

Glycoprotein biomarkers for the detection of pancreatic ductal adenocarcinoma

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Abstract

Pancreatic cancer (PaC) shows a clear tendency to increase in the next years and therefore represents an important health and social challenge. Currently, there is an important need to find biomarkers for PaC early detection because the existing ones are not useful for that purpose. Recent studies have indicated that there is a large window of time for PaC early detection, which opens the possibility to find early biomarkers that could greatly improve the dismal prognosis of this tumor. The present manuscript reviews the state of the art of the existing PaC biomarkers. It focuses on the anomalous glycosylation process and its role in PaC. Glycan structures of glycoconjugates such as glycoproteins are modified in tumors and these modifications can be detected in biological fluids of the cancer patients. Several

studies have found serum glycoproteins with altered glycan chains in PaC patients, but they have not shown enough specificity for PaC. To find more specific cancer glycoproteins we propose to analyze the glycan moieties of a battery of glycoproteins that have been reported to increase in PaC tissues and that can also be found in serum. The combination of these new candidate glycoproteins with their aberrant glycosylation together with the existing biomarkers could result in a panel, which would expect to give better results as a new tool for early diagnosis of PaC and to monitor the disease.

Key words: Pancreatic cancer; Aberrant glycosylation; Glycoproteins; Biomarkers; Diagnosis

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Core tip: There is an urgent need to find new biomarkers for pancreatic cancer (PaC) diagnosis. The review focuses on the field of glycoproteomics and describes serum glycoproteins that have been identified up to date as potential biomarkers and their limitations basically due to the fact that they are neither pancreatic specific nor cancer specific. The review proposes new glycoprotein candidates that have been described to be overexpressed in PaC tissues and that are secreted into serum. The combination of the candidate protein levels and their glycan moieties, which could be altered in cancer, could improve their potential as PaC biomarkers.

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INTRODUCTION

Pancreatic cancer

Cancer still represents both a tremendous social and healthcare problem and its incidence is increasing in developed and developing countries. In pancreatic ductal adenocarcinoma (PaC), total cancer deaths are projected to increase dramatically to become the second leading cause of cancer-related deaths before 2030^[1-4].

Nowadays, PaC presents the lowest five-year survival rate, being surgery the only possible curative option, but this is only possible for 20% of patients. The current therapeutic treatments for PaC are not very effective, extending the overall survival by only 8.5 mo as much^[2].

PaC has a poor prognosis due to delayed diagnosis. In most cases, more than 90% of patients with PaC are diagnosed at stages III and IV^[5]. Symptoms of PaC are often unnoticed as they are not very specific for the disease. The most common symptoms are abdominal unspecific pain, back pain, nausea, diarrhea and jaun-

dice^[6]. New-onset diabetes mellitus (DM) and acute exacerbation of DM can also be signs of PaC. Thus, in all these cases a detailed examination is needed^[7].

It is particularly disturbing that there are no effective detection assay systems and treatment protocols for PaC, advances that have improved the management of other important carcinomas. It is therefore necessary an outstanding effort to study this tumor, to analyze its complexity, and to establish efficient diagnostic systems and treatment options. Today there is a big improvement in the knowledge of the biology of PaC, but the disease still remains difficult to analyze, to detect and to treat^[2]. PaC is a very aggressive tumor presenting a great desmoplastic area that creates a very dense stroma that prevents the arrival of therapeutic drugs. Genetically, PaC is a very complicated disease with at least 63 genetic alterations that promote resistance to the therapies^[2,8,9].

Fortunately, a long period of time before the establishment of metastatic PaC has been indicated by Yachida *et al*^[10]. Further, Poruk *et al*^[11] have indicated that "a reasonable amount of time exists that allow for an early diagnosis of PaC" which indicates that it is possible to early detect the tumor. It is also worthy to mention that an incidental diagnosis of PaC is synonymous with an improved prognosis^[12].

ON THE ROAD TO THE DISCOVERY OF NEW, MORE EFFECTIVE AND USEFUL BIOMARKERS

Early detection remains one of the most promising approaches to improve the long-term survival of cancer patients, and this may be achieved by efficient screening of biomarkers in biological fluids. The objective of a screening program is to detect cancer at a curable stage, before symptoms develop^[13,14].

For many malignancies, biomarkers have become an established part of patient management, and are included in many clinical guidelines. As a consequence, the search for new and better biomarkers has become an integral component of contemporary cancer research. Biomarkers serve multiple purposes, as early detection, therapeutic monitoring, predicting responses, side-effects, surrogate endpoints, and they guide critical treatment decisions.

The term tumor marker (TM), or biomarker, traditionally has referred to molecules, mainly proteins, which are either directly produced by malignant cells, or are produced by other cells in response to certain malignant or other non-malignant conditions. Most clinically relevant biomarkers have been discovered serendipitously, or by trial and error.

Current tumor markers may be grouped into a variety of categories, including proteins, glycoproteins, onco-fetal antigens, hormones, receptors, genetic markers, RNA molecules and metabolites. Despite advances in laboratory technology and an enormous expansion in relevant literature (768000 papers indexed in PubMed in

Table 1 Commonly used serum tumor markers in clinical practice

Biomarker	Tumor class	Glycosylated protein
Alpha-feto protein (AFP)	Testicular, hepatocellular	Yes
Human chorionic gonadotropin	Testicular	Yes
CA 15-3	Breast	Yes
CA 19-9	Pancreatic	Yes
CA 27-29	Breast	Yes
CA 125	Ovarian	Yes
Carcinoembryonic antigen	Colon	Yes
Human epidermal growth factor receptor 2 (HER-2)	Breast	Yes
Prostate specific antigen (PSA)	Prostate	Yes
Thyroglobulin	Thyroid	Yes

Extracted from references 13, 14, 16, 17, 18, 20.

2016 related to biomarkers), we are far from widespread clinical use of biomarkers. In fact, only a few dozen clinical relevant biomarkers useful for cancer exist nowadays (Table 1). The number of biomarkers receiving FDA approval has declined substantially over the last ten years to less than one protein biomarker per year. After almost two decades of intensive research using advanced genomics and proteomics technologies, only a handful of tumor markers have been translated into patient care. Of the proteins that could be differentially expressed in human cancers, only nine have been approved as tumor associated antigens^[15]. All of these cancer biomarkers are glycosylated proteins^[16-20]. Despite their clinical usefulness, they present limitations in terms of sensitivity and specificity as the case of carbohydrate antigen 19-9 (CA 19-9) for the diagnosis of PaC, as discussed later.

TMs are minimally invasive and convenient, and their associated costs are low. Single TMs were traditionally used but these have come under scrutiny due to their low sensitivity and specificity. Recent research has shown superior performance using a combination of multiple TMs as a panel that also incorporates other clinical factors as imaging systems^[21,22]. In the past few years it has become increasingly clear that no single biomarker can be reliably used for cancer diagnosis.

BIOMARKERS IN PAC

Serum CA 19-9 was described after obtaining monoclonal antibodies against human colorectal cancer cells. It is a carbohydrate antigen called sialyl-Lewis a (SLe^a), which is embedded on cell surface molecules, as gangliosides and mucins^[23]. CA 19-9 is routinely used as a biomarker of PaC, with an 82% of sensitivity and a 90% of specificity. But CA 19-9 is also elevated in other situations as pancreatitis, jaundice, hepatic and pancreatic cysts, and other cancers as colorectal and breast. CA 19-9 is not expressed in the 10%-20% of the Caucasian population due to a genetic deficiency of a fucosyltransferase. The marker was ineffective for general population screening and diagnosis, but CA 19-9 has been extensively studied in PaC, and it is continuously requested by clinicians, especially for monitoring. Recently, it has been shown that CA 19-9 is upregulated up to 2 years before PaC

diagnosis^[24], and it seems to be a good companion with other markers improving the diagnostic accuracy of PaC, as for instance, when used together with albumin and IGF, it allows the distinction between PaC and chronic pancreatitis (ChrP) with high sensitivity and specificity^[25].

Imaging techniques

Detection of asymptomatic PaC has only been achieved incidentally by imaging techniques^[11,26]. Significant progress has been made over the past two decades in imaging technology and at present, available techniques for pancreas imaging have a key role in PaC diagnosis as well as in cancer staging, assessment of the treatment response and detection of metastatic lesions. Pancreatic imaging tests include several modalities: Ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and endoscopic ultrasonography (EUS)^[27-31].

Ultrasonography: Ultrasonography (US) may have some limitations in the early diagnosis of PaC. The entire pancreas is difficult to visualize clearly on ultrasonography especially for small tumors located in the head or tail^[32].

Multidetector computed tomography: After the direct suspicion of PaC, the initial diagnosis and staging of PaC mainly relies on a multi-detector row CT^[22]. Overall, CT is reported to have a sensitivity of 89%-97% for PaC diagnosis, although it is less effective in diagnosing small (< 2 cm) tumors with a sensitivity of 65%-75%^[33,34].

Since CT has a wide anatomic coverage, it also shows a high performance for the evaluation of vascular structures invasion (100% sensitivity and 72% specificity), which is a key factor for predicting the tumor resectability^[35,36]. CT is also a primary imaging modality for monitoring the response to treatment^[34].

Magnetic resonance imaging: MRI can be useful in imaging for PaC in patients when EUS or CT findings are not diagnostic^[37].

Diffusion-weighted MRI (DWI) helps to detect solid pancreatic neoplasms with extremely dense cellularity^[38,39]. However, DWI may not be capable of definitively distinguishing between inflammatory/neo-

plastic lesions.

Endoscopic ultrasonography: CT of the pancreas can be complemented by Endoscopic ultrasonography (EUS), which is the most sensitive imaging method for the detection of malignant pancreatic lesions. EUS sensitivity has been reported to be higher than 95%^[40,41].

EUS is particularly useful for identification of small tumors (< 3 cm diameter) that are not visualized by other imaging modalities (93% sensitivity)^[42]. Besides providing important information for tumor detection and staging, EUS allows to obtain tissue samples for histological diagnosis using fine-needle aspiration (FNA) during the same examination. EUS-guided FNA has been shown to have a sensitivity and specificity for PaC of 85% and 98%, respectively^[43,44]. In addition, EUS is equivalent to CT at determining the surgical resectability of PaC^[45].

Positron emission tomography: PaC assessment can be improved using PET techniques. PET relies on the enhanced metabolism of glucose in cancer cells, which overexpress glucose transporter 1, leading to glucose accumulation in tumors. Fluorine 18-fluorodeoxyglucose (FDG), a glucose analogue, is normally used as radiotracer. The reported sensitivity and specificity of FDG-PET for the identification of PaC are low^[46]. The main limitations of PET for PaC diagnosis are low-false negative results in hyperglycemia and false-positive results caused by a physiologic FDG uptake by normal tissues or inflammatory masses (pancreatitis)^[47].

However, FDG-PET has been described to be more sensitive than CT for monitoring the response to treatment and for detecting tumor recurrence after resection^[48,49]. One of the main advantages of PET is its wide anatomic coverage, which allows the detection of possible metastases in the entire body^[35,50].

Although imaging techniques have provided significant advances in the diagnosis and follow up of PaC patients, they still have limitations in the early diagnose of small primary pancreatic tumors and metastases, which is crucial for the effectiveness of therapeutic interventions. Further improvement could be made with a combination of imaging techniques and biomarkers, since the presence of biomarkers in serum could be a very early finding that would increase the ability of imaging techniques to diagnose pancreatic carcinoma.

Blood-based biomarkers

Currently, the only FDA-approved blood-based biomarker for PaC is CA 19-9 which is recommended for monitoring the disease or following up. Therefore, discovering new biomarkers to diagnose PaC, especially in early stages, is mandatory to improve PaC patients live expectancy.

In this context, apart from glycoproteomic strategies to identify novel PaC biomarkers based on altered glycosylation of particular proteins, which will be discussed in depth later on, real efforts have been made to identify new specific biomarkers for the early detection of

PaC^[12, 51-54]. Harsha *et al*^[55] have collected an extensive list of potential new protein PaC biomarkers (207 proteins over-expressed), but many candidates have not worked differentiating between benign and malignant situations. Proteomic analysis of exosomes and tissues of PaC patients have also been investigated^[56-58].

Other approaches including molecules different than proteins are also under investigation. Detection of specific PaC mutations on cell-free circulating tumor DNA (ctDNA) or circulating tumor cells (CTC) has been explored^[59-63]. Some commonly mutated genes in PaC are KRAS, TP53, SMAD4 and CDKN2A^[64-66]; which allow classifying PaC into four different genetic subtypes that might have potential clinical relevance^[65]. These mutations have also been detected in ctDNA or CTCs^[62-63]. The study of Bettgowda *et al*^[59] has shown that about 40% of patients with stages I to III of PaC have detectable ctDNA, a value that increases up to 90% for patients with stage IV tumor. On the other hand, Kinugasa *et al*^[60] have reported the detection of mutated KRAS in ctDNA of 63% PaC patients (all stages), 20% of patients with ChrP and 5% of healthy controls (HC). Thus, these approaches could be interesting for developing an early detection PaC biomarker, although nowadays liquid biopsies still have some drawbacks, such as low diagnostic sensitivity or limitations on the detection techniques, and are not yet suitable to substitute tissue biopsies^[61,67-68]. Other genetic changes such as epigenetic alterations^[62,69-70] or non-coding RNAs expression^[71-75] have also been detected in circulating free nucleic acids. Several miRNAs present better performance for PaC diagnosis than CA 19-9^[76]. A recent study analyzing the blood levels of a panel of microRNAs previously associated with PaC did not show differential miRNA expression levels among patients with localized PaC and metastatic PaC; however, some miRNAs (miR-10b, miR-21-5p and miR-30c) present a consistent association between over-expression in plasma collected years before PaC diagnosis and risk, interesting for follow-up purposes^[74]. The detection of miRNAs in circulating exosomes has also been explored and showed diagnostic potential for PaC, since the level of miR-10b, miR-20a, miR-21, miR-30c, miR-106b and miR-let7a (significantly different in PaC patients plasma compared to HC and ChrP patients) normalized after tumor resection^[77]. Also, another especially interesting mRNA is miR-107, which might be useful not only as a diagnostic biomarker (AUC = 0.851, PaC patients compared with HC) but also as a target for PaC treatment^[75].

Marker combinations: The combination of these novel biomarkers among them or with those already in use (mainly CA 19-9) might achieve the sensitivity and specificity required for early PaC diagnosis for high-risk population screening^[25,78-81]. The recent study of Sefrioui *et al*^[80] compares the diagnostic performance of CA 19-9, CTCs and KRAS mutational status detected

on ctDNA in patients who also underwent endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). The detection of CA 19-9 in combination with ctDNA and CTC analysis improved the sensitivity and specificity (78% and 91% respectively) in comparison with EUS-FNA alone (73% and 88% respectively). Moreover, unlike CA 19-9, the combined markers improved the diagnosis accuracy compared with CA 19-9 alone in cholestasis cases. Another study performed with plasma from PaC patients, ChrP patients and HC showed that the combination of relative abundances of miR-16, miR-196a and CA 19-9 levels (AUC = 0.979) improved in comparison with the analysis of CA 19-9 alone (AUC = 0.903) and the miRNA panel alone (AUC = 0.895). Similar results were obtained when comparing PaC patients against ChrP patients, and PaC patients against ChrP patients plus HC. The same study shows that the miR panel is more effective than CA 19-9 for early diagnosis of PaC^[78]. Some combinatory analyses have been patented^[82]. For example, the combination of plasma tissue factor pathway inhibitor (TFPI), tenascin C (TNC-FN III-C) and CA 19-9 levels, which presented a strong capacity in distinguishing all early-stage cancer from both HC and ChrP^[81].

The value of all these findings is still uncertain since they lack proper robust clinical trials and the subsequent multicenter trials with larger cohorts to validate the data obtained. Moreover, it has to be taken into account that the methodologies used to discover novel PaC biomarkers would need to be simplified to apply them into clinic laboratories^[82].

THE WORLD OF GLYCOBIOLOGY

Anomalous glycosylation associated to cancer development was already described 50 years ago. During the last decades the field of glycobiology and our understanding of the central importance of glycans in biology have grown dramatically. Glycans are directly involved in virtually every disease affecting humankind, including inflammation, infectious diseases, cancer, diabetes and neurodegeneration^[20,83-85].

Protein glycosylation is one of the most common protein post translational modifications. Approximately 50% of cell proteins are glycosylated. A number of N-linked glycoprotein changes have been identified in association with different diseases using glycoproteomic approaches. These glycoproteins have been shown to have aberrant glycosylation patterns in malignancy, but only their total protein levels are clinically monitored (Table 1).

Changes in glycosylation are believed to be a main feature in oncogenic transformation as glycans are known to be continuously involved in several processes, such as protein folding and clearance rates, cell signaling, angiogenesis, differentiation, cell growth, cell-matrix interactions, immune modulation, tumor cell dissociation, invasion, epithelial-mesenchymal transition (EMT), and metastasis.

Glycans expressed in several types of glycoconjugates are known to change during cancer genesis and progression^[84,86]. Glycosylation changes that are commonly associated with cancer transformation include sialylation, fucosylation, increased GlcNAc-branching of N-glycans and over-expression of truncated mucin-type O-glycans^[87]. These changes increase the structural glycan heterogeneity and alter the function of cells. These alterations become more marked as the tumor acquires a more aggressive phenotype. In a malignant cell, almost every glycoprotein will be affected by aberrant glycosylation, and cells usually synthesize an array of glycoforms of every protein.

Changes in glycosylation tend to be more pronounced than alterations in protein expression. Glycan modifications change rapidly and dramatically in response to a disease. This makes glycan alterations more reliable qualitative biomarkers in terms of the predictive value^[88]. Some of the glycoproteins with altered glycosylation in cancer cells can be shed into the bloodstream so that their tumor associated glycoforms can potentially be specific cancer biomarkers^[89,90]. Therefore, new tumor markers based on the detection of altered glycoforms of a particular glycoprotein, regardless of their levels, may have a much higher specificity for the detection of cancer than variable levels of the proteins themselves, which up to date have shown limited clinical utility. That is why glycoproteomics technologies are of particular interest to identify glycoproteins with altered glycoforms, caused by tumor transformation, as potential tumor markers.

The feasibility of this approach has been demonstrated for α -fetoprotein (AFP), which is a glycoprotein used as a marker for the diagnosis of hepatocellular carcinoma (HCC). AFP serum levels are increased in hepatocellular carcinoma, but also in other non-malignant chronic hepatic diseases such as chronic hepatitis or liver cirrhosis, which limits AFP specificity for HCC detection. The detection of an AFP fucosylated glycoform produced by HCC cells, which corresponded to an N-glycan with a disialylated biantennary structure with internal fucose (core-fucose), called AFP-L3, has been shown to improve the specificity of AFP as a marker of HCC. Thus, the ratio of fucosylated AFP to total AFP (AFP-L3%) has been approved by the FDA as a biomarker for the risk assessment of patients with chronic liver disease for the development of HCC^[91,92].

In the same context, we have described changes in glycosylation at the level of core fucose and the type of sialic acid linkage of the prostate-specific antigen (PSA) in prostate cancer^[93,94]. The increase of α 2,3-sialylated PSA glycoforms and the decrease of core fucosylated PSA glycoforms have been reported in cohorts of patient sera, and have proven useful in differentiating high-risk or aggressive prostate cancer from indolent prostate cancer and benign prostatic hyperplasia^[95,96], which is not possible to achieve with the current PSA tests. The fact that PSA is a specific protein of the prostate facilitates that these glycosylation changes on PSA are

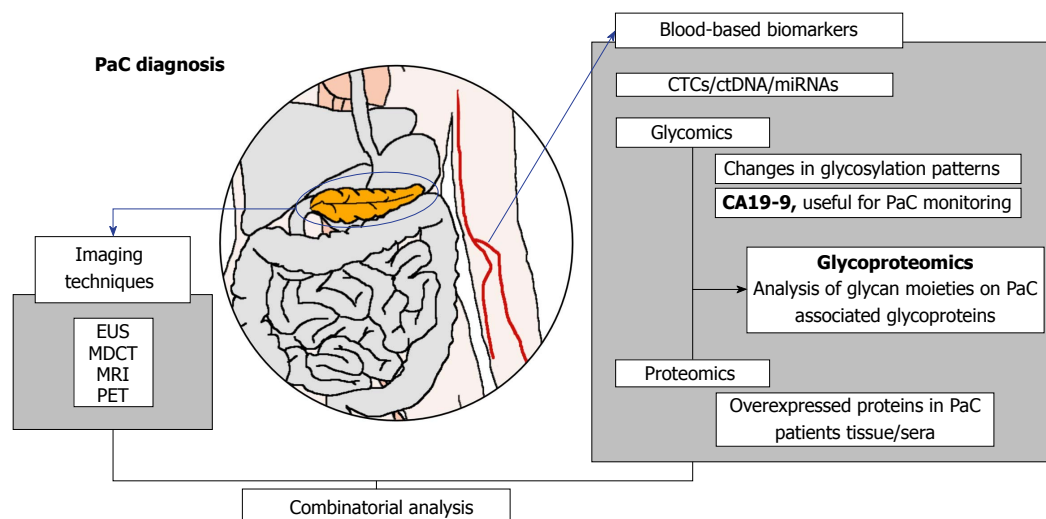


Figure 1 Overview of pancreatic cancer diagnosis methods: imaging techniques and the existing and new potential blood-based biomarkers.

specific to the prostate tumor, and their detection can be transferred into the clinic, in a similar way to that described for AFP.

ANOMALOUS GLYCOSYLATION PATTERNS IN PAC

Identification and detection of abnormal glycosylated proteins in bodily fluids of PaC patients may allow the discovery of new markers for early diagnosis. A large number of proteomic studies in PaC have been reported. Also, many glycomic studies have revealed an aberrant N-glycosylation profile on human pancreatic carcinomas, and human PaC cells show large variation in their N-glycosylation status^[53,97-100].

Zhao *et al.*^[101] observed increased branching of N-linked oligosaccharides, as well as an increasing in protein fucosylation and sialylation in the sera of PaC patients. The increase in Lewis and blood group glycans is a near universal feature of PaC^[102,103]. Truncated O-linked glycosylation resulting in the Tn and sialyl-Tn antigens occurs in almost all epithelial cancers, and in PaC in particular^[104].

Objectives

In this review we will describe the scientific advances and the forthcoming challenges to identify glycoproteins with altered glycosylation in PaC that could be useful as biomarkers, either alone or in combination with other biomarkers.

Results

Proteomic studies have led to identification of panels of proteins with potential as PaC biomarkers. However, in recent years, glycoproteomic strategies in biomarker discovery have become more important than just proteomics because they combine both the protein expression levels and glycan changes associated with PaC, thus

increasing the protein potential for cancer diagnosing^[105].

Although it is well-known that glycosylation plays an important role in epithelial cancers, the glycoproteome of the human pancreas and the aberrant glycosylation associated to PaC has not been deeply addressed. It should be noted that analysis of the glycoproteome is much more complex than the proteome because contrary to proteins, in which the amino acid sequences are unique and coded in the genome, no template exists for glycans. Glycosylation comprises a big heterogeneity of oligosaccharide residues in different sequences and linkages giving rise to a high amount of existing structures. In the present review, we focus on the strategies that have been proposed for the detection and/or quantification of glycobiomarkers in biological fluids and tissues from PaC patients. The summary of the approaches and/or glycoproteins proposed in this article intended to provide glycoproteins candidates with tumor associated glycans that should be explored for the development of a highly PaC sensitive and specific test (Figure 1).

Reported serum glycoproteins with altered glycosylation in PaC patients

The gold standard biomarker for diagnosing cancer should be ideally identified in easily accessible biological fluids, being for this reason, plasma and sera the samples most analyzed. Several plasma glycoproteins display altered N-glycan patterns in tumors. This was shown by N-glycan analysis of whole serum proteins (total serum N-glycome) from several cancer types^[106] and of individual proteins shed or secreted from cancer tissues into the blood^[89]. Some of these serum glycoproteins may also present glycan alterations in chronic inflammatory diseases. For this reason, finding a cancer specific glycoform in a serum glycoprotein, which was not present in benign diseases or in healthy individuals, would improve pancreatic cancer diagnosis.

In the last decade, several studies have identified

serum glycoproteins with altered glycosylation in PaC patients using different glycoproteomic approaches. Most authors have usually taken the serum as a starting point and have used either lectins or antibodies against specific glycan structures, or mass-spectrometry (MS) techniques to identify the differential glycosylation of serum glycoproteins in PaC patients (summarized in Table 2). However, candidate biomarkers are expected to exist in a very low concentration and serum samples are very abundant in others glycoproteins like immunoglobulins and other high-abundance proteins (acute-phase proteins) that can be removed before the analysis with multiple available commercial options (Table 2).

Although the molecular mechanisms underlying PaC-associated glycosylation events have not been perfectly understood, the most common glycan methodologies developed are focused on the analysis of alterations in branching, fucosylation^[107,108] and sialylation^[97]. It has also been reported that specific N-glycosylation occupancy in certain glycoproteins can be significantly altered^[109].

Using glycoprotein microarrays with multi-lectin detection techniques, an increase in both fucosylation and sialylation of some serum glycoproteins was described for PaC patients compared to HC and pancreatitis patients^[110,111]. Serum depletion to remove the most abundant proteins (albumin, IgGs and acute-phase proteins) to identify specific PaC glycoproteins with changes in glycosylation in PaC patients did not proportionate any pancreatic specific glycoprotein. However, several acute-phase proteins liver-derived, such as α 1- β -glycoprotein, showed an increase in the affinity to *Sambucus nigra agglutinin* (SNA) lectin, which binds α 2,6-sialic acid, in most of the PaC serum samples, which were stage III/IV, and allowed to discriminate them from ChrP patients^[111,112].

More recently, Kontro *et al.*^[97] analyzed N-glycopeptides from albumin-depleted serum of PaC, ChrP, and HC. They showed significant changes in the concentration of the α 2,6 sialylated N-glycopeptides in both PaC and ChrP. The glycopeptides corresponded to acute-phase proteins [haptoglobin (HPT), α -1-antitrypsin (A1AT), transferrin, ceruloplasmin, α -1-acid-glycoprotein (AGP), fetuin A] and immunoglobulins.

Using electrophoresis and western blotting of albumin and IgG-depleted serum samples, we identified serum glycoproteins with an increased sialyl-Lewis^x (SLe^x) level in advanced PaC. However, the glycoproteins identified: AGP, HPT and transferrin, which are acute-phase proteins, also showed a SLe^x increase in ChrP patients, suggesting that the increase in SLe^x was related to inflammation rather than cancer itself^[113]. In the search for more specific PaC glycoproteins with an increased SLe^x level, further serum depletion that included the twelve most abundant serum proteins was performed in order to remove the previously described acute-phase proteins. Other glycoproteins with SLe^x increase were identified, but turned out to be acute-

phase proteins. Among them, ceruloplasmin was further analyzed in a bigger cohort of PaC serum samples. The ratio of SLe^x on ceruloplasmin related to ceruloplasmin levels showed an increased tendency in the sera from PaC compared to HC and ChrP patients^[114].

Looking for other potential cancer associated glycoforms, our group performed the N-glycan sequencing of some major serum acute-phase proteins. An increase in core fucosylation was found in HPT and AGP in PaC patients compared to the ChrP and HC groups^[113]. Other authors also analyzed HPT fucosylation in a larger cohort of patients. Although the increase of fucosylated HPT did not show significant differences in PaC vs pancreatitis patients, its combination with CA 19-9 gave higher diagnostic potential compared to CA 19-9 alone^[115].

Recently, we have addressed the analysis of AGP fucosylation using several complementary analytical methodologies, including lectin and MS analyses, and have shown an increase in AGP α 1,3-fucose glycoforms in advanced PaC compared to ChrP and HC patients^[116-118].

The expression of fucosylated glycoproteins in PaC depleted serum samples was analyzed using *Aleuria aurantia lectin* (AAL)^[119]. α 1-Antichymotrypsin (ACT), trombospondin-1 and HPT were proposed as the best fucosylated candidates and were further validated with ELISAs or lectin ELISAs. Their combination with CA 19-9 resulted to be very promising for distinguishing PaC from other conditions with or without obstructive jaundice, indicating a high diagnostic potential^[119].

The study of N-linked core fucosylated glycopeptides with potential use in PaC diagnosis has also been addressed performing nanoLC-MS/MS analysis of iTRAQ labelled glycopeptides. One core fucosylated peptide, belonging again to an acute phase glycoprotein, ACT, presented significant differences between healthy, ChrP and PaC groups^[108].

In a previous study, our group addressed the analysis of serum ribonuclease 1 (RNase 1), which is an acinar pancreatic protein found in serum. However, this protein is also secreted by endothelial cells into serum. Serum pancreatic RNase 1 was found to be much more core fucosylated in PaC than in HC^[120], which suggests that the quantification of core fucosylation in some PaC serum glycoproteins might be useful as a PaC biomarker. Nakata *et al.*^[121] showed that N-glycosylation at Asn-88 in serum RNase 1 was significantly increased in patients with PaC and proposed the serum RNase 1 N-glycosylation occupancy at Asn-88 site as a novel diagnostic marker for PaC.

Besides fucosylation, other authors focused on N-glycan branching of serum glycoproteins, which is one of the most common altered glycosylation features found in most types of cancers. After albumin/IgG depletion, Drabik *et al.*^[122] performed a glycoprotein enrichment using *Phaseolus vulgaris leucoagglutinin* (PHA-L). Then, using two-dimensional capillary chromatography combined with tandem mass spectrometry, they found four glycoproteins (LIFR, CE350, VP13A and HPT) that carried

Table 2 Serum glycoproteins with altered glycosylation identified in pancreatic cancer patients

Techniques	Serum cohort (n)	Serum depletion (Yes/No)	Glycosylation change	Main glycoproteins identified	Ref.
ConA chromatography. Tryptic digestion. N-glycan MS analysis of the N-glycopeptides	2 PaC	No	Increased branching, fucosylation and sialylation		[101]
ConA and WGA chromatography. Glycoprotein separation and array with lectins (SNA, PNA, MAL, AAL).	6 PaC 8 ChrP 10 HC	Yes (IgY 12) (albumin, IgG and major acute-phase proteins)	Increased fucosylation and sialylation	Hemopexin Serum amyloid P component Antithrombin-III Haptoglobin (HPT)-related protein β -2-glycoprotein 1 α 1- β -glycoprotein.	[110]
Antibody microarray to α 1- β -glycoprotein, amyloid P and antithrombin followed by lectin detection (SNA). MS glycoprotein identification.	22 PaC (III/IV) 35 ChrP 89 HC 37 diabetic		Increase affinity to SNA (α 2,6-sialic acid) by 69% in PaC patients	ROC curve (cancer <i>vs</i> non cancer samples), 100% sensitivity 98% specificity. AUC = 0.998	[111]
Bead-based antibody-lectin (SNA, Con A) multiplex assay-for determining SNA and Con A reactivity of α 1- β -glycoprotein, and amyloid P component.	20 PaC (III/IV) 20 ChrP 20 HC		SNA affinity (α 2,6-sialic acid)	α 2,6-sialic glycoforms of α 1- β -glycoprotein Differentiation of ChrP <i>vs</i> PaC ($P = 0.035$)	[112]
SNA affinity chromatography to enrich sialylated glycopeptides and compared their relative abundance by ultra performance LC-MS	10 PaC (II-III) 5 Acute Pancreatitis 16 HC	Yes (albumin depleted)	SNA (α 2,6-sialic acid)	Sialylated glycopeptides of HPT, α 1-antitrypsin (A1AT), transferrin, ceruloplasmin, α 1-acid-glycoprotein (AGP), fetuin A and Igs. Change in acute pancreatitis and PaC	[97]
2DE followed by N-glycan sequencing	9 PaC (I-IV) 3 ChrP 5HC		SLe ^x Fucosylation	Increase in SLe ^x on AGP, HPT and transferrin in advanced PaC and ChrP Increase in core fucosylation of HPT and AGP in PaC <i>vs</i> ChrP and HC	[113]
Electrophoresis (IDE) followed by WB with anti SLe ^x . Immunoprecipitation of ceruloplasmin and SLe ^x detection	20 PaC (II a-IV) 14 ChrP 13 HC	Yes (IgY 12) (albumin, IgG and major acute-phase proteins)	SLe ^x	Ceruloplasmin Tendency to an increase of SLe ^x on ceruloplasmin in PaC <i>vs</i> HC and ChrP	[114]
Lectin (AAL)-antibody ELISA	72 PaC 22 HC 63 pancreatitis		AAL (fucosylation).	Increase of fucosylated HPT in advanced PaC	[115]
AGP purification	19 PaC (I-IV)		α 1,3 fucosylation	Increase of fucosylated AGP in advanced PaC	[116-118]
MS analysis of AGP N-glycans and AAL ELISA	6 ChrP 6 HC				
N-glycan sequencing of human serum ribonuclease (RNase 1).	2 PaC 2 HC		Core fucosylation	Increase of core fucosylation in RNase 1 in PaC	[120]
ELISA to measure N-glycosylation	91 PaC		Asn-88 N-glycosylation	Increase in N-glycosylated Asn-88 of RNase 1 (normalized to RNase 1) in PaC.	[121]
Asn-88 site occupancy of serum RNase 1	60 HC				
AAL to enrich fucosylated glycoproteins LC-MS/MS analyses	20 IPMN 10 MCN	IgY-14 LC10 columns	Fucosylation (AAL)	ACT trombospondin-1 HPT	[119]
ELISA/lectin ELISAs	37 PaC (I-IV) 30 HC 30 ChrP 22 OJ 30 Type II DM			High diagnostic potential combined with CA 19-9	
nanoLC-MS/MS analysis of iTRAQ labelled glycopeptides.	13 HC 13 ChrP 13 PaC 1 Std	IgY-14 LC10 column	Core-fucosylation	One core fucosylated peptide from ACT different between groups	[108]
PHA-L lectin to enrich complex N-glycoproteins	26 HC (include ChrP + pseudo cysts)	Albumin/IgG depletion	Increased fucosylation N211 Novel glycosylation site N64	HPT Leukemia inhibitory factor receptor LIFR	[122]
2D nanoLC-MS/MS analyses	76 PaC		New N-glycosylated side at N2336 in PaC N-glycosylation N877	Centrosome-associate protein 350 CE350 Vacuolar protein sorting-associated protein 13A VP13A	
Western Blot with biotinylated PHA-L nanoLC-MS/MS of tryptic digested gel bands that corresponded to specific lectin interactions on Western blot					

2D-LC-MS/MS	231 serum women samples pooled in groups: time-to-diagnosis	Yes	N-glycosylation occupancy	A1AT HPT AGP	[123]
Lectin (CCL2)-antibody ELISA	109 PaC 91 control (plasma)	No	CCL2 (3' fucosylation)	MUC5AC	[99]
Antibody lectin sandwich array	156 PaC (I -IV) 160 control (plasma)	No	SLe ^a related	MUC5AC MUC16	[127]
Antibody microarray capture of proteins. Glycan analysis with lectins (AAL, WGA) and CA 19-9	23 PaC (I -IV) 23 HC	No	CA 19-9	MUC1 CEA	[128]
Antibody lectin sandwich array	23 PaC (I -IV) 23 HC	No	SLe ^a (CA 19-9)	MUC1 MUC5AC	[129]
Antibody array	285 PaC (I -IV) 102 ChrP 144 HC (serum & plasma)	No	CA 19-9	MUC1 MUC5AC MUC16	[130]

MS: Mass-spectrometry; PaC: Pancreatic cancer; WGA: Wheat germ agglutinin; SNA: Sambucus nigra agglutinin; AAL: Aleuria aurantia lectin; ChrP: Chronic pancreatitis; HC: Healthy controls.

an altered N-glycan structure in PaC compared to HC. Recently, Krishnan *et al.*^[123] carried out a pilot proteomic study to evaluate changes in serum N-glycoproteins that could be potential biomarkers prior to clinical presentation of PaC being inflammatory response, coagulation and immune-related proteins the most promising ones. One of the top-scoring glycoproteins identified, A1AT, was quantified by ELISA. After analyzing 120 control and 92 PaC cases they concluded that whilst A1AT levels performed reasonably well for discriminating prediagnosis cancer cases, it is unlikely to be specific for detecting PaC.

Another group of highly glycosylated proteins with high potential as novel clinical PaC biomarkers are mucins. In this regard, altered glycosylation pattern of glycoproteins belonging to members of mucin (MUC) families have also been associated with PaC in several studies using PaC tissues as well as serum samples (Table 2). MUC4 altered glycosylation was identified in PaC by immunohistochemistry, as only one out of two antibodies directed towards different MUC4-Tn glycoforms showed staining in PaC tissues^[104]. These authors also identified changes in MUC1 glycosylation, as the glycoform MUC1-T observed in normal pancreas became MUC1-STn in neoplastic stages. Recently, the study of immunoprecipitated MUC1 glycoforms using lectins has exposed its potential to discriminate between PaC and cholangiocarcinoma patients^[124]. As mucins can be cleaved from cells and released to the bloodstream, all the specific glycoforms detected in tissues could be potentially detected in serum to be exploited as biomarkers. In addition, in cyst fluid samples, MUC5AC specific glycosylation detected with the *Wheat germ agglutinin* (WGA) lectin was reported to discriminate malignant from benign cases in pre-cancerous lesions

as pancreatic cysts^[125]. We also reported recently the increased expression of MUC1-SLe^x and MUC5AC-SLe^x in PaC tissues compared to benign controls^[126].

Some of these mucin glycoforms described as PaC specific in tissues were also analyzed in sera (see Table 2). Although most of the well-established serological biomarkers are based on immunological methods to detect mucin glycoproteins such as CA125 assay detecting MUC16 in ovarian cancer or CA15-3 assay detecting MUC1 in breast cancer, the exact structural epitope recognized is unknown. Moreover, the structural characterization of O-glycans in mucins have been overlooked due to the analytical limitations (high molecular weight (MW) proteins with high number of glycosylation sites, high glycan heterogeneity in each individual site and lack of enzymes able to release O-glycans).

Regarding MUC5AC, increased levels of specific glycoforms carrying 3' fucose (for instance SLe^x or Le^y) were observed in serum from PaC patients compared to benign controls^[99]. Further, a panel of serum glycans and different MUC5AC glycoforms (including SLe^a and SLe^x) showed improved accuracy over the standard CA 19-9 assay for detecting PaC^[127].

Chen *et al.*^[128], using antibody microarrays to capture multiple serum glycoproteins followed by detection with lectins or glycan-binding antibodies, described cancer-associated glycan alterations on MUC1 and CEA in serum of PaC patients. Using antibody-lectin sandwich arrays, MUC1 and MUC5AC were shown to carry distinct glycan alterations in PaC patients, in particular MUC1 carried the CA 19-9 antigen (SLe^a) in a majority of PaC patients compared to HC^[129]. Using antibody arrays^[130], they noticed that the detection of CA 19-9 on individual carriers, like MUC5AC or MUC16, did not exceed the performance of the CA 19-9 assay. Notwithstanding,

Table 3 List of proposed glycoproteins as possible biomarkers for pancreatic cancer

Glycoprotein	Glycosylation sites	Reported glycosylation	Serum auto-antibodies	Ref.
Mesothelin (cleaved form)	3 N-glycosylation sites	Reduction of MW after PNGase F digestion of A431 cancer cells	Yes	[133-144]
IGFBP-3	3 N-glycosylation sites O-glycosylation	Increase of N-glycosylation levels in tumor tissues Increase of biantennary complex type N-glycans having more mannose, fucose, bisecting GlcNAc and terminal sialic acid in breast cancer serum	Not described	[109,145]
IGFBP-2	O-glycosylation	Not described	Not described	[141,146]
REG 1A	O-glycosylation	Increase protein glycoform diversity in pancreatic ductal fluid of PaC by western blot	Not described	[147-151]
REG 1B	O-glycosylation			
REG 3A	1 potential N-glycosylation site			
REG 4	1 N-glycosylation site			
TIMP-1	2 N-glycosylation sites	Core fucosylated, biantennary N-glycans with Gal or GalNAc in HEK293. Some of the glycans are sialylated, and many have outer arm fucosylation.	Not described	[152-156]
HER-2	7 N-glycosylation sites O-glycosylation	Aberrant N-glycosylation in colon cancer Altered N-glycosylation in breast cancer cells. Altered O-glycans (Tn and T) in PaC cells	Yes	[157-162]

IGFBP: Insulin-like growth factor binding protein; REG: Regenerating protein; PaC: Pancreatic cancer.

these analyses could discriminate between PaC and ChrP patients in low CA 19-9 individuals, so the combined analysis of CA 19-9 plus its detection on specific mucins could yield improved sensitivity for PaC detection.

The studies above reported (summarized in Table 2) consisted on preclinical exploratory works to compare tumor and non-tumor serum samples, to generate potential biomarker candidates for PaC detection. Using serum or depleted serum as starting material to find glycoproteins with altered glycosylation in PaC patients has resulted mainly in the identification of acute-phase glycoproteins mostly liver derived, as well as mucins such as MUC1 and MUC5AC. The glycosylation analysis of some of these reported individual glycoproteins, mainly focused on changes in sialic acid, fucose, or SLe^{x/a}, in PaC patients, showed usually significant changes in advanced PaC, but not in low PaC stages, and in many cases in inflammatory conditions, which limits their use as biomarkers of PaC. The high levels in serum of these acute-phase proteins, which are not tumor specific, have hindered the identification of minor proteins with altered glycosylation from a pancreatic tumor origin that could be more PaC specific, which should be one of the challenges of next investigations.

Some of the best and most widely recognized cancer bio-markers are highly tissue-specific, such as PSA, hCG and AFP. Although proteins with such characteristics are quite rare, we consider that pancreatic tissues are the ideal biological sample for studying the tumor-specific glycoproteins. Once the pancreatic tissue-specific glycoproteins or glycoforms can be found, their presence in serum could be explored.

Proteomics information to identify new potential PaC glycobiomarkers

Although the high number of biomarker studies reported in the last years, no valuable marker for this lethal cancer has been translated into clinics. The detection of the tumor associated glycans on selected glycoproteins,

overexpressed or neo expressed specifically in PaC tissues, could improve the biomarker performance of the glycoprotein.

Although the search of potential biomarkers in PaC cultured cells is a good starting sample, PaC stromal components represent up to 90% of the pancreatic tumor volume, which implies that cell line models are not completely representative of the physiological environment. For that, proteomic analysis of tumor tissues is proposed as starting point to provide glycoprotein candidates that could be secreted or shed into blood for further biomarker analysis in serum or plasma. In this review we have focused on those glycoproteins that have been described in the literature to be differentially expressed and/or oversecreted in PaC cells, tissues, or pancreatic juice and that can also be found in plasma or serum.

The main protein carriers of CA 19-9 described are MUC1, MUC5AC and MUC16 and as we have mentioned previously, the study of their altered glycosylation is of interest for improving the performance of the CA 19-9 test. However, the analysis of these high MW glycoproteins (MW over 200 KDa with a glycan component that contributes to more than 50% of the MW) in serum presents a big challenge. Despite being one of the main families of proteins with potential as cancer biomarkers, in this study we will concentrate on other non-mucin glycoproteins that may be analyzed easily.

Among the large number of proteins described in different proteomic approaches, we have focused on glycoproteins different from acute-phase proteins because these last ones are usually increased in inflammation conditions as discussed previously. In our selection, the proteins associated to inflammatory processes have also been discarded because their abnormal expression may be associated with more than one type of cancer (lack of specificity). The most promising glycoprotein candidates are listed in Table 3 and may be analyzed in detail to determine whether a specific

glycoform could be PaC associated and be used alone or together with CA 19-9 for the detection of early-stage PaC.

It has been described that the immune system reacts to developing tumors and generates auto-antibodies against tumor-associated antigens. These serum auto-antibodies can be detected in early stages and have recently emerged as early stage bio-markers for different types of cancer, including PaC. Possibly auto-antibodies signatures might therefore be best used as a screening tool to detect the presence of cancer in general, to be followed by more specific diagnostic measures in case of positive results^[131].

Cancer patients produce auto-antibodies to proteins that are either mutated, misfolded, over-expressed, or to proteins that show altered post-translational modifications like glycosylation. The mechanisms of auto-antibody production are not fully clear. Secretion of abnormal glycoforms into blood induces the immune system to produce antibodies which results in the amplification of an early malignant alteration. Therefore, the detection of these antibodies indicates that the affected tissue shed these glycoproteins in the blood and may allow the diagnosis in an early stage of the disease, when there are no clinical signs yet, and the amount of anomalous glycan antigens is still very low. Some studies have evaluated serological auto-antibodies in PaC and the combination of four auto-antibodies including anti-mesothelin showed a reasonable diagnostic performance^[132]. The finding of serum auto-antibodies recognizing some of the proposed glycoprotein candidates reinforce them as potential PaC biomarkers (Table 3).

Mesothelin, one of the potential candidates, is highly expressed in some common epithelial cancers. Its expression by immunohistochemistry has been reported in approximately 100% of malignant mesotheliomas^[133], and in other cancers such as ovarian^[134] and lung adenocarcinomas^[135]. Regarding PaC, Zheng *et al.*^[136], reported mesothelin expression by western blot and RT-PCR assay in human PaC cell lines, and Argani *et al.*^[137], reported its expression in the majority of the PaC tissues analyzed by immunohistochemistry. Interestingly, mesothelin is present on normal mesothelial cells, peritoneum and pericardium, but it is not expressed either in normal pancreatic tissues or in ChrP^[138]. Bournet *et al.*^[139] also demonstrated the overexpression of the mesothelin gene in biopsies of PaC samples when compared to ChrP. Although mesothelin is attached to the cell membrane, it can be shed like many other cell membrane proteins. Some authors have identified an increase in mesothelin serum levels when compared with ChrP and HC samples^[140,141]. However, this protein has been also detected in sera from patients with ovarian carcinoma^[142] and malignant mesothelioma^[143]. Mature mesothelin presents three potential glycosylation sites and although mesothelin glycosylation has not been analyzed yet, Onda *et al.*^[144] observed a change in the SDS-PAGE mobility of mesothelin from cell extracts

after being treated with PNGase F.

Some members of the insulin-like growth factor binding protein (IGFBP) family have also been proposed as potential candidates. It is known that six IGF-binding proteins interact with IGFs and modulate their tissue distribution and access to cell receptors. Serum levels of IGFBP-3 are associated with several carcinomas. IGFBP-3 incorporates altered glycan chains in breast cancer (containing increased amounts of bi-antennary chains, fucose and sialic acid residues)^[145], as well as in hepatocellular carcinoma and in PaC. In this regard, Pan *et al.*^[109], using a quantitative glycoproteomics approach with tissue proteins labelled with stable isotope and N-glycopeptide enrichment with hydrazide beads, observed that IGFBP-3, among other proteins, had elevated N-glycosylation level in PaC compared with healthy pancreas.

Chen *et al.*^[52] performed a quantitative proteomic approach in pancreatic juice and identified IGFBP-2 as a novel PaC target. The IGFBP-2 overexpression was further validated by western blotting in pancreatic juice and pancreatic tissues compared to normal and ChrP samples. IGFBP-2 has also been studied as a serum PaC biomarker in combination to mesothelin^[141]. In particular, this secreted glycoprotein does not contain any N-glycosylation site but the presence of O-glycosylation has been reported^[146].

Searching for a more organ-specific glycoprotein we included lithostathine, also known as regenerating protein (REG) in the biomarkers' list. It is a glycosylated protein which is normally found in the exocrine pancreas and its glycan chains have already been characterized^[147,148]. It is the major non-enzymatic component of pancreatic juice, and its presumed role is to prevent stone formation in pancreatic ducts. It is expressed in pancreatic ductal carcinoma, as well as in colon cancer^[149]. REG1 is only present in acinar pancreatic cells and malignant ductal cells and is not expressed in normal ducts or islets cells. REG1 is upregulated in PaC working as a growth factor, and is increased in serum and urine levels in PaC patients^[150,151]. REG1 presents an altered glycosylation pattern in PaC ductal fluid, so its potential as a glycoprotein biomarker should be considered in future investigations^[148].

Mammalian tissue inhibitors of metalloproteinases (MMPs) are also considered biomarker candidates. They are endogenous secreted proteins that inhibit all metalloproteinases and are crucial cancer regulators^[152]. TIMP-1 is a MMP inhibitor and it is expressed in many organs. TIMP-1 levels are altered in many cancers, as lung, prostate, breast, colon and pancreas, and its serum and urine levels are increased in more advanced tumors^[21,153,154]. TIMP-1 contains two N-glycosylation sites that present an altered pattern in colon, lung and prostate cancer and some of these aberrant glycan chains have already been sequenced^[155,156].

Another proposed candidate is HER-2, a transmembrane protein belonging to the EGFR family that possesses intrinsic tyrosine kinase activity. Its expression

has been described in normal and cancerous human pancreas. However, its overexpression has been associated with PaC malignant phenotype and bad prognosis^[157,158]. In regard to glycosylation, some authors analyzed HER-2 N-glycans from human breast cancer cell lines using a mass spectrometry based approach^[159-161]. Although several reports have described N-glycosylation structures of HER-2 from breast cell lines, the N-glycans attached to this protein in PaC cells or tissues remain to be elucidated. In regards to O-glycosylation, Chugh *et al.*^[162] described that the loss of GALNT3 in poorly differentiated PaC cells produced altered O-glycans (Tn and T) on EGFR and HER-2, resulting in an increase in phosphorylation and tumor aggressiveness. The glycosylation of HER-2 could influence in the signalling pathway of PaC cells. The search of aberrant HER-2 glycoforms in PaC sera may be used as a biomarker and also as a target for PaC treatment.

Although there is no single ideal method for the detection of new cancer biomarkers in complex bodily fluids and tissues, we propose the combination of a large variety of analytical methods to develop new approaches based on the glycan analysis of the candidate glycoproteins in a similar way that the ones that have been established for the detection of AFP and PSA in HCC and prostate cancer respectively. The most promising approaches include lectin based methods and mass spectrometry technology. The lectin-based methods commonly adopted in cancer biomarker discovery research are lectin affinity chromatography, enzyme-linked lectin assays, lectin histochemistry, lectin blotting and new lectin arrays^[163]. Some efforts have also been made to achieve glycan-specific antibodies and/or to produce antibodies that can recognize a specific glycoform in a glycopeptide or glycoprotein. However, glycan-specific antibodies are rare, compared with antibodies recognizing peptide epitopes, because carbohydrates have been shown to be poor immunogens.

CONCLUSION

Early detection of PaC using glyco-biomarkers could provide a solution and future directions for PaC management. Simultaneous measurement of specific glycoforms combined with protein levels might increase the diagnostic potential of the candidate glycoproteins. However, we have to consider that the aberrant glycosylation is disease specific but not tissue specific, which means that some alterations such as core fucosylation levels or sialylation degree of the candidate glycoprotein could be a common epithelial cancer feature. Furthermore, the use of a multimarker panel composed of the candidate glycoproteins combined with CA 19-9, as well as other medical approaches (as imaging), could enhance the lack of discriminatory power of a single marker.

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