described to be commonly expressed over 80% [33, 34] and 77% [27] respectively, while they are not detected in healthy pancreatic specimens [33, 35]. Moreover, SLe^x and STn expression have been associated with tumour progression and metastasis [36, 37]. STn is an *O*-linked disaccharide which consists of an α 2,6-linked N-acetylneuraminic acid attached to N-acetylgalactosamine and is often aberrantly expressed in the neoplastic lesions including PDAC [32]. SLe^x epitope is a terminal structure (N-acetylneuraminic acid α 2,3-Galactose β 1,4[Fucose α 1,3]N-acetylglucosamine) that can be found in either *N*- or *O*-glycosylated molecules [32, 38].

Mucins are known to be major carriers of tumour associated carbohydrate antigens and, since they are overexpressed in adenocarcinomas, these glycan alterations are highly amplified on the surface of tumour cells [12]. In gastric tissues, a recent study that characterizes mucin *O*-glycosylation, reports the presence of sialylated glycans, including sialyl-Lewis antigens. A higher level of sialylation and sulfation on gastric *O*-glycans were found in cancerous tissue compared to healthy tissue [18]. In colon cancer, an increase expression of a core 3 sialyl-Le^x hexasaccharide, which appeared to compete with its sulfo-Le^x counterpart in normal tissue, was described on MUC2, suggesting its use as a potential marker of malignant transformation [15]. Therefore, the detection of the cancer related glycosylation on mucins could give rise to more specific and /or sensitive cancer diagnostic markers. The expression of STn antigen on MUC1 and MUC5AC has been previously described in stomach, colon, lung, breast and ovarian tumours [39]. Recently, Remmers and coauthors have shown the expression of Tn/STn on MUC1 in PDAC tissues (91%) using an antibody directed to Tn/STn MUC1

glycoforms [40, 41]. The SLe^x epitope on MUC1 and MUC5AC has been identified in colon, gastric, ovarian and breast carcinomas [17, 39, 42-45], but to our knowledge it has not been investigated in PDAC tissues.

Here we have used the *in situ* proximity ligation assay (PLA) to evaluate the glycan epitopes SLe^x and STn on MUC1 and MUC5AC mucins in a cohort of PDAC tissues. *In situ* PLA provides information of two epitopes in close proximity and therefore has been used to report the colocalization of two antigens indicating, in our case, the expression of a particular glycan epitope on a specific mucin. Our results showed that the expression of SLe^x on MUC1 and MUC5AC was observed in 68% and 84% of the mucin expressing PDAC tissues, respectively. In a second approach, PDAC tissues were assayed for MUC5AC and SLe^x by western blotting and 75% of them showed positivity for both markers. An immunoprecipitation protocol using specific antibodies against MUC5AC was set up. MUC5AC was immunocaptured in selected PDAC tissues that were individually positive for MUC5AC and SLe^x. These immunoprecipitated samples were then probed for SLe^x, and showed that the SLe^x epitope was present on MUC5AC.

2. Materials and methods

2.1 Human tissue samples

Pancreatic tissues were obtained from the Hospital Dr. Josep Trueta (Girona, Spain) from patients undergoing surgical resection following the standard operating procedures of its Ethics Committee. Normal pancreatic tissue refers to adjacent, non-tumour pancreatic tissue from patients with pancreatic-related disorders. The histopathologic features of the resected specimens were confirmed by biopsy or image examination by the Digestive and Pathology Units and classified according to the Tumour Node Metastasis Classification of Malignant Tumours of the International Union Against Cancer (UICC) 7th edition [46]. For immunohistochemistry and PLA, 21 PDAC and three normal tissues were used. PDAC tissues were from 15 males and 6 females, ranging 49–81 years old; six were stage IIA (1 well differentiated, 4 moderately differentiated and one poorly differentiated), thirteen stage IIB (five well differentiated, six moderately differentiated and two poorly differentiated), one stage II* (unclassified A or B due to the lack of available information of the nodule affection,

moderately differentiated), and one stage IV (well differentiated). Tissues were fixed in 10% formalin, embedded in paraffin and cut into 5 µm serial sections for immunohistochemistry (IHC) and *in situ* Proximity Ligation Assays (PLAs). Hematoxilin eosin staining of the tissues was performed in the same tissue blocks but not in the consecutive serial slides of the IHC and PLA assays. For protein lysates, eight PDAC and four normal tissues were used. PDAC tissues were from seven males and one female; ranging 59-79 years old. Six tissues were stage IIB and two stage IV. A small piece of each tissue was immediately frozen in liquid nitrogen and kept at -80°C until their use.

2.2 Immunohistochemistry

Paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 min, and then sections were incubated with normal rabbit serum diluted 1/5 in PBS containing 10% BSA. Normal serum excess was removed and replaced by specific primary antibody in the appropriate concentration (Table 1) in PBS with 5% BSA and incubate overnight at 4 °C. Slides were then washed in PBS and incubated for 30 min with secondary biotinylated rabbit anti-mouse antibody (Dako, Glostrup, Denmark) diluted 1/200 in PBS containing 5% of BSA. The slides were subsequently washed in PBS and incubated for 30 min with avidin-biotin complex (Vectastain Elite ABC kit, Burlingame, CA) according to the manufacturer's recommendations. Staining was performed with 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) containing 0.02% hydrogen peroxide. Counterstaining of the nucleus was done with Mayer's hematoxylin solution. Slides were examined under a bright-field microscope AH2 Vanox-T microscope Olympus. Images were acquired using an Olympus microscope digital camera DP73 and the CellSens Standard 1.11 software.

SLe^x and MUC1-M8 immunostainings were performed using the KM93 and M8 monoclonal detection antibodies, and a biotinylated goat anti-mouse IgM at 1/200 and a biotinylated horse anti-IgG at 1/100 antibodies (Vectastain Elite ABC kit) in 1.5% of goat serum or 1.5% of horse serum, respectively in PBS, following the protocol described in [47]. CSLEX antibody (at 1/20 dilution) (BD Biosciences, San Jose, CA) was used to compare the SLe^x immunostaining obtained with the KM93 antibody.

Staining intensity was graded to the frequency of positive cells as: strongly positive (>60% stained cells), moderately positive (25-60% stained cells), faintly positive (<25% stained cells), sparse cells and negative. The histological analysis and scoring of the tissues were performed by two independent expert pathologists of the Hospital Dr. Josep Trueta of Girona.

2.3 Purification and quantification of monoclonal antibodies

VU4H5 (MAb anti-MUC1), CLH2 (MAb anti-MUC5AC) and TKH2 (MAb anti-STn) were purified with HiTrap Protein G sepharose columns (GE Healthcare, Amersham Biosciences, Uppsala, Sweden), according to the manufacturer's protocol. Desorption buffer was exchanged into phosphate buffer saline (PBS, pH 7.4) using Amicon Ultra-0.5 10K Centrifugal Filters (Millipore, Billerica, MA).

2.4 *In situ* Proximity Ligation Assay

In situ Proximity Ligation Assay (PLA) was performed in paraffin sections from controls and PDAC cases for colocalization analysis of SLe^x and STn antigens with the MUC1 and MUC5AC mucins.

Firstly, Duolink *In Situ* Probemaking kit (Sigma-Aldrich, St. Louis, MO) was used to conjugate the PLA oligonucleotide arms directly to the purified primary antibodies. TKH2 (anti-STn) was conjugated with the MINUS probes (Olink DUO920010, Olink Bioscience, Uppsala, Sweden), and VU4H5 (anti-MUC1) and CLH2 (anti-MUC5AC) with the PLUS probes (Olink DUO92009) according to the manufacturer's instructions. KM93 (MAb anti-SLe^x) antibody was not labelled with PLA probe due to its IgM nature. Thus, the secondary antibodies AffiniPure goat anti-mouse IgG (subclass 1) and AffiniPure goat anti-mouse IgM (both from Jackson ImmunoResearch, West Grove, PA) were labelled using the previously detailed oligonucleotides Olink DUO920010 and Olink DUO92009, respectively.

Deparaffined tissue sections were pre-treated according to the immunohistochemistry protocol explained above. Then, sections were incubated for 30 min at 37°C with the Blocking Solution (Duolink *In Situ* Probemaking kit). For colocalization of mucins with STn epitope, primary antibodies conjugated with oligoprobes were used. Conjugated MAbs to MUC1 (1/40) or MUC5AC (1/100) and

STn (1/40) were diluted in PBS with 5% BSA containing 1/20 of assay reagent (Duolink *In Situ* Probemaking kit) and slides were incubated overnight at 4 °C. Slides were then washed with a filtered solution of 0.01 M Tris, 0.15 M NaCl and 0.05% Tween 20 (Wash buffer A) prior to the ligation step. The ligation solution was prepared diluting the two oligonucleotides 1/5 and the ligase 1/40 (Duolink *In Situ* Detection Reagents Orange, Sigma-Aldrich) in pure water, and was added to slides and incubated for 30 minutes at 37 °C in order to hybridize with the PLA probes and to form a closed circle. After a wash with buffer A, the amplification was performed in darkness adding the amplification solution to the samples, consisting of nucleotides and fluorescently labelled oligonucleotides, together with the polymerase both diluted in pure water 1/5 and 1/80, respectively. The slides were washed with 0.2 M Tris and 0.1 M NaCl (Wash buffer B) and then were incubated with DAPI to visualise nuclei (Sigma-Aldrich, 0.4 mg/mL). After washing slides with 0.01x wash buffer B, samples were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). All slides were examined under a Zeiss Imager.Z1 Axio fluorescence microscope (Zeiss, Welwyn Garden City, UK). Images were acquired using a Zeiss Axio cam MRm and the AxioVision Release 4.8.1 software.

The analyses of the colocalization of MUC1 and MUC5AC with SLe^x epitope were performed using secondary antibodies with oligoprobes. The procedure was similar to as previously described for the PLA with incubations of VU4H5, CLH2 (both IgGs subtypes) and KM93 (IgM subtype) primary antibodies (same conditions as for the immunohistochemistry) followed by 1h incubation at 37 °C with the goat anti-mouse IgG1 and goat anti-mouse IgM oligoprobe secondary antibodies, diluted 1/300.

Imaging analysis of the PLA signal was performed with the Duolink Image Tool software (Sigma-Aldrich). These quantifications were performed for the imaging analysis of the PLA results of SLe^x-MUC1 and SLe^x-MUC5AC colocalization for each of the tissues. Briefly, several representative images per case were subjected to the software analysis and the median of positive blobs (positive PLA events) was set up for each case. Then, the median of positivity for each PLA combination was determined and 3 categories were established as follows: +++ (positive cases whose median is higher than 75% of the overall median); ++ (positive cases higher than 25% and lower than 75%); and + (positive cases lower than 25%).

2.5 Tissue protein lysate

Frozen PDAC and normal pancreatic tissues were thawed, mixed with RIPA buffer [(20 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100 (pH 7.4,)] supplemented with a protease inhibitor cocktail Complete Ultra tablets (Roche, Basel, Switzerland), and homogenised with lysing matrix beads D in a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA). Tissue homogenates were centrifuged 5 min at 14000g at 4 °C. The supernatant was collected and total protein quantified with bicinchoninic acid assay (BCA) (Thermo Scientific Pierce, Waltham, MA).

2.6 Immunoprecipitation

For immunoprecipitation assays, cell lysates containing equal amounts of protein (500 µg) were reduced in buffer containing dithiothreitol such that the final concentration when mixed with sample was 10 mM. After 2 h incubation at 37 °C, fresh iodoacetamide was added to a final concentration of 25 mM and samples were incubated at room temperature for 30 minutes in the dark.

After reduction and alkylation, samples were desalted and concentrated using Amicon Ultra-0.5 3kDa Centrifugal Filter Devices (Millipore) which had been previously passivated with 5% Brij-35 (Sigma). Next, samples were diluted in incubation buffer, 50 mM Tris pH 7.4, 150 mM NaCl and 1% Triton X-100, 5mM EDTA and protease inhibitors (complete ultratablets from Roche), and precleared with 100 µL of Protein G agarose resin for two hours at 4 °C. The precleared samples were incubated with 20 µL of anti-MUC5AC polyclonal antibody (Lum5.1) for two hours at 4 °C. The immunocomplexes were then captured by incubating samples with Protein G agarose resin packed into a spin column (Corning Costar Spin-X, New York, NY) overnight at 4 °C. Protein G column were centrifuged and washed three times with washing buffer (50 mM Tris pH 7.4, 150 mM NaCl and 1% Triton X-100). MUC5AC bound to the Lum5.1 antibody was eluted after boiling the sample with Laemmli buffer containing 1.25% β-mercaptoethanol.

2.7 Western Blot

Twenty micrograms of each protein lysate or immunoprecipitated MUC5AC from tissues were prepared on Laemmli sample buffer containing 1.25% β -mercaptoethanol and heated at 95°C for 5 min. Samples were then loaded in duplicate and separated with a 3% stacking 5% resolving gel and transferred to a PVDF membrane for 4 h at 100 V in Towbin buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) containing 0.1% SDS to promote transfer of high molecular weight proteins. Afterwards, membranes were blocked with 3% BSA in TBST (10 mM Tris, 100 mM NaCl, 0.05% v/v Tween-20, pH 7.4) overnight at RT with shaking.

Membranes were washed, cut in two separated pieces, one was incubated with anti-MUC5AC (Lum5.1) diluted 1/1.000 in 3% BSA in TBST and the other one with anti-sLe^x monoclonal antibody (Clone KM93) diluted 1/60 in 0.5% BSA in TBST for two hours (Table 1). Next, the former membrane was washed and incubated with a goat horseradish peroxidase-conjugated anti-rabbit antibody (Pierce Biotechnology, Rockford, IL) and the latter was incubated with a goat F(ab')₂ anti-mouse IgG+IgM+IgA horseradish peroxidase-conjugated antibody (Abcam, Cambridge, UK) diluted 1/40.000 in 0.5% BSA in TBST for one hour. After washing, membranes were incubated for 5 min with Immobilon Western horseradish peroxidase substrate solution (Millipore, Billerica, MA). Chemiluminescence was visualized using the imaging system Fluorochem SP (AlphaInnotech, San Leandro, CA) under non-saturating conditions.

Western Blots with anti-STn, diluted in 0,5% BSA in TBST, anti-MUC1 antibody (M8, Table 1) diluted 1/10 in 3% BSA in TBST and anti-vinculin antibody (Santa Cruz Biotechnology, Dallas TX) diluted 1/200 in 0.5% skimmed milk in TBST were performed as described above using a goat F(ab')₂ anti-mouse IgG+IgM+IgA horseradish peroxidase-conjugated antibody (Abcam) for STn and MUC1 detection and horseradish peroxidase-conjugated mouse IgG κ light chain binding protein diluted 1/5000 in 0.5% skimmed milk in TBST for vinculin detection (Santa Cruz Biotechnology).

3. Results

3.1 Expression of sialylated glycan epitopes (SLe^x and STn) in pancreatic tissues

First, we analysed the presence of the carbohydrate antigens SLe^x and STn by immunohistochemistry. Positive SLe^x staining was the criteria to select the cohort of PDAC tissues of this work. From our

initial cohort of 25 PDAC tissues, 21 were SLe^x positive corresponding to 84% of the total samples, which is in agreement with the literature [33, 34]. Since several studies have described SLe^x expression using CSLEX antibody, we compared the immunostaining pattern of the antibody KM93 with that of CSLEX on four PDAC tissues and a control tissue. Both antibodies showed the same staining pattern in the luminal and cytoplasmatic areas of ductal cancer cells from the four PDAC tissues and no staining was detected in the control tissue (Fig.S1). From the 21 SLe^x positive tissues, 16 were also positive for STn and two were STn positive staining in few sparse cells (Tables 2 and 3). The 21 selected tissues were analysed for mucin expression and PLA experiments. As expected, both tumour-associated carbohydrate antigens were not expressed in normal pancreas tissues (Fig.1).

3.2 Expression of MUC1 and MUC5AC in pancreatic tissues

The immunohistochemical analysis with VU4H5 antibody showed MUC1 expression in normal and in most of the PDAC tissues (Tables 3 and 4). To our knowledge, this is the first time that the VU4H5, which is specific against APDTRPAP in the hypoglycosylated VNTR region of MUC1, has been tested in pancreatic tissues. In normal pancreas, MUC1 was stained in the acinar cells and faintly in some duct cells (Fig. 2A-C), as previously described [20]. Most PDAC tissues (Fig. 2D-I) were positively stained, however two cases stained only in few sparse cells. These two samples were then assessed using M8 antibody (anti-MUC1, Table 1), which targets the DTR within the VNTR extracellular domain [48] and it was observed higher positivity in tumour cells when compared with VU4H5 staining (data not shown). VU4H5 recognises the non-glycosylated APDTRPAP epitope whereas M8 antibody also recognises this epitope when it is glycosylated [49] and this can explain the increase in MUC1 positivity when using the M8 antibody. PDAC cells from well to poor differentiated tumours presented cytoplasmic staining with more intense membranous staining when compared to controls. MUC1 was also detected in secretions.

MUC5AC is a mucin which is neo-expressed in pancreas under malignant conditions. The expression of this mucin in the three controls were negative (Fig. 2J-L) while 90% of the PDAC samples were positively stained (Fig. 2M-R). MUC5AC staining was localized in the mucus and in some cases within the secretory compartments of ductal cells of cancer tissues. This indicates that epithelial cells

of PDAC are producing and secreting MUC5AC. The CLH2 antibody recognizes a tandem repeat sequence of the MUC5AC independently of glycosylation.

3.3 Expression of sialylated antigens on MUC1 and MUC5AC in PDAC tissues.

In situ PLA is a technique based on the detection of two epitopes located in close proximity employing oligonucleotide-conjugated antibodies (PLA probes). When two antigens are in close proximity, a bridging sequence links the two oligonucleotide sequences present in the different antibodies and a template for a rolling circle amplification is formed. Fluorescently labelled oligonucleotides hybridise with the product of the amplification generating easily detected fluorescent signal [50].

All tissues of the cohort including control and negative samples (with negative staining for at least one of the four antigens analysed) were PLA tested. All healthy tissues presented negative PLA results since it was not possible to detect SLe^x or STn by IHC.

In situ PLA showed colocalization of SLe^x with MUC1 in 68% of the PDAC tissues that were individually positive for both antigens (Table 5, Fig. 3A-F). Staining intensity results for each of the tissues examined by PLA and subjected to subsequent imaging analysis are shown in Table 6. The colocalization was detected in the apical area of the well and moderately differentiated PDAC ducts and in the secreted mucus (Fig. 3C). Moreover, MUC1 bearing SLe^x could be detected in the tumour cells of poorly differentiate tissues (Fig. 3F). Among the positive tissues for MUC1-SLe^x colocalization, about one third of the tissues were faintly positive.

Regarding MUC5AC-SLe^x PLA, 16 out of 19 PDAC tissues (84%) that expressed individually both MUC5AC and SLe^x were MUC5AC-SLe^x positive (Tables 5 and 6, Fig. 4A-I). Regarding the 3 negative tissues for MUC5AC-SLe^x, 2 of them were faintly positive (<25%) and one moderately positive (25-60%) for MUC5AC by immunohistochemistry. The colocalization was also found in the apical area of the well and moderately differentiated PDAC ducts (Fig. 4C), in the secreted mucus (Fig. 4F) and in the tumour cells of poorly differentiated tumours (Fig. 4I). The faintly positive tissues for MUC5AC-SLe^x represented 19% (3/16) of the positive cases.

The study of the colocalization of the mucins MUC1 and MUC5AC with the STn antigen showed only four marginally positive cases for MUC1-STn (Table 5, Fig. 5C) and two positive samples presented

some isolated fluorescent dots in the mucus for MUC5AC-STn (Table 5, Fig. 5F). The tissues included in this work were mostly stage IIA and IIB. None of the IIA cases presented colocalization for STn with MUC1 or MUC5AC.

3.4 Determination of MUC5AC-SLe^x glycoform in pancreatic cancer tissues by immunoprecipitation followed by Western blot.

The expression pattern of SLe^x on MUC5AC, which showed the most promising results by PLA in this work, was further examined by a different approach. Western blot analyses of twenty micrograms of protein lysates from pancreatic adenocarcinoma and control pancreatic tissues were performed in duplicate in the same gel using Lum5.1 (anti-MUC5AC) and KM93 (anti-SLe^x) antibodies. MUC5AC and SLe^x were absent in normal pancreas (4/4), while 75% of the PDAC tissues (6 out of 8) presented a high molecular weight reactive band corresponding to MUC5AC (Fig. 6A). All tissues that were positive for MUC5AC showed also positive staining for SLe^x at the same molecular weight.

Remarkably, similar expression levels and band pattern for MUC5AC and SLe^x were observed in each positive sample. The presence of vinculin, a constitutively expressed protein, of MUC1 and STn were also analysed by Western Blot on 20 micrograms of the protein lysates (Fig. S2). Vinculin expression was found similar among the samples. MUC1 was expressed in the normal pancreas and in most of the pancreatic cancer tissues and STn was not found in the control tissue and was expressed in some of the tumour tissues at different levels, which was in accordance with the IHC results. These results show that the differences found in mucins and carbohydrate antigen expression were intrinsic of each PDAC sample and not due to differences in the amount of total protein. Hence, suggesting that MUC5AC could be a major carrier of SLe^x in PDAC tissues.

In order to validate that MUC5AC was bearing the SLe^x epitope, a MUC5AC immunoprecipitation protocol from PDAC tissues was set up. Gel-forming mucins have an oligomeric structure stabilized by disulphide bonds and can be depolymerized by treatment with a reducing agent. Therefore, considering the complex nature of MUC5AC, reduction and alkylation of the lysed samples were performed to facilitate the immunocapture of MUC5AC. Although CLH2 and Lum5.1 anti-MUC5AC

antibodies were both assessed for immunoprecipitation, only the latter worked properly with our immunoprecipitation protocol (described in section 2.6). After sample immunoprecipitation of selected PDAC tissues, which expressed individually both MUC5AC and SLe^x, a high molecular weight band above 250 kDa corresponding to MUC5AC was detected. Western blotting of the immunoprecipitated mucin probed with anti-SLe^x antibody confirmed the presence of this epitope on MU5CAC (Fig. 6B). Overall, MUC5AC and SLe^x colocalization was observed using both PLA and immunoprecipitation followed by western blotting.

4. Discussion

In this work we assessed the expression of MUC1 and MUC5AC as well as the cancer associated glycans SLe^x and STn in pancreatic cancer tissues. Our results show the expression of SLe^x in MUC5AC in most of the pancreatic cancer tissues as a promising new biomarker of disease. Secreted mucins, such as MUC5AC, protect epithelial cells from different harsh microenvironments forming a protective layer that allows the selective passage of molecules. Transmembrane mucins, like MUC1, apart from being sensors of the external environment, are engaged in signal transduction. The cytoplasmic tail of MUC1 is involved in several signalling pathways including those concerning Ras, β -catenin, p120 catenin, p53 and estrogen receptor α [51]. In tumour state, cancer cells express normal and new mucin core proteins with aberrant glycan structures that arise during disease progression to acquire new capabilities [8, 22, 40]. Studies with interference RNA specifically targeting MUC1 and MUC5AC showed a reduction of PDAC cells to adhere and invade [52, 53], demonstrating the role of these mucins in PDAC progression and metastasis. Mucins are considered a potential tool for the diagnosis and therapy of a wide range of cancers. More than 200 clinical studies have been conducted over the last three decades to evaluate mucins as therapeutic constituents or prognostic indicators [11, 54-56]. PDAC is characterized by a lack of blood supply to tumours, whose surfaces are often coated with layers of dense, fibrous capsules, making the delivery of chemotherapy drugs difficult [7]. Target therapy using anti-MUC1 antibodies conjugated to radioactive elements and/or cytotoxic drugs that have shown to increase efficacy than radioimmunotherapy alone in prostate and ovarian cancers could

be potentially useful in PDAC [6, 11]. Moreover, several studies show that MUC1 [57] and STn epitope [58] can be successful in activating the immune system when injected as vaccines in MUC1 transgenic mice.

There are several antibodies against MUC1 displaying different staining patterns and percentages of positivity in normal pancreas and PDAC tissues [59, 60]. To our knowledge, this is the first time that the monoclonal antibody VU4H5 against MUC1 was used in the IHC analysis of pancreatic tissues. Considering that the specificity described for VU4H5 is against the non-glycosylated APDTRPAP epitope within the tandem repeat sequence of MUC1 [60-62], the low expression found in some tissues in this study could be explained by a possible glycosylation present on the MUC1 in PDAC. To address this issue, we have also used antibody M8 for the evaluation of MUC1 expression in the low MUC1 expressing cases, and higher positivity was observed. These results are in agreement with results from Hinoda and collaborators that found an increase in MUC1 detection in cancer tissues using an antibody directed against the core protein after deglycosylation [63].

On the other hand, MUC5AC is being considered an interesting target for PDAC detection due to its neo-expression in pancreas during malignant transformation [6, 64]. Herein we showed the neo-expression of MUC5AC in PDAC in agreement with other authors [27-29, 40].

Regarding the expression of tumour-associated carbohydrate antigens SLe^x and STn, the detection in PDAC tissues and not in normal pancreas is in agreement with the reported literature [33-35]. The cancer associated expression of SLe^x in pancreatic adenocarcinoma can be attributed to changes in glycosyltransferase expression [65]. An increase in α 2,3-sialyltransferases such as ST3GAL4 and in α 1,3-fucosyltransferases such as FUT3 and FUT6 have been described in pancreatic adenocarcinoma tissues [66]. In addition, changes in nucleotide donor transporters and hypoxia have been reported to regulate SLe expression in colon cancer [67-69]. Since PDAC tumours are usually hypoxic due to poor vascularization [70] it is possible to postulate that the highly hypoxic environment of PDAC could be one of the factors inducing an increase in the glycosyltransferase expression and leading to an augment of SLe^x expression.

In this work we have addressed whether SLe^x and/or STn could be carried on MUC1 and MUC5AC mucins in PDAC. The presence of the sialylated epitopes SLe^x and STn on MUC1 and MUC5AC has

been previously described by other authors in gastric, colon, lung, breast and ovarian cancer tissues [17, 39, 42-45] as well as in pancreatic cancer cell line [71], using PLA and other antibody-based approaches. Since an increase of truncated structures is a key feature of tumour cells [72], we expected an increase in STn expression on MUC1 and MUC5AC. Although the expression of MUC1 bearing Tn/STn, detected by a specific anti-MUC1-Tn/STn antibody, in PDAC tissues has been reported by Remmers and collaborators [40], our results do not show the same tendency. This could be due to the use of different antibodies, different methodology and different cohort. We have used a specific anti-STn antibody and PLA technique to colocalize this antigen within the mucin proteins. Regarding the SLe^x epitope on these mucins, our results show a wide colocalization of SLe^x on MUC1 and especially on MUC5AC in PDAC tissues. Furthermore, the presence of SLe^x on MUC5AC was confirmed by immunoprecipitation of PDAC tissue protein lysates and subsequent Western blotting analysis using anti-SLe^x antibodies. Our results show that both mucins are carriers of SLe^x antigen in most PDAC tissues that expressed individually SLe^x and the corresponding mucin. However, other glycoconjugates could also be carriers of SLe^x antigen.

Most of the currently used cancer biomarkers assays take advantage of changes in the biodistribution of tumour cell derived products [12, 20, 32], such as the diagnostic serum tests that are based on changes in the level of the circulating mucins, MUC16 (CA125) [73, 74] and MUC1 (CA15-3) [75], or changes in the mucin specific terminal glycan structures sialyl-Lewis a (CA19-9)[76, 77] or STn (CA72-4) [78, 79]. To date, there is no specific tumour marker to diagnose PDAC. The only marker approved by FDA to manage PDAC is CA19-9 that detects sialyl-Lewis a, an epitope found mainly on mucins [80]. Some mucin-based markers lack specificity due to their detection also in benign conditions. Glycan-based markers, on the other hand, present low tissue specificity since aberrant *O*-glycans can be found in many carcinoma tissues. Therefore, our strategy was the detection of a specific glycoform on a specific mucin that could circumvent these weaknesses and generate a biomarker with potential application for specific PDAC detection.

A good biomarker should be a molecule that can be found in blood or other fluids. However, due to the pathophysiology and micro-architecture of PDAC, which is poorly perfused and vascularized, secreted proteins that could be candidates for biomarkers have rarely been detected in circulation [81].

On the other hand, PDAC tissue contains higher concentration of PDAC-specific markers and is the most direct source for the finding of cancer biomarkers [82, 83]. Thus, our strategy has been to analyse pancreatic tissues to identify candidates for their future validation in fluids, such as serum of PDAC patients. In the present study, the colocalization of MUC1 and MUC5AC with the tumour epitope SLe^x presented a high specificity due to SLe^x absence in non-malignant control tissue and has potential to be considered a PDAC biomarker. In this regard, further analyses of the glycan epitope SLe^x on MUC1 and MUC5AC in serum from PDAC patients are warranted, particularly the MUC5AC-SLe^x glycoform that showed 84% of expression in the MUC5AC expressing PDAC tumours, including poorly, moderately and highly differentiated tissues.

Conclusions

In this study, we have evaluated the anti-MUC1 VU4H5 antibody in pancreatic tissues and we have also studied the colocalization of the tumour associated antigens SLe^x and STn within the mucins MUC1 and MUC5AC in pancreatic cancer tissues. We have shown a wide expression of SLe^x on MUC1 and MUC5AC in the tumour cells and in the secretions. We have confirmed, by immunoprecipitation of MUC5AC followed by SLe^x probing, the presence of SLe^x on MUC5AC in PDAC tissues. Since mucins can reach the bloodstream and be detected in fluids such as pancreatic juice or serum, the herein presented results suggest that the glycoforms MUC1-SLe^x and MUC5AC-SLe^x could be good candidates for pancreatic cancer serum biomarkers.

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Table 1: Antibodies used for immunohistochemistry and Western blotting.

Antibody	Isotype	Concentration	Antigen and reference
CLH2	IgG2	0.60 ng/μL	MUC5AC [84]
KM93	IgM	2.5 ng/μL IHC/PLA 3.3 ng/μL western blotting	SLe ^x (Merck Millipore, Darmstadt, Germany)
LUM5.1	polyclonal	Rabbit polyclonal serum diluted 1/1000 western blotting	MUC5AC [85]
M8	IgG1	Supernatant diluted 1/2	MUC1 [86-88]
TKH2	IgG1	0.95 ng/μL	STn [89]
VU4H5	IgG1	1.19 ng/μL	MUC1 [60-62]

Table 2: Carbohydrate epitopes SLe^x and STn expression in pancreatic tissues.

			PDAC category and stage (TNM system)			
			Normal pancreas	Stage IIA (T3N0M0)	Stage IIB (T3N1M0)	Stage II* (T3NxM0)
SLe ^x	negative	3/3 (100%)	-	-	-	-
	sparse cells	-	-	-	-	-
	faintly positive	-	-	1/13 (8%)	1/1 (100%)	-
	moderately positive	-	5/6 (83%)	7/13 (54%)	-	-
	strongly positive	-	1/6 (17%)	5/13 (38%)	-	1/1 (100%)
STn	negative	3/3 (100%)	1/6 (17%)	2/13 (15%)	-	-
	sparse cells	-	-	2/13 (15%)	-	-
	faintly positive	-	4/6 (67%)	-	-	-
	moderately positive	-	1/6 (17%)	6/13 (46%)	-	1/1 (100%)
	strongly positive	-	-	3/13 (23%)	1/1 (100%)	-

The stage II* refers to a patient that the N (regional lymph nodes) cannot be addressed.

Table 3. Carbohydrate epitopes SLe^x and STn and mucins MUC1 and MUC5AC expression in each of the pancreatic tissues analysed.

Category and stage (TNM system)	Cases	Differentiation grade ¹	Gender	Age	SLe ^x	STn	MUC1	MUC5AC
Normal Pancreas	3	-	M	65	-	-	+++	-
		-	F	76	-	-	+++	-
		-	F	61	-	-	+++	-
PDAC	Stage IIA (T3N0M0)	MD	M	70	++	+	+++	+++
		MD	M	65	++	+	++	++
		MD	M	61	++	++	++	++
		PD	M	65	++	++	+++	+
		WD/MD	M	71	+++	-	sc	-
		MD/PD	M	70	++	+	+	+
	Stage IIB (T3N1M0)	MD	M	57	++	++	++	+
		MD	M	72	+++	++	+++	++
		WD	F	70	++	+++	++	+++
		PD	M	58	++	++	+++	++
		WD	M	72	+++	++	++	+
		WD	F	49	+++	-	+	-
		WD	F	78	+++	+++	+	+++
		PD	M	81	++	sc	+++	+
		MD	F	71	++	++	sc	++
		MD	M	67	++	++	+++	++
		WD/MD	F	66	++	-	++	++
		MD	F	57	+	sc	++	+
		MD	M	79	++	+++	+++	+++
	Stage II* (T3NxM0)	MD	M	65	+	+++	+++	+++
	Stage IV (T3N1M1)	WD	M	76	+++	++	+++	+++

Staining intensity is graded as: negative: -, sparse cells: sc; faintly positive (10-25% stained cells): +; moderately positive (25-60% stained cells): ++; and strongly positive (>60% stained cells): +++.

The stage II* refers to a patient that the N (regional lymph nodes) cannot be addressed.

¹Differentiation grade: PD: poorly differentiated, MD: moderately differentiated, and WD: well differentiated.

Table 4: Mucins MUC1 and MUC5AC expression in pancreatic tissues.

			Normal pancreas	PDAC category and stage (TNM system)			
			Stage IIA (T3N0M0)	Stage IIB (T3N1M0)	Stage II* (T3NxM0)	Stage IV (T3N1M1)	
MUC1	negative	-	-	-	-	-	
	sparse cells	-	1/6 (17%)	1/13 (8%)	-	-	
	faintly positive	-	1/6 (17%)	1/13 (8%)	-	-	
	moderately positive	-	2/6 (33%)	6/13 (46%)	-	-	
	strongly positive	3/3 (100%)	2/6 (33%)	5/13 (38%)	1/1 (100%)	1/1 (100%)	
MUC5AC	negative	3/3 (100%)	1/6 (17%)	1/13 (8%)	-	-	
	sparse cells	-	-	-	-	-	
	faintly positive	-	2/6 (33%)	4/13 (31%)	-	-	
	moderately positive	-	2/6 (33%)	5/13 (38%)	-	-	
	strongly positive	-	1/6 (17%)	3/13 (23%)	1/1 (100%)	1/1 (100%)	

The stage II* refers to a patient that the N (regional lymph nodes) cannot be addressed.

Table 5: Immunohistochemistry results for mucins and tumour-associated antigens and their colocalization by *in situ* PLA.

	Immunohistochemistry				<i>In situ</i> PLA			
	MUC1	MUC5AC	SLe ^x	STn	MUC1-SLe ^x	MUC5AC-SLe ^x	MUC1-STn	MUC5AC-STn
Normal pancreas	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Tumour	19/21 (90%)	19/21 (90%)	21/21 (100%)	16/21 (76%)	13/19 (68%)	16/19 (84%)	4/15 [#] (27%)	2/16 (13%)

[#]15 PDAC tissues were positive for both MUC1 and STn by IHC.

Table 6: **Immunohistochemistry results for mucins and SLe^x and their colocalization by *in situ* PLA.**

Category and stage (TNM system)		Cases	Differen. grade ¹	SLe ^x	MUC1	PLA MUC1-SLe ^x	MUC5AC	PLA MUC5AC-SLe ^x
Normal Pancreas		3	-	-	+++	-	-	-
			-	-	+++	-	-	-
			-	-	+++	-	-	-
PDAC	Stage IIA (T3N0M0)	6	MD	++	+++	++	+++	+
			MD	++	++	+++	++	+
			MD	++	++	++	++	++
			PD	++	+++	+	+	-
			WD/MD	+++	sc	-	-	-
			MD/PD	++	+	-	+	+++
	Stage IIB (T3N1M0)	13	MD	++	++	+	+	++
			MD	+++	+++	++	++	-
			WD	++	++	-	+++	+++
			PD	++	+++	+++	++	++
			WD	+++	++	-	+	-
			WD	+++	+	-	-	-
			WD	+++	+	+++	+++	+++
			PD	++	+++	-	+	+
			MD	++	sc	-	++	+++
			MD	++	+++	++	++	+++
			WD/MD	++	++	-	++	+
			MD	+	++	+	+	+
			MD	++	+++	++	+++	+++
	Stage II* (T3NxM0)	1	MD	+	+++	+++	+++	+
	Stage IV (T3N1M1)	1	WD	+++	+++	+++	+++	+++
Overall result	Normal		0/3	3/3	0/3	0/3	0/3	
PDAC		21/21	19/21	13/19	19/21	16/19		

For IHC: negative: -, sparse cells: sc; faintly positive (5-25% stained cells): +; moderately positive (25-60% stained cells): ++; and strongly positive (>60% stained cells): +++.

For PLA: +++: positive cases whose median is higher than 75% of the overall median; ++: positive cases higher than 25% and lower than 75%; and + positive cases lower than 25%.

Figure Captions

Figure 1: Expression of SLe^x and STn in PDAC. Immunohistochemistry staining of the SLe^x epitope at different magnification (x40 and x200) and the corresponding hematoxylin eosin staining (x40) of normal pancreas (control) (A-C) and two representative pancreatic cancer tissues (well differentiated, D-F and poorly differentiated, G-I) with KM93 antibody. Immunohistochemistry staining of the STn antigen at different magnification (x40 and x200) and the corresponding hematoxylin eosin staining (x40) of a control (J-L) and two representative pancreatic cancer tissues (well differentiated, M-O and poorly differentiated, P-R) with TKH2 antibody.

Figure 2: Expression of MUC1 and MUC5AC in PDAC. Immunohistochemistry staining at different magnification (x40 and x200) and the corresponding hematoxylin eosin staining (x40) of normal pancreas (control) (A-C), and two representative pancreatic cancer tissues (well differentiated, D-F and poorly differentiated G-I) with the anti-MUC1 antibody VU4H5. Immunohistochemistry staining of a control (J-L) and two representative pancreatic cancer tissues (well differentiated, M-O and poorly differentiated P-R) with the anti-MUC5AC antibody CLH2.

Figure 3: Expression of MUC1-SLe^x in PDAC. Expression of MUC1 (A and D) and SLe^x antigen (B and E) and their colocalization by *in situ* PLA in moderately differentiated (C) and poorly differentiated (F) PDAC tissues. x200 magnification.

Figure 4: Expression of MUC5AC-SLe^x in PDAC. Expression of MUC5AC (A, D and G) and SLe^x antigen (B, E and H) and their colocalization by *in situ* PLA in tumour ducts (C), mucus (F) and poorly differentiated tumour (I). x200 magnification.

Figure 5: Expression of MUC1-STn and MUC5AC-STn in PDAC. Expression of MUC1 (A) and MUC5AC (D) and STn antigen (B and E) and its corresponding colocalization by *in situ* PLA in pancreatic cancer tissues (C and F). x200 magnification.

Figure 6: A. MUC5AC and SLe^x western blots of pancreatic tissue lysates. Western blots corresponding to four control (C1-C4) and eight PDAC tissue lysates (T1-T8) with Lum5.1 antibody to detect MUC5AC (top panel) and KM93 to detect SLe^x (bottom panel) are shown. **B. Detection of SLe^x on immunoprecipitated MUC5AC from a PDAC tissue lysate (T1).** Representative Western blots with Lum5.1 antibody to detect MUC5AC (top panel) or with KM93 to detect SLe^x (bottom

panel) are shown. Lane 1: PDAC lysate. Lane 2: Immunoprecipitated (IP) PDAC lysate with Lum5.1 antibody.

MUC1

SLe^x

MUC1-SLe^x

MUC5AC

SLe^x

MUC5AC-SLe^x

MUC1

STn

MUC1-STn

MUC5AC

STn

MUC5AC-STn

A **B**

Supplementary figures

Figure S1. SLe^x staining pattern using KM93 and CSLEX antibodies.

Immunohistochemistry staining of the SLe^x epitope at different magnification (x40 and x200) of two representative pancreatic adenocarcinoma tissues. A-D corresponds to a IIB, moderately differentiated PDAC (PDAC A) and E-H corresponds to a IIB, well differentiated PDAC (PDAC B)

Figure S2: Expression pattern of MUC5AC, MUC1, SLe^x, STn and vinculin in six of the the pancreatic tissue lysates. Western blots of 20 µg of one control (C1) and of five PDAC tissue lysates (T1, T4, T5, T7, T8) to detect mucins (MUC5AC and MUC1), carbohydrate antigens (SLe^x and STn) and vinculin.