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Review article
Pregledni članak

GLYCANS AS A TARGET IN THE DETECTION OF REPRODUCTIVE TRACT CANCERS

GLIKANI KAO METE U DETEKCIJI KANCERA REPRODUKTIVNOG TRAKTA

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Summary: The significance of changes in glycosylation for the beginning, progress and outcome of different human diseases is highly recognized. In this review we summarized literature data on the alteration of glycans in cancer, especially glycoforms of tumor markers of reproductive tract cancers: prostate-specific antigen (PSA) and cancer antigen 125 (CA125). We aimed to highlight the diagnostic potential and relevance of glycan microheterogeneity and to present some novel methods for cancer detection. A computerized search of articles published up to 2007 was performed through the PubMed database. Search terms utilized included prostate/ovarian cancer glycosylation, prostate/ovarian cancer detection, PSA/CA125 glycosylation. Additional sources were identified through cross-referencing and researching in available biomedical books. The comparative studies of sugar chain structures of the PSA and CA125 indicated specific structural alterations associated with malignant transformation, in relation to glycan branching, sialylation and fucosylation. These glycan modifications should be better in distinguishing between benign and malignant conditions than the measurement of marker concentrations alone, which is widely used in practice. Cancer-associated changes in the glycosylation could yield more sensitive and discriminative diagnostic tests for reproductive tract cancer detection, i.e. for improvement of the clinical utility of known tumor markers or the discovery of new ones.

Keywords: CA125, glycosylation, ovary, prostate, PSA, tumor marker

Kratak sadržaj: Poznat je značaj promena glikozilacije za nastanak, razvoj i ishod različitih bolesti. U ovom radu sumirani su podaci iz literature o promenama glikana kod kancera, posebno glikoformi tumorskih markera kancera reproduktivnog trakta: specifičnog antigena prostate (PSA) i kancerskog antigena 125 (CA125). Cilj ovog rada je da se naglase dijagnostički potencijal i značaj mikroheterogenosti glikana kao i da se prezentuju neke nove metode za detekciju kancera. Izvršena je pretraga radova objavljenih do 2007. godine u bazi podataka PubMed, pomoću termina »prostate/ovarian cancer glycosylation«, »prostate/ovarian cancer detection«, »PSA/CA125 glycosylation«. Kao dodatni izvor podataka korišćene su i dostupne biomedicinske knjige. Uporedna studija strukture šećernih lanaca PSA i CA125 ukazala je na specifične promene povezane sa malignom transformacijom, koje se tiču grananja, sijalinizacije i fukozilacije glikana. Ovakve glikanske modifikacije tumorskih markera mogle bi imati veći potencijal u smislu diferencijalne dijagnostike benignih i malignih stanja nego samo merenje njihove koncentracije, koje se sada široko primenjuje u praksi. Promene glikozilacije, vezane za kancer, mogu biti osnov za razvoj senzitivnijih i specifičnijih dijagnostičkih testova za detekciju kancera reproduktivnog trakta, tj. poboljšati kliničku upotrebu poznatih ili doprineti pronalaženju novih tumorskih markera.

Ključne reči: CA125, glikozilacija, jajnici, prostata, PSA, tumorski markeri

Introduction

In humans, 500 genes, i.e. 0.5–1 % of the translated genome, participate in oligosaccharide synthesis and function (1). Glycosylation, as the most common and ubiquitous posttranslational modification, leads to the formation of an enormous number of extremely complex glycan (oligosaccharide) structures, existing in many shapes and sizes (2, 3). There are three major types of carbohydrate-peptide link-

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age: N-glycosidic, O-glycosidic and glypiated linkage. In addition to them, α-mannose attached C-glycosidically to tryptophan and a phosphoglycosyl (GlcNAc-1-P) bond attached to serine can also occur occasionally (2). Both N- linked and O-linked glycans are heterogeneous and exist in different forms. N-glycans comprise high mannose, complex and hybrid types of oligosaccharide chains, sharing a common pentasaccharide core consisting of two N-acetylglucosamine (GlcNAc) and three mannose (Man) residues, and may have branches, i.e. antennae, arising from the core mannoses. O-glycans comprise three main types of oligosaccharide chains: the most abundant mucin-type, and the collagen- and glycosaminoglycan-type.

Both N-linked and O-linked glycans can be attached to individual glycoproteins and heterogeneous glycosylation may occur (2, 3). This means that the structural composition can vary from molecule to molecule, resulting in the existence of different glycoforms of a particular glycoprotein, which may have different molecular properties. The differences may be related to increased branching, or loss of a sugar from one branch of a glycan. Minor changes include gain or loss of a terminal sugar or changes in the linkage between the sugars.

The meaning of the extreme structural diversity of glycans is explained by the introduction of the concept of a sugar code (4, 5). According to this, glycans act as information-storage molecules, and carbohydrates are assigned functional significance. Much biological information can reside in complex glycan structures, due to the enormous coding potential, which is far beyond the coding potential of amino acids or nucleotides. This is based not only on variation in the sequence of monosaccharides in oligosaccharide chains, but also on the possibility of postglycosylation modification of the sugars, as well as the formation of different linkage isomers and branching patterns. Carbohydrate-binding proteins, i.e. lectins, play a significant role in decoding the information content of glycans by recognizing and specifically binding to certain glycosylated ligands (6). Lectins and glycans represent an evolutionary conserved system, which controls the fundamental aspects of cell-cell and cell-environment communication responsible for cell homeostasis. Thus, glycans are involved in recognition phenomena during fertilization, implantation, development, differentiation, morphogenesis, infection, leukocyte trafficking, blood clotting, attachment and invasion of cancer cells and apoptosis (7–10).

The significance of alterations in glycosylation for the initiation, progress and outcome of different human diseases is highly recognized (11, 12). The disease implications of glycopeptide bonds may be related to either N- or O- bonds, as well as glypiated linkages. Generally, the defects in enzymatic transfer of saccharides to protein or defects and changes in glycosidase activities lead to an altered glyco-pattern of a particular molecule, which may result in its accumulation or deficiency. Pronounced changes in cell and tissue glycophenotype are also associated with essentially all types of experimental and human cancers (13, 14). They can occur in an early phase of oncogenesis, as well as during tumor growth and progression, i.e. metastasis. Changes in glycans influence cell adhesion properties, leading to loss of density-dependent growth inhibition and spreading of neoplastic cells (15). They also influence immunogenicity, i.e. they may elicit an immune response in cancer patients or have anti-recognition effects and help the cell to escape immune surveillance (16). In addition, glycans influence greatly the signal and recognition properties of cells or molecules, acting as specific markers of various pathophysiological conditions in relation to their type and differentiation stage (17–22).

So far, glycobiological investigations have resulted in much medicinally relevant information, enabling us to trace new routes in cancer research, concerning detection of the disease and clinical manipulation, with the intention of creating new strategies for cancer prevention and therapy (23, 24). Carbohydrate-based anti-cancer vaccines as well as carbohydrate-based drugs, already in the market or in various phases of clinical trials, emerge as promising candidates for medical agents in the battle against specific cancers (25, 26). In contrast to their clinical application as therapeuticals, which is still in its infancy, the glycoproteins are widely used as biological markers of different pathophysiological conditions, including cancer.

In this review we summarize literature data on the alteration of glycans in cancer with special emphasis on the glycoforms of two tumor markers of reproductive tract cancers: prostate-specific antigen (PSA) and cancer antigen 125 (CA125). We have aimed to highlight the diagnostic potential and relevance of glycan microheterogeneity and present some emerging methods and innovative approaches to cancer detection.

Methods

A computerized search of articles published up to 2007 was performed through the PubMed database, a service of the National Library of Medicine and the National Institutes of Health. Retrieval provides bibliographic data with an abstracted summary, obtained by a combination of keywords. Search terms utilized included prostate/ovarian cancer glycosylation (77/66 studies identified), prostate/ovarian cancer detection (4391/2053 studies identified), and PSA/CA125 glycosylation (29/18 studies identified). Additional sources were identified through cross-referencing and by researching in available biomedical books.

Results

Glycosylation changes common to cancer

Complex regulatory mechanisms in the biosynthesis of glycans are characteristically changed in cancer. Although the biochemical basis of the alterations is still not elucidated and reports are often conflicting, some generalizations can be deduced (*Table I*). Thus, certain glycosylation pathways are favored, resulting in the expression and secretion of aberrantly or incompletely glycosylated molecules, or the appearance of completely new structures (13, 14, 17, 27). Glycosylation abnormalities of both N-linked and O-linked glycans mainly result from rearrangements of their biosynthetic pathways due to the changes in the activity and expression pattern of glycosyltransferases acting in the Golgi apparatus (13, 14, 17, 28). In some cases, these modifications may be linked to the activation of particular oncogenes (12, 29).

One of the most prominent changes is related to β-N-acetylglucosaminyltransferase isoenzymes (GnT) (30). Among six GnT (I-VI) which add βGlcNAc at different positions of the trimannosyl core, GnT III, -IV, -and –V play roles in the structural alteration of the complex-type sugar chains during cancer (17, 31, 32). Specifically, GnT-V is essential for tumor growth and metastasis. Thus, in some cancer types, increased β1,6-branching of the complex type N-linked sugar chains was found to be the basis for metastatic spread and correlated with a poor prognosis (17, 31, 32).

As for O-glycans, characteristic alterations are reported to be related to the appearance of unusual structures, as well as increased production of truncated mucin-type oligosaccharides carrying blood groups and other related carbohydrate antigens (12, 13). For instance, undersulfated sialomucins are predominantly increased in cancer. Increased sialylation, associated with both N-linked and O-linked glycans, results from changed activity of differentially expressed α-2,3-sialyltransferase (ST3Gal III) and α-2,6-sialyltransferase (ST6Gal I) in neoplastic tissue (33–35). Thus, sialic acid can occur in several different linkages, while increases or decreases of derivatives, such as O-acetyl and N-glycolyl-neuraminic acid (Neu 5Gc), are also observed.

Fucosylation changes in cancer are generally related to low activity of the α1,2-fucosyltransferase (α2FucT), and increased activity of serum α1,3-fucosyltransferase (α3FucT). Changes in α2FucT activity influence the expression of blood group H, Lewis^b and Lewis^y antigens (36). α3FucT, which catalyses the synthesis of Lewis^x and Lewis^y, is found to be higher in cancer cells than in normal cells (37). Thus, increased expression of certain glycosyltransferases, i.e. sialyl- and fucosyltransferases, can be responsible for changes in Lewis antigen expression.

Generally, carbohydrate antigenic moieties comprising blood group- and tissue-related antigens may be added to different substrates: N-glycans, O-glycans and glycosphyngolipids. Lewis antigens: Lewis^a, Lewis^b, Lewis^x, Lewis^y, some with various modifications in terms of sialylation and sulfation, may appear, i.e. there is an increase or decrease in serum or on the surface of various tumor cells (38, 39). Increases in another group of carbohydrate antigens comprising T and Tn antigen, either singly or together, are also typical for malignant transformation (40). Specifically, the occurrence of increased sialyl-Tn is typical of well differentiated advanced stage and poor prognosis cancer (41).

As for blood group-associated carbohydrate antigens, they can be lost, increased, decreased, or have an aberrant structure (12, 39). Moreover, some

Table I Common cancer-associated changes of N- and O-glycan structures and enzymes related to their biosynthesis.

N- and O-glycans		Glycosyltransferases	
Structure	Expression	Enzyme	Expression
Highly branched complex type N-linked sugar chains	▲	ß-N-acetylglucosaminyltransferases (GnT)	▲
β1,6 branching: GlcNAc (β1,6) [GlcNAc(ß1,2)]Man(α1,6)Manß	▲	GnT-V	▲
Sialyl-motifs	▲	Sialyltransferases (ST)	
Sialic acid α2,3Galβ1,3/4GlcNAc	▲	ST3Gal III	▲
Sialic acid α2,6Galβ1,4GlcNAc	▲	ST6Gal I	▲
Sialyl Tn antigen: Siaα2,6GalNAc-O-Ser/Thr	▲	ST6GalNAc II	
Fucosyl-motifs	▲▼	Fucosyltransferases (FucT)	▲▼
Core fucosylation of N-linked sugar chains: Fucα1,6GlcNAc	▲	Coreα6FucT	
Human blood group H: Fucα1,2Galβ1,3GlcNAc	▲▼	α2FucT	▼
Human blood group Lewis: Galβ1,3/4[Fucα1,3/4]GlcNAc-	▲▼	α3/4FucT (FucIII)	▲
Sialyl Lewis^x / Lewis^x: Siaα2,3Galβ1,4[Fucα1,3]GlcNAc-	▲	FucT-IV, -V, -VI	
Galactosyl-motifs		Galactosyltransferases (GalT)	
Poly-N-acetyllactosamine: [Gal(β1,4)GlcNAc(β1,3)]$_n$	▲	β4GalT	▲

▲ increased ▼decreased

tumors can express a histo-blood group which is not compatible with the erythrocytic blood group due to aberrant synthesis by blood-group-dependent glycosyltransferases (42).

One change common to cancer is increased activity of serum galactosyltransferases (GalT), i.e., specifically, an alteration in the activity, size, glycosylation and secretion of β4GalT (43, 44). However, galactosylation can also be changed in a variety of benign diseases.

Glycans and reproductive tract cancers

Reproductive tract cancers are complex and heterogeneous, comprising diseases with diverse origin, clinical symptoms and outcome. Among them, prostate cancer (PCa) is the primary cause of cancer-related death in males, whereas ovarian cancer, as the most aggressive, has the highest fatality rate among all gynecological cancers. In contrast to the wealth of literature data on prostate cancer and ovarian cancer dealing with different clinical and pharmacological examinations, there are few studies on glycobiological aspects. Generally, in relation to glycans, the synthetic and secretory activity of both tissues is profoundly changed at an early stage as well as during tumor progression (45).

Glycopattern of prostate cancer

Concerning the prostate tissue-related oligosaccharide pattern, lectin histochemisty has revealed an increase of sialic acid, galactose and fucose in human prostatic carcinoma compared to normal tissue (46). Moreover, increased expression of the GalNAcα1, 3GalNAc-Gal moiety and triantennary complex-type oligosaccharide units in dysplastic cells and androgen-independent tumor were also observed when the glycosylation patterns of normal, dysplastic and androgen-independent prostate cancer in rats were compared (47). At the cell level, glycan heterogeneity also exists among different prostatic cells and carcinoma cell lines, i.e. there is a relationship between cell-surface oligosaccharide structures and phenotypic variants within a defined tumor-cell population. The pattern of oligosaccharide expression, identified by lectin-binding (*Table II*), was found to differentiate clearly between the tumor sublines and to distinguish them from normal rat prostatic epithelium (48).

Human prostatic carcinoma cell lines differ in their expression of FucT. Thus, the LNCaP cell line is unique in expressing α1,2 FucT as the exclusive FucT (49). It has a catalytic role in the expression of blood group H, Lewis^b and Globo H antigen. ABO blood groups and Lewis antigens are characteristicaly changed in prostatic cancer and might be potential markers of disease. Lewis^y and sialyl Lewis^x antigen are absent or present in low amounts in benign tissue, but are more highly expressed in cancer (50). Moreover, sialyl Lewis^x may be an important marker in patients with metastatic hormone-resistant cancer and correlates with a poor prognosis (51).

β4GalT can also increase in prostate cancer (52). Among different related changes, an abnormality in galactosylation of serum IgG oligosaccharides chains has been found with prostate cancer progression (53). Monogalactosyl oligosaccharides and digalactosyl oligosaccharides decreased significantly, while agalactosyl IgG oligosaccharides increased with PCa tumor progression.

Some of the reported molecular markers of PCa are glycosylated, but there are no data on their structural changes in cancer (54). Among them, prostate-specific antigen is the most useful tumor marker in the detection and monitoring of prostate cancer. It is known to exhibit remarkable heterogeneity and is currently the object of intensive structural investigations aimed at improving its diagnostic usefulness.

Table II Lectins commonly used for glycan analysis.

Abbreviation	Lectin	Major carbohydrate-binding specificity
Con A	*Canavalia ensiformis* agglutinin	high mannose, hybrid and biantenary complex type N-glycans
PHA-E	*Phaseolus vulgaris* erythroagglutinin	GlcNAcβ1,4 linked to the β-mannosyl residue of the trimannosyl core
PHA-L	*Phaseolus vulgaris* leukoagglutinin	2,2,6 tri- and 2,2,4,6- tetraantennary complex type N-glycans
WGA	*wheat germ* agglutinin	GlcNAcβ1,4GlcNAcβ1,4GlcNAc
RCA I	*Ricinus communis* agglutinin I	Galβ1,4GlcNAc
UEA I	*Ulex europaeus* agglutinin I	Fucα1,2Gal
AAA	*Aleuria aurantia* agglutinin	Fucα1,6GlcNAc
SNA	*Sambucus nigra* agglutinin	sialic acid α2,6Gal/GalNAc
MAA	*Maackia amurensis* agglutinin	sialic acid α2,3Galβ1,4GlcNAc
SBA	*soybean* agglutinin	GalNAcα1-Ser/Thr
PNA	*Arachis hypogaea* agglutinin	Galβ1,3GalNAcα1-Ser/Thr
WFA	*Wisteria floribunda* agglutinin	GalNAcβ1,4GlcNAc

Glycoforms of prostate-specific antigen

Prostate specific antigen (PSA) or human kallikrein 3 is an extracellular serine protease predominantly expressed by the epithelial cells of the prostate (55). Studies for the establishment of an international PSA standard defined PSA from seminal plasma (SP PSA) as a single-chain polypeptide, carrying an N-linked oligosaccharide chain of the N-acetyllactosamine type, with α2,6 linked sialic acid groups at the end of two branches (56). According to these results of NMR spectroscopy, oligosaccharides represent 8.3% of the total molecular mass.

However, accumulated experimental evidence has indicated that it comprises heterogenous molecules differing in their polypeptide backbone as well as in the carbohydrate composition, resulting in the existence of different molecular forms, i.e different PSA isoforms (55, 57–60). Besides the major PSA subpopulation bearing biantennary N-glycan, PSA molecules with uncommon N-glycan structures, such as monoantennary glycan chains or different outer chain moieties, were detected (61–63). Thus, enzymatic digestion and subsequent fractionation of oligosaccharides indicated that two seminal plasma PSA isoforms have both mono- and biantennary glycans, differing in the ratio of mono- and disialylated sugars and the ratio of outer chain moieties: Galβ1-4GlcNAcβ1-, GlcNAcβ1- and GalNAcβ1-4GlcNAcβ-1 (62).

A catalog of molecular forms of SP PSA, established by means of two-dimensional electrophoresis, mass spectrometry and amino-acid sequencing, indicated approximately twenty PSA proteins and fragments, of which eleven were glycosylated (64). They contained the glycosylation site (Asn 45) and full-length PSA, although one full-length PSA was found not to be glycosylated. Thus, comparative analysis revealed that PSA from non-cancerous tissue has molecular masses from 6 to 28 kDa, whereas PSA from cancerous material did not contain lower molecular mass forms (64). However, in both cases, there was evidence for the presence of non-glycosylated as well as glycosylated forms. In addition to this study indicating a full length non-glycosylated PSA form, a PSA transcript produced by alternative splicing and lacking the glycosylation site was also detected (65).

A further additional detailed study based on mass spectrometric analysis confirmed the presence of mono- or disialylated N-glycans associated with SP PSA isoforms (63). Both α2,6 and α2,3 linked sialic acid moieties were found, while core fucosylation was detected in 80% and GalNAc was present in 25% of SP PSA molecules. Subsequent studies based on the lectin-binding patterns of SP PSA favor the proposed structure regarding sialylation, fucosylation and glycan branching (66–69).

Nevertheless, literature data obtained for SP PSA, and PSA from serum, prostate tissue, prostate cancer cell lines and urine, indicated differences and heterogeneity related to the examined sources, as well as pronounced changes associated with the pathological conditions of benign prostatic hyperplasia (BPH) and prostate cancer, in both the peptide and glycan parts of the PSA molecule (*Table III*). For instance, the precursor form of PSA, pro-PSA, is more evident in PCa than in BPH. A specific form of clipped free PSA known as bPSA is typical of BPH (70, 71). Thus, BPH PSA consists of molecules with multiple internal cleavage sites, and lower chymotrypsin-like activity than SP PSA (55, 71). BPH PSA isoforms have a lower pI than PCa PSA, and it is suggested that an irregular glycosylation process in the dysplastic cells of the prostate might be responsible for this difference (64).

Generally, based on chemical analysis and the lectin-binding patterns of serum (72–74), prostate

Table III Alterations in N-linked sugar-chain of PSA during BPH and PCa.

Glycostructure	Relative amounts[5]
biantennary complex type chain with/without Fucα1,6GlcNAc	decreased in PCa PSA[1,2] compared to BPH PSA[1,2]
multiantennary complex type chain with branched GlcNAcβ1,4Man	increased in PCa PSA[1,2] compared to BPH PSA[1,2]
sialic acid (total)	undetected/low in LNCaP[3] PSA
	decreased in PCa PSA[2,4] and BPH PSA[2]
sialic acid α2,3 /α2,6Gal (ratio)	decreased in PCa PSA[2,4] compared to SP PSA
Fucα1,6GlcNAc	increased in LNCaP PSA compared to SP PSA
	decreased in PCa PSA[4] compared to SP PSA
	decreased in PCa PSA[2] compared to BPH PSA[2]
Fucα1,2Gal (H2 epitope)	only in LNCaP PSA
	undetected in PCa PSA[4]
terminal GalNAc	increased in LNCaP PSA compared to SP PSA
	undetected in PCa PSA[4]

[1] prostate tissue extract
[2] urine
[3] LNCaP cell line
[4] serum
[5] estimated by chemical analysis or lectin-binding patterns

tissue extract (66) and urine (68, 75), PSA of cancer origin showed decreased glycosylation compared to PSA from BPH and seminal plasma. Seven PSA fractions bearing different oligosaccharide structures were resolved from both BPH- and PCa-tissue extracts by means of serial lectin affinity-chromatography (66). It was suggested that the ratio of these distinct glycans can be related to a higher level of expression of GnT IV, and a generally altered glycosylation process during malignant transformation. Thus, the amounts of PSA passing through columns of ConA, PHA-L and PHA-E were significantly greater for PCa PSA than BPH PSA. Except for Con A reactivity, a similar decrease in lectin-binding was also observed when comparing urinary forms of BPH PSA and PCa PSA (68). Generally, PSA of cancer origin (tissue or LNCaP cell line) was found to have more branched glycans, i.e. it contained a higher percentage of multiantennary chains (bi-, tri- and possibly tetraantennary), compared to BPH PSA (62, 66), or SP PSA (61, 63). However, the results regarding the sialylation of cancer-associated PSA are different depending on the source examined and in some cases they are conflicting. For instance, a total absence of sialylation (63) and the existence of α2,3 linked-sialic acid in PSA from the LNCaP carcinoma cell line (67) were reported. Specifically, serum PCa PSA was recently observed to be sialylated in a ratio similar to SP PSA, but with a lower proportion of α2,3/α2,6 linked sialic acid, than SP PSA (69). The results of lectin-affinity chromatography of serum PCa PSA also indicated the presence of both types of sialic acid linkage, but the abundance of α2,6 linked-sialic acid was lower in metastatic PCa PSA in comparison to PCa PSA from localized cancer (74). In addition, no significant difference in binding related to α2,3 linked sialic acid was noticed except for the loss of distinct reactive molecular subpopulations.

Regarding fucosylation, an increase in core fucosylation was found in LNCaP PSA (~100%) compared to SP PSA (80%), whereas the presence of H2 epitope (Fuc α1,2Gal) was detected only in 10–15% of LNCaP PSA (63). However, serum PCa PSA was found to have a lower fucose content than SP PSA (69, 74). Compared to SP PSA, the results of lectin-affinity chromatography of urinary BPH PSA and urinary PCa PSA, as well as metastatic serum PCa PSA, indicated a decrease in reactivity related to the core fucosylated structure, i.e. α1,6-linked Fuc, but not to fucose in the outer chain position, i.e. α1,2-linked Fuc (68, 74). In addition, pronounced serum PCa PSA heterogeneity in respect to α1,6 Fuc in the core was observed, with no significant differences between localized and metastatic prostate cancer (74).

PCa PSA was also reported to differ from SP PSA in GalNAc content. LNCaP PSA was found to have a higher GalNAc content (65%) than SP PSA (25%), whereas serum PCa PSA had no terminal GalNAc (63, 69).

Glycopattern of ovarian cancer

The ovarian cancer glycophenotype shares the characteristics found in other reproductive tract cancers, but there are also some typical structural alterations. Thus, similarly to other gynecological cancers, an increase in FucT activity was found to be associated with ovarian cancer (76). The elevated fucose content observed in haptoglobin and α-1 proteinase inhibitor may be related to the rise in α1,2-, α1,3- and particularly α1,4-FucT activities (77).

These two proteins are acute phase reactants and also exhibit changes in N-linked glycan branching and sialylation. It is thought that this happens via different mechanisms. In the glycan chains of α-1-proteinase inhibitor, there are more biantennary chains, fewer branches ending in α2,3 sialic acid and more branches ending in α2,6 sialic acid. There is also more fucose, probably linked α1,6 to the core region. On the other hand, haptoglobin shows increased branching (more triantennary chains). More branches end in α2,3 sialic acid, fewer branches end in α2,6 sialic acid, and there is more fucose, probably in α1,3 linkage at the end of the chains. Thus, concerning the expression of sialyltransferases (ST) in ovarian cancers, the mRNA of three STs, including ST3Gal III, ST3Gal IV, and ST3Gal VI, was significantly decreased in patients with ovarian cancers (78). By contrast, mRNA expression of ST3Gal I and ST6Gal I was increased in ovarian cancer tissues. These alterations may be of importance in malignant ovarian cancers, and an increased expression of ST3Gal I may contribute directly to increased α2,3-linked sialylation in ovarian serous carcinoma.

Some studies suggested that the risk of ovarian cancer is associated with the ABO blood group, i.e. that it is higher in blood group A patients (79). As for the other carbohydrate antigens, the expression of sialyl-Tn antigen (NeuAcα2,6GalNAc) is greatly increased in various types of ovarian cancer and is associated with a poor prognosis (41). In some cases, Tn antigen is also increased. In addition, indicators of the malignant potential of ovarian cancer have been reported to be related to the occurrence of antibodies to mucin MUC 1 core protein, and altered proteoglycan gene expression in primary ovarian carcinoma tumors and secondary metastases (80, 81).

The occurrence and elevated activity of β4Gal transferase isoforms as well as the presence of aberrant tumor-reactive IgG populations resulting from altered glycosylation of one of the two Fc chains were found to be associated with ovarian tumors, similarly as in prostate cancer (82, 83). Intensive investigations and great analytical efforts are currently directed to the search for genetic as well as protein biomarkers of ovarian cancer (84). CA125 antigen, known as the hallmark of serous epithelial ovarian carcinoma, is far from being fully explored regarding the diagnostic potential of its glycans (85, 86).

Glycoforms of CA 125 antigen

CA 125, a coelomic epithelium-related antigen, is defined by the monoclonal antibody termed OC125 (87). This antibody was obtained by somatic hybridization of spleen cells from mice immunized with the ovarian carcinoma cell line OVCA433. Estimates of the size of the molecule ranged from 200 kDa for the core subunit, i.e. precursor form, to much larger mature secreted species of over 1–4 MDa (88). Moreover, lower molecular mass species have been revealed in ovarian tissue and cell preparation. As for the structure of CA125, it was claimed to be a glycoprotein with Asn-linked sugar chains (89), and a glycosylphosphoinositol-linked glycoprotein (90). Currently, it is categorized to the mucin family and is designated as MUC 16, based on molecular cloning of a part of its gene (91).

The CA125 molecule is composed of an extracellular domain containing N-terminal domain and a tandem repeat region, a transmembrane region and an intracellular domain (91, 92). The N-terminal domain is extremely large and made up of over 10 000 amino acids. The tandem repeat region is dominated by 40–60 repeats made up of 156 amino acids, each of them having a disulphide bridge loop of 19 amino acids. Although the overall structure is well conserved, only a few of repeats are identical in terms of amino acid sequence. The cytoplasmic tail is relatively short, consisting of 256 amino acids, and it possesses a phosphorylation site.

The primary structure of CA125 revealed numerous potential glycosylation sites. They are related to motifs known to be often associated with O-glycosylation such as clustered serine and threonine residues adjacent to or nearby a proline residue, located in the tandem repeat region (93). In addition, motifs associated with N-glycosylation were found to be located throughout the CA 125 sequence (94). The initial investigations regarding CA125 glycosylation indicated that the molecule contains 28% of carbohydrates, with a predominance of O-linked oligosaccharide chains forming a hairbrush structure at the N-terminal domain, but the presence of N-linked sugar chains was also confirmed (95, 96). The mannose content was found to vary between molecules of different origin. Thus, OVCAR-3 cell line-derived CA125 has a lower mannose content than the OVCA-433 cell line-derived antigen (94, 96).

The glycan composition of OVCAR-3 carcinoma cell line-derived CA125 has been extensively analysed (96). The analysed glycans were found to be mostly O-linked. Core type 1 (Galβ1,3GalNAc) was predominant, but core type 2[GlcNAcβ1,6 (Galβ1,3)GalNAc] was also present in these glycans. A specific feature of branched core type 1 glycans is the presence of either sialic acid or fucose. Core type 2 structures were found to have up to two polylactosamine chains. The least abundant forms of O-glycans are difucosylated O-linked side chains. They may carry either Lewis^x or Lewis^y epitopes or blood group H-epitopes. Earlier reports have suggested that both Lewis^x and Lewis^y epitopes are present at a single CA125 antigenic moiety (94).

Regarding N-linked oligosaccharides, high mannose (more than 80%) and complex type bisecting structures were detected. Mono-fucosylated biantennary complex type glycans were found to be the most abundant, whereas tri- and tetraantennary type glycans were present in lower amounts. Sialic acid, attached to galactose at the 3-linked and 6-linked positions, occured in both N- and O-glycans. However, the level of sialylation was low and no components carrying more than one sialic acid were detected.

Different glycosylation patterns and possible variations in the production of CA125 glycoprotein in reproductive and related tissues have been presumed for some time (97, 98, 99). Data on glycosylation of OVCAR-3 cell line-derived CA125 in combination with the data on both pregnancy- and cancer-associated CA125, obtained using lectin-affinity chromatography as a method for structural assessment, point to the existence of glycosylation differences (*Table IV*) (97). Strong binding of both pregnancy-associated and cancer-derived CA125 to WGA (wheat germ

Table IV Differences in glycosylation of cancer-associated CA125 and pregnancy-associated CA125.

Glycostructure[1]	Relative amounts[2]
high mannose/biantennary complex type N-glycans	increased in cCA125
GlcNAcβ1,4 linked to the β-mannosyl residue of the trimannosyl core	increased in cCA125
2,2,6 tri- and 2,2,4,6- tetraantennary complex type N-glycans	low in cCA125, undetected in pCA125
Fucα1,6GlcNAc	comparable in pCA125 and cCA125
Galβ1,4GlcNAc	increased in cCA125
GalNAcα1-Ser/Thr	increased in cCA125
sialic acid α2,3Gal	comparable in pCA125 and cCA125
sialic acid α2,6Gal/GalNAc	increased in cCA125

[1] according to lectin-binding specificity

[2] assessed by lectin-binding patterns

agglutinin) was observed, which is consistent with its high affinity towards mucine type molecules. WGA, RCA (*Ricinus communis* agglutinin I), PNA (*Arachis hypogaea* agglutinin) and SBA (soybean agglutinin) reactivity allowed us to deduce the presence of polylactosamine structures, as well as Galβ1,3GalNAc and GlcNAcβ1,6(Galβ1,3)GalNAc structures as O-glycan-associated structures. SBA-reactivity was more pronounced towards the cancer-derived antigen. Structural differences related to the presence of oligosaccharides with terminal GalNAcβ1,4GlcNAc in cancer-derived CA125 antigen were also detected (97).

The ConA (lectin from *Canavalia ensiformis*), PHA-E (*Phaseolus vulgaris* erythroagglutinin) and PHA-L (*Phaseolus vulgaris* leukoagglutinin) binding patterns, used to assess the composition of N-glycans, suggested a lower content of high mannose glycans in pregnancy-related CA125 compared to the cancer-related antigen. In addition, differences in PHA-E reaction indicated an increase in GlcNAcβ1,4 linked to the β-mannosyl residue of the trimannosyl core in cancer-derived CA125.

Assessment of the extent and type of fucosylation indicated the presence of fucose linked α1,6 to core mannose and fucose linked α1,3 to side chains, with no great difference between the cancer- and pregnancy-related forms. The presence of sialic acid linked α2,6 and α2,3 to galactose on both the cancer- and pregnancy-associated CA125 forms was confirmed, the former one exhibiting an increase in sialic acid linked α2,6. Thus, glycosylation of pregnancy- and cancer-associated CA125 assessed by experimentally obtained lectin-binding patterns points out the main differences attributed to N-glycan-associated structures reactive with ConA and PHA-E and O-glycan-associated structures reactive with SBA, SNA and WFA.

Discussion

Diagnostic potential of tumor marker-associated glycans

Measurement of serum concentrations of tumor markers for reproductive tract cancers, widely used in laboratory practice, is often associated with the problem of low specificity of cancer detection (100). One reason is that their concentrations can be elevated during both benign or malignant processes. For instance, a rise in serum PSA within the concentration range of 4–10 μg/L found in both PCa and BPH does not allow us to distinguish between these pathological processes (55). Similarly, an elevated concentration of CA125 antigen, known as the hallmark of serous epithelial ovarian carcinoma, has also been confirmed in a variety of benign or malignant diseases that stimulate peritoneal synthesis, conditions that affect the endometrium, or even in healthy subjects (85, 101). This influences the clinical usefulness of CA125 for screening or monitoring the clinical course and disease status in patients who have histologically confirmed ovarian malignancies.

So far, glycosylation analysis has pointed to extreme heterogeneity, i.e. the existence of many different molecular glycoforms of PSA and CA125. The biological meaning of this heterogeneity is not yet understood and, due to the structural complexity, more experiments are needed to gain complete insight into all existing forms and for their complete characterization. However, the accumulated experimental evidence indicates that the structural properties of these tumor markers may be more relevant in distinguishing between benign and malignant conditions than measurement of their concentrations alone. Thus, serum PSA-Con A reactivity has already been suggested to be useful in the monitoring of prostate function, but has suffered from inconsistent results (72, 73). Recent results suggest that differences in sialylation, fucosylation and branching of the PSA molecule may be more relevant for distinguishing BPH from PCa. Thus, PSA-lectin interactions await more extensive studies with larger numbers of patients, and should be additionally addressed in other detection systems to make possible their exploration in clinical evaluation of prostate health. The same holds true for CA125 which, in contrast to PSA, has a far more complex structure and has not been so extensively investigated from the basic molecular aspects.

Thus, clinical expectations related to cancer detection are primarily related to discovering highly sensitive and specific tumor markers, in order to eliminate any doubts and controversies, which are often encountered in laboratory practice when interpreting assay results. The use of a panel of different tumor markers seems to be very promising for the elimination of many false positive results (54, 84, 102). However, there is still a need for better differential diagnostic tools. Cancer-associated changes in the glycosylation of tumor markers and other proteins provide a basis for the development of more sensitive and discriminative clinical tests, i.e. for improving the clinical utility of known tumor markers or for finding new ones. In this respect, the comparative study of sugar chain structures has attracted attention and is expected to provide information on glycomarkers useful for differential diagnosis, the follow-up of patient response to therapy and the scoring of the metastatic potential. The diagnostic significance of microheterogeneity in various glycoproteins has been confirmed and is already used to correlate their properties to their origin (12, 17). Various techniques are employed in experimental work, but still there are no wide spectra of commercial assays for the detection of specific glycoforms of tumor markers. The well known and widely used assay for the detection of CA 19-9 antigen is based on the carbohydrate antigenic component corresponding to sialylated-lacto-

N-fucopentaose II, while the assay for the detection of CA15-3 antigen is related to the aberrantly, i.e. less glycosylated form of mucin MUC1.

Glycans as tools and targets hold promise for the use in the field of cancer detection and their full exploration in the pharmaceutical and biotechnological industry is awaited.

Future outlook

The interdisciplinary character of glycobiological investigations is a good basis for cooperation of academic and biotechnological investigators. There is a growing interest in the spread of ideas and industrial partnerships dealing with the development of techniques to support the requirements of human experimental and clinical pathology. Some of the ongoing projects have been raised to the level of national importance and a consortium of international collaboration is being established (103).

Moreover, glycomic profiling of cells and tissues is one focus of investigation in contemporary glycoscience. The term glycome refers to the entire set of glycans in one organism and it was introduced in the post-genomic era analogously to the terms genome and proteome (104). Work on functional and structural glycomics projects is expected not only to answer many fundamental biological questions, but also to highlight the diagnostic and clinical importance of glycans (105, 106). Thus, glycobiological research is continuing to produce significant information and create a wide range of attractive possibilities for biomarker discovery with clinical applications. Structural analysis of glycans is very complex, but there have been significant advances in the field. Several conventional methods, such as chromatography or two-dimensional electrophoresis adapted for oligosaccharide characterization, nuclear magnetic resonance, HPLC with pulsed-amperometric detection and mass spectrometry, are currently in use and many others are being improved or developed to serve these purposes. They include automation of mass spectrometry and various array-based technologies using immobilized glycans or lectins (107).

Nevertheless, in terms of designing a strategy for biomarker discovery, new principles and platforms for the analysis of relatively small amounts of numerous glycoproteins and accurate monitoring on the level of the glycoproteome are needed. Lectins, recognizing specific oligosaccharide structures or carbohydrate-specific monoclonal antibodies, seem to be inevitable participants in the creation of new research tools. Screening of different types of glycomolecules, selection of differentially expressed components, their enrichment and purification or identification are the most challenging parts of experimental and clinical glycoproteomics. They require large-scale technologies enabling high sensitivity, proper standardization and validation of the methods to be used. Considering glycans as tumor markers raises several pressing questions:

- How to cope with glycan heterogeneity in both normal and pathological conditions, i.e. how to pick up relevant changes relating to the heterogenous molecule alone?
- Are we able to detect relevant glyco-changes if they occur at a very low level, i.e. can we catch the early appearance (preceding the cancer) of glyco-markers?
- Can we reach the advantage of using a rapid and cost-effective platform accomodated to everyday use in clinical practice?

Further progress in the field of applied glycoscience requires an integrated systematic approach in order to explore properly all opportunities arising from deciphering the glycocode.

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