Koichi Furukawa
Minoru Fukuda *Editors*

Glycosignals in Cancer

Molecular Assembly and Recognition

Second Edition

Springer

Glycosignals in Cancer

Koichi Furukawa • Minoru Fukuda
Editors

Glycosignals in Cancer

Molecular Assembly and Recognition

Second Edition

Springer

Editors
Koichi Furukawa
Department of Biomedical Sciences
Chubu University College of Life and Health Sciences
Kasugai, Aichi, Japan

Minoru Fukuda
Glycobiology Unit
Sanford-Burnham Medical Research Institute
La Jolla, CA, USA

ISBN 978-981-19-7731-2 ISBN 978-981-19-7732-9 (eBook)
https://doi.org/10.1007/978-981-19-7732-9

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Contents

Part I
Search for Novel Tumor Epitopes Related to Carbohydrates

The Ganglioside Structures: Chemistry and Biochemistry

Laura Mauri and Sandro Sonnino

Abstract Gangliosides are the main glycosphingolipids of the central nervous system where they are involved in the processes of cell signaling through interactions with membrane proteins, many of which are receptors. The history of gangliosides started from the end of the nineteenth century, but the clear understanding of their structure only took place from the 1940s to the 1960s of the following century.

In this article we report on the chemical, physicochemical, and biochemical properties of gangliosides.

1 Gangliosides

The name of "gangliosides" was introduced at the end of nineteenth century, by J.L.W. Thudichum (Thudichum 1962) who reported for the first time on the isolation from human brain of unknown compounds that for some of their features resulted "mysterious." The name was clearly associated to the mystery of questions posed by the Sphinx to the wayfarers entering into the city of Thebes. Originally, the first compounds that were characterized resulted to be the simple cerebrosides, monoglycosylceramides. However, in spite of the fact that the chemistry of sphingolipids gained the attention of several investigators, progress in this field was extremely slow, and more than another half century was needed to establish the correct structure of sialic acid, of sphingosine, and of several gangliosides (Carter et al. 1947; Gottschalk 1955; Kuhn and Wiegandt 1963).

L. Mauri · S. Sonnino (✉)
Department of Medical Biotechnology and Translational Medicine, University of Milan, Segrate, MI, Italy
e-mail: laura.mauri@unimi.it; sandro.sonnino@unimi.it

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_1

2 Ganglioside Chemical Structure

Gangliosides are glycolipids characterized by a sialic acid containing oligosaccharide linked to a lipid moiety called ceramide. Ceramide is double chain lipid composed by a lipidic basic amino chain, trivially called sphingosine, amidically linked to a long chain fatty acid. Figure 1 reports the general structure of gangliosides. Both the oligosaccharide chain, the soluble head group of ganglioside protruding into the cell aqueous environment, and the ceramide, the hydrophobic moiety of ganglioside tenaciously inserted into the cell outer layer, represent, with their structures, a specific code responsible for the biological properties of gangliosides (Lunghi et al. 2021).

2.1 The Ganglioside Oligosaccharide Chain

The oligosaccharide chain of gangliosides is formed by a sequence of neutral sugars present in both α and β configuration and linked each other by glycosidic linkage. The main sugars found in mammals are glucose, galactose, fucose, *N*-acetylgalactosamine, and *N*-acetyl-glucosamine. With a single exception, the ganglioside GM4 (see below), glucose is the sugar with free reducing C1 available for linkage with the ceramide moiety (Chiricozzi et al. 2021).

To the neutral oligosaccharide chain, one to five sialic acid in α configuration, is chetosidically linked to galactose or in much less extent to a hexosamine. Sialic acid (Schauer 1982) is the trivial name for all the derivatives of the neuraminic acid, the 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid. Many sialic acids are present in nature, but in healthy humans, only the 5-*N*-acetyl- and the 5-*N*-acetyl-9-*O*-acetyl-neuraminic acid are present; the latter represents about 10% of total sialic acid (Kamerling and Vliegenthart 1975) (Fig. 2). The 5-*N*-glycolyl-neuraminic acid, quite common in many mammals, has been found in some human cancer tissues (Yu and Ledeen 1972).

Ganglioside oligosaccharide is codified according to the structure of neutral chain as reported in Table 1 and according to the IUPAC-IUB (Chester 1998).

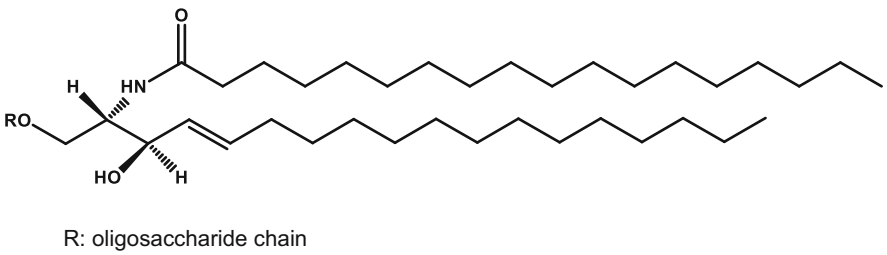


Fig. 1 General structure of gangliosides

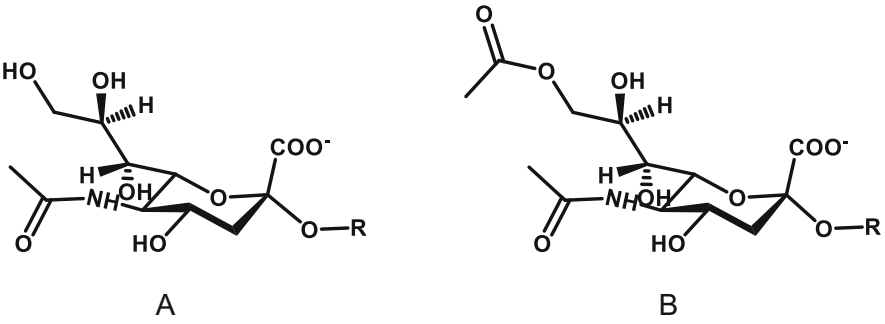


Fig. 2 Structure of Neu5Ac (**a**) and Neu5,9Ac$_2$ (**b**)

Table 1 Structures of the main neutral oligosaccharide chains of gangliosides in vertebrates. Galactose of the Gal series is linked to the position 1 of ceramide (see below). In all the other series, this is done by glucose

Series	Abbreviation	Structure
	Gal	ß-Gal-
	Glc	ß-Glc-
	Lac	ß-Gal-(1-4)-ß-Glc-
Ganglio-3	Gg_3	ß-GalNAc-(1-4)-ß-Gal-(1-4)-ß-Glc-
Ganglio-4	Gg_4	ß-Gal-(1-3)-ß-GalNAc-(1-4)-ß-Gal-(1–4)-ß-Glc-
Ganglio-5	Gg_5	ß-GalNAc-(1-4)-ß-Gal-(1-3)-ß-GalNAc-(1-4)-ß-Gal-(1-4)-ß-Glc-
Globo-3	Gb_3	α-Gal-(1-4)-ß-Gal-(1-4)-ß-Glc-
Globo-4	Gb_4	ß-GalNAc-(1-3)-α-Gal-(1-4)-ß-Gal-(1–4)-ß-Glc-
Globo-5	Gb_5	ß-Gal-(1-3)-ß-GalNAc-(1-3)-α-Gal-(1-4)-ß-Gal-(1-4)-ß-Glc-
Isoglobo-3	iGb_3	α-Gal-(1-3)-ß-Gal-(1-4)-ß-Glc-
Isoglobo-4	iGb_4	β-GalNAc-(1-3)-α-Gal-(1-3)-β-Gal-(1–4)-β-Glc-
Lacto-3	Lc_3	β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-
Lacto-4	Lc_4	ß-Gal-(1-3)-ß-GlcNAc-(1-3)- ß-Gal-(1–4)-ß-Glc-
Neolacto-4	nLc_4	ß-Gal-(1-4)-ß-GlcNAc-(1-3)- ß-Gal-(1-4)-ß-Glc-
Neolacto-6	nLc_6	ß-Gal-(1-4)-ß-GlcNAc-(1-3)-ß-Gal-(1-4)-ß-GlcNAc-(1-3)-ß-Gal-(1-4)-ß-Glc-

Each series linked to ceramide is abbreviated as reported in Table 2.

The additional sugar residues like sialic acid, fucose, or an hexosamine should be named by referral to the root oligosaccharide and locating the additional substituents by a Roman numeral designating the position of the substituent in the root oligosaccharide (counting from the ceramide end) to which the substituent is attached, with an Arabic numeral superscript indicating the position on that residue which is

Table 2 Glycolipid abbreviation according to the oligosaccharide series

Abbreviation of oligosaccharide series	Abbreviation of corresponding glycolipid
Gal	GalCer
Glc	GlcCer
Lac	LacCer
Gg_3	Gg_3Cer
Gg_4	Gg_4Cer
Gg_5	Gg_5Cer
Gb_3	Gb_3Cer
Gb_4	Gb_4Cer
Gb_5	Gb_5Cer
iGb_3	iGb_3Cer
iGb_4	iGb_4Cer
Lc_3	Lc_3Cer
Lc_4	Lc_4Cer
nLc_4	nLc_4Cer
nLc_6	nLc_6Cer

Table 3 Examples of abbreviation for gangliosides and complex gangliosides of the ganglio series

II^3-Neu5Ac-LacCer	α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
II^3-Neu5Ac-Gg_3Cer	β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)-]β-Gal-(1-4)-β-Glc-(1-1)-Cer
IV^3-Neu5Ac-Gg_4Cer	α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
II^3-Neu5Ac-IV^3-Neu5Ac-Gg_4Cer	α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)-]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
II^3-Neu5Ac-IV^3-$(Neu5Ac)_2$-Gg_4Cer	α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-]β-Gal-(1-4)-β-Glc-(1-1)-Cer
II^3-Neu5Ac-Gg_5Cer	ß-GalNAc-(1-4)-ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)-]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
IV^2-α-Fuc-II^3-Neu5Ac-Gg_4Cer	α-Fuc-(1-2)-ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)-]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
IV^6-Neu5Ac-nLc_4Cer	α-Ne5Ac-(2-6)-βGal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
IV^3-Neu5Ac-nLc_4Cer	α-Ne5Ac-(2-3)-βGal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
IV^3-Neu5Ac-Lc_4Cer	α-Ne5Ac-(2-3)-βGal-(1-3)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
III^6-Neu5Ac-Lc_4Cer	βGal-(1-3)-[3]β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer

substituted. The anomeric configuration should also be specified. That of sialic acid is always α and can be omitted. Table 3 reports a few examples.

Lars Svennerholm (Svennerholm 1980) introduced a simpler nomenclature for gangliosides extracted from the brain and belonging to the Gal, Lac, and ganglio series, now worldwide used and accepted by the IUPAC-IUB.

In this system, the fact that it is dealing with gangliosides is indicated by the letter G; the number of sialic acid residues is stated by M for mono-, D for di-, T for tri-, and Q for tetra- and P for penta-sialosylglycosphingolipids. A number is then assigned to the individual compound.

Number **1** was assigned to the structure β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer, number **2** to the structure β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer, number **3** to the structure β-Gal-(1-4)-β-Glc-(1-1)-Cer and number **4** to the structure β-Gal-(1-1)-Cer.

GM1 is available in two structures designed with letters **a** and **b:** GM1a often, named only GM1, is the β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Ne5Ac-(2-3)-]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1b is the α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer.

In disialosyl-, trisialosyl-, and tetrasialosylgangliosides, where residue(s) of sialic acid can be linked to the galactose IV, of the series **1**, the letter **a** refers to one residue of sialic acid linked to the galactose at position II, the letter **b** refers to two residues of sialic acid linked to galactose at position II, and the letter **c** refers to three residue of sialic acid linked to galactose at position II.

In disialosyl- and trisialosylgangliosides of the series **2** and **3**, where the residues of sialic acid are linked to galactose, no letters are used.

Much later, trivial abbreviation involving the Greek letter α has been assigned to gangliosides with residue(s) of sialic acid linked to GalNAc.

Table 4 reports the abbreviation according to Svennerholm' nomenclature for the main gangliosides and a few additional trivial code for additional gangliosides. Table 5 reports the abbreviation for a few gangliosides having Neu5Ac linked to hexosamine. Finally, Table 6 reports graphical information for the main sugars occurring in gangliosides and Fig. 3 some examples of ganglioside graphical representation (Neelamegham et al. 2019).

2.2 The Ganglioside Ceramide Structures

Ceramides are *N*-acylated long-chain aliphatic amino alcohols called sphingoids.

The most commonly occurring sphingoid is the unsaturated compound (2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol, worldwide reported as *d*18:1 or C18-sphingosine (Carter et al. 1947; Karlsson 1970; Carter et al. 1961). The corresponding saturated compound originally named "dihydrosphingosine" should now be as sphinganine, C18-sphinganine, or *d*18:0. Figure 4 shows the correct representation for a 2*S*,3*R*,4*E* sphingoid (Fig. 4a) and the corresponding ceramide (Fig. 4b).

The second more abundant mammalian sphingoid contains 20 carbon atoms, the eicosasphingosine, or *d*20:1 or C20-sphingosine.

The fatty acids of naturally occurring ceramides range in chain length from about C_{16} to about C_{26} and may contain one or more double bonds and/or hydroxyl substituents at C-2. The fatty acid linked to the sphingosine of the nervous system

Table 4 Abbreviation of the main gangliosides

Abbreviation according to Svennerholm	Abbreviation according to IUPAC-IUB
GM4	Neu5Ac-GalCer
GM3	II^3Neu5Ac-LacCer
GD3	$II^3(Neu5Ac)_2$-LacCer
O-acetyl-GD3	II^3[Neu5,9Ac_2-(2-8)-Neu5Ac]-LacCer
GM2	II^3Neu5Ac-Gg_3Cer
GD2	$II^3(Neu5Ac)_2$-Gg3Cer
GM1a	II^3Neu5Ac-Gg_4Cer
GM1b	IV^3Neu5Ac-Gg_4Cer
Fuc-GM1	IV^2αFucII^3Neu5Ac-Gg_4Cer
GalNAc-GM1	II^3Neu5Ac-Gg_5Cer
GD1a	IV^3Neu5AcII^3Neu5Ac-Gg_4Cer
GalNAc-GD1a	IV^3Neu5AcII^3Neu5Ac-Gg_5Cer
GD1b	$II^3(Neu5Ac)_2$-Gg_4Cer
GD1b-lactone	II^3[Neu5Ac-(2-8,1-9)-Neu5Ac]-Gg_4Cer
Fuc-GD1b	IV^2αFuc$II^3(Neu5Ac)_2$-Gg4Cer
GT1a	$IV^3(Neu5Ac)_2II^3$Neu5Ac-Gg_4Cer
GT1b	IV^3Neu5Ac$II^3(Neu5Ac)_2$-Gg_4Cer
O-Acetyl-GT1b	IV^3Neu5AcII^3[Neu5,9Ac_2-(2-8)-Neu5Ac]-Gg_4Cer
GT1c	$II^3(Neu5Ac)_3$–Gg_4Cer
GQ1b	$IV^3(Neu5Ac)_2II^3(Neu5Ac)_2$-$Gg_4$Cer
GQ1c	IV^3Neu5Ac$II^3(Neu5Ac)_3$-Gg_4Cer
GP1c	$IV^3(Neu5Ac)_2$II3$(Neu5Ac)_3$-Gg_4Cer

Table 5 Trivial abbreviations of gangliosides with sialic acid linked to GalNAc

GD1α	IV^3Neu5AcIII^6Neu5AcGg$_4$Cer
GT1α	IV^3Neu5Ac$III^6(Neu5Ac)_2Gg_4$Cer
GQ1α	$IV^3(Neu5Ac)_2III^6(Neu5Ac)_2Gg_4$Cer
Chol-1β	III^6Neu5Ac$II^3(Neu5Ac)_2Gg_4$Cer
Chol-1α-a	IV^3Neu5AcIII^6Neu5AcII^3Neu5AcGg$_4$Cer
Chol-1α-b	IV^3Neu5AcIII^6Neu5Ac$II^3(Neu5Ac)_2Gg_4$Cer

ganglioside ceramide is predominantly stearic acid, representing the 90–95% of the total fatty acids, together with a scant quantity of palmitic and the 20-carbon arachidic acid. In the gangliosides of extra-nervous system, the 24-carbon lignoceric acid is quite abundant. The same is for unsaturated fatty acids. Figure 5 reports the scheme of the synthesis of gangliosides containing *d*18:1 e *d*20:1.

The complete chemical name for a specific ceramide includes the sphingoid and fatty acyl substituents. For example, a ceramide containing 2-hydroxyoctadecanoic acid and *d*18:1 should be abbreviated as *d*18:1, 2-OH 18:0.

Table 6 Graphical information

Name	Abbreviation	Shape	Color	CMYK color assignment	RGB color assignment
Glucose	Glc	Filled circle	Blue	100/50/0/0	0/114/188
Galactose	Gal	Filled circle	Yellow	0/15/100/0	255/212/0
Fucose	Fuc	Filled triangle	Red	0/100/100/0	237/28/36
N-acetylglucosamine	GlcNAc	Filled square	Blue	100/50/0/0	0/114/188
N-acetylgalactosamine	GalNAc	Filled square	Yellow	0/15/100/0	255/212/0
N-acetylneuraminic acid	Neu5Ac	Filled diamond	Purple	38/88/0/0	165/67/153
N-glycolylneuraminic acid	Neu5Gc	Filled diamond	Light blue	41/5/3/0	143/204/233

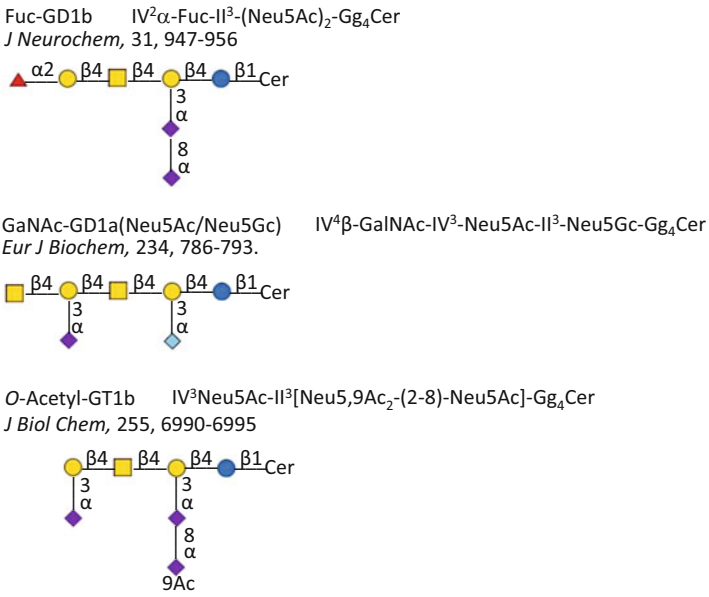


Fig. 3 Graphical representation of the three gangliosides Fuc-GD1b, GalNAc-GD1a(Neu5Ac/Neu5Gc), and *O*-acetyl-GT1b. The three gangliosides are represented with colored and shaped sugars according to Symbol Nomenclature for Glycans (SNFG)-NCBI (nih.gov)

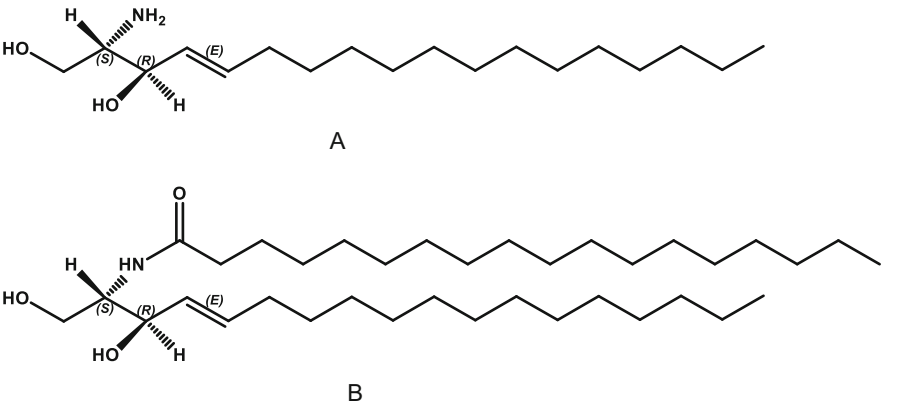


Fig. 4 Stereochemical representation of sphingosine (**a**) and Ceramide (**b**)

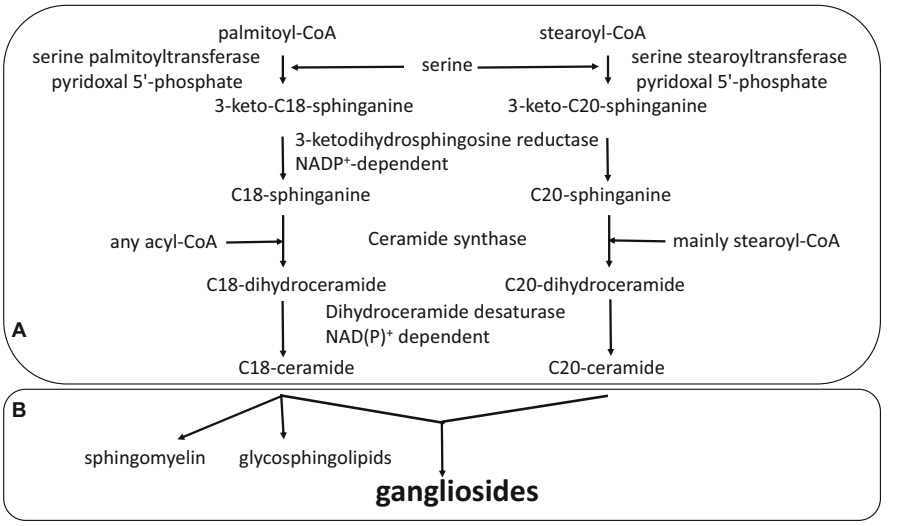


Fig. 5 Scheme of the reactions for the synthesis of ceramide (**a**) and gangliosides (**b**) containing *d*18:1 and *d*20:1

3 Physicochemistry of Ganglioside

Gangliosides are amphiphilic compounds. As components of our cell membranes, they are strongly associated to the plasma membrane outer layer with the ceramide potion and with the soluble oligosaccharide chain free to interact through hydrogen bond with the neighboring molecules.

Gangliosides are considered to carry the driving forces necessary to stabilize membrane domains, worldwide known as lipid rafts, highly enriched of sphingolipids, cholesterol, and dipalmitoylphosphatidylcholine, but containing a

Table 7 The main ganglioside features that favor the formation and stabilization of membrane lipid domains

– The strong hydrophobic interactions with the other hydrophobic membrane components
– The organization at the water-lipid interface of a net of hydrogen bonds both like donor and acceptor, involving the amide linkage of ceramide
– The large surface area required by the monomers to remain inserted into the membrane, due to their big dynamic hydrophilic head group
– The association of water to the head group useful to form bridges with the neighboring membrane components
– Their exclusive association to the outer layer of the plasma membrane favors positive convex curvature useful to stabilize the edges of membrane invaginations

few proteins. This is due to several features associated to their structure that, all together, thermodynamically favor their aggregation (Sonnino et al. 2006, 2007) (see Table 7).

But gangliosides must be carefully manipulated when available in pure form after tissue extraction or when added to cells for any type of studies.

As amphiphilic compounds with very well-balanced hydrophilic-hydrophobic effects, they form aggregates in water solutions (Sonnino et al. 1994). Aggregates are of variable shape, from small quite spherical micelles of GT1b to vesicles of GM3, this depending essentially by the number of sugars in the head group. Differences in the ceramide structure, changing the ganglioside hydrophobic volume, participate in addressing the final aggregate shape.

Thus, in water solutions the number of monomers is always very low, from 10^{-5} M for GT1b down to 10^{-10} M, or less, for GM3.

Gangliosides added to the medium of cultured cells interact with the cell surface as monomers and become membrane components and located correctly into the lipid raft already enriched of sphingolipids and cholesterol. Then these gangliosides, now indistinguishable from the endogenous ones, enter into the metabolic pathway together with the endogenous membrane gangliosides.

Aggregates of gangliosides can interact with membrane proteins remaining weakly associated to their protruding soluble portion. But with time and depending by their shape, they are endocytosed and directed to lysosomes.

All this above, considering that the half-life of monomer release from the aggregate is very long, says that only a very minor portion of gangliosides added to cells in culture become new cell membrane components.

Ganglioside monomers stick strongly to a number of surfaces used in any laboratory, such as glass, dialysis tubes, and plastics. Gangliosides dissolved in organic solvents, like dimethylsulfoxide and ethanol, are often present as monomers or dimers. This property is used to prepare micro titration plate useful to analyze sera for their anti-ganglioside antibody content.

4 Biochemistry of Gangliosides

Gangliosides are components of all the cell membranes but are particularly abundant in the plasma membranes of neurons. They are present on the cell surface, as a pattern of structures differing in both the oligosaccharide and ceramide portions. Their structure and quantity depend on four separate processes in equilibrium with each other, i.e., (1) the de novo biosynthesis, (2) the structural modification on the cell surface, (3) the catabolism, and (4) the intracellular trafficking (Tettamanti 2004). Figure 5 reports a scheme of the synthesis of gangliosides and Fig. 6 the ganglioside complex metabolic pathways.

4.1 The De Novo Biosynthesis of Gangliosides

The de novo biosynthesis of gangliosides begins with the synthesis of ceramide occurring in the endoplasmic reticulum. The enzyme acyl-CoA-serine acyl transferase is associated to vitamin B6 and bounds a palmitoyl or a stearoyl chain to serine forming the C18 and the C20 3-ketosphinganine, respectively. At the beginning of life, the enzyme hardly recognizes the stearoyl-CoA, but this progressively occurs, and in aged mammals, gangliosides containing C20 sphingosine are particularly abundant. Following, the 3-keto compound is reduced and acylated to form the

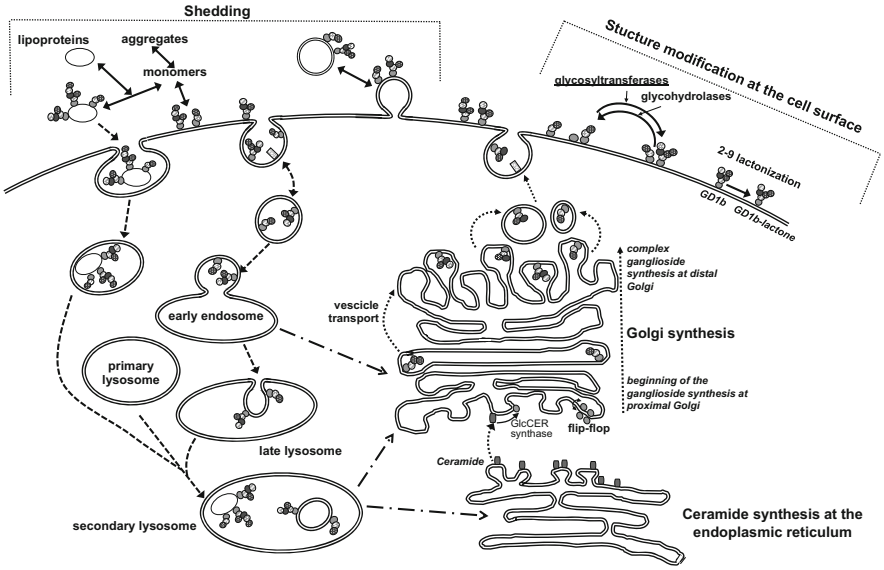


Fig. 6 Cartoons representing the ganglioside metabolic processes: the synthesis at the endoplasmic reticulum and Golgi apparatus, the structural changes occurring at the cell surface, the lysosomal catabolism, and the intracellular/extracellular trafficking

dihydroceramide, and the dihydroceramide is oxidized to ceramide in a reaction NADP dependent Fig. 5. A reports the scheme of reactions for the synthesis of ceramide that can be summarized in the sequence acyl-CoA + serine → 3-keto-sphinganine → sphinganine → dihydroceramide → ceramide (Vanier et al. 1973; Mansson et al. 1978; Chigorno et al. 1997a, b).

Ceramide is complexed with proteins (CERT) or forms vesicles (Giussani et al. 2008). Both the aggregates reach the Golgi apparatus, where the synthesis of the oligosaccharide starts. The type of the aggregate and the structure of the ceramide seem to direct to the synthesis of glycosphingolipids or of sphingomyelin. For the oligosaccharide synthesis, specific glycosyltransferases recognize the substrates and the nucleotide-activated sugars. Nucleotide-activated sugars are synthesized in the nucleus and transported into the cytoplasm and then to the Golgi apparatus by specific transporters. Ceramide is firstly glycosylated to glucosylceramide on the cytosolic leaflet of *cis*-Golgi membranes by glucosylceramide synthase. From this site, the glucosylceramide reaches the lumen of the late Golgi by vesicular transport, flip-flop, and/or a transport FAPP2-mediated. In this compartment, the synthesis of more complex glycosphingolipids and gangliosides takes place. Sialic acid is attached to the neutral sugar chain by sialyltransferases. Several sialyltransferases specific for different acceptors of sialic acid from cytosine monophosphate-*N*-acetyl neuraminic acid (CMP-Neu5Ac) have been identified in the *trans*-Golgi (D'Angelo et al. 2007; Halter et al. 2007; Lannert et al. 1998; Buton et al. 2002).

The final gangliosides move via vesicular transport to the plasma membranes, and after fusion process, they become components of the external leaflet.

4.2 The Structural Modifications at the Cell Surface

Several reports have erased the notion that the glycosyltransferases are exclusively associated to the Golgi apparatus and that the glycosylhydrolases are exclusively associated to lysosomes. Now we know that some of these enzymes are also associated with the plasma membrane (Sonnino et al. 2010). Probably, the first information on this goes back to 40 years ago, when it was reported that sialyltransferases, working on both glycoproteins and glycolipids, were associated to the cell surface (Roseman 1970; Preti et al. 1980).

But very important, the more recent information that couples of enzymes capable to catalyze opposite reactions are associated to the plasma membranes suggest that changes of glycosphingolipid structures at this site could be the way to change rapidly the GSL plasma membrane concentration, to modify the glycosphingolipid pattern, and to modulate cell functions.

Up today, several enzymes of the ganglioside metabolism have been found associated with the plasma membranes: sialidase and sialyltransferase, β-hexosaminidase and β-*N*-acetylgalactosaminyltransferase, and β-galactosidase and β-glucosidase (Aureli et al. 2011). For the synthesis of gangliosides, CMP-Neu5Ac is necessary. It seems that the specific transporter of it is capable of

moving it from the nucleus to cytosol and from cytosol to the extracellular environment. In any case, CMP-Neu5Ac has been found outside cells and working in association with the plasma membrane-associated sialyltransferase to produce GD3 from GM3 (Crespo et al. 2010; Vilcaes et al. 2011). Sialidase Neu3, an enzyme not present in the lysosomes, seems mainly responsible for the hydrolysis of sialic acid linked to the external galactose or with less extent to a sialic acid linked to galactose. Neu3 modulates the cell surface glycolipid composition acting by *trans* interactions (Papini et al. 2004), being capable to hydrolyze substrates belonging to the surface of neighboring cells. Nevertheless, it is necessary to recall that other sialidases have been found associated to the plasma membranes.

The plasma membrane-associated glycosylhydrolases modify the ganglioside structure and are capable of modulating the plasma membrane concentration of ceramide, by sequential action of Neu3, β-galactosidase, and β-glucosidase, thus modulating the processes ceramide dependent (Aureli et al. 2014). The structure of the plasma membrane-associated β-galactosidase is not known, but probably, and as for β-hexosaminidase and β-glucocerebrosidase, it is the lysosomal enzyme that is transferred to the plasma membrane during a process of fusion of lysosomes with plasma membranes. Two different β-glucocerebrosidases are associated with the plasma membranes. One, the GBA1 (Beutler 1992) derives from lysosomes; the other, the GBA2 (Boot et al. 2007), displays a structure different from that of lysosomes. The lysosomal-plasma membrane-associated enzymes work well under acidic pH; this is determined by the activity of the cell surface proton pumps, located together with gangliosides and glycosylhydrolases in the same lipid raft.

4.3 Ganglioside Catabolism

Lysosomes are the site for ganglioside catabolism. Gangliosides reach the lysosomes through endocytic vesicles. Many vesicles are formed with the means of maintaining the membrane structure by a great membrane replacing. But only a minor portion of them becomes lysosomes, while the larger part rapidly reassociates with the plasma membrane. Several catabolites such as sphingosine, sialic acid, and *N*-acetylgalactosamine escape the lysosomes by means, in some cases, of specific transporters and are recycled for de novo biosynthesis. The catabolic process starts from a free non-reducing end and progressively reduces the length of the chain. Ceramide glycanases capable of removing the total oligosaccharide chain from gangliosides have never been found in mammals. The lysosomal glycosylhydrolases work on gangliosides associated with the outer layer of intralysosomal vesicles whose formation is necessary for a correct presentation of the substrates. In addition to this, specific enzyme "supporters," cofactors or saposins, are necessary for the hydrolysis. The most known within them is the GM2-activator protein that is necessary for the detachment of the *N*-acetylgalactosamine from GM2 and GalNAc-GD1a. Its absence or incorrect structure determines the AB variant of Sandhoff disease (Sandhoff and Sandhoff 2018).

4.4 Ganglioside Intracellular Trafficking

The glycosphingolipid intracellular trafficking and the complete metabolic pathway, involving both biosynthesis and catabolism, are under continuous investigations.

It is claimed that the glycolipid biosynthesis is mainly regulated at the transcriptional level controlling the expression of glycosyltransferases and transporter proteins. In confirmation of this, we know that the changes of ganglioside content and pattern during neuronal differentiation and oncogenic transformation are associated with changes to the expression of the corresponding glycosyltransferases. But, on the other hand, other processes such as different glycosphingolipid intracellular flows, half-life, and shedding influence the resulting ganglioside patterns and seem to be very important to modify the plasma membrane ganglioside concentration and pattern in response to extracellular signals. This would supply the slowness of the processes of de novo biosynthesis and transport to the plasma membrane. As an example, the ganglioside half-life is short in neurons, from half to a few hours (Prinetti et al. 2001), but it is long in the majority of cells, in fibroblasts, having been calculated in the order of 2–3 days. In addition to this, both cultured neuronal and non-neuronal cells lose 7–8% of the total cell sphingolipids by shedding processes every day. Thus, taking in account that the recycling of sphingosine is quantitatively a very important process for the de novo biosynthesis of gangliosides, the de novo biosynthesis of sphinganine is necessary to replace the shed compounds.

References

Aureli M, Loberto N, Chigorno V, Prinetti A, Sonnino S (2011) Remodeling of sphingolipids by plasma membrane associated enzymes. Neurochem Res. 36:1636–1644

Aureli M, Samarani M, Murdica V, Mauri L, Loberto N, Bassi R, Mauri L, Loberto N, Bassi R, Prinetti A, Sonnino S (2014) Gangliosides and cell surface ganglioside glycohydrolases in the nervous system. In: Yu RK, Schengrund C-L (eds) Glycobiology of the nervous system. Springer, New York, NY, pp 223–244

Beutler E (1992) Gaucher disease: new molecular approaches to diagnosis and treatment. Science 256:794–799

Boot RG, Verhoek M, Donker-Koopman W, Strijland A, van Marle J, Overkleeft HS et al (2007) Identification of the non-lysosomal glucosylceramidase as beta-glucosidase 2. J Biol Chem 282: 1305–1312

Buton X, Hervé P, Kubelt J, Tannert A, Burger KN, Fellmann P et al (2002) Transbilayer movement of monohexosylsphingolipids in endoplasmic reticulum and Golgi membranes. Biochemistry. 41:13106–13115

Carter H, Glick F, Norris W, Philips G (1947) BIiochemistry of the sphingolipids. 3. Structure of Sphingosine. J Biol Chem 170:285–294

Carter HE, Rothfus JA, Gigg R (1961) Biochemistry of the sphingolipids: XII. conversion of cerebrosides to ceramides and sphingosine; structure of Gaucher cerebroside. J Lipid Rese 2: 228–234

Chester MA (1998) IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature of glycolipids—recommendations. Eur J Biochem 257:293–298

Chigorno V, Negroni E, Nicolini M, Sonnino S (1997a) Activity of 3-ketosphinganine synthase during differentiation and aging of neuronal cells in culture. J Lipid Res 38:1163–1169
Chigorno V, Riva C, Valsecchi M, Nicolini M, Brocca P, Sonnino S (1997b) Metabolic processing of gangliosides by human fibroblasts in culture—formation and recycling of separate pools of sphingosine. Eur J Biochem 250:661–669
Chiricozzi E, Aureli M, Mauri L, Di Biase E, Lunghi G, Fazzari M, Valsecchi M, Carsana EV, Loberto N, Prinetti A, Sonnino S (2021) Glycosphingolipids. Adv Exp Med Biol 1325:61–102
Crespo PM, Demichelis VT, Daniotti JL (2010) Neobiosynthesis of glycosphingolipids by plasma membrane-associated glycosyltransferases. J Biol Chem 285:29179–29190
D'Angelo G, Polishchuk E, Di Tullio G, Santoro M, Di Campli A, Godi A et al (2007) Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. Nature 449:62–67
Giussani P, Colleoni T, Brioschi L, Bassi R, Hanada K, Tettamanti G et al (2008) Ceramide traffic in C6 glioma cells: evidence for CERT-dependent and independent transport from ER to the Golgi apparatus. Biochim Biophys Acta 1781:40–51
Gottschalk A (1955) Structural relationship between sialic acid, neuraminic acid and 2-carboxy-pyrrole. Nature 176:881–882
Halter D, Neumann S, van Dijk SM, Wolthoorn J, de Mazière AM, Vieira OV et al (2007) Pre- and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. J Cell Biol 179: 101–115
Kamerling JP, Vliegenthart JF (1975) Identification of O-cetylated N-acylneuraminic acids by mass spectrometry. Carbohydr Res 41:7–17
Karlsson KA (1970) On the chemistry and occurrence of sphingolipid long-chain bases. Chem Phys Lipids 5:6–43
Kuhn R, Wiegandt H (1963) Die Konstitution der Ganglio-N-tetraose und des Gangliosids GI. Chemische Berichte 96:866–880
Lannert H, Gorgas K, Meissner I, Wieland FT, Jeckel D (1998) Functional organization of the Golgi apparatus in glycosphingolipid biosynthesis. Lactosylceramide and subsequent glycosphingolipids are formed in the lumen of the late Golgi. J Biol Chem 273:2939–2946
Lunghi G, Fazzari M, Di Biase E, Mauri L, Chiricozzi E, Sonnino S (2021) The structure of gangliosides hides a code for determining neuronal functions. FEBS Open Bio 11:3193–3200
Mansson JE, Vanier MT, Svennerholm L (1978) Changes in the fatty acid and sphingosine composition of the major gangliosides of human brain with age. J Neurochem 30:273–275
Neelamegham S, Aoki-Kinoshita K, Bolton E, Frank M, Lisacek F, Lütteke T et al (2019) Updates to the symbol nomenclature for glycans guidelines. Glycobiology 29:620–624
Papini N, Anastasia L, Tringali C, Croci G, Bresciani R, Yamaguchi K, Miyagi T, Preti A, Prinetti A, Prioni S, Sonnino S, Tettamanti G, Venerando B, Monti E (2004) The plasma membrane-associated sialidase MmNEU3 modifies the ganglioside pattern of adjacent cells supporting its involvement in cell-to-cell interactions. J Biol Chem 279:16989–16995
Preti A, Fiorilli A, Lombardo A, Caimi L, Tettamanti G (1980) Occurrence of sialyltransferase activity in the synaptosomal membranes prepared from calf brain cortex. J Neurochem 135:281–296
Prinetti A, Chigorno V, Prioni S, Loberto N, Marano N, Tettamanti G, Sonnino S (2001) Changes in the lipid turnover, composition, and organization, as sphingolipid-enriched membrane domains, in rat cerebellar granule cells developing in vitro. J Biol Chem 276:21136–21145
Roseman S (1970) The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion. Chem Phys Lipids 5:270–297
Sandhoff R, Sandhoff K (2018) Emerging concepts of ganglioside metabolism. FEBS Lett. 592: 3835–3864
Schauer R (1982) Sialic acids. Chemistry, metabolism and function. Cell Biology Monographs, vol Cell Biol. Springer, Wien
Sonnino S, Cantù L, Corti M, Acquotti D, Venerando B (1994) Aggregative properties of gangliosides in solution. Chem Phys Lipids. 171:21–45

Sonnino S, Prinetti A, Mauri L, Chigorno V, Tettamanti G (2006) Dynamic and structural properties of sphingolipids as driving forces for the formation of membrane domains. Chem Rev. 106:2111–2125

Sonnino S, Mauri L, Chigorno V, Prinetti A (2007) Gangliosides as components of lipid membrane domains. Glycobiology. 17:1R–13R

Sonnino S, Aureli M, Loberto N, Chigorno V, Prinetti A (2010) Fine tuning of cell functions through remodeling of glycosphingolipids by plasma membrane-associated glycohydrolases. FEBS Lett. 584:1914–1922

Svennerholm L (1980) Ganglioside designation. Adv Exp Med Biol. 25:11

Tettamanti G (2004) Ganglioside/glycosphingolipid turnover: new concepts. Glycoconj J. 20:301–317

Thudichum JLWA (1962) A Treatise on the Chemical Constituents of the Brain (a fascimile edition of the original). Bailliere, London

Vanier MT, Holm M, Månsson JE, Svennerholm L (1973) The distribution of lipids in the human nervous system—V. Gangliosides and allied neutral glycolipids of infant brain. J Neurochem. 21:1375–1384

Vilcaes AA, Demichelis VT, Daniotti JL (2011) Trans-activity of plasma membrane-associated ganglioside sialyltransferase in mammalian cells. J Biol Chem. 286:31437–31446

Yu RK, Ledeen RW (1972) Gangliosides of human, bovine, and rabbit plasma. J Lipid Res. 13(5): 680–686

Fucosylated Proteins as Cancer Biomarkers

Eiji Miyoshi, Kazutoshi Fujita, Koichi Morishita, Tsunenori Ouchida, Tsutomu Nakagawa, Shinji Takamatsu, and Jumpei Kondo

Abstract Fucosylation, a type of glycosylation associated with cancer and inflammation, involves the attachment of fucose residues to glycans. Fucosylation is regulated by the expression of various glycogenes, such as those encoding fucosyltransferase, GDP-fucose-synthesizing enzymes, and GDP-fucose transporter. Levels of serum fucosylated proteins are increased in cancer patients, and fucosylated α-fetoprotein (AFP-L3) is used as a biomarker for hepatocellular carcinoma. We previously reported that fucosylated haptoglobin (Fuc-Hp) is elevated in patients with pancreatic cancer, and we developed a lectin-antibody enzyme-linked immunosorbent assay to determine serum Fuc-Hp levels. The molecular mechanisms underlying increased serum fucosylated proteins include abnormal production and secretion of fucosylated proteins in cancer cells and their surrounding tissues. In accordance with the abnormal secretion theory, we found that fucosylated prostate-specific antigen is a novel biomarker for malignant prostate cancer. Recently, we established novel glycan antibodies for AFP-L3 and Fuc-Hp that enable the detection of cells that produce these molecules. In this review, we summarize recent progress in the research of fucosylated proteins as cancer biomarkers.

E. Miyoshi (✉) · K. Morishita · T. Ouchida · S. Takamatsu · J. Kondo
Department of Molecular Biochemistry & Clinical Investigation, Osaka University Graduate School of Medicine, Suita, Japan
e-mail: emiyoshi@sahs.med.osaka-u.ac.jp; shinjit@sahs.med.osaka-u.ac.jp; jumpeko@sahs.med.osaka-u.ac.jp

K. Fujita
Department of Urology, Kindai University Faculty of Medicine, Osaka, Japan
e-mail: kfujita@med.kindai.ac.jp

T. Nakagawa
Department of Pharmaceutics, School of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Hokkaido, Japan
e-mail: tsutomunakagawa@hoku-iryo-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_2

Abbreviations

10-7G mAb	10-7G monoclonal antibody
AAL	*Aleuria aurantia* lectin
AFP	α-Fetoprotein
AFP-L3	Fucosylated AFP
AOL	*Aspergillus oryzae* L-fucose-specific lectin
AUC	Area under the curve
ELISA	Enzyme-linked immunosorbent assay
FasMab	Fucosylated AFP-associated monoclonal antibody
Fuc-Hp	Fucosylated haptoglobin
FUT8	α1–6 Fucosyltransferase
HCC	Hepatocellular carcinoma
Hp	Haptoglobin
PhoSL	*Pholiota squarrosa* lectin
PSA	Prostate-specific antigen
ROC	Receiver operating characteristic curve

1 Introduction

Fucosylation is the attachment of a fucose moiety to oligosaccharides on *N*-/*O*-glycans and glycolipids. Many fucosyltransferases (such as FUTs 1–13), their donor substrate GDP-fucose-synthesizing enzymes, and GDP-fucose transporters are involved in fucosylated glycan synthesis (Miyoshi et al. 2008). Increases in cellular fucosylation and serum fucosylated proteins are observed in cancer, inflammation, and liver diseases (Blomme et al. 2009). One of the master genes of cellular fucosylation is hepatocyte nuclear factor 1α (HNF1α), which regulates the expression of FUT and GDP-fucose synthesis genes (Lauc et al. 2010). Recently, increased terminal fucosylation of serum glycoproteins has been associated with maturity-onset diabetes of the young diabetes mellitus, the pathogenesis of which involves HNF1α abnormalities (Demus et al. 2021).

We have reported that serum fucosylated haptoglobin (Fuc-Hp) is a novel type of cancer biomarker (Okuyama et al. 2006). Mass spectrometry analysis has shown that site-specific glycan changes in cancer-associated haptoglobin (Nakano et al. 2008; Takahashi et al. 2016) and serum Fuc-Hp levels are increased in a patients with a variety of cancer (Zhang et al. 2016). To determine the molecular mechanisms underlying increased cellular fucosylation in the liver, we purified and cloned the α1–6 fucosyltransferase (Fut8) gene (Uozumi et al. 1996; Yanagidani et al. 1997), established a quantitative assay of intracellular GDP-fucose (Noda et al. 2002, 2003), and investigated factors important for increased cellular fucosylation. In hepatocellular carcinoma (HCC) cells, the GDP-fucose transporter is a key factor for cellular fucosylation (Moriwaki et al. 2007). Recently, we confirmed these results

with mathematical analyses and found a common transcription factor involved in the regulation of fucosylated regulatory genes (Kondo et al. 2022).

Selected apical secretion of fucosylated proteins has been observed in the liver (Nakagawa et al. 2012). Fut8-deficient mice show extremely low levels of glycoproteins produced from hepatocytes into the bile duct (Nakagawa et al. 2006). Fucosylated α-fetoprotein (AFP-L3) is a specific biomarker widely used for HCC. Although dysregulated apical secretion of AFP-L3 in HCC cells is speculated to be involved, expression of Fut8 is observed both in HCC tissue and its surrounding cirrhotic tissue (Noda et al. 1998). Thus far, which factors regulate the sorting and selective secretion of fucosylated proteins in cancerous and non-cancerous hepatocytes remain unknown.

Fucosylation in apical protein sorting and secretion also occurs in other organs, such as the kidney and prostate, that have cellular polarity. Prostate-specific antigen (PSA) is the gold standard for prostate cancer biomarker-based diagnosis (Van Poppel et al. 2021). However, PSA alone is not sufficient to avoid invasive biopsy in borderline-high PSA cases and low-risk prostate cancer groups. Recently, we identified core-fucosylated PSA as a specific prostate cancer biomarker candidate (Fujita et al. 2021). Core-fucosylated PSA can distinguish more aggressive prostate cancer.

A recent topic in glycobiology has been next-generation glycan antibodies, which recognize both specific peptide and characteristic glycan structure (Nishino et al. 2018; Egashira et al. 2019). Using these antibodies, we can identify which cells in cancer patient tissue produce glycan biomarkers. In this review, we summarize recent progress in the research of fucosylated proteins as cancer biomarkers.

1.1 Fucosylated Haptoglobin Is a Novel Glycan Biomarker for Cancer and Inflammatory Diseases

Aleuria aurantia lectin (AAL) blotting and *N*-terminal sequencing were used to find fucosylated haptoglobin (Fuc-Hp) in patients with pancreatic cancer (Okuyama et al. 2006). As shown in Fig. 1a, levels of a protein approximately 40 kD in size were almost the same, although binding capacity to AAL was dramatically increased in patients with pancreatic cancer. Mass spectrometry analysis confirmed the attachment of fucose in *N*-glycan to haptoglobin purified from cancer patient sera. Next, we established lectin-antibody ELISA to measure serum levels of Fuc-Hp (Matsumoto et al. 2010). In cases of pancreatic cancer, serum levels of Fuc-Hp were significantly increased at stage III and stage IV (Fig. 1b), and ROC analysis showed a high AUC value in 300 patients with pancreatic cancer compared to 315 normal controls (Fig. 1c) (Kamada et al. 2013a, 2013b). Combination of carbohydrate antigen 19–9 (CA19–9), a sign of pancreatic cancer, and Fuc-Hp determined that PhoSL-antibody ELISA showed a better AUC value for differential diagnosis of pancreatic cancer from normal volunteers and chronic pancreatitis

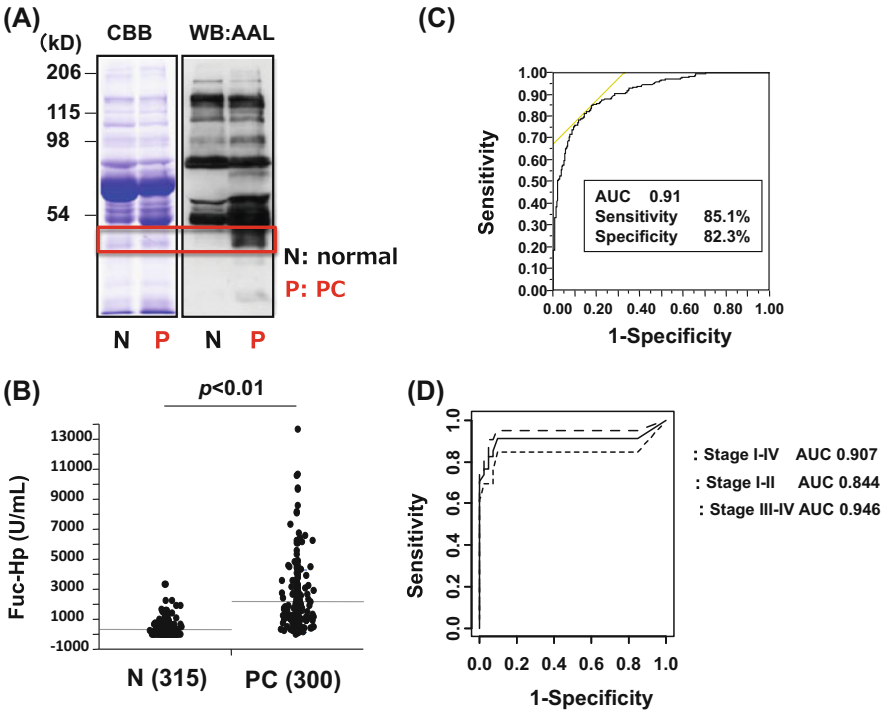


Fig. 1 Fucosylated haptoglobin (Fuc-Hp) as a cancer biomarker for pancreatic cancer. (**a**) Identification of Fuc-Hp with AAL blot followed by *N*-terminal sequencing. (**b**) ELISA data of Fuc-Hp in 315 healthy volunteers and 300 pancreatic cancer patients. (**c**) ROC analysis of Fuc-Hp for diagnosis of pancreatic cancer. (**d**) Combination of Fuc-Hp determined with PhoSL-antibody ELISA and CA19–9 showed better AUC values. These data are based on our previous papers with slight modifications (Okuyama et al. 2006; Kamada et al. 2013a, 2013b; Kusama et al. 2017)

(Fig. 1d) (Matsumoto et al. 2010; Kusama et al. 2017). Serum Fuc-Hp levels were increased in patients with a variety of cancers, as well as those with non-alcoholic steatohepatitis, which entails ballooning hepatocytes (Takeda et al. 2012; Kamada et al. 2015, 2013a,b). Regarding clinical stage, serum Fuc-Hp levels were higher at advanced stages (Matsumoto et al. 2010), suggesting that inflammation-related cytokines and cancer metastasis are associated with the production of Fuc-Hp. For example, we reported that interleukin 6 treatments induce Fuc-Hp production in hepatoma cells (Narisada et al. 2008).

Haptoglobin has four *N*-linked glycosylation sites. To identify characteristic sites with fucosylated glycans, we used mass spectrometry to perform site-directed *N*-glycan analysis of haptoglobin purified from the sera of pancreatic cancer patients (Nakano et al. 2008). We found that, while all *N*-glycosylation sites of haptoglobin are fucosylated, only site 3 has characteristic glycans with fucosylation. Because lectin-antibody ELISA measures the fucosylation of other glycoproteins binding to

haptoglobin, site-directed *N*-glycan analysis of haptoglobin is more accurate for determining Fuc-Hp levels. However, clinical examination should be simple and reliable in every measurement. Fucosylated AFP (AFP-L3) is a specific biomarker for HCC and is measured with an automated immunoassay system that uses on-chip electrokinetic reaction and separation by affinity electrophoresis (Kagebayashi et al. 2009). We applied this assay system to measure Fuc-Hp. While AFP has only one N-linked glycan, haptoglobin has four N-linked glycans, resulting in no separation of Fuc-Hp on lectin affinity chromatography (data not shown). More convenient methods of Fuc-Hp measurement except lectin-antibody ELISA will be required for clinical laboratory examination.

2 Core Fucosylation Is an Apical Sorting Signal

Fut8 is the only fucosyltransferase involved in the synthesis of core fucosylation. Knockout of Fut8 in HepG2 cells reduces the production of AFP-L3 (Egashira et al. 2019), and expression of Fut8 is observed in both HCC tissue and its surrounding cirrhotic tissue (Fig. 2a). In contrast, levels of GDP fucose, a donor substrate of Fut8, were increased in HCC tissue compared to surrounding tissue of chronic liver diseases (Fig. 2b) (Noda et al. 2003). Accordingly, serum AFP-L3 levels are increased in HCC patients but not patients with chronic liver diseases (Noda et al. 1998). In the mouse liver, Fut8 is a major fucosyltransferase, as murine Fut6 is pseudogene. Fut8 knockout mice show extremely low levels of glycoproteins produced from hepatocytes in bile but similar levels of bile albumin, which has no *N*-glycans, suggesting that core fucosylation of hepatic glycoproteins is an apical sorting signal in the bile duct (Fig. 2c). The theory of apical sorting and fucosylation was not clearly observed in HepG2 cells, although mild suppression of AFP-L3 secretion into the bile duct structure was observed in HepG2 cells with knockdown of fucosylation regulatory genes (Nakagawa et al. 2012). High levels of fucosylated proteins were observed in human bile compared to sera of the same patient, suggesting that both core and terminal fucosylation are apical sorting signals (Fig. 2d) (Nakagawa et al. 2006).

3 Development of More Convenient Assays to Evaluate Aberrant Glycosylation of PSA

The PSA test is the gold standard for prostate cancer biomarkers. The detection of elevated blood PSA can prompt prostate needle biopsy for the diagnosis of prostate cancer. However, up to 40% of patients newly diagnosed with prostate cancer are categorized as lowrisk (Barocas et al. 2008). Patients with low-risk prostate cancer have a minimal likelihood of disease progression and do not require definitive

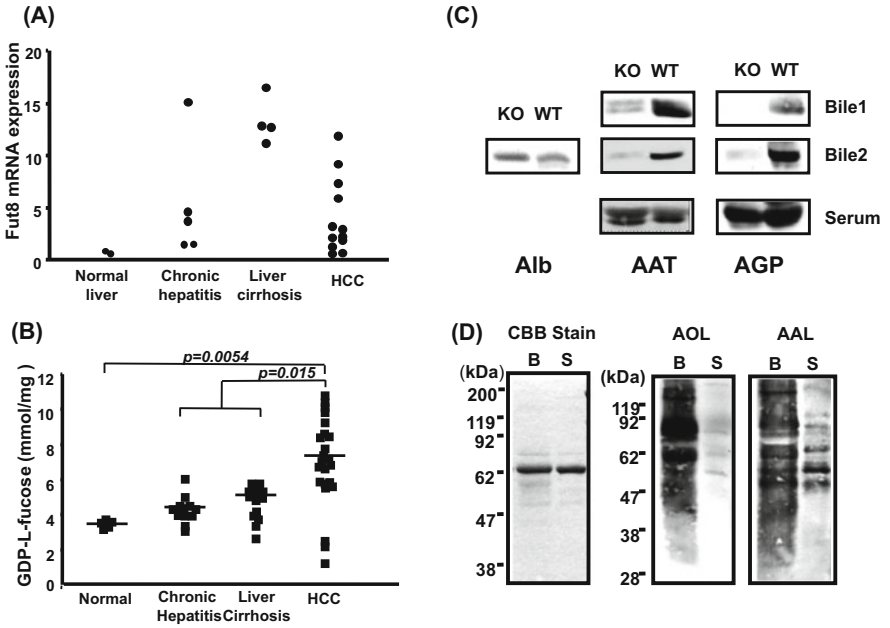


Fig. 2 Molecular mechanism for increased fucosylation in HCC. (**a**) mRNA expression of Fut8 with Northern blot analysis. (**b**) Levels of GDP-L-fucose in tissue from patients with HCC or chronic liver diseases. (**c**) Minimal secretion of hepatic glycoproteins such as α1 anti-trypsin (AAT) and α1-acid glycoprotein (AGP) in the bile of Fut8 KO mice. (**d**) Increased fucosylated protein in bile (B) compared to sera (S) in patients with choledocholithiasis. These data are based on our previous papers with slight modifications (Noda et al. 1998; Nakagawa et al. 2006)

therapy. Unfortunately, the PSA test cannot discriminate high-risk prostate cancer, which needs definitive therapy, from low-risk prostate cancer. PSA levels are also elevated by benign prostate hypertrophy or prostatitis, resulting in low specificity for the accurate diagnosis of prostate cancer. To avoid unnecessary prostate biopsies, the development of new markers for high-risk prostate cancer is needed.

PSA is a glycoprotein with one *N*-glycosylation site (Asn-69), and 70% of PSA molecules contain a fucose (Bélanger et al. 1995). Studies have reported using fucosylated PSA for the detection of prostate cancer. According to fucosylation and apical sorting theory, fucosylated PSA should be secreted into urine in benign prostate diseases. To investigate this hypothesis, we measured levels of urine fucosylated PSA with lectin-antibody ELISA. As expected, levels of urine fucosylated PSA were decreased in patients with prostate cancer compared to patients with benign prostate hypertrophy or prostate inflammation (Fig. 3a). Interestingly, urine fucosylated PSA levels were lower in cases of advanced stage prostate cancer. The analysis of urine PSA in prostate cancer patients was not reproducible in urine sample of prostate cancer patients in the USA, suggesting that stock conditions of urine are important for precise measurement of urine fucosylated PSA levels because PSA belongs to a serine-protease.

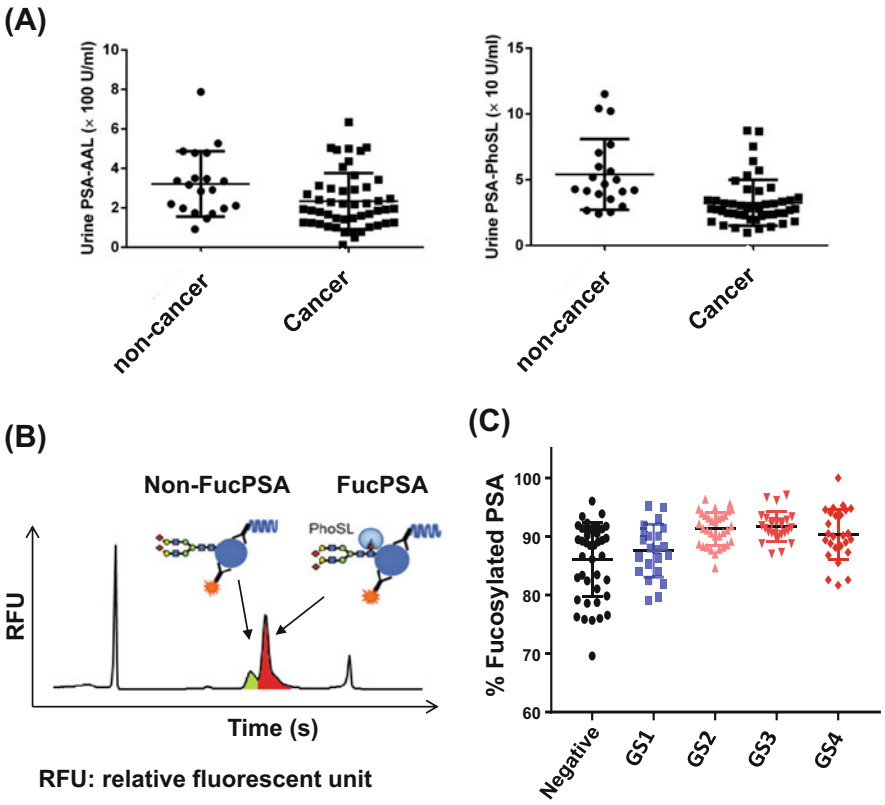


Fig. 3 Fucosylated PSA is a biomarker for prostate cancer. (**a**) Lectin-antibody ELISA using urine samples. (**b**) Microcapillary electrophoresis-based immunoassay system with PhoSL to detect fucosylated PSA (FucPSA). (**c**) Serum levels of FucPSA in terms of Gleason score (GS). These data are based on our previous papers with slight modifications (Fujita et al. 2016; Fujita et al. 2021)

In contrast, elevated expression of α1,2-fucosylated PSA in prostate cancer has been reported using *Trichosanthes japonica* agglutinin II (TJA-II) column chromatography (Fukushima et al. 2010). In addition, serum α1,2-fucosylated PSA is significantly higher in men with prostate cancer than men with benign prostate hypertrophy, as found using enzyme-linked immunosorbent lectin assay with Ulex Europaeus (UEA-1) (Dwek et al. 2010). However, for the clinical application of fucosylated PSA, the development of a highly sensitive and automated system is needed. For example, trace PSA can be detected in sera of cancer patients by ELISA (the normal range of PSA is less than 3–4 ng/mL). A magnetic bead-based immunoassay with AAL was used to determine that the total fucosylated PSA is significantly increased in high-risk prostate cancer and associated with the Gleason score (Li et al. 2015). The microcapillary electrophoresis-based immunoassay system with PhoSL enables the stable and rapid measurement of serum fucosylated PSA

(Fig. 3b). The ratio of serum core-type fucosylated free PSA can differentiate high-risk prostate cancer from biopsy-negative or low-risk prostate cancer (Fig. 3c) (Fujita et al. 2021). Free serum PSA from patients with prostate cancer exhibits increased binding to *Maackia amurensis* agglutinin (MAA) lectin, which recognizes α2, 3-linked sialic acid, compared to free serum PSA from patients with benign prostate hypertrophy (Ohyama et al. 2004). Automated micro-total immunoassay systems were developed to measure the ratio of α2, 3-linked sialyl *N*-glycan-carrying PSA (S2,3PSA) using MAL (Ishikawa et al. 2017). This group found that the serum S2,3PSA ratio can predict prostate cancer better than serum PSA and that serum S2,3PSA levels are not correlated with serum levels of core fucosylated PSA (FucPSA) in patients with prostate cancer. Thus, the simultaneous measurement of S2,3PSA and FucPSA increases the accuracy of detection of high-risk prostate cancer (Hatano et al. 2021). Fucosylated PSA, especially core-type fucosylated PSA, is a promising biomarker for prostate cancer.

4 Novel Pathophysiology of Disease-Associated Glycosylation Explored with Next-Generation Antibodies

To develop a more convenient assay to measure serum Fuc-Hp, we established a novel glycan antibody for Fuc-Hp. The conventional glycan antibody recognizes only the sugar chain structure, such as Sialyl Lewis x antigen. On the other hand, next-generation glycan antibody recognizes both the characteristic glycan structure and amino acids and the novel peptide epitope resulting from aberrant glycosylation. After we prepared non-fucosylated haptoglobin and Fuc-Hp, mice were immunized with Fuc-Hp. Original screening was used to obtain a novel glycan antibody for Fuc-Hp, referred to as 10-7G mAb, using HCT116 cells (Moriwaki et al. 2009). Column chromatography of 10-7G mAb revealed that 10-7G mAb recognized fucosylated forms of both mature haptoglobin and its precursor, prohaptoglobin (proHp), which was also fucosylated (Nishino et al. 2018). Most haptoglobin is produced from hepatocytes as prohaptoglobin, followed by cleavage with C1rL, a protease for maturation of haptoglobin. In cases of lymphocytes and macrophages, most haptoglobin is secreted as prohaptoglobin.

Western blot analysis was used to find that 10-7G mAb detects proHp. To determine the epitope of 10-7G mAb, deletion mutants of haptoglobin α chain were used (Morishita et al. 2020). As shown in Fig. 4a, the epitope of 10-7G mAb is hidden by haptoglobin β chain. However, aberrant glycosylation of haptoglobin induces morphological changes, resulting in the appearance of the epitope for 10-7G mAb. Levels of serum fucosylated haptoglobin measured with 10-7G mAb ELISA (10-7G value) are increased in patients with pancreatic cancer. Interestingly, the genotype of haptoglobin gene affected the result (Morishita et al. 2018). As shown in Fig. 4b, 10-7G values are below the level of detection in the Hp1–1 phenotype. This

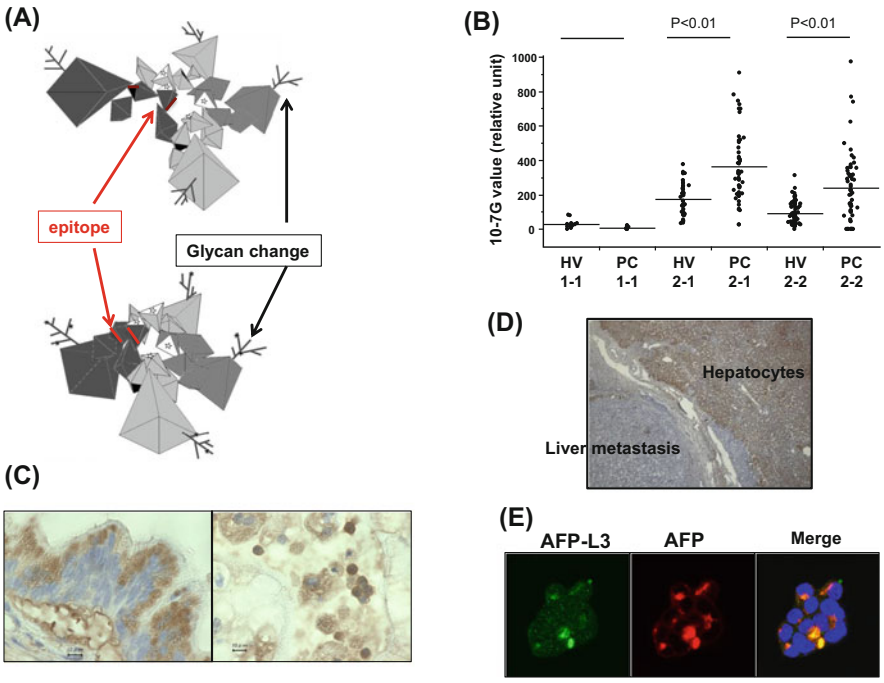


Fig. 4 Study of next-generation glycan antibodies with 10-7G mAb and FasMab. (**a**) Schematic structure of haptoglobin in terms of 10-7G mAb epitope. (**b**) Serum levels of Fuc-Hp with 10-7G mAb ELISA show different 10-7G values in each haptoglobin phenotype (Hp1–1, Hp1–2, and Hp2–2). 10-7G values are increased pancreatic cancer (PC) patients compared to normal volunteers (HV), except in Hp1–1. Immunohistochemical study of 10-7G mAb in pancreatic cancer tissue (**c**) and liver metastasis of pancreatic cancer (**d**). (**e**) Immunocytochemical study of AFP-L3 in HepG2 cells with FasMab and anti-AFP Ab. These data are based on our previous papers with slight modifications (Morishita et al. 2020; Morishita et al. 2018; Ito et al. 2021; Nishino et al. 2018)

is because proHp is not produced in the Hp1–1 phenotype, and fucosylated haptoglobin measured with lectin-antibody ELISA is also below the level of detection.

To determine which cells and tissues produce Fuc-Hp and proHp, an immunohistochemical study using 10-7G mAb was performed (Nishino et al. 2018; Ito et al. 2021). In pancreatic cancer tissue, only 1 out of 21 cases shows positive staining of 10-7G mAb in carcinoma cells (Fig. 4c, left panel). On the other hand, approximately 50% of infiltrated immune cells surrounding cancer tissue show positive staining of 10-7G mAb, indicating that proHp is produced in the lesion of inflammation (Fig. 4c, right panel). In fact, robust staining of 10-7G mAb is observed in the intestinal lymphoid tissue of patients with inflammatory bowel diseases (IBD) (Motooka et al. 2021). 10-7G values are consistent with the severity of endoscopic examination in IBD patients. In contrast, 10-7G mAb shows negative staining of hepatocytes in the normal liver. However, hepatocytes surrounding metastatic cancer show positive staining of 10-7G mAb (Fig. 4d). Interleukin 6 produced from

liver metastasis of pancreatic cancer and disruption of cellular polarity of hepatocytes induces the accumulation of Fuc-Hp in hepatocytes (Narisada et al. 2008).

We have succeeded in establishing a next-generation glycan antibody for AFP-L3, referred to as FasMab, which recognizes both core fucose and AFP peptides (Egashira et al. 2019). As expected, FasMab recognizes AFP produced from parental HepG2 cells but does not recognize AFP produced from Fut8-deficient HepG2 cells. Preliminary ELISA systems using FasMab show increases in fucosylated AFP in HCC patients compared to benign liver disease patients. AFP-L3 levels measured with FasMab are consistent with those measured with conventional lectin affinity chromatography (Egashira et al. 2019). Immunostaining using FasMab shows positive staining of AFP-L3 in the bile duct-like structure of HepG2 cells but not in Fut8 knockout HepG2 cells (Fig. 4e).

5 Closing

A long history of cancer and fucosylation research has given rise to published studies of novel biomarkers, cancer biology, and future glycotherapies. For example, next-generation antibodies will allow the novel pathological analysis of cancer and inflammatory diseases. To further advance fucosylation research, a cargo-receptor that regulates apical sorting of fucosylated proteins must be identified, and the molecular mechanisms of membrane trafficking regulated by glycosylation must be clarified.

Conflicts of Interest and Source of Funding The authors have no potential conflicts relevant to the manuscript. This study was supported by a KAKENHI grant (19H03562 and A20H05675a) from the Japan Society for the Promotion of Science and Management Expenses grant of Osaka University.

References

Barocas DA, Cowan JE, Smith JA Jr (2008) Carroll PR: what percentage of patients with newly diagnosed carcinoma of the prostate are candidates for surveillance? An analysis of the CaPSURE database. J Urol 180:1330–1334.; discussion 1334-1335. https://doi.org/10.1016/j.juro.2008.06.019

Bélanger A, van Halbeek H, Graves HC, Grandbois K, Stamey TA, Huang L, Poppe I, Labrie F (1995) Molecular mass and carbohydrate structure of prostate specific antigen: studies for establishment of an international PSA standard. Prostate 27:187–197. https://doi.org/10.1002/pros.2990270403

Blomme B, Van Steenkiste C, Callewaert N, Van Vlierberghe H (2009) Alteration of protein glycosylation in liver diseases. J Hepatol 50:592–603. https://doi.org/10.1016/j.jhep.2008.12.010

Demus D, Jansen BC, Gardner RA, Urbanowicz PA, Wu H, Štambuk T, Juszczak A, Medvidović EP, Juge N, Gornik O, Owen KR, Spencer DIR (2021) Interlaboratory evaluation of plasma N-glycan antennary fucosylation as a clinical biomarker for HNF1A-MODY using liquid

chromatography methods. Glycoconj J 38:375–386. https://doi.org/10.1007/s10719-021-09992-w

Dwek MV, Jenks A, Leathem AJ (2010) A sensitive assay to measure biomarker glycosylation demonstrates increased fucosylation of prostate specific antigen (PSA) in patients with prostate cancer compared with benign prostatic hyperplasia. Clin Chim Acta 411:1935–1939. https://doi.org/10.1016/j.cca.2010.08.009

Egashira Y, Suganuma M, Kataoka Y, Higa Y, Ide N, Morishita K, Kamada Y, Gu J, Fukagawa K, Miyoshi E (2019) Establishment and characterization of a fucosylated α-fetoprotein-specific monoclonal antibody: a potential application for clinical research. Sci Rep 9:12359. https://doi.org/10.1038/s41598-019-48821-x

Fujita K, Hayashi T, Matsuzaki K, Nakata W, Masuda M, Kawashima A, Ujike T, Nagahara A, Tsuchiya M, Kobayashi Y, Nojima S, Uemura M, Morii E, Miyoshi E, Nonomura N (2016) Decreased fucosylated PSA as a urinary marker for high Gleason score prostate cancer. Oncotarget 7:56643–56649. https://doi.org/10.18632/oncotarget.10987

Fujita K, Hatano K, Tomiyama E, Hayashi Y, Matsushita M, Tsuchiya M, Yoshikawa T, Date M, Miyoshi E, Nonomura N (2021) Serum core-type fucosylated prostate-specific antigen index for the detection of high-risk prostate cancer. Int J Cancer 148:3111–3118. https://doi.org/10.1002/ijc.33517

Fukushima K, Satoh T, Baba S, Yamashita K (2010) Alpha1,2-Fucosylated and beta-N-acetylgalactosaminylated prostate-specific antigen as an efficient marker of prostatic cancer. Glycobiology 20:452–460. https://doi.org/10.1093/glycob/cwp197

Hatano K, Yoneyama T, Hatakeyama S, Tomiyama E, Tsuchiya M, Nishimoto M, Yoshimura K, Miyoshi E, Uemura H, Ohyama C, Nonomura N, Fujita K (2021) Simultaneous analysis of serum α2,3-linked sialylation and core-type fucosylation of prostate-specific antigen for the detection of high-grade prostate cancer. Br J Cancer 148(12):3111–3118. https://doi.org/10.1038/s41416-021-01637-x

Ishikawa T, Yoneyama T, Tobisawa Y, Hatakeyama S, Kurosawa T, Nakamura K, Narita S, Mitsuzuka K, Duivenvoorden W, Pinthus JH, Hashimoto Y, Koie T, Habuchi T, Arai Y, Ohyama C (2017) An automated micro-Total immunoassay system for measuring cancer-associated α2,3-linked Sialyl N-glycan-carrying prostate-specific antigen may improve the accuracy of prostate cancer diagnosis. Int J Mol Sci 18:470. https://doi.org/10.3390/ijms18020470

Ito N, Yamada M, Morishita K, Nojima S, Motooka K, Sakata N, Asuka T, Otsu R, Takamatsu S, Kamada Y, Mori S, Akita H, Eguchi H, Morii E, Miyoshi E (2021) Identification of fucosylated haptoglobin-producing cells in pancreatic cancer tissue and its molecular mechanism. Glycoconj J 38:45–54. https://doi.org/10.1007/s10719-020-09970-8

Kagebayashi C, Yamaguchi I, Akinaga A, Kitano H, Yokoyama K, Satomura M, Kurosawa T, Watanabe M, Kawabata T, Chang W, Li C, Bousse L, Wada HG, Satomura S (2009) Automated immunoassay system for AFP-L3% using on-chip electrokinetic reaction and separation by affinity electrophoresis. Anal Biochem 388:306–311. https://doi.org/10.1016/j.ab.2009.02.030

Kamada Y, Akita M, Takeda Y, Yamada S, Fujii H, Sawai Y, Doi Y, Asazawa H, Nakayama K, Mizutani K, Fujii H, Yakushijin T, Miyazaki M, Ezaki H, Hiramatsu N, Yoshida Y, Kiso S, Imai Y, Kawada N, Takehara T, Miyoshi E (2013a) Serum fucosylated haptoglobin as a novel diagnostic biomarker for predicting hepatocyte ballooning and nonalcoholic steatohepatitis. PLoS One 8:e66328. https://doi.org/10.1371/journal.pone.0066328

Kamada Y, Kinoshita N, Tsuchiya Y, Kobayashi K, Fujii H, Terao N, Kamihagi K, Koyama N, Yamada S, Daigo Y, Nakamura Y, Taniguchi N, Miyoshi E (2013b) Reevaluation of a lectin antibody ELISA kit for measuring fucosylated haptoglobin in various conditions. Clin Chim Acta 417:48–53. https://doi.org/10.1016/j.cca.2012.12.014

Kamada Y, Ono M, Hyogo H, Fujii H, Sumida Y, Mori K, Tanaka S, Yamada M, Akita M, Mizutani K, Fujii H, Yamamoto A, Takamatsu S, Yoshida Y, Itoh Y, Kawada N, Chayama K, Saibara T, Takehara T, Miyoshi E (2015) A novel noninvasive diagnostic method for

nonalcoholic steatohepatitis using two glycobiomarkers. Hepatology (Baltimore, Md) 62:1433–1443. https://doi.org/10.1002/hep.28002

Kondo J, Sakata N, Morishita K, Hayashibara A, Sakon D, Takamatsu S, Asakura N, Suzuki T, Miyoshi E (2022) Transcription factor SP1 regulates haptoglobin fucosylation via induction of GDP-fucose transporter 1 in the hepatoma cell line HepG2. Biochem Biophys Rep. 32101372-S2405580822001728:101372. https://doi.org/10.1016/j.bbrep.2022.101372

Kusama K, Okamoto Y, Saito K, Kasahara T, Murata T, Ueno Y, Kobayashi Y, Kamada Y, Miyoshi E (2017) Reevaluation of Pholiota squarrosa lectin-reactive haptoglobin as a pancreatic cancer biomarker using an improved ELISA system. Glycoconj J 34:537–544. DOI: 10.1007/s10719-017-9772-9

Lauc G, Essafi A, Huffman JE, Hayward C, Knezevic A, Kattla JJ, Polasek O, Gornik O, Vitart V, Abrahams JL, Pucic M, Novokmet M, Redzic I, Campbell S, Wild SH, Borovecki F, Wang W, Kolcic I, Zgaga L, Gyllensten U, Wilson JF, Wright AF, Hastie ND, Campbell H, Rudd PM, Rudan I (2010) Genomics meets glycomics-the first GWAS study of human N-Glycome identifies HNF1alpha as a master regulator of plasma protein fucosylation. PLoS Genet 6: e1001256. https://doi.org/10.1371/journal.pgen.1001256

Li QK, Chen L, Ao MH, Chiu JH, Zhang Z, Zhang H, Chan DW (2015) Serum fucosylated prostate-specific antigen (PSA) improves the differentiation of aggressive from non-aggressive prostate cancers. Theranostics 5:267–276. https://doi.org/10.7150/thno.10349

Matsumoto H, Shinzaki S, Narisada M, Kawamoto S, Kuwamoto K, Moriwaki K, Kanke F, Satomura S, Kumada T, Miyoshi E (2010) Clinical application of a lectin-antibody ELISA to measure fucosylated haptoglobin in sera of patients with pancreatic cancer. Clin Chem Lab Med 48:505–512. https://doi.org/10.1515/CCLM.2010.095

Miyoshi E, Moriwaki K, Nakagawa T (2008) Biological function of fucosylation in cancer biology. J Biochem 143:725–729. https://doi.org/10.1093/jb/mvn011

Morishita K, Ito N, Koda S, Maeda M, Nakayama K, Yoshida K, Takamatsu S, Yamada M, Eguchi H, Kamada Y (2018) Haptoglobin phenotype is a critical factor in the use of fucosylated haptoglobin for pancreatic cancer diagnosis. Clin Chim Acta 487:84–89. https://doi.org/10.1016/j.cca.2018.09.001

Morishita K, Maki Y, Takamatsu S, Ito N, Koda S, Motooka K, Kamada Y, Kajihara Y, Miyoshi E (2020) Identification of the epitope of 10-7G glycan antibody to recognize cancer-associated haptoglobin. Anal Biochem 593:113588. https://doi.org/10.1016/j.ab.2020.113588

Moriwaki K, Noda K, Nakagawa T, Asahi M, Yoshihara H, Taniguchi N, Hayashi N, Miyoshi E (2007) A high expression of GDP-fucose transporter in hepatocellular carcinoma is a key factor for increases in fucosylation. Glycobiology 17:1311–1320. https://doi.org/10.1093/glycob/cwm094

Moriwaki K, Noda K, Furukawa Y, Ohshima K, Uchiyama A, Nakagawa T, Taniguchi N, Daigo Y, Nakamura Y, Hayashi N, Miyoshi E (2009) Deficiency of GMDS leads to escape from NK cell-mediated tumor surveillance through modulation of TRAIL signaling. Gastroenterology 137: 188–198, 198 e181–182. https://doi.org/10.1053/j.gastro.2009.04.002

Motooka K, Morishita K, Ito N, Shinzaki S, Tashiro T, Nojima S, Shimizu K, Date M, Sakata N, Yamada M, Takamatsu S, Kamada Y, Iijima H, Mizushima T, Morii E, Takehara T, Miyoshi E (2021) Detection of fucosylated haptoglobin using the 10-7G antibody as a biomarker for evaluating endoscopic remission in ulcerative colitis. World J Gastroenterol 27:162–175. https://doi.org/10.3748/wjg.v27.i2.162

Nakagawa T, Uozumi N, Nakano M, Mizuno-Horikawa Y, Okuyama N, Taguchi T, Gu J, Kondo A, Taniguchi N, Miyoshi E (2006) Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. J Biol Chem 281:29797–29806. https://doi.org/10.1074/jbc.M605697200

Nakagawa T, Moriwaki K, Terao N, Nakagawa T, Miyamoto Y, Kamada Y, Miyoshi E (2012) Analysis of polarized secretion of fucosylated alpha-fetoprotein in HepG2 cells. J Proteome Res 11:2798–2806. https://doi.org/10.1021/pr201154k

Nakano M, Nakagawa T, Ito T, Kitada T, Hijioka T, Kasahara A, Tajiri M, Wada Y, Taniguchi N, Miyoshi E (2008) Site-specific analysis of N-glycans on haptoglobin in sera of patients with pancreatic cancer: a novel approach for the development of tumor markers. Int J Cancer 122: 2301–2309. https://doi.org/10.1002/ijc.23364

Narisada M, Kawamoto S, Kuwamoto K, Moriwaki K, Nakagawa T, Matsumoto H, Asahi M, Koyama N, Miyoshi E (2008) Identification of an inducible factor secreted by pancreatic cancer cell lines that stimulates the production of fucosylated haptoglobin in hepatoma cells. Biochem Biophys Res Commun 377:792–796. https://doi.org/10.1016/j.bbrc.2008.10.061

Nishino K, Koda S, Kataoka N, Takamatsu S, Nakano M, Ikeda S, Kamamatsu Y, Morishita K, Moriwaki K, Eguchi H, Yamamoto E, Kikkawa F, Tomita Y, Kamada Y, Miyoshi E (2018) Establishment of an antibody specific for cancer-associated haptoglobin: a possible implication of clinical investigation. Oncotarget 9:12732–12744. https://doi.org/10.18632/oncotarget.24332

Noda K, Miyoshi E, Uozumi N, Yanagidani S, Ikeda Y, Gao C, Suzuki K, Yoshihara H, Yoshikawa K, Kawano K, Hayashi N, Hori M, Taniguchi N (1998) Gene expression of alpha1-6 fucosyltransferase in human hepatoma tissues: a possible implication for increased fucosylation of alpha-fetoprotein. Hepatology (Baltimore, Md) 28:944–952. https://doi.org/10.1002/hep.510280408

Noda K, Miyoshi E, Nakahara S, Ihara H, Gao CX, Honke K, Yanagidani S, Sasaki Y, Kasahara A, Hori M, Hayashi N, Taniguchi N (2002) An enzymatic method of analysis for GDP-L-fucose in biological samples, involving high-performance liquid chromatography. Anal Biochem 310: 100–106. https://doi.org/10.1016/s0003-2697(02)00313-5

Noda K, Miyoshi E, Gu J, Gao CX, Nakahara S, Kitada T, Honke K, Suzuki K, Yoshihara H, Yoshikawa K, Kawano K, Tonetti M, Kasahara A, Hori M, Hayashi N, Taniguchi N (2003) Relationship between elevated FX expression and increased production of GDP-L-fucose, a common donor substrate for fucosylation in human hepatocellular carcinoma and hepatoma cell lines. Cancer Res 63:6282–6289. https://pubmed.ncbi.nlm.nih.gov/14559815/

Ohyama C, Hosono M, Nitta K, Oh-eda M, Yoshikawa K, Habuchi T, Arai Y, Fukuda M (2004) Carbohydrate structure and differential binding of prostate specific antigen to Maackia amurensis lectin between prostate cancer and benign prostate hypertrophy. Glycobiology 14: 671–679. https://doi.org/10.1093/glycob/cwh071

Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K, Murata K, Ohigashi H, Yokoyama S, Eguchi H, Ishikawa O, Ito T, Kato M, Kasahara A, Kawano S, Gu J, Taniguchi N, Miyoshi E (2006) Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. Int J Cancer 118:2803–2808. https://doi.org/10.1002/ijc.21728

Takahashi S, Sugiyama T, Shimomura M, Kamada Y, Fujita K, Nonomura N, Miyoshi E, Nakano M (2016) Site-specific and linkage analyses of fucosylated N-glycans on haptoglobin in sera of patients with various types of cancer: possible implication for the differential diagnosis of cancer. Glycoconj J 33(3):471–482. https://doi.org/10.1007/s10719-016-9653-7

Takeda Y, Shinzaki S, Okudo K, Moriwaki K, Murata K, Miyoshi E (2012) Fucosylated haptoglobin is a novel type of cancer biomarker linked to the prognosis after an operation in colorectal cancer. Cancer 118:3036–3043. https://doi.org/10.1002/cncr.26490

Uozumi N, Yanagidani S, Miyoshi E, Ihara Y, Sakuma T, Gao CX, Teshima T, Fujii S, Shiba T, Taniguchi N (1996) Purification and cDNA cloning of porcine brain GDP-L-Fuc:N-acetyl-beta-D-glucosaminide alpha1-->6fucosyltransferase. J Biol Chem 271:27810–27817. https://doi.org/10.1074/jbc.271.44.27810

Van Poppel H, Roobol MJ, Chapple CR, Catto JWF, N'Dow J, Sønksen J, Stenzl A, Wirth M (2021) Prostate-specific antigen testing as part of a risk-adapted early detection strategy for prostate cancer: European Association of Urology Position and Recommendations for 2021. Eur Urol 80:703–711. https://doi.org/10.1016/j.eururo.2021.07.024

Yanagidani S, Uozumi N, Ihara Y, Miyoshi E, Yamaguchi N, Taniguchi N (1997) Purification and cDNA cloning of GDP-L-Fuc:N-acetyl-beta-D-glucosaminide:alpha1-6 fucosyltransferase

(alpha1-6 FucT) from human gastric cancer MKN45 cells. J Biochem 121:626–632. https://doi.org/10.1093/oxfordjournals.jbchem.a021631

Zhang S, Shang S, Li W, Qin X, Liu Y (2016) Insights on N-glycosylation of human haptoglobin and its association with cancers. Glycobiology 26:684–692. https://doi.org/10.1093/glycob/cww016

Part II
Assembly of Glycoconjugates

Substantial Basis for Glyco-Assembly: Siglec7 and Synthetic Sialylpolymers

Sayo Morishita, Masaya Hane, Ken Kitajima, and Chihiro Sato

Abstract Sialic acid is one of the tags that are recognized as "self," when immune cells survey "non-self" items using the sialic acid-recognizing lectins, Siglecs, on their cell surface. These self-non-self discriminating machineries give not only an advantage to the host cells in innate immune system but also a disadvantage because they allow cancer cells to escape from the immune cells. Siglec7 is one of such machineries predominantly expressed in natural killer (NK) cells and acts as an inhibitory receptor in a sialic acid-dependent manner; however, how Siglec7 binds the sialic acid residues on its counter-receptors has still remained unknown. Since 1999, many reports have described the binding properties of Siglec7 using various sialic acid-containing materials, mostly focusing on the sialyl linkage and the types of core glycan structure. Very recently, two new features of Siglecs binding have been reported: One is the discovery of the new binding sites in various Siglec molecules different than the conventional binding site; the other is the finding of the mucin-type ligands as a natural counter-receptor of Siglec7. These new features thus led us to overview the new binding sites of Siglec7, structural aspects and affinity for the SIglec7 ligands, and natural ligands. In this chapter, we describe the synthetic and natural ligands for Siglec7 and discuss their ligand-binding properties, which are important for therapeutic drug development especially under the spot light of multivalency.

S. Morishita
Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Japan

Bioscience and Biotechnology Center, Nagoya University, Chikusa, Nagoya, Japan

M. Hane · K. Kitajima · C. Sato (✉)
Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Japan

Bioscience and Biotechnology Center, Nagoya University, Chikusa, Nagoya, Japan

Integrated Glyco-BioMedical Research Center (iGMED), Institute for Glycocore Research (iGCORE), Nagoya University, Chikusa, Nagoya, Japan
e-mail: chi@agr.nagoya-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_3

1 Introduction

Siglecs are sialic acid (Sia)-binding immunoglobulin (Ig)-like lectins (Crocker et al. 2007) that comprise a Sia-binding N-terminal V-set domain, 1–16 C2-set Ig domains, a transmembrane domain, and a cytosolic region. The V-set domain is important for the interaction of Sia with the immune system (Fig. 1). The crystal structure of Siglec with a Sia analogue demonstrates the critical Sia binding at the Arg (R) residue on the F strand of Siglec; all Siglecs except for Siglec12 have this essential R residue (Alphey et al. 2003; Crocker et al. 2007) (Fig. 2, 1O7V, 2HRL). The majority of Siglecs are predominantly expressed in immune cells, and their expression is cell type-specific. The cytosolic regions of almost all Siglecs have an immunoreceptor tyrosine-based inhibition motif (ITIM) and/or an ITIM-like motif

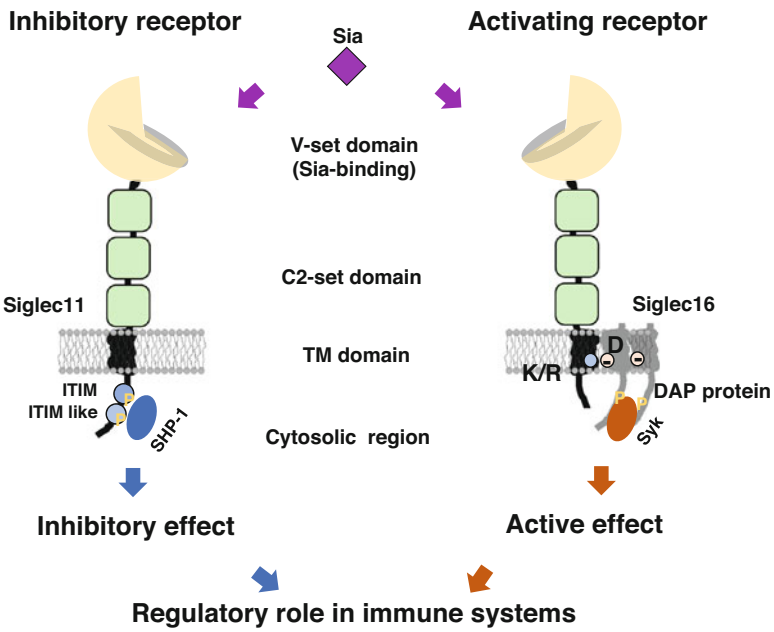


Fig. 1 Schematic structure of Siglec. Siglecs are categorized into two groups based on their functions as follows: an inhibitory receptor and an activating receptor. Extracellular domain of Siglecs is common. All Siglecs have one V-set domain, 1–16 C2-set domains, a transmembrane domain, and a cytosolic region. The V-set domain is the critical region for Sia (Neu5Ac) (ligand, purple diamond) binding and is involved in distinguishing between self and non-self. Inhibitory Siglec has an ITIM or/and ITIM-like motif where SHP-1 is recruited via phosphorylation (P, tyrosine residues) of both motifs. Inhibitory receptor suppresses the immune response to regulate the signal. Activating receptor has an R or K residue in the transmembrane domain, and DAP is bound to Siglec via a D residue in its transmembrane domain. DAP recruits Syk and activates the immune response. At times, Siglecs shows paired receptors, as observed in case of Siglec11 (for inhibitory receptor) and Siglec16 (for activating receptor). The V-set domains of Siglec11 and Siglec16 are the same; therefore, the binding property toward Sia is the same, but the immune response is reverse

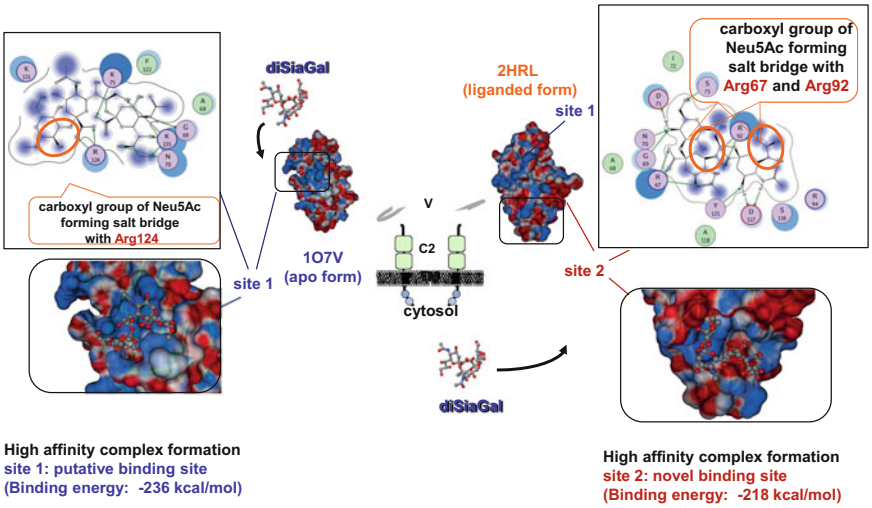


Fig. 2 Schematic structure of Siglec. Molecular modeling of Siglec7. Crystal information of apo-Siglec7 (1O7V) and the ligand form of Siglec7 (2HRL) was prepared. After removal of GT1b analogue ligand information from that of 2HRL, both structures were docked with diSia structure. The highest energy models are shown. Left, 1O7V with diSia structure. Right, 2HRL with diSia structure. The 1O7V residue showed docking with the primary Sia-binding site, and the carboxyl group of Neu5Ac formed a salt bridge with R124. Siglec7 model calculated using 2HRL as a template showed the docking with the secondary Sia-binding site; the carboxyl groups of Neu5Ac formed salt bridges with R67 and R92. In in vitro experiments, R67 but not R92 was shown to be involved in Sia binding. R92 is somehow related to ligand binding. Interestingly, R94 was later shown to be the common amino acid among all Siglecs (see Fig. 3). Considering the location, site 1, primary Sia-binding site, might be related to the trans-ligand, and site 2 or the secondary Sia-binding site might be related to the cis-ligand

(s) to regulate signal inhibition; therefore, such Siglecs are inhibitory receptors that recruit signaling molecules such as SHP1 via ITIM and ITIM-like regions. There are several paired Siglecs such as Siglec11 and Siglec16 where one acts as an inhibitory receptor and the other serves as an activating receptor (Fig. 1). The extracellular domains of these paired Siglecs are identical. The striking difference in the activating receptor is the transmembrane and cytosolic region, especially the presence of an Arg residue within the transmembrane region that mediates the recruitment of the adaptor protein and DAP to regulate signal activity via signaling modules such as Syk (Fig. 1). Siglecs are important for immune cell functions via Sia binding, and any disturbance in the interactions between Sia and Siglec alters the state of the immune system and may lead to infection, inflammation, autoimmunity, and cancer-escaping system (Crocker et al. 2007). In general, Siglecs are considered to be receptors for distinguishing self from non-self; however, the precise mechanism of action of the "self"-"non-self" distinguishing system, natural ligands, molecular basis of the catch and release of ligands, and recycling remains unclear.

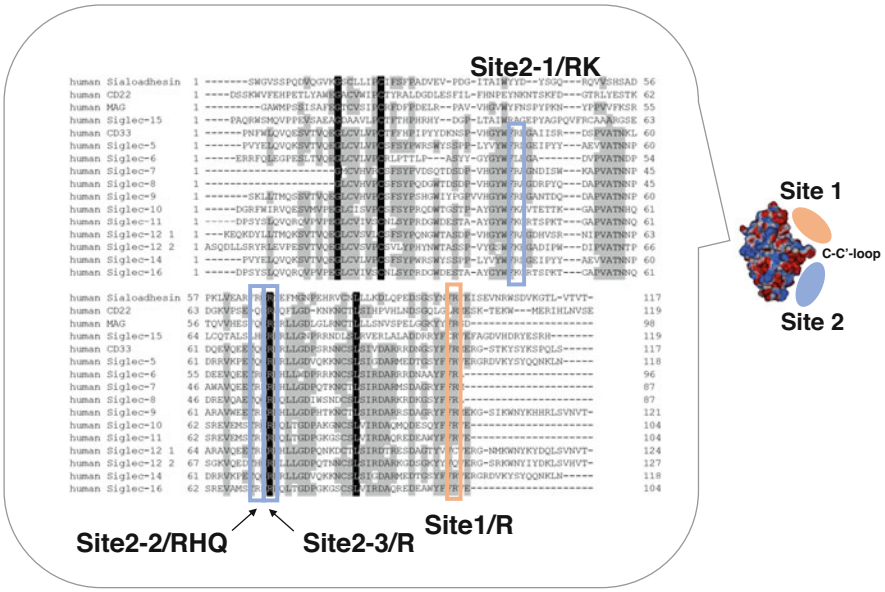


Fig. 3 Amino acid sequence of Siglec. Amino acid sequences of human Siglec1–16 were aligned. The common amino acid sequences among all Siglecs are shown in black. Site 1 is shown in pink and site 2 in blue. Right panel shows the crystal structure of Siglec7. The Siglec12, that is considered to have no Sia-binding ability, has no R in site1

As Siglecs are important for the immune system and cancer-escaping system, the understanding of the role of ligands and ligand-binding systems of Siglecs in biological processes or disease pathogenesis is very important. Recent research on Sia-based chemistry has focused on the establishment of biochemical probes with high affinity for Siglecs as well as specific probes for clinical use (Büll et al. 2016; Hudak et al. 2014; Ohira et al. 2017; Prescher et al. 2017; Yamaguchi et al. 2017) because ligands or ligand-binding systems are keys for medicinal targets.

Siglec7 (CDw328), a member of human CD33-related Siglecs, comprises an extracellular N-terminal V-set domain, two C2-type Ig repeat domains, transmembrane domain, an ITIM, and an ITIM-like motif in the cytosolic region (Fig. 2) (Angata and Varki 2000; Nicoll et al. 1999). It is well known that Siglec7 localizes to NK cells and monocytes and functions as an inhibitory receptor. The expression of Siglec7 is downregulated on an NK cell subset ($CD56^{bright}$) in obese humans (Rosenstock et al. 2017). Downregulation of Siglec7 expression during infection with hepatitis C virus leads to dysfunctional NK cell phenotypes, reduced degranulation, and cytokine secretion (Varchetta et al. 2016). Decreased Siglec7 level is also observed in patients with human immunodeficiency virus (HIV) infection (Brunetta et al. 2009). Therefore, Siglec7-expressing NK cells are more functional than Siglec7-negative NK cells and show higher expression of several activation markers and increased cytokine production (Shao et al. 2016). In monocytes, Siglec7 participates in generating a monocyte-mediated inflammatory outcome following

pathogen recognition (Varchetta et al. 2012). In the case of T cells, Siglec7 is expressed on a small subset of $CD8^+/CD3^+$ T cells and Jurkat cells; T cell activation is inhibited via R124 of Siglec7 (Ikehara et al. 2004). Siglec7 has recently been considered to be a target molecule via regulation of Sia-dependent protection of carcinomas from NK cells because cancer cells are protected by the overexpression of Sia on their surfaces from the survey of immune cells. A hypersialylation state on the surface easily helps a cell to escape from NK cells based on the self (Sia)-recognition by Siglec.

Many reports on the function of Siglec7 were appeared as described above; however, there are not so many reports on the understanding the regulatory mechanism of ligands for Siglec7 and on the identification of natural ligands for Siglec7. In this chapter, we described the recent advance on the establishing the synthetic Sia compounds, understating the molecular regulatory mechanism of ligands, and natural ligands.

2 Ligand-Binding Site of Siglec7

Several crystal structures of Siglec7 with or without ligand(s) have been solved so far (Attrill et al. 2006b; Dimasi et al. 2004; Alphey et al. 2003). The highly variable C–C′ loop (residues 70–75 in Siglec7: Fig. 3) of the V-set domain is a key determinant of Siglec specificity, as observed in domain-swapping experiments (Yamaji et al. 2002). The crystal structure of Siglec7 complexed with a synthetic oligosaccharide corresponding to the α2,8-disialylated ganglioside GT1b revealed the involvement of R124 in Sia binding and the marked conformational change in the C–C′ loop (Alphey et al. 2003). Accumulating data from the crystal structure of Siglec7 with or without ligands allow prediction of its ligands using computational analyses. For instance, docking results using two different Siglec7V structures (an apo form, 1O7V, and a liganded form, 2HRL) demonstrated interesting aspects for ligand recognition as follows: first, a new binding site (site 2, region containing R67 and R92) other than the essential binding site (site 1, region containing R124) was suggested; second, an additional glycan group under the diSia epitope influenced the affinity toward ligand; third, the presence of a new binding site may be regulated by ligand interaction; and fourth, the flexible C–C′ loop transition might be the key for stable interaction. Subsequent biochemical analyses confirmed and supported these observations. In particular, in 2020, a new Sia-binding site other than a primary Sia-binding site (site 1, region containing R124) predicted by in silico analysis was shown by in vitro analyses, equilibrium dialysis, and double reciprocal plot using different ligands for Siglec7 (Yamakawa et al. 2020) (Fig. 2). The new binding site was named as site 2, which is a region containing R67 (site 2–1, Fig. 2). Interestingly, another R residue, R94 (site 2–3, Fig. 2), was found to be conserved among all Siglecs (Yoshimura et al. 2021b). R67 and R94 near the edge of the C–C′ loop are thought to regulate this loop. R92 at site 2 (site 2–2, Fig. 2) was also speculated to bind to the ligand for Siglec7; however, no significant changes aside

from slight modifications were observed by in vitro analysis (Yoshimura et al. 2021b). Mutual regulation of sialic acid binding regions, sites 1 and 2, may be the key mechanism underlying the complexity of these ligands.

3 Interaction Between Siglec7 and Synthetic Glycans

Siglec7 was firstly cloned in 1999 from a human primary dendritic cell cDNA library, and its Sia-based binding was confirmed by the interaction between Siglec7-expressing COS-1 cells and erythrocytes (Nicoll et al. 1999). In addition, the authors used biotinylated polyacrylamide glycoconjugates containing Neu5Acα2,3Galβ1,4Glc (2,3-PAA), Neu5Acα2,6Galβ1,4Glc (2,6-PAA), and Galβ1,4Glc (Lac-PAA) (Fig. 4a) and observed stronger binding with 2,6-PAA than with 2,3-PAA; no binding was observed with Lac-PAA. Interestingly, even after sialidase treatment to remove cis-ligands as sialoglycans on COS-1 cells, Siglec-7 showed the same binding property toward 2,6-PAA and upregulated binding toward 2,3-PAA, unlike other Siglecs (Siglec1, CD22, and Siglec5). These phenomena might be attributed to the different binding sites for 2,3Sia and 2,6Sia, as described in Sect. 2 (Fig. 2). In 2000, the same Siglec7 gene was cloned from human peripheral blood mononuclear cells, and the binding specificity was analyzed using a biotin-conjugated polyacrylamide array (Angata and Varki 2000) of Neu5Acα2,6Galβ1,4Glc (2,6Lac-PAA), Neu5Acα2,3Galβ1,4Glc (2,3Lac-PAA), Neu5Acα2,3Galβ1,3GlcNAc (2,3LacNAc(I)-PAA), Neu5Acα2,3Galβ1,4GlcNAc (2,3LacNAc(II)-PAA), Neu5Acα2,3Galβ1,3GalNAc, Neu5Acα2,6GalNAc (2,6Core1-PAA), Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAc (sLe^x-PAA), and Neu5Acα2,3Galβ1,4(Fucα1,3)GlcNAc (sLe^a-PAA) (Fig. 4a). The highest binding was observed for 2,6Lac-PAA but not for 2,6Core1-PAA, indicating, as described in Sect. 2, that the underlying non-Sia glycan structure or the distance from the backbone polymer structure was also important. In addition, the authors showed the requirement of the glycerol side chain (C_7-C_9) of Sia by periodate treatment. In 2002, ganglioside glycan probes were used for binding analysis (Yamaji et al. 2002). The oligosaccharide part of the ganglioside was released from the native ganglioside by ozonolysis, and the released glycan was conjugated to streptavidin (SA) by reductive amination. The oligosaccharide-SA was then coupled with biotinylated and radioiodinated bovine serum albumin (BSA) to obtain multivalent ganglioside glycans (Fig. 4b). Binding analysis was performed using CHO cells expressing Siglec7, and GD3, GT1b, GD1a, LSTa, LSTb, LSTc, and GM1 probes were prepared. Siglec7 could bind to GD3, GT1b, and LSTb probes (Fig. 4b); it is also bound to GD1a and LSTc, but the binding was only observed for sialidase-treated Siglec7-expressing CHO cells, indicating that the ligand-binding pocket of Siglec7 on CHO cells was already occupied and only opened after sialidase treatment. The presence of different Sia-binding sites, as described in Sect. 2, can be considered. One pocket, probably site 2, may be for a cis-ligand such as GD1a- and LST1c-like molecules. As the GD1a is one of the most abundant gangliosides in the cells, it is

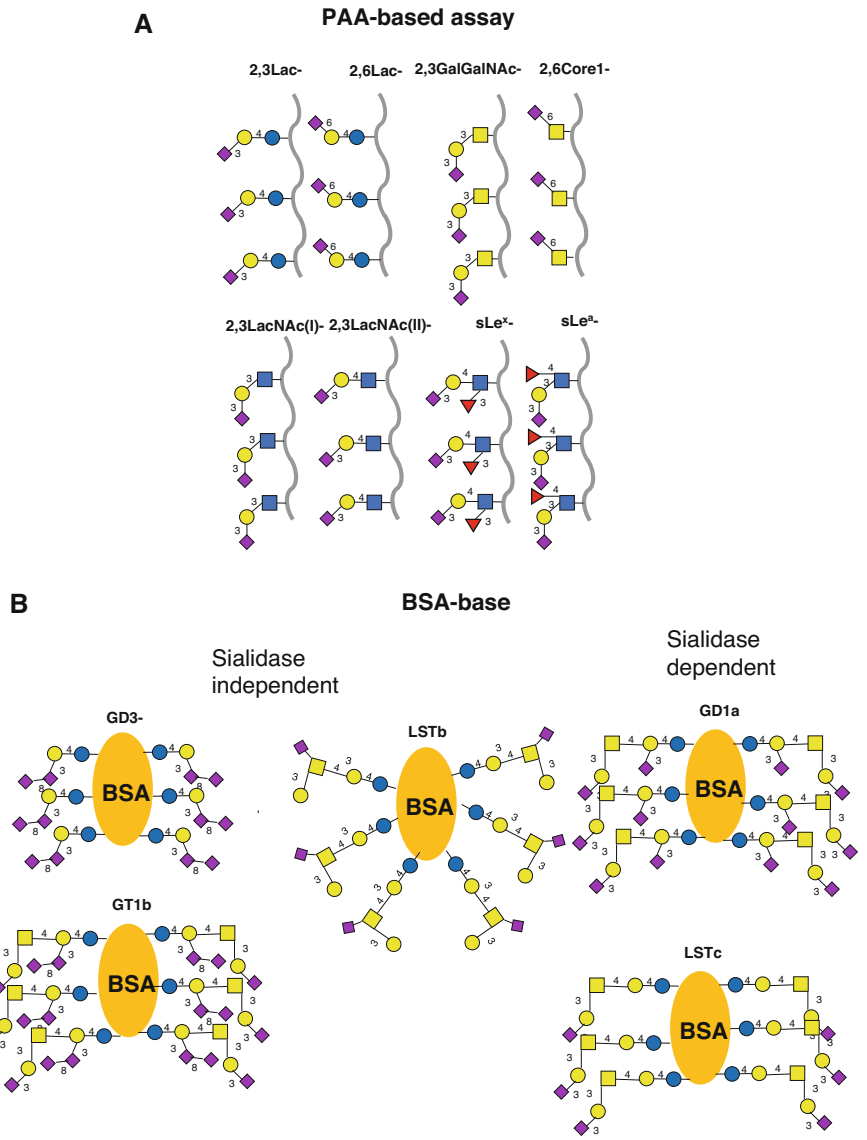


Fig. 4 Siglec ligands used for binding or inhibition assay. (**a**) Polyacrylamide (PAA)-based ligands. 2,3Lac-, 2,6Lac, 2,3GalGalNAc-, 2,6Core1-, 2,3LacNAc(I/II), sLex, and sLea were used, and binding activities were observed. (**b**) BSA-based ligands. Ganglioside oligosaccharides were conjugated to BSA, and multivalent soluble ligands were used for binding assay in Siglec7-expressing cells. GD3, GT1b, and LSTb-BSA probes can bind to Siglec7-expressing cells with any sialidase treatment. On the other hand, GD1a and LSTc showed sialidase-dependent binding. (**c**) ELISA-based ligands. Typical gangliosides were used for the interaction with Siglec7. GD3, GD2, GT1b, GQ1b, GT1a, and 2,6SPG were shown to bind to Siglec7. Disialic, trisialic, and tetrasialic acid containing phosphatidylethanolamine (PE) (Sato et al. 1995) were synthesized and shown to bind to Siglec7. (**d**) Array-based ligands. The glycan array system was used for the analysis of the interaction. Siglec7 showed high activity toward GD3, GD2, triSia, and GT3 and intermediate activity toward Neu5Acα2,6-(Neu5Acα2,3)-GalNAc, 6-sulfosLex, and 6'sulfosLex. (**e**) Ligands used for inhibition. DiSia-octyl, poly-diSia, and diSia-dex were used for the inhibition analysis (see Fig. 5). Only diSia-dex could release Siglec7 from the GD3 plate

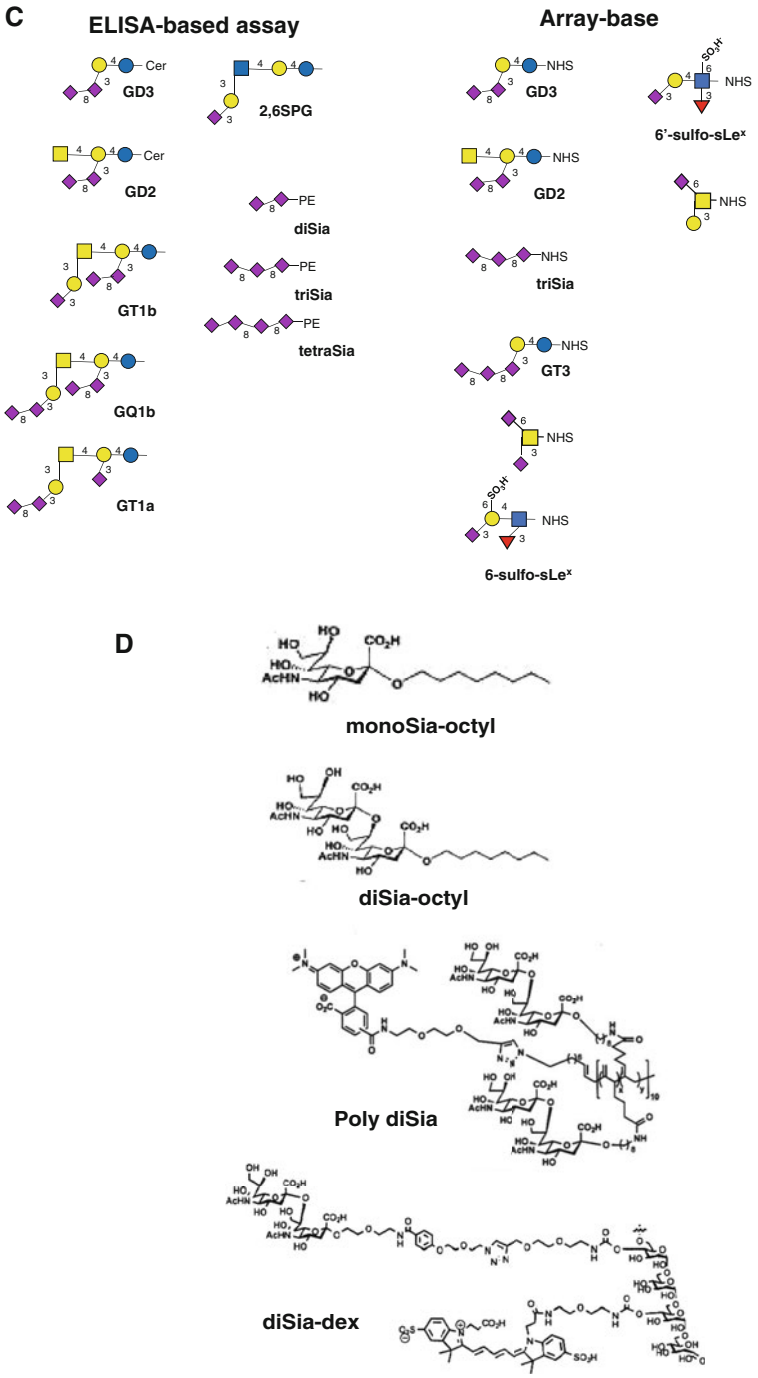


Fig. 4 (continued)

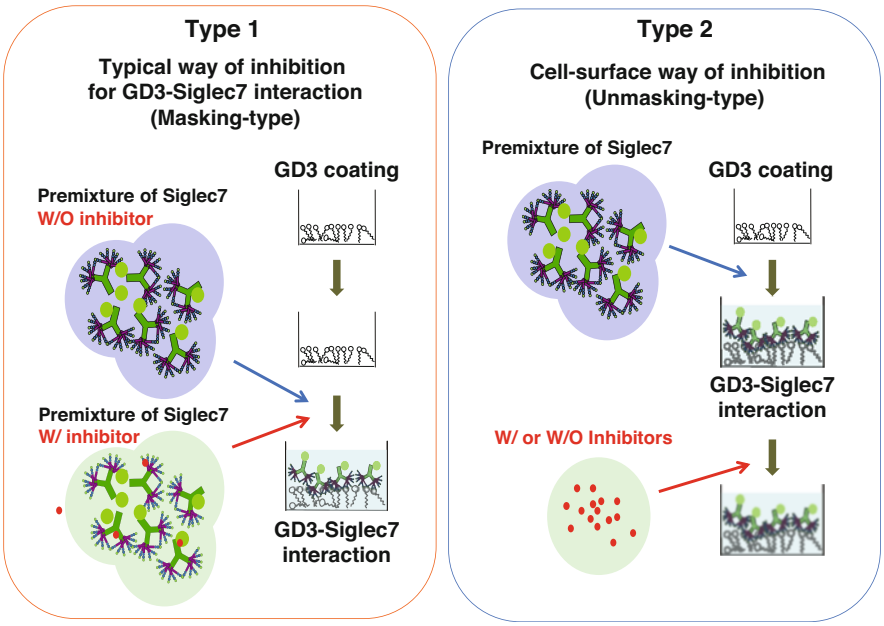


Fig. 5 Inhibition analysis of Siglec7. Two types of inhibition assays were used. Type I inhibition assay is a typical way of inhibition analysis of Siglec7. Type II inhibition assay is a cell surface-type inhibition, which releases the binding site of Siglec7 masked with the ligand on host cells

reasonable that GD1a is the self-signal. Interestingly, Siglec9 did not show any binding toward these multivalent probes, indicating that multivalency is critical feature for Siglec7 binding. In the inhibition assay, free GD3 oligosaccharides inhibited the binding between Siglec7 and gangliosides other than GD1a and LST1c, with IC_{50} values of 5–10 mM. Inhibition analysis using GD3 glycan derivatives such as GD3-C_1 amide, GD3-(C_1-OH), and GD3-(C_7-OH) showed lower inhibition than that observed with GD3 glycan, indicating that C_1 and C_7 positions are important. Chimeric analyses of Siglec7 and Siglec9 revealed a small region (NDISWK) of Siglec7 localized in the C–C′ loop (Fig. 3) responsible for ligand specificity. Interestingly, the three upstream amino acids, R, were shown to form a second Sia-binding site (Figs. 2 and 3) (Yamakawa et al. 2020; Yoshimura et al. 2021b). The binding of GD3, GT1b, and GQ1b was also demonstrated using Siglec7-expressing CHO cells, although the baselines toward these gangliosides were very high (Rapoport et al. 2003) (Fig. 4c).

In 2001, various authentic gangliosides (GD3, GD2, GT1b, GD1a, GD1b, GM2, and GM3), α2,3/6SPG, α2,3-SnLc$_6$, sLe^x, and sLe^a, were analyzed for their interactions with Siglec7. Among these, GD3, GD2, and GT1b appeared to bind Siglec7 (Fig. 4c). In addition, α2,6-SPG could also bind Siglec7 (Ito et al. 2001) (Fig. 4c). Other samples showed no interaction with Siglec7, although GM3 has the same sequence as Neu5Acα2,3Galβ1,4Glc-, which was used to determine the binding using the glycan array system (Nicoll et al. 1999; Angata and Varki 2000). Based on

the solid-phase analysis, the disialyl (diSia) epitope on gangliosides (GD3, GD2, GT1a, GT1b, and GQ1b) was focused on the ligand for Siglec7 (Fig. 4c). Newly synthesized oligoSia-containing glycolipids, oligoSia-PE (Fig. 4c), were analyzed for the requirement of Siglec7 interaction, and di-, tri-, and tetra-Sia containing phosphatidylethanolamine were found to interact with Siglec7 even before sialidase treatment (Yamakawa et al. 2020). The glycan specificity of Siglec7 binding was also analyzed using the glycan array system supplied by the Consortium for Functional Glycomics (Avril et al. 2006a) that showed a new sulfated epitope, (su)-sLe^x (Fig. 4d).

Based on the reports of the interaction between ganglioside glycan and Siglec7, synthetic ganglioside glycans were used to understand the crystal as a ligand-bound conformational form by structural analysis of GT1b and Siglec7 (Attrill et al. 2006a, b). First, oxamide-Neu5Ac was prepared from the methyl α-glycoside of 9-amino-9-deoxy-Neu5Ac via reaction with activated oxamic acid. At the same time, DSLc4 was also used (Attrill et al. 2006b). The synthetic GT1b analog (GT1b-trimethylsilyl)ethyl was used for crystal formation (Attrill et al. 2006a). Both analyses confirmed the interaction between ligands and the R124 residue on Siglec7 that was considered important for Sia binding.

4 Interaction Between Siglec7 and Synthetic Glycopolymers

Glycan multivalency was analyzed using 2,3PAA and 2,6PAA, and almost all results were obtained from multivalent-based ligands or assays (Nicoll et al. 1999) (Fig. 4). The glycan multivalency of Siglecs was also confirmed by liposome-based experiments. The interaction between Siglec1 and its ligand was used as the target of the drug toward macrophages expressing Siglec1. The compound 9-*N*-biphenylcarboxyl-NeuAcα2-3Galβ1-4GlcNAc-ethylamine (39-BPCNeuAc) was introduced into hydroxysuccinimide (NHS)-activated pegylated lipids, and Sia analogue-bearing liposomes were prepared (Chen et al. 2012a). The glycan ligand of Siglec1-decorated liposomes was successfully delivered to macrophages, and internalization was achieved. The same approach was adopted toward CD22 (Chen et al. 2012b). These studies, from the viewpoint of drug delivery, clearly showed that Sia-coated liposomal nanoparticles exhibited effective binding and served as therapeutic material targets for Siglecs. A sialoside library in solution and on-chip was also established (Rillahan et al. 2012, 2013), and a high-affinity ligand for Siglec7 was found where the fluorescein group appended to the C_9 position through a triazole linkage. Researchers also succeeded in synthesizing Siglec7 ligand-coated liposomal nanoparticles to deliver liposomes to Siglec7-expressing cells. The diSia-coated nanoparticles could bind to mouse Siglec-E and inhibit the inflammation of human monocytes and macrophages (Spence et al. 2015).

The consideration of the strength of the affinity of the ligand toward Siglec7 first appeared in the inhibition analysis. The IC_{50} value of the GD3 epitope toward GD3-Siglec7 interaction was 5–10 mM (Yamaji et al. 2002). Later, the synthesized

9-*N*-oxamyl sialosides were shown to have an IC_{50} of 1.6 mM, and the native Neu5Ac had an IC_{50} of 8 mM. If the 9-position was subjected to hydrophobic substitution, the IC_{50} changed to 1/58 (140 μM) of the original value (Prescher et al. 2015). The affinity also increased to 1.6 μM following the substitution at the C-9 position (Prescher et al. 2017). These authors used a monoSia structure for binding monovalent Sia.

The affinity of synthetic diSia structure toward Siglec7 was first measured by Tanaka and Sato (Ohira et al. 2017) who showed that the IC_{50} values of fluorescently labeled poly-diSia (degree of polymerization [DP] = 20), fluorescently labeled poly-diSia (DP = 50), and diSia were 10 μM, 3 μM, and greater than 1 mM, respectively. This was the first quantitative analysis to demonstrate that diSia multivalency effectively changed the affinity. After the development of dextran-based new glycopolymers (Dex), the interaction (K_D) between diSia-Dex and Siglec7 was measured by biolayer interference methods (BLI) and found to be 5.87×10^{-10} M (Yamaguchi et al. 2017). When calculated as the diSia unit, the mono-diSia contribution was almost 2×10^{-7} M. The affinity between diSia-Gal and Siglec7 was 2×10^{-6} M when measured by the equilibrium dialysis method (Yamakawa et al. 2020); based on these values, the multivalent effect on affinity was approximately tenfold. This observation suggests that the multivalency effect was synergistic. The authors also analyzed the inhibitory effect of polySia on the diSia-Siglec7 interaction. PolySia consists of Sia (colominic acid) with a DP of ~40 and could be considered as a polymer of diSia (DP = 20). In addition, the non-reducing terminal ends of polySia and diSia may be the same. However, there was no inhibitory effect of colominic acid on Siglec7-GD3 interaction, indicating that the diSia epitope on polySia, even at the non-reducing terminal end, was not the same glycotope of the diSia structure. This observation is important and consistent with the results of antigen specificity of anti-oligo/polySia antibody toward oligo/polySia structures. In addition, inhibitory effects of glycopolymers such as chondroitin, hyaluronan, heparan sulfate, chondroitin sulfate-6S, chondroitin sulfate-4S, keratan sulfate, and dermatan sulfate were not observed, indicating that any negative charge, except for Sia, is not the cause for interaction in the case of Siglec7. In addition, mannose polymer (mannan), glucose polymer (glucan), and GlcN polymer (chitosan) had no binding ability toward Siglec7.

The IC_{50} of diSia-Dex was shown to be ~1 nM (Yamaguchi et al. 2017), which is almost consistent with the K_D value. The inhibition of monoSia-Dex against binding of GD3 and Siglec7 was achieved at 50 nM concentration, and monoSia-Dex only inhibited half of the binding, indicating that there are several binding sites. As it is demonstrated that Siglec7 had a secondary Sia-binding site around R67, based on in silico analyses, mutation analysis, equilibrium dialysis, saturation transfer difference nuclear magnetic resonance analysis, and reciprocal plots of inhibition assay (Yamakawa et al. 2020), monoSia- and diSia-Dex may distinguish the sites. In addition, each site may show different binding affinity.

For the inhibition analysis, two types of experiments were performed using Siglec7. The typical method (type 1) involves pre-inhibition of Siglec7 using an inhibitor and analysis of GD3 binding (Fig. 5). This inhibition mimicked the

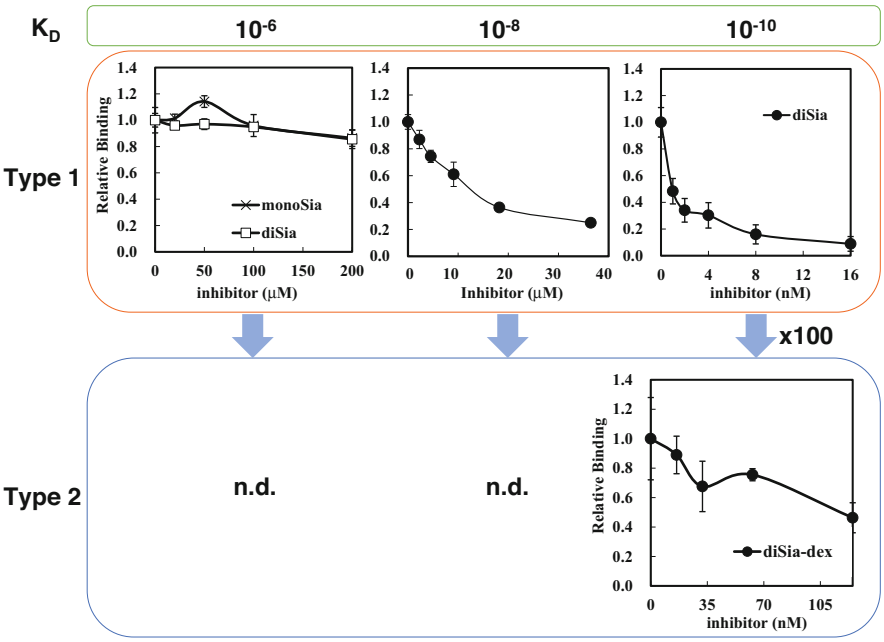


Fig. 6 Summary of the inhibition assay using multivalent ligands. DiSia-octyl, poly-diSia, and diSia-dex were used for the analysis of type I and type II inhibition (see Fig. 5). K_D is also shown. diSia-octyl showed a high K_D value and did not inhibit the interaction between Siglec7 and GD3. Poly-diSia showed only type I inhibition. The K_D is two-order lower than that observed with diSia-octyl. Using diSia-dex, both inhibition types were observed. The K_D was almost 10^{-10} (M), indicating that the affinity greater than 10^{-10} (M) is important for releasing the cis-ligand on host cells from the binding site. Multivalent effect such as mucin-type glycan is necessary

masking effect. In contrast, the type 2 inhibition experiment mimicked the unmasking effect because the inhibitor was used after Siglec7-GD3 binding. These two types of experiments revealed an interesting feature. MonoSia-octyl could not inhibit the interaction (Fig. 6, *upper-left*, monoSia), but 2,3PAA could bind to Siglec7, indicating that the monoSia epitope should be multivalent for Siglec7 binding. This should be a mucin-type O-linked Sia cluster, which was confirmed by thin-layer chromatography (TLC) immunostaining (Yoshimura et al. 2021b). DiSia-octyl also showed no effect on type 1 inhibition (Fig. 6, *upper-left*, diSia), indicating that diSia clusters are required. Unlike GM3, Siglec7 can bind to GD3 ganglioside; thus, the cluster of GD3 localized on lipid rafts may be a ligand candidate. If the diSia epitope increases as a glycopolymer, such as poly-diSia (DP = 20), it shows type 1 inhibition (Fig. 6, *upper-middle*, diSia) and indicates that at least 20 diSia epitopes are required for stable masking. There was no effect on type 2 inhibition using this compound, suggesting that an affinity stronger than that of poly-diSia is required. If the concentration of the diSia epitope in the polymer is increased, as in diSia-Dex, the type I inhibition effect is increased by 100 times. Effective type 2 inhibition was observed using this glycopolymer (Fig. 6, *lower-*

right, diSia), demonstrating that the unmasking effect of diSia clustering requires a K_D of at least 5.87×10^{-10} M based on the BLI-based measurement. Using this glycopolymer, a natural ligand could be identified (Yoshimura et al. 2021a).

For multivalency, GD3 expression experiments are interesting (Hashimoto et al. 2019). A colon cancer cell line, DLD-1, which expresses no ligands for the recombinant protein Siglec7-Fc on the cells, showed no Siglec7 binding even after transfection with *ST8Sia1*, responsible enzyme for GD3. After transfection with *ST8Sia1*, GD3 was confirmed to be expressed in DLD-1 cells using an anti-GD3 antibody. However, the addition of purified GD3 from milk to DLD-1 cells resulted in Siglec7 binding. These authors found that Siglec7 recognizes only GD3-containing regular ceramides, but not phytoceramides. The key enzyme was sphingolipid delta(4)-desaturase/C4-monooxygenase (DES2), and the ceramide moiety involved in raft location (clustering in raft domain) and ligand length from the membrane might be important.

5 Natural Ligands for Siglec7

From the viewpoint of natural ligands, three disialogangliosides, namely, disialylgalactosylgloboside (DSGG), $IV^3Neu5AcIII^6Neu5AcLc_4$ ($DSLc_4$), and $IV^4GalNAcIV^3Neu5AcIII^6Neu5AcLc_4$ ($GalNAcDSLc_4$) (Fig. 7), related to lung metastasis were first identified in renal cell carcinoma (RCC) (Ito et al. 2001). Next, the glycan epitope-responsible genes to synthesize the diSia-epitope were considered. The first gene identified was *ST8SIA1*. Transfection approach showed that Siglec7-mediated Sia recognition modulates NK cell killing activity and has important implications in tumor cell escape from NK cell cytotoxicity (Nicoll et al. 2003). The masking of Siglec7 on NK cells was demonstrated using the 2,8-PAA probe because Siglec7 could bind 2,8-PAA only after sialidase treatment. Activation by interleukin (IL)-2, IL-12, and interferon did not favor any binding toward 2,8-PAA, indicating that Siglec7 was constitutively masked by cis-ligands on NK cells. Overexpression of *ST8Sia1* in P815 cells (target cells) led to higher binding toward Siglec7, suggesting that the newly synthesized GD3 is a potential ligand for Siglec7. Inhibition of NK cell cytotoxicity via DSGB4, a ligand for Siglec7, and Siglec7 interaction was also confirmed (Kawasaki et al. 2010). In 2004, $diSiaLe^a$ was considered as a natural ligand for Siglec7 by gene analysis, and synthetic glycan, $diSiaLe^a$, and $diSiaLe^c$ were used for the analysis of Siglec7 binding (Miyazaki et al. 2004). Although the authors did not purify the glycoconjugates, the results from cell-based and in vitro analyses were consistent.

For the bacterial ligand, *Campylobacter jejuni* strains were analyzed (Avril et al. 2006b). Among the Siglec-tested strains, HS:19($GM1^+$, $GT1a^+$) and HS($GD3^+$) showed binding toward Siglec7 in a Sia-dependent manner. HS($GM1^+$, $GD1a^+$) also showed significant binding properties, but the reaction was Sia-independent (Fig. 7, LPS).

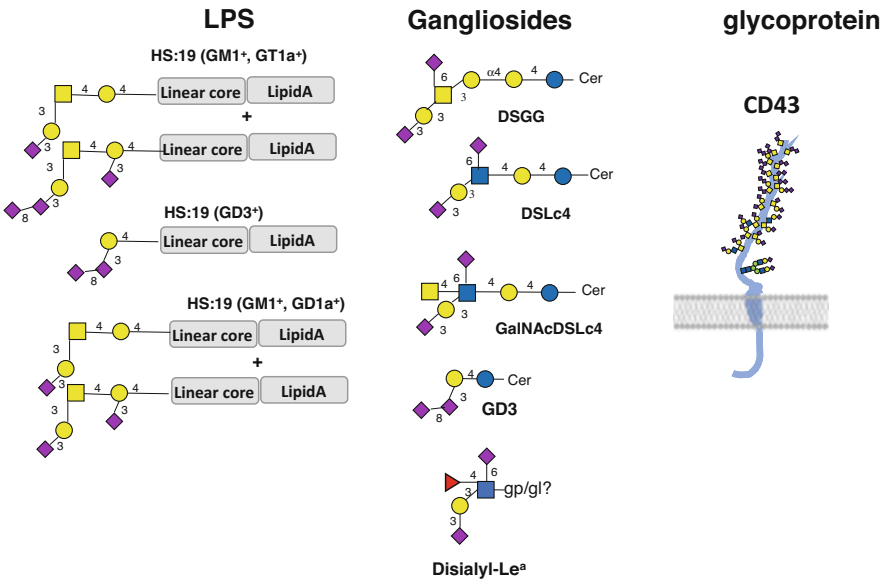


Fig. 7 Natural ligands identified so far. *Campylobacter jejuni* strains have ligands for Siglec7, and HS:19 ($GM1^+$, $GT1a^+$) and HS ($GD3^+$) strains showed binding toward Siglec7 in a Sia-dependent manner. HS ($GM1^+$, $GD1a^+$) also showed significant binding properties but in a Sia-independent manner. Ligands from cancer cells are shown. DSGG, DSLc4, and GalNAcDSLc4 were identified. Using transfection experiments, disialyl Le^a and GD3 were shown to be Siglec7 ligands. From K562 cells, CD43 was identified as a Siglec7 ligand. Ligand on 562 was trapped on a Siglec7 column and specifically eluted using diSia-dex (see Fig. 5). Same results were obtained with the CRISPR system

The natural ligand between NK and K562 cells was analyzed in part. Although NK cells are usually masked by their surface-expressed Sia-containing glycoconjugates, the unmasking system remains unsolved. After unmasking, Siglec7 can bind to tumor cells (K562 cells) because it is a specific inhibitory receptor that checks self and non-self molecules. Therefore, in 2021, proximity labeled molecules on K562 cells were affinity-purified using a Siglec7 column, and specifically bound molecules were eluted with the high-affinity multivalent ligand molecule, diSia-dex (Yamakawa et al. 2020). After mass spectrometry (MS) analysis, the natural ligand of K562 was found to be sialophorin (SPN). At the same time, the possibility of ganglioside ligand expression on K562 cells was analyzed by Siglec7 blotting using TLC immunoblotting; no binding was observed, although the diSia-epitope-containing ganglioside GD3, which was shown to be the Siglec7 ligand using glycan array and enzyme-linked immunosorbent assays (ELISAs), was detected. These data show that the ganglioside on K562 might not be the ligand for Siglec7 and that the trans-natural ligand was SPN. Interestingly, genetically engineered cell-based experiments showed that SPN was a ligand for Siglec7 (Wisnovsky et al. 2021). SPN, also known as leukosialin or CD43, is a type I mucin-type glycoprotein composed of a 19-amino acid signal peptide, an extracellular 255 amino acid region with many O-glycans, and one *N*-glycan near the

transmembrane region (N239). The intracellular 123 amino acid region is related to signal transduction and activation of β-catenin, nuclear factor kappa B, nuclear factor of activated T cells (NFAT), and activator protein-1. The conformational structure of the extracellular domain of SPN shows a rodlike structure, protruding approximately 45 nm from the cell surface. There are approximately 80 *O*-linked glycans in the Ser/Thr-rich region. Based on mutation analysis, it was shown that the Siglec7-binding motif is present at the N terminus of CD43, which is densely glycosylated with disialyl core 1 epitopes and is a favorable location for sensing Siglec7. Further detailed analyses are imperative.

6 Future Perspective

Multivalency is a key feature in understanding natural ligands and drug targeting. Under natural conditions, ligands on glycoproteins and glycolipids are different. In the case of glycoproteins, the natural ligand is localized on the *O*-linked glycan on mucin-type glycoprotein (CD43) (Fig. 8). In the case of glycolipids, especially GD3,

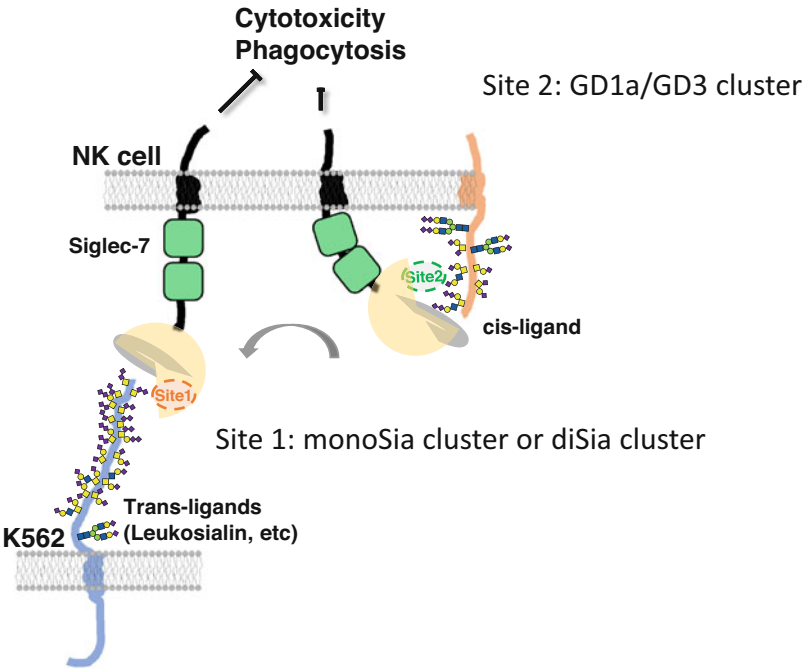


Fig. 8 Hypothesis of the cis/trans ligands of Siglec7. The cells naturally expressing Siglec7, such as NK cells, have masked Siglec7 (masking) because α2,8-PAA showed no binding toward NK cells. Therefore, the cis ligand might be a ganglioside cluster or other glycoproteins on several ligands. NK cells sense cancer cells such as K562 cells when in their vicinity. K562 cells displayed highly dense *O*-lined glycan. NK cells bind to K562 via Siglec7 and mediate immunosuppression. Siglec7 may utilize site 2 and site 1 for cis and trans binding, respectively

the ceramide moiety for localization in the raft and, therefore, the clustering is important. From the viewpoint of synthetic glycopolymers, especially considering binding parameters, clustering ligands such as diSia-dex are the only ligand type that showed the Siglec7-releasing effect from GD3 on the plate. Thus, the affinity of the trans-ligand might be around 10^{10}. Another point is the presence of (at least two) Sia-binding sites on Siglec7 (Fig. 8). Attempts to overcome this affinity have been successful, and in some cases, materials have been used for clinical therapy (Xiao et al. 2016); however, the precise ligand-binding and ligand-releasing mechanisms of Siglecs still remain unclear. A comprehensive consideration of several in silico and in vitro experiments is warranted in the future.

References

Alphey M, Attrill H, Crocker P, van Aalten D (2003) High resolution crystal structures of Siglec-7. Insights into ligand specificity in the Siglec family. J Biol Chem 278:3372–3377

Angata T, Varki A (2000) Siglec-7: a sialic acid-binding lectin of the immunoglobulin superfamily. Glycobiology 10:431–438

Attrill H, Imamura A, Sharma RS, Kiso M, Crocker PR, van Aalten DM (2006a) Siglec-7 undergoes a major conformational change when complexed with the alpha(2,8)-disialylganglioside GT1b. J Biol Chem 281:32774–32783

Attrill H, Takazawa H, Witt S, Kelm S, Isecke R, Brossmer R, Ando T, Ishida H, Kiso M, Crocker PR, van Aalten DM (2006b) The structure of siglec-7 in complex with sialosides: leads for rational structure-based inhibitor design. Biochem J 397:271–278

Avril T, North S, Haslam S, Willison H, Crocker P (2006a) Probing the cis interactions of the inhibitory receptor Siglec-7 with alpha2,8-disialylated ligands on natural killer cells and other leukocytes using glycan-specific antibodies and by analysis of alpha2,8-sialyltransferase gene expression. J Leukoc Biol 80:787–796

Avril T, Wagner ER, Willison HJ, Crocker PR (2006b) Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on campylobacter jejuni lipooligosaccharides. Infect Immun 74:4133–4141

Brunetta E, Fogli M, Varchetta S, Bozzo L, Hudspeth KL, Marcenaro E, Moretta A, Mavilio D (2009) The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia. Blood 114:3822–3830

Büll C, Heise T, Adema GJ, Boltje TJ (2016) Sialic acid mimetics to target the sialic acid-Siglec axis. Trends Biochem Sci 41:519–531

Chen WC, Kawasaki N, Nycholat CM, Han S, Pilotte J, Crocker PR, Paulson JC (2012a) Antigen delivery to macrophages using liposomal nanoparticles targeting sialoadhesin/CD169. PLoS One 7:e39039

Chen WC, Sigal DS, Saven A, Paulson JC (2012b) Targeting B lymphoma with nanoparticles bearing glycan ligands of CD22. Leuk Lymphoma 53:208–210

Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. Nat Rev Immunol 7:255–266

Dimasi N, Moretta A, Moretta L, Biassoni R, Mariuzza RA (2004) Structure of the saccharide-binding domain of the human natural killer cell inhibitory receptor p75/AIRM1. Acta Crystallogr D Biol Crystallogr 60:401–403

Hashimoto N, Ito S, Tsuchida A, Bhuiyan RH, Okajima T, Yamamoto A, Furukawa K, Ohmi Y (2019) The ceramide moiety of disialoganglioside (GD3) is essential for GD3 recognition by the sialic acid-binding lectin SIGLEC7 on the cell surface. J Biol Chem 294:10833–10845

Hudak JE, Canham SM, Bertozzi CR (2014) Glycocalyx engineering reveals a Siglec-based mechanism for NK cell immunoevasion. Nat Chem Biol 10:69–75

Ikehara Y, Ikehara SK, Paulson JC (2004) Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. J Biol Chem 279:43117–43125

Ito A, Handa K, Withers DA, Satoh M, Hakomori S (2001) Binding specificity of siglec7 to disialogangliosides of renal cell carcinoma: possible role of disialogangliosides in tumor progression. FEBS Lett 504:82–86

Kawasaki Y, Ito A, Withers DA, Taima T, Kakoi N, Saito S, Arai Y (2010) Ganglioside DSGb5, preferred ligand for Siglec-7, inhibits NK cell cytotoxicity against renal cell carcinoma cells. Glycobiology 20:1373–1379

Miyazaki K, Ohmori K, Izawa M, Koike T, Kumamoto K, Furukawa K, Ando T, Kiso M, Yamaji T, Hashimoto Y, Suzuki A, Yoshida A, Takeuchi M, Kannagi R (2004) Loss of disialyl Lewis(a), the ligand for lymphocyte inhibitory receptor sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7) associated with increased sialyl Lewis(a) expression on human colon cancers. Cancer Res 64:4498–4505

Nicoll G, Ni J, Liu D, Klenerman P, Munday J, Dubock S, Mattei MG, Crocker PR (1999) Identification and characterization of a novel Siglec, Siglec-7, expressed by human natural killer cells and monocytes. J Biol Chem 274:34089–34095

Nicoll G, Avril T, Lock K, Furukawa K, Bovin N, Crocker P (2003) Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. Eur J Immunol 33:1642–1648

Ohira S, Yasuda Y, Tomita I, Kitajima K, Takahashi T, Sato C, Tanaka H (2017) Synthesis of end-functionalized glycopolymers containing α(2,8) disialic acids via π-allyl nickel catalyzed coordinating polymerization and their interaction with Siglec-7. Chem Commun (Camb) 53: 553–556

Prescher H, Gütgemann S, Frank M, Kuhfeldt E, Watzl C, Brossmer R (2015) Synthesis and biological evaluation of 9-N-oxamyl sialosides as Siglec-7 ligands. Bioorg Med Chem 23: 5915–5921

Prescher H, Frank M, Gütgemann S, Kuhfeldt E, Schweizer A, Nitschke L, Watzl C, Brossmer R (2017) Design, synthesis, and biological evaluation of Small, high-affinity Siglec-7 ligands: toward novel inhibitors of cancer immune evasion. J Med Chem 60:941–956

Rapoport E, Mikhalyov I, Zhang J, Crocker P, Bovin N (2003) Ganglioside binding pattern of CD33-related siglecs. Bioorg Med Chem Lett 13:675–678

Rillahan CD, Schwartz E, McBride R, Fokin VV, Paulson JC (2012) Click and pick: identification of sialoside analogues for siglec-based cell targeting. Angew Chem Int Ed Engl 51:11014–11018

Rillahan CD, Schwartz E, Rademacher C, McBride R, Rangarajan J, Fokin VV, Paulson JC (2013) On-chip synthesis and screening of a sialoside library yields a high affinity ligand for Siglec-7. ACS Chem Biol 8:1417–1422

Rosenstock P, Horstkorte R, Gnanapragassam VS, Harth J, Kielstein H (2017) Siglec-7 expression is reduced on a natural killer (NK) cell subset of obese humans. Immunol Res 65:1017–1024

Sato C, Kitajima K, Inoue S, Seki T, Troy FA, Inoue Y (1995) Characterization of the antigenic specificity of four different anti-(alpha 2-->8-linked polysialic acid) antibodies using lipid-conjugated oligo/polysialic acids. J Biol Chem 270:18923–18928

Shao JY, Yin WW, Zhang QF, Liu Q, Peng ML, Hu HD, Hu P, Ren H, Zhang DZ (2016) Siglec-7 defines a highly functional natural killer cell subset and inhibits cell-mediated activities. Scand J Immunol 84:182–190

Spence S, Greene MK, Fay F, Hams E, Saunders SP, Hamid U, Fitzgerald M, Beck J, Bains BK, Smyth P, Themistou E, Small DM, Schmid D, O'Kane CM, Fitzgerald DC, Abdelghany SM, Johnston JA, Fallon PG, Burrows JF, McAuley DF, Kissenpfennig A, Scott CJ (2015) Targeting Siglecs with a sialic acid-decorated nanoparticle abrogates inflammation. Sci Transl Med 7: 303ra140

Varchetta S, Brunetta E, Roberto A, Mikulak J, Hudspeth KL, Mondelli MU, Mavilio D (2012) Engagement of Siglec-7 receptor induces a pro-inflammatory response selectively in monocytes. PLoS One 7:e45821

Varchetta S, Mele D, Lombardi A, Oliviero B, Mantovani S, Tinelli C, Spreafico M, Prati D, Ludovisi S, Ferraioli G, Filice C, Aghemo A, Lampertico P, Facchetti F, Bernuzzi F, Invernizzi P, Mondelli MU (2016) Lack of Siglec-7 expression identifies a dysfunctional natural killer cell subset associated with liver inflammation and fibrosis in chronic HCV infection. Gut 65:1998–2006

Wisnovsky S, Möckl L, Malaker SA, Pedram K, Hess GT, Riley NM, Gray MA, Smith BAH, Bassik MC, Moerner WE, Bertozzi CR (2021) Genome-wide CRISPR screens reveal a specific ligand for the glycan-binding immune checkpoint receptor Siglec-7. Proc Natl Acad Sci U S A 118:e2015024118

Xiao H, Woods EC, Vukojicic P, Bertozzi CR (2016) Precision glycocalyx editing as a strategy for cancer immunotherapy. Proc Natl Acad Sci U S A 113:10304–10309

Yamaguchi S, Yoshimura A, Yasuda Y, Mori A, Tanaka H, Takahashi T, Kitajima K, Sato C (2017) Chemical synthesis and evaluation of a Disialic acid-containing dextran polymer as an inhibitor for the interaction between Siglec 7 and its ligand. Chembiochem 18:1194–1203

Yamaji T, Teranishi T, Alphey MS, Crocker PR, Hashimoto Y (2002) A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to alpha 2,8-disialyl and branched alpha 2,6-sialyl residues. A comparison with Siglec-9. J Biol Chem 277:6324–6332

Yamakawa N, Yasuda Y, Yoshimura A, Goshima A, Crocker PR, Vergoten G, Nishiura Y, Takahashi T, Hanashima S, Matsumoto K, Yamaguchi Y, Tanaka H, Kitajima K, Sato C (2020) Discovery of a new sialic acid binding region that regulates Siglec-7. Sci Rep 10:8647

Yoshimura A, Asahina Y, Chang LY, Angata T, Tanaka H, Kitajima K, Sato C (2021a) Identification and functional characterization of a Siglec-7 counter-receptor on K562 cells. J Biol Chem 296:100477

Yoshimura A, Hatanaka R, Tanaka H, Kitajima K, Sato C (2021b) The conserved arginine residue in all Siglecs is essential for Siglec-7 binding to sialic acid. Biochem Biophys Res Commun 534:1069–1075

Role of Sialyl-Tn Antigen in Cancer Metastasis

Ruka Ito, Keisuke Nagao, and Kazuaki Ohtsubo

Abstract Glycosylation is the most prominent posttranslational modification of proteins that regulates various protein functions and characteristics. Protein glycosylation is regulated by the cellular expression profile of enzymes regarding glycan and substrate synthesis and the cellular metabolism reflecting cellular status and microenvironments. In the disease process, the glycan synthesis pathway is altered, and aberrant glycans can be generated. Sialyl-Tn (sTn) antigen is a cancer-associated glycan, which is formed on the *O*-glycans of various proteins. The expression of the sTn antigen correlates with poor prognosis and cancer metastasis, in which sTn antigen plays a pivotal role in the cancer progression. ST6GalNAc-I is a glycosyltransferase responsible for the synthesis of the sTn antigen, the expression of which is induced under hypoxic conditions. In cancer progression, cancer cells benefit from intratumoral microenvironments by exchanging signals to the microenvironments and stromal cells through sTn antigen and its receptor proteins. The function of the sTn antigen is relevant to immune suppression, epithelial-mesenchymal transition, remodeling of the extracellular matrix, invasion, generation and maintenance of cancer stem cells, and metastasis. In this chapter, we introduce the accumulated evidences of the pathophysiological function and significance of the sTn antigen and discuss its role in cancer metastasis.

R. Ito · K. Nagao
Department of Analytical Biochemistry, Graduate School of Health Sciences, Kumamoto University, Kumamoto, Japan

K. Ohtsubo (✉)
Department of Analytical Biochemistry, Graduate School of Health Sciences, Kumamoto University, Kumamoto, Japan

Department of Analytical Biochemistry, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
e-mail: kohtsubo@kumamoto-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_4

Abbreviations

ARNT	Aryl hydrocarbon receptor nuclear translocator
cAMP	Cyclic adenosine 3′,5′-monophosphate
COX	Cyclooxygenase
CRE	cAMP response element
CSC	Cancer stem cell
DAP12	DNAX activation protein of 12 KDa
DC	Dendritic cell
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EP	E prostanoid receptor
GalNAc	*N*-acetylgalactosamine
HIF-1	Hypoxia-inducible factor 1
ITAM	Immuno-receptor tyrosine-based activation motif
KLH	Keyhole limpet hemocyanin
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinases
mTORC	Mechanistic target of rapamycin complex
NeuAc	*N*-acetylneuraminic acid
NK cell	Natural killer cell
PGE2	Prostaglandin E2
PHD	Prolyl hydroxylase
PKA	Protein kinase A
ppGalNAcTs	Polypeptide *N*-acetylgalactosaminyltransferases
Siglec	Sialic acid-binding immunoglobulin-like lectin
sTn	Sialyl-Tn
Syk	Spleen trypsin kinase
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinase
VHL	von Hippel-Lindau

1 Introduction

Protein glycosylation is an essential posttranslational modification, which fine-tunes the function of glycoproteins and regulates cellular functions. Dysregulation in glycan synthesis impairs cellular functions and is a part of pathogenesis of various diseases, and aberrant glycans are occasionally detected as biomarkers of disorders (Ohtsubo and Marth 2006). Cellular glycosylation is a comprehensive outcome of portfolio of gene expression and cellular metabolism reflecting cellular signals arising from cellular microenvironments (Ohtsubo and Marth 2006). In the development and progression of cancer, cellular expression profile of

glycosyltransferases, glycosidases, transporters, and chaperonins is altered, and intracellular metabolic pathway is also dramatically shifted that consequently evoke the synthesis of aberrant glycans. Malignant transformation is accompanied by comprehensive increase of cellular sialylation, especially de novo production of α2,6-linked sialic acid, that is functionally relevant to cellular signaling, proliferation, and cellular interaction (Pinho and Reis 2015; Kim and Varki 1997; Sata et al. 1991). The aberrant glycans are often detected as tumor markers associating with tumor progression, malignancy, and metastasis (Kailemia et al. 2017; Angata et al. 2012). Therefore, cancer-associated aberrant glycans can be key links between tumor microenvironment and cellular responses. Sialyl-Tn (sTn) antigen is a glycan epitope bearing the α2,6-linked sialic acid and is used as a clinical tumor marker. There are accumulating findings and evidences indicating that sTn antigen functionally involves in cancer progression and metastasis; however, the underlying mechanism of the sTn antigen-mediated pathogenesis of cancer is still largely unknown. In this chapter, we introduce the sTn antigen-related cellular signal exchange among cells and microenvironments in the immune suppression, epithelial-mesenchymal transition (EMT), remodeling of the extracellular matrix, invasion, generation and maintenance of cancer stem cells, and metastasis.

2 Biogenesis of sTn Antigen

The synthesis of *O*-glycan is initiated by polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts), which transfer *N*-acetylgalactosamine (GalNAc) from a nucleotide sugar donor substrate, UDP-GalNAc, to hydroxy group of serine/threonine residues of peptide. The formed structure is designated as Tn antigen. Normally, the Tn antigen is further glycosylated by a series of glycosyltransferases to form branched, elongated, and complex structures (Fig. 1). In cancer cells, the early termination of the *O*-glycan synthesis is often occurred by 1) malfunction of the *O*-glycan elongation enzyme, Coreβ1,3-galactosyltransferase, caused by the genetic mutation, or the hypermethylation-mediated epigenic down-regulation of its chaperone, COSMC (Ju et al. 2008; Radhakrishnan et al. 2014), 2) aberrant localization of the initiation enzymes of *O*-glycan synthesis, ppGalNAc-Ts, from Golgi apparatus to the endoplasmic reticulum (Gill et al. 2010), 3) out of optimal pH range of glycosyltransferases in Golgi apparatus (Hassinen et al. 2011), and 4) induction of ST6GalNAc-I, which transfers a sialic acid (N-Acetylneuraminic acid, NeuAc) from CMP-NeuAc to the Tn antigen at α2,6-likage and blocks the further elongation of *O*-glycans (Marcos et al. 2004). The resultant disaccharide structure (NeuAcα2,6GalNAcα-*O*-Ser/Thr) is named as sTn antigen (Fig. 1). sTn antigen, a truncated *O*-glycan, is a noted cancer-associated carbohydrate epitope, the expression of which correlates with cancer metastasis and poor prognosis (Ju et al. 2013; Munkley 2016). Besides, the expression of sTn antigen is found in wide variety of cancers (Julien et al. 2012; Ohuchi et al. 1986; Itzkowitz et al. 1989; Thor et al. 1986;

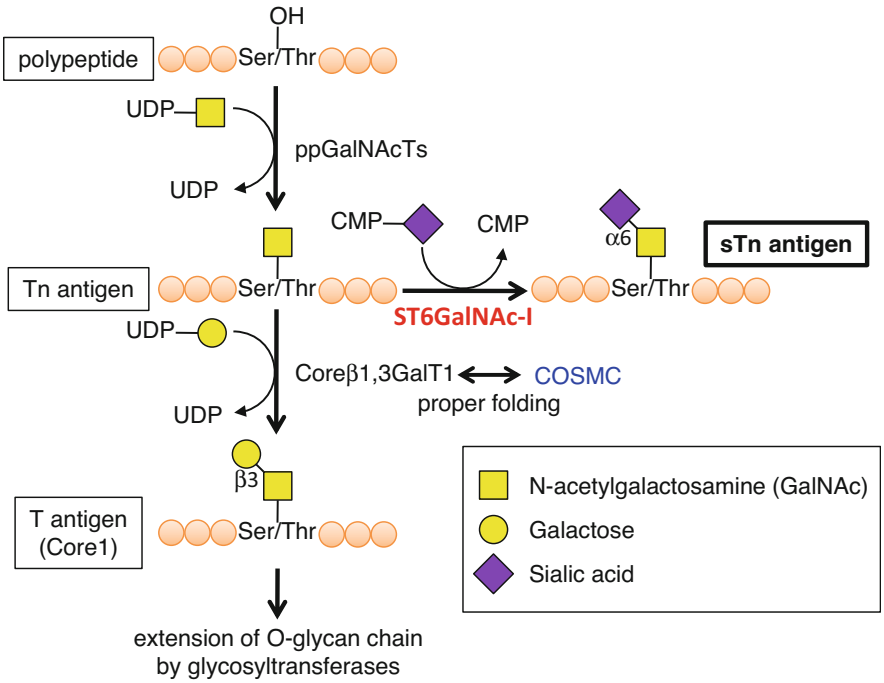


Fig. 1 Early termination of the biosynthesis pathway of *O*-glycans by sTn antigen production. *O*-glycan synthesis is initiated at the hydroxy group of a serine or threonine residue in polypeptide by ppGalNAcTs, which catalyze the transfer of an *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc. The resultant glycan epitope is termed Tn antigen. The Tn antigen is further extended by adding a galactose that is catalyzed by Coreβ1,3-galactosyltransferase (Coreβ1,3GalT). For the acquisition of the enzymatic activity of Coreβ1,3GalT, the proper protein folding by a chaperon, COSMC, is required. The resultant glycan epitope is termed T antigen or core 1 structure and normally further elongated by the sequential reaction of glycosyltransferases. On the other hand, the Tn antigen may receive a sialic acid at α2,6 linkage by ST6GalNAC-I. The resultant glycan epitope is termed sialyl-Tn (sTn) antigen, which terminates the further elongation of the glycan chain. ST6GalNAc-I and Coreβ1,3GalT compete for reaction with the Tn antigen as an acceptor substrate; therefore, enhanced expression of ST6GalNAc-I or malfunction of Coreβ1,3GalT results in the sTn antigen production and the early termination of *O*-glycans

Lottich et al. 1985; Johnston et al. 1986; Flucke et al. 2001; Ching et al. 1993; Genega et al. 2000; Myers et al. 1994; Ferreira et al. 2013; Hashiguchi et al. 2016), so that the detection of sTn antigen in specimens provides clinically important information.

3 Hypoxia-Induced sTn Antigen in Tumor Microenvironments

It has been reported that the expression of sTn antigen is controlled by hypoxia-inducible factor 1 (HIF-1) under hypoxic conditions (Peixoto et al. 2016). Hypoxia is a substantial microenvironment factor heterogeneously generated in growing solid tumor tissues by vascular insufficiency and defective of angiogenesis. Indeed, clinical detection of sTn antigen is correlated with tumor mass, which is usually found in advanced cancers (Kobayashi et al. 1991; Leivonen et al. 2001). Prostate cancer exhibits enhanced expression of ST6GalNAc-I in primary cancer tissues where hypoxic tumor environments should be formed, while the expression is significantly reduced in newly formed metastatic lesions (Munkley et al. 2015). HIF-1 is a heterodimer complex transcription factor, consisting of an oxygen-dependent HIF-α subunit and the aryl hydrocarbon receptor nuclear translocator (ARNT)/HIF-1β subunit. Under normal oxygen concentration, prolyl hydroxylases are constitutively activated, and HIF-1α is hydroxylated at Pro402 and Pro564, which are determinants for the molecular interaction with the von Hippel-Lindau E3 ubiquitin ligase; therefore, HIF-1α is poly-ubiquitinated and degraded by the proteasome pathway (Fig. 2). Under low oxygen concentration, the activity of prolyl hydroxylases is attenuated, so that HIF-1α could escape from degradation and acts as a transcription factor with HIF-1β (Fig. 2) (Myers et al. 1994). HIF-1 is a master regulator controlling expressions of various proteins regarding tumor growth, anti-apoptosis, angiogenesis, immune modulation, invasion, cancer stem cell formation, and cellular metabolic shift from aerobic pathway to anaerobic pathway enabling accommodation in hypoxic tumor microenvironments and contribute tumor progression (Rankin and Giaccia 2016; Vaupel and Multhoff 2018; Zhang et al. 2021). In the hypoxic tumor microenvironments, cancer cells exchange signals with intratumoral congregating cells, including NK cells, macrophages, lymphocytes, neutrophils, platelets, and fibroblasts by juxtacrine, and paracrine fashion. sTn antigen might partly involve in these cellular events through the regulation of function of its carrier proteins.

4 sTn Antigen in Intratumoral Immune Modulation

Several glycoproteins have been previously identified as carrier proteins of sTn antigen, such as MUC1, β1 integrin, CD44, and osteopontin (Clement et al. 2004; Julien et al. 2006, 2009; Ozaki et al. 2012).

MUC1 is a major extracellular matrix (ECM) component providing a functional niche in the tumor microenvironments. MUC1 is a heavily glycosylated protein, and its glycoform is dramatically changed in cancers that include sTn antigen-positive MUC1. The cancer-associated MUC1 is involved in immune modulation. It has been revealed that sTn antigen is a functional determinant of the cancer associated with

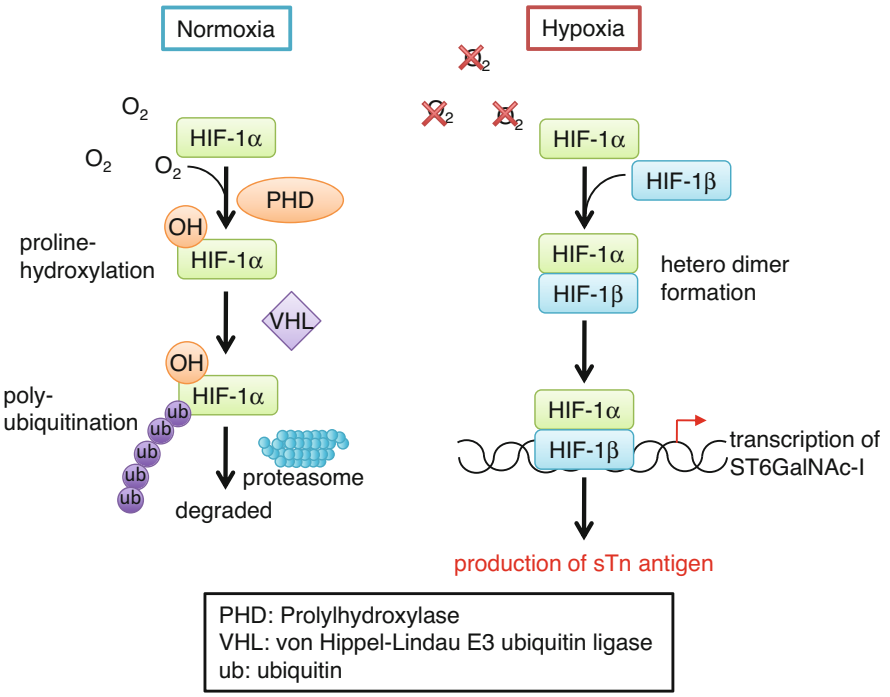


Fig. 2 Hypoxia-induced expression of ST6GalNAc-I. Under normoxic conditions, abundant oxygen allows prolyl hydroxylases (PHD) to react to HIF-1α. The hydroxylated HIF-1α is recognized by von Hippel-Lindau (VHL) E3 ubiquitin ligase and receives poly-ubiquitination. The poly-ubiquitinated HIF-1α is degraded by the proteasome pathway. In contrast, under hypoxic condition, PHD could not obtain enough oxygen for the hydroxylation of target proteins; therefore, HIF-1α could escape from the proteasome-dependent degradations and form a heterodimer with HIF-1β. The heterodimer HIF-1 works as a transcription factor activating various gene expressions, including ST6GalNAc-I. The hypoxia-induced ST6GalNAc-I produces sTn antigen on various glycoproteins

mucin-mediated inhibition of NK cell cytotoxicity, in which removing sTn antigen from mucin by neuraminidase treatment abolished NK cell cytotoxicity (Ogata et al. 1992). These results indicate that sTn antigen is functionally relevant to the immune escape and further suggest the presence of the receptor molecule for the sTn antigen-positive MUC1 on NK cells; however, it has not been identified yet.

In tumor microenvironments, maturation of dendritic cells (DCs) is important for proper immune response for tumor rejection. It has been demonstrated that upregulation of ST6GalNAc-I in bladder cancer coincided with immaturity of DCs (detected as the elevated cellular CD1a levels) that resulted in the lowered secretion of IL-12 and TNF-α and subsequent attenuation of the T lymphocyte priming. Indeed, the sTn antigen-mediated tolerogenic DC function was recapitulated in in vitro observation that DCs, phagocytosed sTn antigen-expressing cancer cells, failed to prime T lymphocyte. Moreover, the induction of tolerance in DCs

encountered with sTn antigen was canceled by treatment with blocking monoclonal antibody against sTn antigen, MUC1, and CD44 (Carrascal et al. 2014), suggesting that the sTn antigen on these carrier proteins is a trigger of the tolerance induction in DCs, at least. These findings indicate that sTn antigen disturbs the DC maturation and suppresses the antitumor response of T lymphocyte. These findings were also recapitulated by another group; cancer-associated MUC1 impaired DC maturation, and T lymphocytes primed by the immature DCs became anergic and act as suppressor and regulatory cells (Monti et al. 2004; Carlos et al. 2005). Moreover, it has been elucidated that cancer-associated MUC1 directly induces anergy in T lymphocyte, in which isolated MUC1 from peritoneal effusion of cancer patients attenuated the proliferation of T lymphocyte that was canceled by the stimulation of T lymphocyte with IL-2 or anti-CD28 monoclonal antibody (Agrawal et al. 1998a). These findings well explain the mechanism of the attenuated immune response for tumor rejection by the intratumoral lymphocytes.

In addition, mucins are important inducers of cyclooxygenase (COX)-2 in tumor microenvironments (Inaba et al. 2003), which is an inflammatory inducible cytoplasmic enzyme that catalyzes the conversion of arachidonic acid into prostaglandins and thromboxanes (van der Donk et al. 2002). One of the major products of COX2 is prostaglandin E2 (PGE2), which is a lipid signal mediator of the inflammation-related biological events associating various immunological reactions, pain reactions, developmental processes, angiogenesis, and tumor progression (Smith et al. 2000; Kaiser et al. 1990). Immunohistochemistry revealed that the deposition of MUC2 in columnar-lined esophagus tissues was overlapped with that of COX2 (Jang and Cho 2005). The biological signal of PGE2 is mediated in autocrine and paracrine manner via E prostanoid (EP) receptors, EP1, EP2, EP3, and EP4, which exhibit different ligand binding affinities and have isoforms generated by alternative splicing; therefore, EPs provide concentration-dependent precise functional regulation of target cells in tumor microenvironments (Abramovitz et al. 2000; Sugimoto and Narumiya 2007). Indeed, enhanced expression of COX2 is relevant to tumoral growth, apoptosis, angiogenesis, and metastasis (Ohno et al. 2005a). It has been reported that the tumoral mucins stimulated intratumoral macrophages and monocytes and induced COX2 production that resulted in PGE2 production (Inaba et al. 2003). The PGE2 secreted from tumoral macrophages should bind to EP2 receptor on cancer cells and induce COX2 production, which evokes various cellular responses. EP2 and EP4 receptors substantially mediate immune-suppressive effects through activation of the cAMP/PKA/CRE (cAMP response element)-binding protein pathway (Subbaramaiah et al. 2008). PGE2 dose-dependently attenuated the proliferation and the following production of interferon-**γ** and interleukin-4 in specific antigen-stimulated helper type 1 (Th1) and helper type 2 (Th2) T lymphocytes in an adenylate cyclase-dependent manner, respectively. Besides, the treatment of PGE2 and EP2 receptor agonist also abolished the specific antigen stimulation-induced production of interferon-γ and interleukin-5 by peripheral blood mononuclear cells (Okano et al. 2006). Taken together, these results imply that PGE2, induced by cancer-associated mucins, stimulates the cAMP-dependent

EP2/EP4-mediated pathway and suppresses Th1- and Th2-polarized antigen-specific T lymphocyte responses in tumor microenvironments.

The cancer-associated mucin-induced COX2 induction and the resulting immune suppression could be implicated in the aberrant glycosylation of mucin. This was evident in endometrial cancer, in which sTn antigen expression was significantly associated with tumoral production of COX2 that coincided with poor infiltration of $CD8^+$ T lymphocyte into the cancer nest (Ohno et al. 2005b).

As described above, cancer-associated mucins receiving aberrant glycosylation, such as sTn antigen, are involved in immune suppression at multiple steps that largely contribute to the escape from immunological tumor rejection.

In addition to the immune suppression, COX2 is involved in the determination of the tropism of metastatic destination organs. In breast cancers, COX2 predominantly regulates Dickkopf (DDK)1 expression, which is a predictive factor whether breast cancer cells metastasize in lung or bone marrow. DDK1 attenuates COX2-mediated recruitment of neutrophils and macrophages in lung metastatic lesion via the non-canonical β-catenin-independent Wnt signaling pathway, and simultaneously DDK1 activates the canonical Wnt/β-catenin signaling pathway in osteoblasts and facilitates bone marrow metastasis (Zhuang et al. 2017).

5 sTn Antigen in Epithelial-Mesenchymal Transition (EMT)

Emergence of truncated *O*-glycans including sTn antigen is found in most of cancers and in various premalignant lesions (Ching et al. 1993; Itzkowitz et al. 1991; Kim et al. 2002). In ovarian cancer, sTn antigen-expressing cells are always found at invasive front edge of tumor tissues and rarely observed in metastatic lesions (Davidson et al. 2000a, b). In breast cancer, sTn antigen is abundantly expressed in primary ductal cancer cells, and lower levels of sTn antigen are found in invasive cancer cells (Schmitt et al. 1995). Indeed, overexpression of sTn antigen in breast and gastric cancer cell lines by introducing the ST6GalNAc-I expression vector resulted in tumor growth and metastasis in murine xenograft models (Julien et al. 2006; Ozaki et al. 2012).

Cellular glycosylation provides various carbohydrate moieties which act as ligands for a wide variety of recognition molecules (Ohtsubo and Marth 2006). In cancer progression, the alterations in cellular glycan structure cause the cellular binding profile of lectins and proteins bearing carbohydrate recognition ability, which results in changes in regulation of cellular responses and in cellular interactions against other cells and ECM (Hauselmann and Borsig 2014). Since sTn antigen has negative charges derived from the intrinsic sialic acid, the expression of sTn antigen terminates *O*-glycan synthesis at early step and simultaneously confers electric charge on the carrier proteins that is implicated in the cellular interactions in microenvironments (Munkley and Elliott 2016).

It has been reported that the sTn antigen expression reduces the cell-cell interaction and releases individual cancer cells from primary cancer nest that were relevant to the altered glycan recognition by lectins (Takenaka et al. 2004; Beatson et al. 2015). And thereby, these findings indicated that the induction of the sTn antigen facilitates tumor metastasis (Munkley et al. 2015) and further suggested that sTn antigen evokes the invasion of cancer cells and consequently facilitates their dissemination by blood and lymph circulation (Pinho et al. 2007). However, these obtained data were insufficient to explain the detailed mechanism of the initiation of the sTn antigen-mediated cancer dissemination.

Most of cancers originate in epithelial tissues. Epithelial cells are closely connected with adjacent cells through engagement of cell adhesion machinery (adherens junctions, tight junctions, and desmosomes) and maintain cellular polarity. These characteristics are inherited to cancer cells. For the initiation of invasion of epithelial cancers, reduction of the cell-cell interactions and activation of the cellular motility are essential that are found in the process of EMT. EMT is a cell-physiological process in which epithelial cells lose their polarity and cell-cell attachment, and therefore cellular motility and invasiveness are increased (Greenburg and Hay 1982; Ribatti et al. 2020). Indeed, the benign tumor cells initially acquire invasive and metastatic features in the process of tumor progression, in which EMT plays a crucial role (Thiery et al. 2009). During EMT process, reduction of E-cadherin, cytokeratins, desmoplakin, and occludin and disappearance of adherens junctions occur that are accompanied with increase of N-cadherin, vimentin, and fibronectin, matrix metalloproteinases (MMP)-2, MMP-3, MMP-9, Snail 1, Snail 2, Twist, FOX C2, and SOX 10 that consequently confer invasive functions and apoptosis resistance on the cells (Garside et al. 2012; Micalizzi et al. 2010; Wells et al. 2008; Ribatti et al. 2020). EMT is regulated by complex cellular signal transduction pathway leading to these synchronized alterations. It has been elucidated that the transforming growth factor (TGF)-β/Smads pathway robustly mediates EMT signals through enhancement of corresponding transcription factors (Xu et al. 2009). Characteristics of EMT are organized under the regulations of transcription factors, Snail1, Snail2, ZEB1, and Twist (Stemmler et al. 2019). Snail1 elicits the reduction of E-cadherin and claudins and the increase of vimentin and fibronectin (Kaufhold and Bonavida 2014). In tumor microenvironments, tumor-associated macrophages are one of the major producers of TGF-β and contribute to the induction of EMT in cancer cells. In the F9-teratocarcinoma-transplanted mouse model, elimination of macrophage by clodronate-liposome treatment reduced mesenchymal gene expression and induced epithelial differentiation of the tumor cells. Moreover, the intratumoral M2-like macrophage was the inducer of the EMT by secreting TGF-β (Bonde et al. 2012).

Glycan-mediated molecular interactions between cancer-associating glycoproteins and lectins on immune cells are deeply involved in the modulation of tumor microenvironments (Aarnoudse et al. 2006; Rabinovich and Croci 2012). In line with these findings, tumoral infiltration of M2-like macrophages was found in sTn antigen expressing ulcerative colitis and colon cancer tissues, where the tumor-associated M2-like macrophage produced IL-13 and CCL17 and stimulated colon

cells that promote the expression of ST6GalNAc-I (Kvorjak et al. 2020). In the tumor microenvironment, M2-like macrophage and sTn antigen expressing cancer cells might exchange signals in paracrine and juxtacrine manner and initiate EMT. For the recognition of sTn antigen, M2-like macrophage might express an endogenous lectin as a receptor molecule. Sialoglycans, which are glycans bearing sialic acid moiety, are usually recognized by lectin family consisting of sialic acid-binding immunoglobulin-like lectins (Siglecs) (Crocker and Varki 2001; Varki and Angata 2006). Siglecs are receptor-type lectins mainly expressed on immune cell linage and exert immuno-suppressive effects. Besides, a subfamily of Siglecs lacks the cytoplasmic domain and is associated with signal adaptor molecule DNAX activation protein of 12 KDa (DAP12) at plasma membrane. DAP12 has an immuno-receptor tyrosine-based activation motif (ITAM) and mediates activation signals for the upregulation of the production of cytotoxic cytokines by modifying Toll-like receptor signals in immune cells including macrophages (Lanier 2009). The enhanced expression of DAP12 correlates with poor prognosis in breast cancer (Shabo and Svanvik 2011). Siglec-15 is a member of this subfamily, expressed on macrophages and DCs, and preferentially binds to the sTn antigen (Angata et al. 2007; Ishida-Kitagawa et al. 2012). It has been revealed that the tumoral infiltrating M2-like macrophages express Siglec-15 in clinical cancer tissues of the liver, lungs, and rectum, which suggests the tumor-associated M2-like macrophages have a potential to recognize the sTn antigen on cancer cells (Takamiya et al. 2013). Indeed, coculture of M-CSF-induced human macrophage with the sTn antigen-expressing cancer cells, which were established from human non-small lung carcinoma cells, enhanced TGF-β production. The sTn antigen-expressing cancer cell-induced TGF-β production was recapitulated in genetically engineered THP-1 cells (human promonocytic cell line) expressing Siglec-15 that was significantly reduced by the genetic mutation of Siglec-15 or by the disruption of the sTn antigen on cancer cells by sialidase treatment. These findings indicated that Siglec-15 on macrophages is a responsible receptor molecule for the recognition of the sTn antigen on cancer cells (Takamiya et al. 2013). Extracellular ligand engagement with DAP12-associated cell surface receptor causes the recruitment of spleen trypsin kinase (Syk) to DAP12 and transduces the signal across the plasma membrane in immune cells (Humphrey et al. 2005; Turnbull and Colonna 2007). In consistent with this, the sTn antigen-dependent production of TGF-β was abolished by the treatment of Syk inhibitor (Takamiya et al. 2013). These findings indicate that the tumoral sTn antigen is recognized by Siglec-15 on intratumoral M2-like macrophages in which the cellular signal is mediated by DAP12-Syk pathway (Fig. 3). The mechanism is a key link between the tumoral sTn antigen expression and the induction of EMT in tumor microenvironments.

For releasing cancer cells from the tumor nest, as well as the reduction of cell-cell interaction, the degrading and remodeling of the ECM surrounding cancer cells are also essential initial steps in metastatic processes. A family of proteinases, MMPs, is deeply involved in the degradation and remodeling of ECM in tumor progression (Egeblad and Werb 2002; Page-McCaw et al. 2007). Humans have 23 MMPs consisting of 17 soluble MMPs and 6 membrane-associated MMPs (Quesada et al.

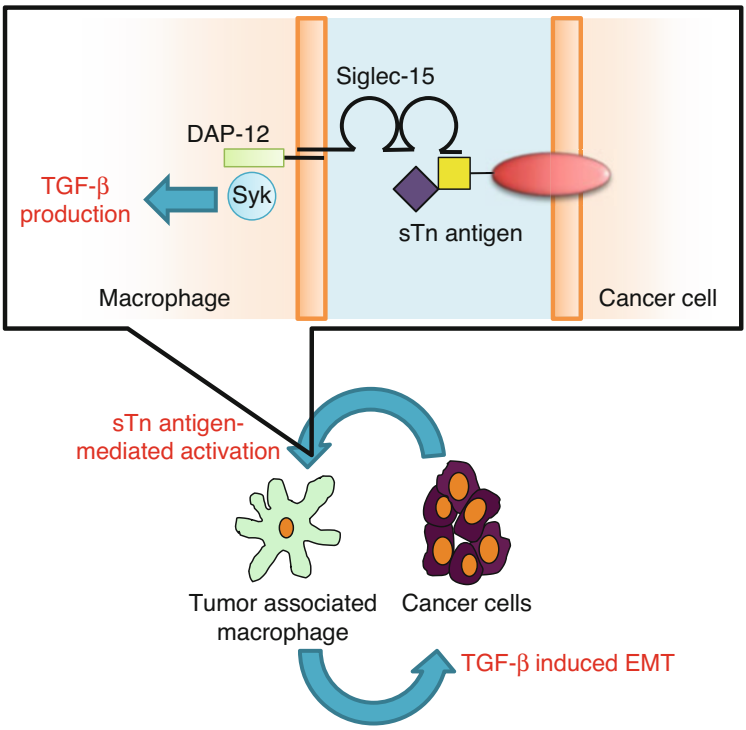


Fig. 3 sTn antigen-mediated activation of tumor-associated macrophage and the induction of EMT. Cancer cell presents sTn antigen on cell surface glycoproteins. The sTn antigen is recognized by sialic acid-binding immunoglobulin-like lectin (Siglec)-15 on the tumor-associated macrophage. The ligand-binding signal is transduced to spleen trypsin kinase (Syk) through an adaptor molecule, DNAX activation protein of 12 KDa (DAP12) at plasma membrane. The activated Syk signal results in the production of TGF-β. TGF-β is a strong inducer of epithelial-mesenchymal transition (EMT) of cancer cells that facilitates metastasis and cancer progression

2009). They are further classified by domain architecture, substrate specificity, and their spatial and temporal expression profiles (Radisky and Radisky 2010). The catalytic domain of MMPs is composed of an active center, and a substrate binding pocket is the target of the endogenous tissue inhibitors of metalloproteinases (TIMPs); thereby, MMP activity is physiologically regulated by the production of TIMPs (Nagase et al. 2006). Moreover, MMPs are produced as cryptic proenzymes, in which an N-terminal short prodomain structurally blocks active site (Tallant et al. 2009; Visse and Nagase 2003). The sequential cleavage of MMPs by proteinases removes the prodomain and releases the active MMPs (Visse and Nagase 2003; Rosenblum et al. 2007). Expression of MMPs is regulated through various signal cascades in response to the tumor microenvironments. It has been reported that the expressions of MMP-2, MMP-12, and MMP-13 were transcriptionally enhanced in the breast cancer cells undergoing TGF-β-induced EMT (Janda et al. 2002; Jechlinger et al. 2003). In addition, the TGF-β treatment of glioma cell lines

exhibited the induction of MMP-2 expression and the reduction of TIMP-2 expression that resulted in enhanced cellular invasion (Wick et al. 2001). Besides, hypoxia also induces expression of MMPs: MMP-2 and MT1-MMP in breast cancer (Muñoz-Nájar et al. 2006), MMP-2 in oral squamous cell carcinoma (Ma et al. 2019), MMP-2 and MMP-9 in breast cancer cells (Choi et al. 2011; Lv et al. 2017), MT1-MMP in ovarian cancer cells (Sun et al. 2015), MMP-1 in bladder cancer cells (Shin et al. 2015), MMP-2 in prostate cancer cells (Dai et al. 2011), MMP-2 and MMP-9 in monocytes (Wan et al. 2011), and MMP-9 in vascular smooth muscle cells (Revuelta-López et al. 2013). These findings indicate that the emerging intratumoral microenvironments, such as sTn antigen-related TGF-β-induced EMT and hypoxia, induce the expression of MMPs to degrade and remodel the ECM and then enable cancer cells to detach from tumor nest.

6 sTn Antigen Expression and Integrin-Mediated Tumor Invasion

As well as reducing cell-cell interactions and degrading ECM to release cancer cells from tumor nest, enhancing automotility to invade into surrounding tissues is a fundamental metastatic process. For activating cellular motility, the continuous interaction with the ECM components and modulation of cellular signals are required. ECM is the complex network of specialized glycoproteins, including collagen, fibronectin, laminin, and vitronectin. The cellular adhesion signal of these macromolecules regulates various cell-physiological events: cellular proliferation, differentiation, survival, and migration (Berrier and Yamada 2007). These cellular activities are required for metastasis. For recognition of ECM proteins and exchange signals, mammalian cells express cell surface receptor molecules, integrins. Integrins form a heterodimeric complex consisting of an α integrin and a β integrin, which have a large extracellular domain and a short cytoplasmic tail. The extracellular domains of integrin subunits together form a head-domain having a ligand-binding activity (Zaidel-Bar et al. 2007). In vertebrates, 24 distinct integrin complexes are identified as the selective pairing of 18-α integrins and 8-β integrins; they have different ligand preferences and affinities (Luo et al. 2007). β integrins determine the subcellular distribution of the integrin heterodimer at focal adhesion sites (Akiyama et al. 1994; Reszka et al. 1992). The signal of the engagement of the ECM ligands is mediated at the cytoplasmic tail by associating with adaptors bridging integrin to kinase, cytoskeletal proteins, and other plasmalemmal receptors that evoke the assembly of actin filaments (Giancotti and Ruoslahti 1999). The function of integrin heterodimer is conformationally regulated. In the inactive state, the integrin heterodimer takes bent form bearing low affinity to ECM ligands. If the ECM ligand binds to integrin, the conformation of the integrin heterodimer is changed to upright-close form bearing moderate affinity to ECM ligands. Subsequently, the conformation is further changed to the upright-open form (the β-tail

domain and the cytoplasmic tail domain of β integrin are separated from α integrin) bearing high affinity to ECM ligands (Barkan et al. 2008). Conversely, the molecular interaction of an activator, e.g., talin and kindlin, with the cytoplasmic tail of β integrin also induces alterations in the conformation of the integrin heterodimer that enlarge the affinity to ECM ligands (Barkan et al. 2008). Following the initial activation of integrin with ECM, the large mature focal adhesion complex is orchestrated to generate signals for cellular proliferation, differentiation, survival, and migration (Barkan et al. 2008; Shibue and Weinberg 2009). These findings suggest that the conformation of the cytoplasmic tail and the β-tail domain is important to regulate the activation status of integrins. In line with this speculation, mutations in these regions of either integrin resulted in the constitutive activation (O'Toole et al. 1991, 1994).

Since integrins are involved in cell migration and invasion in pathophysiological events (Huttenlocher and Horwitz 2011; Paul et al. 2015), most of the biological functions of integrins have been investigated in cancer cells. As β1 integrin is the most abundant β integrin subunit (Sastry and Horwitz 1993) and acts as a connector to actin in the cytoplasmic tail (Hynes 1992), many studies have suggested the pathophysiological significance of β1 integrin in cancer progression (Hamidi et al. 2016). Indeed, the blocking antibody treatment against β1 integrin suppressed cancer cell proliferation in vivo and in vitro that was accompanied with significant impairment of tumoral angiogenesis (Park et al. 2006; Bhaskar et al. 2007).

It has been considered that the glycosylation is one of the major regulators of integrin function (Janik et al. 2010; Yoon et al. 2005; Gu and Taniguchi 2004). Especially in the cancer progression, altered glycosylation is associated with intracellular distribution and function of integrins. It has been reported that overexpression of ST6GalNAc-I in murine mammary adenocarcinoma cell line resulted in the expression of the sTn antigen on β1 integrin that altered cellular morphology and cellular behaviors (Clement et al. 2004). Moreover, exposure of bladder tumor cell lines to hypoxia induced production of sTn antigen on various glycoproteins, including β1 integrin that was coincident with enhanced cellular migration and invasion (Peixoto et al. 2016). sTn antigen is considered to be functionally involved in the activation of the cellular signals downstream of β1 integrin; however, the sTn antigen-dependent regulation mechanism of β1 integrin remains unclear. Although the *O*-glycosylation sites of β1 integrin have not been identified, potential *O*-glycosylation sites can be predicted by in silico analyses using NetOGlyc 4.0 program (https://services.healthtech.dtu.dk/service.php?NetOGlyc-4.0) (Steentoft et al. 2013). The prediction of the *O*-glycosylation site analyses revealed that β1 integrin has potential *O*-glycosylation sites, including two sites in hybrid domain, three sites in B-I domain, three sites in I-EGF1 domain, one site in I-EGF4 domain, and three sites in β-tail domain (Fig. 4). Noteworthy, as this prediction, β1 integrin receives the sTn antigen in the β-tail domain, which is important for the conformational regulation, and in B-I domain, which contains a metal binding motif. sTn antigen has an intrinsic negative charge which may cause intramolecular structural changes modulating the ligand affinity and may produce

A

```
  1 MNLQPIFWIGLISSVCCVFAQTDENRCLKANAKSCGECIQAGPNCGWCTNSTFLQEGMPTSARCDDLEALKKKGCPPDDI 80
 81 ENPRGSKDIKKNKNVTNRSKGTAEKLKPEDITQIQPQQQLVLRLRSGEPQTFTLKFKRAEDYPIDLYYLMDLSYSMKDDLE 160
161 NVKSLGTDLMNEMRRITSDFRIGFGSFVEKTVMPYISTTPAKLRNPCTSEQNCTSPFSYKNVLSLTNKGEVFNELVGKQR 240
241 ISGNLDSPEGGFDAIMQVAVCGSLIGWRNVTRLLVFSTDAGFHFAGDGKLGGIVLPNDGQCHLENNMYTMSHYYDYPSIA 320
321 HLVQKLSENNIQTIFAVTEEFQPVYKELKNLIPKSAVGTLSANSSNVIQLIIDAYNSLSSEVILENGKLSEGVTISYKSY 400
401 CKNGVNGTGENGRKCSNISIGDEVQFEISITSNKCPKKDSDSFKIRPLGFTEEVEVILQYICECECQSEGIPESPKCHEG 480
481 NGTFECGACRCNEGRVGRHCECSTDEVNSEDMDAYCRKENSSEICSNNGECVCGQCVCRKRDNTNEIYSGKFCECDNFNC 560
561 DRSNGLICGGNGVCKCRVCECNPNYTGSACDCSLDTSTCEASNGQICNGRGICECGVCKCTDPKFQGQTCEMCQTCLGVC 640
641 AEHKECVQCRAFNKGEKKDTCTQECSYFNITKVESRDKLPQPVQPDPVSHCKEKDVDDCWFYFTYSVNGNNEVMVHVVEN 720
721 PECPTGPDIIPIVAGVVAGIVLIGLALLLIWKLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK  798
```

B

Position	Prediction score	Position	Prediction score
96	0.656	468	0.522
99	0.638	504	0.62
197	0.858	509	0.639
198	0.735	602	0.615
199	0.694	660	0.5
432	0.665	675	0.561
440	0.5		

C

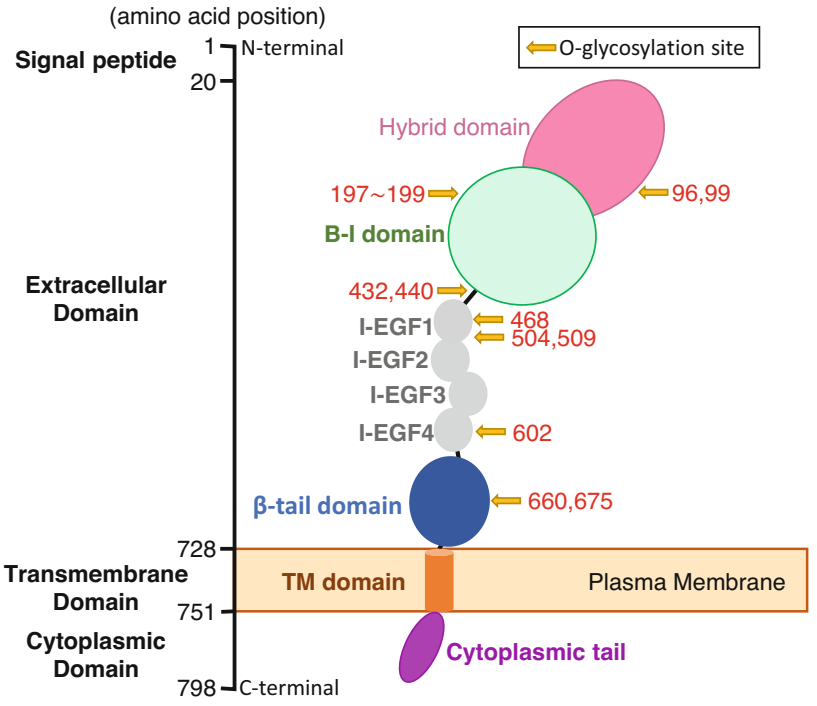


Fig. 4 Prediction of *O*-glycosylation sites of human β1 integrin. (**a**) Amino acid sequence of human β1 integrin with potential *O*-glycosylation sites. *O*-glycosylation sites of human β1 integrin were analyzed by NetOGlyc 4.0 program (Steentoft et al. 2013). Predicted *O*-glycosylation sites are indicated as red-colored characters in amino acid sequences of β1 integrin. Each domain of β1 integrin is indicated as the respective color corresponding to the domain of β1 integrin illustrated in (**c**). (**b**) Prediction score of individual deduced *O*-glycosylation site. The obtained data from the analyses by NetOGlyc 4.0 program was summarized in a chart. (**c**) Schematic diagram of human β1 integrin. The hybrid domain, B-I domain, I-EGF1 ~ 4 domains, β-tail domain, TM domain, and cytoplasmic domains are illustrated. The position of the individual predicted *O*-glycosylation site is

the intermolecular repulsive force between α and β integrin subunits that may lead to the conformational change to the open form of the integrin heterodimer.

Glycoproteomic analyses of integrins will confirm the glycosylation profile of integrins and should provide detailed information about the structure-function relationships of integrin glycans; these investigations will reveal the regulation mechanism of integrins and provide molecular insight into the pathophysiological role of integrins.

7 sTn Antigen Expression and Cancer Stem Cells

In solid tumors, hypoxia is a major feature of tumor microenvironment, which provides an essential niche for development and accumulation of cancer stem cells (CSCs). CSCs are poorly differentiated cells, possessing the intensive invasiveness and tumorigenicity that directly and indirectly contribute to the generation of metastatic cells (Shiozawa et al. 2013). CSCs have been detected in various cancers: breast cancer (Quinn et al. 2021), hepatocellular carcinoma (Tomuleasa et al. 2010), glioblastoma (Ishii et al. 2021), melanoma (Fattore et al. 2020), and head and neck cancer (Yoshikawa et al. 2013). Although the origin of CSCs has not been fully identified yet, the source of the cells is considered to be generated in the process of EMT and under the intratumoral hypoxic conditions, in which differentiated and non-stem cells might acquire the characters like CSCs (Li et al. 2019; Nejad et al. 2021). Intratumoral hypoxia acts as an inducer of the CSC marker protein, CD133, via the HIF-1α-mediated enhancing of the expression of OCT3/4 and SOX2 (Iida et al. 2012). Since CSCs differ from differentiated cancer cells in the gene expression profile and cellular metabolism, CSCs should express specific glycans, which allow distinguishing CSCs from non-cancer stem cells (Alisson-Silva et al. 2014; Lanctot et al. 2007). It has been demonstrated that the glycosylation pattern of CD133 might be involved in the maintenance of cancer stem cells (Bidlingmaier et al. 2008).

As hypoxia is a common inducer of sTn antigen expression and development of CSCs, some populations of cancer stem cells express sTn antigen. It has been reported that a subset of human ovarian cancer cells is positive for CD133 and sTn antigen that exhibited the features of CSC: enhanced colony and sphere formation and elevated chemoresistance (Starbuck et al. 2018). As described above, sTn antigen bearing MUC1 enhances the expression of COX2, which produces PGE2. PGE2 is also important for cancer stem cell maintenance; PGE2 induces IL-6 in the triple-negative breast cancer cell lines that regulate the renewal of CSCs (Basudhar et al. 2017). It has been revealed that the proliferation and stemness properties of CSCs are regulated by PI3K-Akt signal pathway in breast cancer (Gargini et al.

Fig. 4 (continued) indicated as an arrow with numbers of amino acid positions from N-terminal. The structure of β1 integrin is illustrated based on the UniProt database (http://www.uniprot.org/)

2015, 2016), glioma (Gargini et al. 2016), and colorectal cancer (Todaro et al. 2014). In colorectal cancers, sTn antigen is involved in the regulation of Akt pathway. sTn antigen is expressed on CD44 which induces the phosphorylation of Akt cooperating with galectin-3. The Akt activation results in the reduction of GSK3β and subsequently promotes the β-catenin-mediated transcription. The Akt signal is branched to mTORC1 which causes phosphorylation of S6 and enhances protein synthesis. Both of the downstream of the Akt signals evoke stemness in cancer cells (Ogawa et al. 2017). These findings indicate that sTn antigen plays a curtail role in generation, maintenance, and renewal of CSCs at multiple steps in tumor microenvironments that substantially contribute to the generation of metastatic cells.

8 Conclusion and Future Perspectives

In the present chapter, we have provided current knowledge of the function of sTn antigen in cancer metastasis and progression, which are closely related to immune suppression, EMT, remodeling the extracellular matrix, enhancing cellular motility, generation, and maintenance of cancer stem cells. In these cancer-related events, sTn antigen and its carrier proteins evoke various alterations in cellular functions through the exchange of cellular signals that contribute to the facilitation of metastasis. Therefore, these molecules and cellular signals are good candidates for the therapeutic targets. Vaccination with sTn-keyhole limpet hemocyanin (KLH) was clinically successful to induce production of sTn antigen-specific IgGs (Miles et al. 2011; Miles and Papazisis 2003; Ibrahim and Murray 2003). Moreover, sTn-KLH vaccination induced sTn antigen-specific antibodies and eliminated tumors in murine models (Julien et al. 2005). Theratope (sTn-KLH vaccination) could induce specific immune response against sTn antigen and exhibited a significantly improved survival in phase II clinical trials for metastatic breast cancer patients (Miles et al. 1996; MacLean et al. 1996a, b; Holmberg and Sandmaier 2004; Reddish et al. 1996); however, double-blind phase III clinical trial of Theratope failed to improve survival of patients, and no overall benefit in time to progression was observed (Miles et al. 2011). These findings indicate that immunotherapy with sTn antigen has a potential to induce specific immune responses in cancer patients, but there are difficulties in obtaining successful clinical outcomes that need to be clarified in the future. Besides, the pharmacological intervention to inhibit the ST6GalNAc-I or nucleic acid drugs to suppress ST6GalNAc-I expression in cancer cells might be capable to suppress cancer progression and metastasis, because the administration of the ST6GalNAc-I siRNA-liposome significantly suppressed peritoneal dissemination and improved the survival of the mice receiving gastric cancer xenografts (Tamura et al. 2016). As the expression of ST6GalNAc-I is specifically expressed in cancer cells, these approaches may be tolerated by patients with little side effects. Other than targeting the sTn antigen or ST6GalANc-I, vaccination targeting MUC1 exhibited a favorable toxicity profile in the initial phase I and II trials (Agrawal et al. 1998b; Palmer et al. 2001; North and Butts 2005), and COX2 inhibitor (celecoxib) treatment exhibited

positive outcome in clinical trials of colorectal cancer, breast cancer, gastric cancer, and head and neck cancer (Tołoczko-Iwaniuk et al. 2019). As these target molecules and cellular signals are commonly and deeply involved in the progression and metastasis of various cancers, the medications targeting them in cancer therapy seem to be justified. Future studies elucidating the detailed molecular functions of sTn antigen in the regulation of its carrier proteins and exploring the sTn antigen-recognizing receptors will contribute to better understand the role of sTn antigen in cancer metastasis and to the development of new drugs and therapeutic approaches.

Acknowledgments This work was partly supported by JSPS KAKENHI Grant Number JP21K06082 (K.O.).

Declaration of Conflict of Interest None declared.

References

Aarnoudse CA, Garcia Vallejo JJ, Saeland E, van Kooyk Y (2006) Recognition of tumor glycans by antigen-presenting cells. Curr Opin Immunol 18:105–111. https://doi.org/10.1016/j.coi.2005.11.001

Abramovitz M, Adam M, Boie Y, Carriere M, Denis D, Godbout C, Lamontagne S, Rochette C, Sawyer N, Tremblay NM, Belley M, Gallant M, Dufresne C, Gareau Y, Ruel R, Juteau H, Labelle M, Ouimet N, Metters KM (2000) The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. Biochim Biophys Acta 1483:285–293. https://doi.org/10.1016/S1388-1981(99)00164-X

Agrawal B, Krantz MJ, Reddish MA, Longenecker BM (1998a) Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. Nat Med 4:43–49. https://doi.org/10.1038/nm0198-043

Agrawal B, Krantz MJ, Reddish MA, Longenecker BM (1998b) Rapid induction of primary human CD4+ and CD8+ T cell responses against cancer-associated MUC1 peptide epitopes. Int Immunol 10:1907–1916. https://doi.org/10.1093/intimm/10.12.1907

Akiyama SK, Yamada SS, Yamada KM, LaFlamme SE (1994) Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. J Biol Chem 269:15961–15964. https://doi.org/10.1016/s0021-9258(17)33955-8

Alisson-Silva F, de Carvalho RD, Vairo L, Asensi KD, Vasconcelos-dos-Santos A, Mantuano NR, Dias WB, Rondinelli E, Goldenberg RC, Urmenyi TP, Todeschini AR (2014) Evidences for the involvement of cell surface glycans in stem cell pluripotency and differentiation. Glycobiology 24:458–468. https://doi.org/10.1093/glycob/cwu012

Angata T, Tabuchi Y, Nakamura K, Nakamura M (2007) Siglec-15: an immune system Siglec conserved throughout vertebrate evolution. Glycobiology 17:838–846. https://doi.org/10.1093/glycob/cwm049

Angata T, Fujinawa R, Kurimoto A, Nakajima K, Kato M, Takamatsu S, Korekane H, Gao C-X, Ohtsubo K, Kitazume S, Taniguchi N (2012) Integrated approach toward the discovery of glyco-biomarkers of inflammation-related diseases. Ann N Y Acad Sci 1253:159–169. https://doi.org/10.1111/j.1749-6632.2012.06469.x

Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ, Liu ZY, Costes SV, Cho EH, Lockett S, Khanna C, Chambers AF, Green JE (2008) Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. Cancer Res 68:6241–6250. https://doi.org/10.1158/0008-5472.CAN-07-6849

Basudhar D, Glynn SA, Greer M, Somasundaram V, No JH, Scheiblin DA, Garrido P, Heinz WF, Ryan AE, Weiss JM, Cheng RYS, Ridnour LA, Lockett SJ, McVicar DW, Ambs S, Wink DA

(2017) Coexpression of NOS2 and COX2 accelerates tumor growth and reduces survival in estrogen receptor-negative breast cancer. Proc Natl Acad Sci U S A 114:13030–13035. https://doi.org/10.1073/pnas.1709119114

Beatson R, Maurstad G, Picco G, Arulappu A, Coleman J, Wandell HH, Clausen H, Mandel U, Taylor-Papadimitriou J, Sletmoen M, Burchell JM (2015) The breast cancer-associated glycoforms of MUC1, MUC1-Tn and sialyl-Tn, are expressed in COSMC wild-type cells and bind the C-type lectin MGL. PLoS One 10:e0125994. https://doi.org/10.1371/journal.pone.0125994

Berrier AL, Yamada KM (2007) Cell-matrix adhesion. J Cell Physiol 213:565–573. https://doi.org/10.1002/jcp.21237

Bhaskar V, Zhang D, Fox M, Seto P, Wong MHL, Wales PE, Powers D, Chao DT, DuBridge RB, Ramakrishnan V (2007) A function blocking anti-mouse integrin alpha5beta1 antibody inhibits angiogenesis and impedes tumor growth in vivo. J Transl Med 5:61. https://doi.org/10.1186/1479-5876-5-61

Bidlingmaier S, Zhu X, Liu B (2008) The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells. J Mol Med 86:1025–1032. https://doi.org/10.1007/s00109-008-0357-8

Bonde AK, Tischler V, Kumar S, Soltermann A, Schwendener RA (2012) Intratumoral macrophages contribute to epithelial-mesenchymal transition in solid tumors. BMC Cancer 12:35. https://doi.org/10.1186/1471-2407-12-35

Carlos CA, Dong HF, Howard OM, Oppenheim JJ, Hanisch FG, Finn OJ (2005) Human tumor antigen MUC1 is chemotactic for immature dendritic cells and elicits maturation but does not promote Th1 type immunity. J Immunol 175:1628–1635. https://doi.org/10.4049/jimmunol.175.3.1628

Carrascal MA, Severino PF, Guadalupe Cabral M, Silva M, Ferreira JA, Calais F, Quinto H, Pen C, Ligeiro D, Santos LL, Dall'Olio F, Videira PA (2014) Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells. Mol Oncol 8:753–765. https://doi.org/10.1016/j.molonc.2014.02.008

Ching CK, Holmes SW, Holmes GK, Long RG (1993) Comparison of two sialosyl-Tn binding monoclonal antibodies (MLS102 and B72.3) in detecting pancreatic cancer. Gut 34:1722–1725. https://doi.org/10.1136/gut.34.12.1722

Choi JY, Jang YS, Min SY, Song JY (2011) Overexpression of MMP-9 and HIF-1alpha in breast cancer cells under hypoxic conditions. J Breast Cancer 14:88–95. https://doi.org/10.4048/jbc.2011.14.2.88

Clement M, Rocher J, Loirand G, Le Pendu J (2004) Expression of sialyl-Tn epitopes on β1 integrin alters epithelial cell phenotype, proliferation and haptotaxis. J Cell Sci 117:5059–5069. https://doi.org/10.1242/jcs.01350

Crocker PR, Varki A (2001) Siglecs, sialic acids and innate immunity. Trends Immunol 22:337–342. https://doi.org/10.1016/S1471-4906(01)01930-5

Dai Y, Bae K, Siemann DW (2011) Impact of hypoxia on the metastatic potential of human prostate cancer cells. Int J Radiat Oncol Biol Phys 81:521–528. https://doi.org/10.1016/j.ijrobp.2011.04.027

Davidson B, Berner A, Nesland JM, Risberg B, Kristensen GB, Trope CG, Bryne M (2000a) Carbohydrate antigen expression in primary tumors, metastatic lesions, and serous effusions from patients diagnosed with epithelial ovarian carcinoma: evidence of up-regulated Tn and sialyl Tn antigen expression in effusions. Hum Pathol 31:1081–1087. https://doi.org/10.1053/hupa.2000.9776

Davidson B, Gotlieb WH, Ben-Baruch G, Kopolovic J, Goldberg I, Nesland JM, Berner A, Bjamer A, Bryne M (2000b) Expression of carbohydrate antigens in advanced-stage ovarian carcinomas and their metastases-a clinicopathologic study. Gynecol Oncol 77:35–43. https://doi.org/10.1006/gyno.1999.5708

Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174. https://doi.org/10.1038/nrc745

Fattore L, Mancini R, Ciliberto G (2020) Cancer stem cells and the slow cycling phenotype: how to cut the gordian knot driving resistance to therapy in melanoma. Cancers 12:3368. https://doi.org/10.3390/cancers12113368

Ferreira JA, Videira PA, Lima L, Pereira S, Silva M, Carrascal M, Severino PF, Fernandes E, Almeida A, Costa C, Vitorino R, Amaro T, Oliveira MJ, Reis CA, Dall'Olio F, Amado F, Santos LL (2013) Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol 7:719–731. https://doi.org/10.1016/j.molonc.2013.03.001

Flucke U, Zirbes TK, Schroder W, Monig SP, Koch V, Schmitz K, Thiele J, Dienes HP, Holscher AH, Baldus SE (2001) Expression of mucin-associated carbohydrate core antigens in esophageal squamous cell carcinomas. Anticancer Res 21:2189–2193

Gargini R, Cerliani JP, Escoll M, Antón IM, Wandosell F (2015) Cancer stem cell-like phenotype and survival are coordinately regulated by Akt/FoxO/Bim pathway. Stem Cells 33:646–660. https://doi.org/10.1002/stem.1904

Gargini R, Escoll M, García E, García-Escudero R, Wandosell F, Antón IM (2016) WIP drives tumor progression through YAP/TAZ-dependent autonomous cell growth. Cell Rep 17:1962–1977. https://doi.org/10.1016/j.celrep.2016.10.064

Garside VC, Chang AC, Karsan A, Hoodless PA (2012) Co-ordinating notch, BMP, and TGF-β signaling during heart valve development. Cell Mol Life Sci 70:2899–2917. https://doi.org/10.1007/s00018-012-1197-9

Genega EM, Hutchinson B, Reuter VE, Gaudin PB (2000) Immunophenotype of high-grade prostatic adenocarcinoma and urothelial carcinoma. Modern Pathol 13:1186–1191. https://doi.org/10.1038/modpathol.3880220

Giancotti FG, Ruoslahti E (1999) Integrin signaling. Science 285:1028–1032. https://doi.org/10.1126/science.285.5430.1028

Gill DJ, Chia J, Senewiratne J, Bard F (2010) Regulation of O-glycosylation through Golgi-to-ER relocation of initiation enzymes. J Cell Biol 189:843–858. https://doi.org/10.1083/jcb.201003055

Greenburg G, Hay ED (1982) Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol 95:333–339. https://doi.org/10.1083/jcb.95.1.333

Gu J, Taniguchi N (2004) Regulation of integrin functions by N-glycans. Glycoconj J 21:9–15. https://doi.org/10.1023/B:GLYC.0000043741.47559.30

Hamidi H, Pietila M, Ivaska J (2016) The complexity of integrins in cancer and new scopes for therapeutic targeting. Br J Cancer 115:1017–1023. https://doi.org/10.1038/bjc.2016.312

Hashiguchi Y, Kasai M, Fukuda T, Ichimura T, Yasui T, Sumi T (2016) Serum sialyl-Tn (sTn) as a tumor marker in patients with endometrial cancer. Pathol Oncol Res 22:501–504. https://doi.org/10.1093/annonc/mdu338.53

Hassinen A, Pujol FM, Kokkonen N, Pieters C, Kihlstrom M, Korhonen K, Kellokumpu S (2011) Functional organization of Golgi N- and O-glycosylation pathways involves pH-dependent complex formation that is impaired in cancer cells. J Biol Chem 286:38329–38340. https://doi.org/10.1074/jbc.M111.277681

Hauselmann I, Borsig L (2014) Altered tumor-cell glycosylation promotes metastasis. Front Oncol 4:28. https://doi.org/10.3389/fonc.2014.00028

Holmberg LA, Sandmaier BM (2004) Vaccination with Theratope® (sTn-KLH) as treatment for breast cancer. Expert Rev Vaccines 3:655–663. https://doi.org/10.1586/14760584.3.6.655

Humphrey MB, Lanier LL, Nakamura MC (2005) Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. Immunol Rev 208:50–65. https://doi.org/10.1111/j.0105-2896.2005.00325.x

Huttenlocher A, Horwitz AR (2011) Integrins in cell migration. Cold Spring Harb Perspect Biol 3:a005074. https://doi.org/10.1101/cshperspect.a005074

Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25. https://doi.org/10.1016/0092-8674(92)90115-S

Ibrahim NK, Murray JL (2003) Clinical development of the sTn-KLH vaccine (Theratope®). Clin Breast Cancer 3:S139–S143. https://doi.org/10.3816/CBC.2003.s.003

Iida H, Suzuki M, Goitsuka R, Ueno H (2012) Hypoxia induces CD133 expression in human lung cancer cells by up-regulation of OCT3/4 and SOX2. Int J Oncol 40:71–79. https://doi.org/10.3892/ijo.2011.1207

Inaba T, Sano H, Kawahito Y, Hla T, Akita K, Toda M, Yamashina I, Inoue M, Nakada H (2003) Induction of cyclooxygenase-2 in monocyte/macrophage by mucins secreted from colon cancer cells. Proc Natl Acad Sci U S A 100:2736–2741. https://doi.org/10.1073/pnas.0435410100

Ishida-Kitagawa N, Tanaka K, Bao X, Kimura T, Miura T, Kitaoka Y, Hayashi K, Sato M, Maruoka M, Ogawa T, Miyoshi J, Takeya T (2012) Siglec-15 regulates the formation of functional osteoclasts in concert with DNAX-activating protein of 12 KDa (DAP12). J Biol Chem 287:17493–17502. https://doi.org/10.1074/jbc.M111.324194

Ishii H, Mimura Y, Zahra MH, Katayama S, Hassan G, Afify SM, Seno M (2021) Isolation and characterization of cancer stem cells derived from human glioblastoma. Am J Cancer Res 11:441–457

Itzkowitz SH, Yuan M, Montgomery CK, Kjeldsen T, Takahashi HK, Bigbee WL, Kim YS (1989) Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. Cancer Res 49:197–204

Itzkowitz S, Kjeldsen T, Friera A, Hakomori S, Yang US, Kim YS (1991) Expression of Tn, sialosyl Tn, and T antigens in human pancreas. Gastroenterology 100:1691–1700. https://doi.org/10.1016/0016-5085(91)90671-7

Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downwar J, Beug H, Grünert S (2002) Ras and TGFβ cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol 156:299–313. https://doi.org/10.1083/jcb.200109037

Jang TJ, Cho MY (2005) Cyclooxygenase-2 expression and cell proliferation are increased in MUC2-positive area of columnar- lined esophagus. Pathol Int 55:546–549. https://doi.org/10.1111/j.1440-1827.2005.01867.x

Janik ME, Litynska A, Vereecken P (2010) Cell migration-the role of integrin glycosylation. Biochim Biophys Acta 1800:545–555. https://doi.org/10.1016/j.bbagen.2010.03.013

Jechlinger M, Grunert S, Tamir IH, Janda E, Lüdemann S, Waerner T, Seither P, Weith A, Beug H, Kraut N (2003) Expression profiling of epithelial plasticity in tumor progression. Oncogene 22:7155–7169. https://doi.org/10.1038/sj.onc.1206887

Johnston WW, Szpak CA, Thor A, Schlom J (1986) Phenotypic characterization of lung cancers in fine needle aspiration biopsies using monoclonal antibody B72.3. Cancer Res 46:6462–6470

Ju T, Lanneau GS, Gautam T, Wang Y, Xia B, Stowell SR, Willard MT, Wang W, Xia JY, Zuna RE, Laszik Z, Benbrook DM, Hanigan MH, Cummings RD (2008) Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc. Cancer Res 68:1636–1646. https://doi.org/10.1158/0008-5472.CAN-07-2345

Ju T, Wang Y, Aryal RP, Lehoux SD, Ding X, Kudelka MR, Cutler C, Zeng J, Wang J, Sun X, Heimburg-Molinaro J, Smith DF, Cummings RD (2013) Tn and sialyl-Tn antigens, aberrant O-glycomics as human disease markers. Proteomics Clin Appl 7:618–631. https://doi.org/10.1002/prca.201300024

Julien S, Lagadec C, Krzewinski-Recchi MA, Courtand G, Le Bourhis X, Delannoy P (2005) Stable expression of sialyl-Tn antigen in T47-D cells induces a decrease of cell adhesion and an increase of cell migration. Breast Cancer Res Treat 90:77–84. https://doi.org/10.1007/s10549-004-3137-3

Julien S, Adriaenssens E, Ottenberg K, Furlan A, Courtand G, Vercoutter-Edouart AS, Hanisch FG, Delannoy P, Le Bourhis X (2006) ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology 16:54–64. https://doi.org/10.1093/glycob/cwj033

Julien S, Picco G, Sewell R, Vercoutter-Edouart AS, Tarp M, Miles D, Taylor-Papadimitriou J, Clausen H, Burchell JM (2009) Sialyl-Tn vaccine induces antibody-mediated tumour protection in a relevant murine model. Br J Cancer 100:1746–1754. https://doi.org/10.1038/sj.bjc.6605083

Julien S, Videira PA, Delannoy P (2012) Sialyl-Tn in cancer: (how) did we miss the target? Biomol Ther 2:435–466. https://doi.org/10.3390/biom2040435

Kailemia MJ, Park D, Lebrilla CB (2017) Glycans and glycoproteins as specific biomarkers for cancer. Anal Bioanal Chem 409:395–410. https://doi.org/10.1007/s00216-016-9880-6

Kaiser E, Chiba P, Zaky K (1990) Phospholipases in biology and medicine. Clin Biochem 23:349–370. https://doi.org/10.1016/0009-9120(90)90051-U

Kaufhold S, Bonavida B (2014) Central role of Snail1 in the regulation of EMT and resistance in cancer: a target for therapeutic intervention. J Exp Clin Cancer Res 33:62. https://doi.org/10.1186/s13046-014-0062-0

Kim YJ, Varki A (1997) Perspectives on the significance of altered glycosylation of glycoproteins in cancer. Glycoconj J 14:569–576. https://doi.org/10.1023/A:1018580324971

Kim GE, Bae HI, Park HU, Kuan SF, Crawley SC, Ho JJ, Kim YS (2002) Aberrant expression of MUC5AC and MUC6 gastric mucins and sialyl Tn antigen in intraepithelial neoplasms of the pancreas. Gastroenterology 123:1052–1060. https://doi.org/10.1053/gast.2002.36018

Kobayashi H, Terao T, Kawashima Y (1991) Clinical evaluation of circulating serum sialyl Tn antigen levels in patients with epithelial ovarian cancer. J Clin Oncol 9:983–987. https://doi.org/10.1200/JCO.1991.9.6.983

Kvorjak M, Ahmed Y, Miller ML, Sriram R, Coronnello C, Hashash JG, Hartman DJ, Telmer CA, Miskov-Zivanov N, Finn OJ, Cascio S (2020) Cross-talk between colon cells and macrophages increases ST6GALNAC1 and MUC1-sTn expression in ulcerative colitis and colitis-associated colon cancer. Cancer Immunol Res 8:167–178. https://doi.org/10.1158/2326-6066.CIR-19-0514

Lanctot PM, Gage FH, Varki AP (2007) The glycans of stem cells. Curr Opin Chem Biol 11:373–380. https://doi.org/10.1016/j.cbpa.2007.05.032

Lanier LL (2009) DAP10- and DAP12-associated receptors in innate immunity. Immunol Rev 227:150–160. https://doi.org/10.1111/j.1600-065X.2008.00720.x

Leivonen M, Nordling S, Lundin J, von Boguslawski K, Haglund C (2001) STn and prognosis in breast cancer. Oncology 61:299–305. https://doi.org/10.1159/000055337

Li N, Babaei-Jadidi R, Lorenzi F, Spencer-Dene B, Clarke P, Domingo E, Tulchinsky E, Vries RGJ, Kerr D, Pan Y, He Y, Bates DO, Tomlinson I, Clevers H, Nateri AS (2019) An FBXW7-ZEB2 axis links EMT and tumour microenvironment to promote colorectal cancer stem cells and chemoresistance. Oncogenesis 8:13. https://doi.org/10.1038/s41389-019-0125-3

Lottich SC, Johnston WW, Szpak CA, Delong ER, Thor A, Schlom J (1985) Tumor-associated antigen TAG-72: correlation of expression in primary and metastatic breast carcinoma lesions. Breast Cancer Res Treat 6:49–56. https://doi.org/10.1007/BF01806010

Luo BH, Carman CV, Springer TA (2007) Structural basis of integrin regulation and signaling. Annu Rev Immunol 25:619–647. https://doi.org/10.1146/annurev.immunol.25.022106.141618

Lv Y, Chen C, Zhao B, Zhang X (2017) Regulation of matrix stiffness on the epithelial-mesenchymal transition of breast cancer cells under hypoxia environment. Naturwissenschaften 104:38. https://doi.org/10.1007/s00114-017-1461-9

Ma TT, Wang L, Wang JL, Liu YJ, Chen YC, He HJ, Song Y (2019) Hypoxia-induced cleavage of soluble ephrinA1 from cancer cells is mediated by MMP-2 and associates with angiogenesis in oral squamous cell carcinoma. Onco Targets Ther 12:8491–8499. https://doi.org/10.2147/OTT.S213252

MacLean GD, Reddish MA, Koganty RR, Longenecker BM (1996a) Antibodies against mucin-associated sialyl-Tn epitopes correlate with survival of metastatic adenocarcinoma patients undergoing active specific immunotherapy with synthetic sTn vaccine. J Immunother Emphas Tumor Immunol 19:59–68. https://doi.org/10.1097/00002371-199601000-00007

MacLean GD, Miles DW, Rubens RD, Reddish MA, Longenecker BM (1996b) Enhancing the effect of Theratope® sTn-KLH cancer vaccine in patients with metastatic breast cancer by pretreatment with low-dose intravenous cyclophosphamide. J Immunother Emphas Tumor Immunol 19:309–316. https://doi.org/10.1097/00002371-199607000-00006

Marcos NT, Pinho S, Grandela C, Cruz A, Samyn-Petit B, Harduin-Lepers A, Almeida R, Silva F, Morais V, Costa J, Kihlberg J, Clausen H, Reis CA (2004) Role of the human ST6GalNAc-I and ST6GalNAc-II in the synthesis of the cancer-associated sialyl-Tn antigen. Cancer Res 64:7050–7057. https://doi.org/10.1158/0008-5472.CAN-04-1921

Micalizzi DS, Farabaugh SM, Ford HL (2010) Epithelial-mesenchymaltransition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia 15:117–134. https://doi.org/10.1007/s10911-010-9178-9

Miles D, Papazisis K (2003) Rationale for the clinical development of sTn-KLH (Theratope®) and anti-MUC-1 vaccines in breast cancer. Clin Breast Cancer 3:S134–S138. https://doi.org/10.3816/cbc.2003.s.002

Miles DW, Towlson KE, Graham R, Reddish M, Longenecker BM, Taylor-Papadimitriou J, Rubens RD (1996) A randomised phase II study of sialyl-Tn and DETOX-B adjuvant with or without cyclophosphamide pretreatment for the active specific immunotherapy of breast cancer. Br J Cancer 74:1292–1296. https://doi.org/10.1038/bjc.1996.532

Miles D, Roche H, Martin M, Perren TJ, Cameron DA, Glaspy J, Dodwell D, Parker J, Mayordomo J, Tres A, Murray JL, Ibrahim NK, Theratope® Study Group (2011) Phase III multicenter clinical trial of the sialyl-Tn (sTn)-keyhole limpet hemocyanin (KLH) vaccine for metastatic breast cancer. Oncologist 16:1092–1100. https://doi.org/10.1634/theoncologist.2010-0307

Monti P, Leone BE, Zerbi A, Balzano G, Cainarca S, Sordi V, Pontillo M, Mercalli A, Di Carlo V, Allavena P, Piemonti L (2004) Tumor-derived MUC1 mucins interact with differentiating monocytes and induce IL-10highIL-12low regulatory dendritic cell. J Immunol 172:7341–7349. https://doi.org/10.4049/jimmunol.172.12.7341

Munkley J (2016) The role of Sialyl-Tn in cancer. Int J Mol Sci 17:275. https://doi.org/10.3390/ijms17030275

Munkley J, Elliott DJ (2016) Sugars and cell adhesion: the role of ST6GalNAc1 in prostate cancer progression. Cancer Cell Microenviron 3:e1174. https://doi.org/10.14800/ccm.1174

Munkley J, Oltean S, Vodak D, Wilson BT, Livermore KE, Zhou Y, Star E, Floros VI, Johannessen B, Knight B, McCullagh P, McGrath J, Crundwell M, Skotheim RI, Robson CN, Leung HY, Harries LW, Rajan P, Mills IG, Elliott DJ (2015) The androgen receptor controls expression of the cancer-associated sTn antigen and cell adhesion through induction of ST6GalNAc1 in prostate cancer. Oncotarget 6:34358–34374. https://doi.org/10.18632/oncotarget.6024

Muñoz-Nájar UM, Neurath KM, Vumbaca F, Claffey KP (2006) Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. Oncogene 25:2379–2392. https://doi.org/10.1038/sj.onc.1209273

Myers RB, Meredith RF, Schlom J, LoBuglio AF, Bueschen AJ, Wheeler RH, Stockard CR, Grizzle WE (1994) Tumor associated glycoprotein-72 is highly expressed in prostatic adenocarcinomas. J Urol 152:243–246. https://doi.org/10.1016/S0022-5347(17)32870-7

Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69:562–573. https://doi.org/10.1016/j.cardiores.2005.12.002

Nejad AE, Najafgholian S, Rostami A, Sistani A, Shojaeifar S, Esparvarinha M, Nedaeinia R, Javanmard SH, Taherian M, Ahmadlou M, Salehi R, Sadeghi B, Manian M (2021) The role of hypoxia in the tumor microenvironment and development of cancer stem cell: a novel approach to developing treatment. Cancer Cell Int 21:62. https://doi.org/10.1186/s12935-020-01719-5

North S, Butts C (2005) Vaccination with BLP25 liposome vaccine to treat non-small cell lung and prostate cancers. Expert Rev Vaccines 4:249–257

O'Toole TE, Mandelman D, Forsyth J, Shattil SJ, Plow EF, Ginsberg MH (1991) Modulation of the affinity of integrin alpha IIb beta 3 (GPIIb-IIIa) by the cytoplasmic domain of alpha IIb. Science 254:845–847. https://doi.org/10.1126/science.1948065

O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V, Loftus JC, Shattil SJ, Ginsberg MH (1994) Integrin cytoplasmic domains mediate inside-out signal transduction. J Cell Biol 124:1047–1059. https://doi.org/10.1083/jcb.124.6.1047

Ogata S, Maimonis PJ, Itzkowitz SH (1992) Mucins bearing the cancer-associated sialosyl-Tn antigen mediate inhibition of natural killer cell cytotoxicity. Cancer Res 52:4741–4746

Ogawa T, Hirohashi Y, Murai A, Nishidate T, Okita K, Wang L, Ikehara Y, Satoyoshi T, Usui A, Kubo T, Nakastugawa M, Kanaseki T, Tsukahara T, Kutomi G, Furuhata T, Hirata K, Sato N, Mizuguchi T, Takemasa I, Torigoe T (2017) ST6GALNAC1 plays important roles in enhancing cancer stem phenotypes of colorectal cancer via the Akt pathway. Oncotarget 8:112550–112564. https://doi.org/10.18632/oncotarget.22545

Ohno S, Ohno Y, Suzuki N, Inagawa H, Kohchi C, Soma G, Inoue M (2005a) Multiple roles of cyclooxygenase-2 in endometrial cancer. Anticancer Res 25:3679–3687

Ohno Y, Ohno S, Suzuki N, Kamei T, Inagawa H, Soma G, Inoue M (2005b) Role of cyclooxygenase-2 in immunomodulation and prognosis of endometrial carcinoma. Int J Cancer 114:696–701. https://doi.org/10.1002/ijc.20777

Ohtsubo K, Marth JD (2006) Glycosylation in cellular mechanisms of health and disease. Cell 126: 855–867. https://doi.org/10.1016/j.cell.2006.08.019

Ohuchi N, Thor A, Nose M, Fujita J, Kyogoku M, Schlom J (1986) Tumor-associated glycoprotein (TAG-72) detected in adenocarcinomas and benign lesions of the stomach. Int J Cancer 38:643–650. https://doi.org/10.1002/ijc.2910380505

Okano M, Sugata Y, Fujiwara T, Matsumoto R, Nishibori M, Shimizu K, Maeda M, Kimura Y, Kariya S, Hattori H, Yokoyama M, Kino K, Nishizaki K (2006) E prostanoid 2 (EP2)/EP4-mediated suppression of antigen-specific human T-cell responses by prostaglandin E2. Immunology 118:343–352. https://doi.org/10.1111/j.1365-2567.2006.02376.x

Ozaki H, Matsuzaki H, Ando H, Kaji H, Nakanishi H, Ikehara Y, Narimatsu H (2012) Enhancement of metastatic ability by ectopic expression of ST6GalNAcI on a gastric cancer cell line in a mouse model. Clin Exp Metastasis 29:229–238. https://doi.org/10.1007/s10585-011-9445-1

Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8:221–233. https://doi.org/10.1038/nrm2125

Palmer M, Parker J, Modi S, Butts C, Smylie M, Meikle A, Kehoe M, MacLean G, Longenecker M (2001) Phase I study of the BLP25 (MUC1 peptide) liposomal vaccine for active specific immunotherapy in stage IIIB/IV non-small-cell lung cancer. Clin Lung Cancer 3:49–57.; discussion 58. https://doi.org/10.3816/CLC.2001.n.018

Park CC, Zhang H, Pallavicini M, Gray JW, Baehner F, Park CJ, Bissell MJ (2006) Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. Cancer Res 66: 1526–1535. https://doi.org/10.1158/0008-5472.CAN-05-3071

Paul NR, Jacquemet G, Caswell PT (2015) Endocytic trafficking of integrins in cell migration. Curr Biol 25:R1092–R1105. https://doi.org/10.1016/j.cub.2015.09.049

Peixoto A, Fernandes E, Gaiteiro C, Lima L, Azevedo R, Soares J, Cotton S, Parreira B, Neves M, Amaro T, Tavares A, Teixeira F, Palmeira C, Rangel M, Silva AM, Reis CA, Santos LL, Oliveira MJ, Ferreira JA (2016) Hypoxia enhances the malignant nature of bladder cancer cells and concomitantly antagonizes protein O-glycosylation extension. Oncotarget 7:63138–63157. https://doi.org/10.18632/oncotarget.11257

Pinho SS, Reis CA (2015) Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer 15:540–555. https://doi.org/10.1038/nrc3982

Pinho S, Marcos NT, Ferreira B, Carvalho AS, Oliveira MJ, Santos-Silva F, Harduin-Lepers A, Reis CA (2007) Biological significance of cancer-associated sialyl-Tn antigen: modulation of malignant phenotype in gastric carcinoma cells. Cancer Lett 249:157–170. https://doi.org/10.1016/j.canlet.2006.08.010

Quesada V, Ordóñez GR, Sánchez LM, Puente XS, López-Otín C (2009) The degradome database: mammalian proteases and diseases of proteolysis. Nucleic Acids Res 37(Database issue):D239–D243. https://doi.org/10.1093/nar/gkn570

Quinn HM, Vogel R, Popp O, Mertins P, Lan L, Messerschmidt C, Landshammer A, Lisek K, Château-Joubert S, Marangoni E, Koren E, Fuchs Y, Birchmeier W (2021) YAP and β-catenin

cooperate to drive oncogenesis in basal breast cancer. Cancer Res 81:2116–2127. https://doi.org/10.1158/0008-5472.CAN-20-2801

Rabinovich GA, Croci DO (2012) Regulatory circuits mediated by lectin- glycan interactions in autoimmunity and cancer. Immunity 36:322–335. https://doi.org/10.1016/j.immuni.2012.03.004

Radhakrishnan P, Dabelsteen S, Madsen FB, Francavilla C, Kopp KL, Steentoft C, Vakhrushev SY, Olsen JV, Hansen L, Bennett EP, Woetmann A, Yin G, Chen L, Song H, Bak M, Hlady RA, Peters SL, Opavsky R, Thode C, Qvortrup K, Schjoldager KT, Clausen H, Hollingsworth MA, Wandall HH (2014) Immature truncated O-glycophenotype of cancer directly induces oncogenic features. Proc Natl Acad Sci U S A 111:E4066–E4075. https://doi.org/10.1073/pnas.1406619111

Radisky ES, Radisky DC (2010) Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer. J Mammary Gland Biol Neoplasia 15:201–212. https://doi.org/10.1007/s10911-010-9177-x

Rankin EB, Giaccia AJ (2016) Hypoxic control of metastasis. Science 352:175–180. https://doi.org/10.1126/science.aaf4405

Reddish MA, MacLean GD, Poppema S, Berg A, Longenecker BM (1996) Pre-immunotherapy serum CA27.29 (MUC-1) mucin level and CD69+ lymphocytes correlate with effects of Theratope® sialyl-Tn-KLH cancer vaccine in active specific immunotherapy. Cancer Immunol Immunother 42:303–309. https://doi.org/10.1007/s002620050287

Reszka AA, Hayashi Y, Horwitz AF (1992) Identification of amino acid sequences in the integrin beta 1 cytoplasmic domain implicated in cytoskeletal association. J Biol Chem 117:1321–1330. https://doi.org/10.1083/jcb.117.6.1321

Revuelta-López E, Castellano J, Roura S, Gálvez-Montón C, Nasarre L, Benitez S, Bayes-Genis A, Badimon L, Llorente-Cortés V (2013) Hypoxia induces metalloproteinase-9 activation and human vascular smooth muscle cell migration through low-density lipoprotein receptor-related protein 1-mediated Pyk2 phosphorylation. Arterioscler Thromb Vasc Biol 33:2877–2887. https://doi.org/10.1161/ATVBAHA.113.302323

Ribatti D, Tamma R, Annese T (2020) Epithelial-mesenchymal transition in cancer: a historical overview. Transl Oncol 13:100773. https://doi.org/10.1016/j.tranon.2020.100773

Rosenblum G, Meroueh S, Toth M, Fisher JF, Fridman R, Mobashery S, Sagi I (2007) Molecular structures and dynamics of the stepwise activation mechanism of a matrix metalloproteinase zymogen: challenging the cysteine switch dogma. J Am Chem Soc 129:13566–13574. https://doi.org/10.1021/ja0739411

Sastry SK, Horwitz AF (1993) Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. Curr Opin Cell Biol 5:819–831. https://doi.org/10.1016/0955-0674(93)90031-K

Sata T, Roth J, Zuber C, Stamm B, Heitz PU (1991) Expression of alpha 2,6-linked sialic acid residues in neoplastic but not in normal human colonic mucosa. A lectin-gold cytochemical study with Sambucus nigra and Maackia amurensis lectins. Am J Pathol 139:1435–1448

Schmitt FC, Figueiredo P, Lacerda M (1995) Simple mucin-type carbohydrate antigens (T, sialosyl-T, Tn and sialosyl-Tn) in breast carcinogenesis. Virchows Arch 427:251–258

Shabo I, Svanvik J (2011) Expression of macrophage antigens by tumor cells. Adv Exp Med Biol 714:141–150. https://doi.org/10.1007/BF00203391

Shibue T, Weinberg RA (2009) Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. Proc Natl Acad Sci U S A 106:10290–10295. https://doi.org/10.1073/pnas.0904227106

Shin DH, Dier U, Melendez JA, Hempel N (2015) Regulation of MMP-1 expression in response to hypoxia is dependent on the intracellular redox status of metastatic bladder cancer cells. Biochim Biophys Acta 1852:2593–2602. https://doi.org/10.1016/j.bbadis.2015.09.001

Shiozawa Y, Nie B, Pienta KJ, Morgan TM, Taichman RS (2013) Cancer stem cells and their role in metastasis. Pharmacol Ther 138:285–293. https://doi.org/10.1016/j.pharmthera.2013.01.014

Smith WL, Dewitt DL, Garavito RM (2000) Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem 69:145–182. https://doi.org/10.1146/annurev.biochem.69.1.145

Starbuck K, Al-Alem L, Eavarone DA, Hernandez SF, Bellio C, Prendergast JM, Stein J, Dransfield DT, Zarrella B, Growdon WB, Behrens J, Foster R, Rueda BR (2018) Treatment of ovarian cancer by targeting the tumor stem cell-associated carbohydrate antigen, Sialyl-Thomsen-nouveau. Oncotarget 9:23289–23305. https://doi.org/10.18632/oncotarget.25289

Steentoft C, Vakhrushev SY, Joshi HJ, Kong Y, Vester-Christensen MB, Schjoldager KT, Lavrsen K, Dabelsteen S, Pedersen NB, Marcos-Silva L, Gupta R, Bennett EP, Mandel U, Brunak S, Wandall HH, Levery SB, Clausen H (2013) Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. EMBO J 32:1478–1488. https://doi.org/10.1038/emboj.2013.79

Stemmler MP, Eccles RL, Brabletz S, Brabletz T (2019) Non-redundant functions of EMT transcription factors. Nat Cell Biol 21:102–112. https://doi.org/10.1038/s41556-018-0196-y

Subbaramaiah K, Hudis C, Chang SH, Hla T, Dannenberg AJ (2008) EP2 and EP4 receptors regulate aromatase expression in human adipocytes and breast cancer cells. Evidence of a BRCA1 and p300 exchange. J Biol Chem 283:3433–3444. https://doi.org/10.1074/jbc.M705409200

Sugimoto Y, Narumiya S (2007) Prostaglandin E receptors. J Biol Chem 282:11613–11617. https://doi.org/10.1074/jbc.R600038200

Sun L, Lin P, Qin Z, Liu Y, Deng LL, Lu C (2015) Hypoxia promotes HO-8910PM ovarian cancer cell invasion via Snail-mediated MT1-MMP upregulation. Exp Biol Med 240:1434–1445. https://doi.org/10.1177/1535370215570205

Takamiya R, Ohtsubo K, Takamatsu S, Taniguchi N, Angata T (2013) The interaction between Siglec-15 and tumor-associated sialyl-Tn antigen enhances TGF-beta secretion from monocytes/macrophages through the DAP12-Syk pathway. Glycobiology 23:178–187. https://doi.org/10.1093/glycob/cws139

Takenaka Y, Fukumori T, Raz A (2004) Galectin-3 and metastasis. Glycoconj J 19:543–549. https://doi.org/10.1023/B:GLYC.0000014084.01324.15

Tallant C, Marrero A, Gomis-Ruth FX (2009) Matrix metalloproteinases: fold and function of their catalytic domains. Biochim Biophys Acta 1803:20–28. https://doi.org/10.1016/j.bbamcr.2009.04.003

Tamura F, Sato Y, Hirakawa M, Yoshida M, Ono M, Osuga T, Okagawa Y, Uemura N, Arihara Y, Murase K, Kawano Y, Iyama S, Takada K, Hayashi T, Sato T, Miyanishi K, Kobune M, Takimoto R, Kato J (2016) RNAi-mediated gene silencing of ST6GalNAc I suppresses the metastatic potential in gastric cancer cells. Gastric Cancer 19:85–97. https://doi.org/10.1007/s10120-014-0454-z

Thiery JP, Acloque H, Huang RYJ, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139:871–890. https://doi.org/10.1016/j.cell.2009.11.007

Thor A, Ohuchi N, Szpak CA, Johnston WW, Schlom J (1986) Distribution of oncofetal antigen tumor-associated glycoprotein-72 defined by monoclonal antibody B72.3. Cancer Res 46:3118–3124

Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, Apuzzo T, Sperduti I, Volpe S, Cocorullo G, Gulotta G, Dieli F, De Maria R, Stassi G (2014) CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. Cell Stem Cell 14:342–356. https://doi.org/10.1016/j.stem.2014.01.009

Tołoczko-Iwaniuk N, Dziemiańczyk-Pakieła D, Nowaszewska BK, Celińska-Janowicz K, Miltyk W (2019) Celecoxib in cancer therapy and prevention - review. Curr Drug Targets 20:302–315. https://doi.org/10.2174/1389450119666180803121737

Tomuleasa C, Soritau O, Rus-Ciuca D, Pop T, Todea D, Mosteanu O, Pintea B, Foris V, Susman S, Kacsó G, Irimie A (2010) Isolation and characterization of hepatic cancer cells with stem-like properties from hepatocellular carcinoma. J Gastrointest Liver Dis 19:61–77

Turnbull IR, Colonna M (2007) Activating and inhibitory functions of DAP12. Nat Rev Immunol 7:155–161. https://doi.org/10.1038/nri2014

van der Donk WA, Tsai AL, Kulmacz RJ (2002) The cyclooxygenase reaction mechanism. Biochemistry 41:15451–15458. https://doi.org/10.1021/bi026938h

Varki A, Angata T (2006) Siglecs-the major subfamily of I-type lectins. Glycobiology 16:1R–27R. https://doi.org/10.1093/glycob/cwj008

Vaupel P, Multhoff G (2018) Hypoxia-/HIF-1alpha-driven factors of the tumor microenvironment impeding antitumor immune responses and promoting malignant progression. Adv Exp Med Biol 1072:171–175. https://doi.org/10.1007/978-3-319-91287-5_27

Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 92:827–839. https://doi.org/10.1161/01.RES.0000070112.80711.3D

Wan R, Mo Y, Chien S, Li Y, Li Y, Tollerud DJ, Zhang Q (2011) The role of hypoxia inducible factor-1α in the increased MMP-2 and MMP-9 production by human monocytes exposed to nickel nanoparticles. Nanotoxicology 5:568–582. https://doi.org/10.3109/17435390.2010.537791

Wells A, Yates C, Shepard CR (2008) E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. Clin Exp Metastasis 25:621–628. https://doi.org/10.1007/s10585-008-9167-1

Wick W, Platten M, Weller M (2001) Glioma cell invasion: regulation of metalloproteinase activity by TGF-beta. J Neuro-Oncol 53:177–185. https://doi.org/10.1023/A:1012209518843

Xu J, Lamouille S, Derynck R (2009) TGF-β–induced epithelial to mesenchymal transition. Cell Res 19:156–172. https://doi.org/10.1038/cr.2009.5

Yoon SO, Shin S, Mercurio AM (2005) Hypoxia stimulates carcinoma invasion by stabilizing microtubules and promoting the Rab11 trafficking of the alpha6beta4 integrin. Cancer Res 65:2761–2769. https://doi.org/10.1158/0008-5472.CAN-04-4122

Yoshikawa M, Tsuchihashi K, Ishimoto T, Yae T, Motohara T, Sugihara E, Onishi N, Masuko T, Yoshizawa K, Kawashiri S, Mukai M, Asoda S, Kawana H, Nakagawa T, Saya H, Nagano O (2013) XCT inhibition depletes CD44v-expressing tumor cells that are resistant to EGFR-targeted therapy in head and neck squamous cell carcinoma. Cancer Res 73:1855–1866. https://doi.org/10.1158/0008-5472.CAN-12-3609-T

Zaidel-Bar R, Itzkovitz S, Ma'ayan A, Iyengar R, Geiger B (2007) Functional atlas of the integrin adhesome. Nat Cell Biol 9:858–867. https://doi.org/10.1038/ncb0807-858

Zhang Q, Han Z, Zhu Y, Chen J, Li W (2021) Role of hypoxia inducible factor-1 in cancer stem cells. Mol Med Rep 23:17. https://doi.org/10.3892/mmr.2020.11655

Zhuang X, Zhang H, Li X, Li X, Cong M, Peng F, Yu J, Zhang X, Yang Q, Hu G (2017) Differential effects on lung and bone metastasis of breast cancer by Wnt signalling inhibitor DKK1. Nat Cell Biol 19:1274–1285. https://doi.org/10.1038/ncb3613

Insights into the Role of Chondroitin Sulfate in Cancer

Satomi Nadanaka and Hiroshi Kitagawa

Abstract Chondroitin sulfate (CS) chains are secreted into the extracellular matrices or are associated with the plasma membranes of virtually all cells. They are covalently attached to the serine (Ser) residues of core proteoglycan proteins. The expression levels and/or fine structure of CS chains are altered in response to environmental cues, and such changes are involved in controlling cell fate decisions. CS chains selectively interact with numerous proteins, including growth factors, morphogens, adhesion molecules, and receptors, and regulate various cellular and/or tissue processes. These interactions between various signaling molecules and the CS moieties of proteoglycans (PGs) are the molecular basis for the functions of PGs as signaling modulators, transductors of signals into cells, and regulators of cellular functions. In addition, it is thought that the interactions between proteins and CS chains are regulated by the fine structure of CSs. The CS chains consist of *N*-acetylgalactosamine (GalNAc) residues alternating in glycosidic linkages with glucuronic acid residues. During the biosynthesis of CS chains, GalNAc residues are sulfated to varying degrees at the 4- and/or 6-positions. Recent studies have indicated that CS chains encode important functional information via the introduction of position-specific sulfate groups. Here, we describe the significance of CS-PGs as signal molecules or co-receptors of soluble factors such as growth factors/morphogens in cancer biology. In addition, we discuss how alterations in CS structures activate cancer-related cell signaling and lead to cancer progression.

1 Structure and Biosynthesis of CS

CS chains attached to a unique core protein are expressed as PGs. Both core proteins and CS chains, particularly the sulfation pattern of CS chains, participate in interactions with many protein ligands, such as growth factors, cytokines, and morphogens

S. Nadanaka · H. Kitagawa (✉)
Laboratory of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe, Japan
e-mail: kitagawa@kobepharma-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_5

implicated in tumor progression. CS biosynthetic or degradative enzymes in a developing tumor may modify the CS structure and thus alter cell function.

CS chains consist of repeating disaccharide units [(-4GlcAβ1-3GalNAcβ1-)$_n$] and are covalently linked to specific Ser residues in any of the core proteins via the glycosaminoglycan-protein linkage region (GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser) (Fig. 1) (Mikami and Kitagawa 2013; Nadanaka and Kitagawa 2008). The repeating disaccharide units are biosynthesized by a combination of six homologous glycosyltransferases—chondroitin (Chn) synthases (ChSy)-1, ChSy-2, and ChSy-3, Chn polymerizing factor (ChPF), and Chn GalNAc transferases (ChGn)-1 and ChGn-2 (also known as CSGALNACT-1 and CSGALNACT-2, respectively) (Fig. 1). The resulting backbones of the CS chains are subsequently modified via sulfation and uronate epimerization (Mikami and Kitagawa 2013). Based on the substrate preferences of Chn sulfotransferases identified to date, the biosynthetic scheme for CS-type sulfation can be classified into initial "4-*O*-sulfation" and "6-*O*-sulfation" pathways. In the initial step, the non-sulfated O unit [GlcA-GalNAc] serves as a common acceptor substrate for two types of sulfotransferases, chondroitin 4-*O*-sulfotransferases (C4ST-1 and C4ST-2) (Nadanaka et al. 2008, 2016, 2011) and chondroitin 6-*O*-sulfotransferase-1 (C6ST-1), forming monosulfated A (GlcA-GalNAc(4-*O*-sulfate)) and C (GlcA-GalNAc(6-*O*-sulfate)) units, respectively (Fig. 1). Subsequent sulfation of the A and C units can also occur via GalNAc 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) or CS-specific uronyl 2-*O*-sulfotransferase (UST) action, resulting in the formation of disulfated disaccharide E (GlcA-GalNAc(4,6-*O*-disulfate)) and D (GlcA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate)) units, respectively (Fig. 1) (Mikami and Kitagawa 2013). Arylsulfatase B (ARSB), which removes 4-*O*-sulfate groups from the non-reducing ends of CS chains, is not only a lysosomal enzyme but is also present on the membranes of human cerebrovascular and epithelial cells (Feferman et al. 2013; Prabhu et al. 2011). A decline in ARSB expression increases the levels of 4-*O*-sulfated chondroitin.

Of these CS biosynthetic enzymes, the expression of ChGn-1, C4ST-1, and GalNAc4S-6ST is upregulated in breast cancer cells (Iida et al. 2015; Ito et al. 2017; Liu et al. 2019). In addition, high expression levels of C4ST-1 (CHST11) have been shown to significantly correlate with poor relapse-free survival in three independent lung cancer cohorts (Salanti et al. 2015). ChGn-1 (CSGALNACT1) is associated with clinical outcome of multiple myeloma (Agnelli et al. 2011) and involved in biosynthesis of a therapeutic cancer target CS (Agnelli et al. 2011; Clausen et al. 2016). Both *ChGn-1* (CSGALNACT1) and *GalNAc4S-6ST* (CHST15) genes have the highest incidence rates (5–6%) of deletion, mutation, and amplification in colorectal cancer (Wu et al. 2021). Moreover, the expression of *C4ST-1* (CHST11) is positively correlated with breast cancer progression (Herman et al. 2015), and the CSs produced by C4ST-1 function as ligands of P-selectin in aggressive breast cancer cells (Cooney et al. 2011). In addition, deregulation of *C4ST-1* probably leads to an affected CS-dependent pathway specific to the hematopoietic lineage and causes B-cell chronic leukemia (Schmidt et al. 2004). Costello syndrome, a pediatric genetic disorder linked to oncogenic germline mutations in the

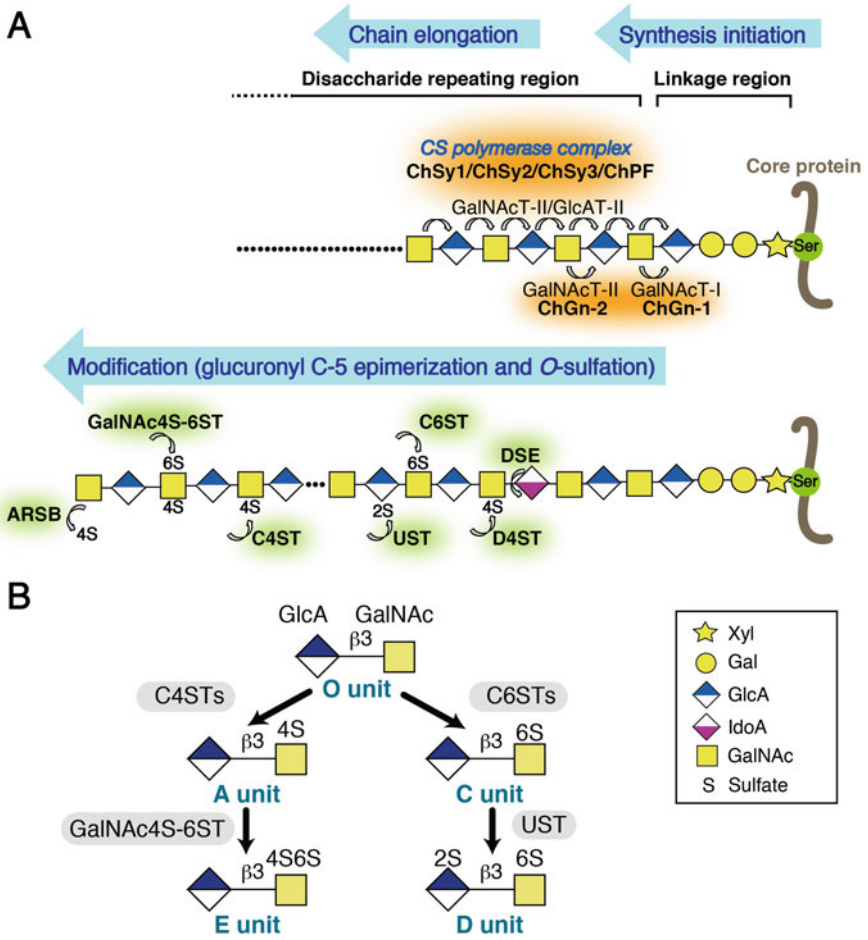


Fig. 1 Scheme for CS biosynthetic pathway. (**a**) A number of glycosyltransferases participate in the synthesis of the linkage regions and disaccharide repeating regions, which are characteristic of the CS chains. The chondroitin backbone is further modified by specific sulfotransferases and epimerases— GalNAcT-I, GalNAc transferase I; GlcAT-II, GlcA transferase-II; GalNAcT-II, GalNAc transferase-II; ChSy, chondroitin synthase; ChPF, chondroitin polymerizing factor; ChGn, chondroitin GalNAc transferase; C4ST, chondroitin 4-*O*-sulfotransferase; C6ST, chondroitin 6-*O*-sulfotransferase; D4ST, dermatan 4-*O*-sulfotransferase; UST, uronyl 2-*O*-sulfotransferase; GalNAc4S-6ST, GalNAc 4-sulfate 6-*O*-sulfotransferase; DSE, GlcA C-5 epimerase (DS epimerase). (**b**) The disaccharide units found in CS chains include GlcA and GalNAc residues. These sugar residues can be esterified by sulfate groups at various positions, as indicated by an "S" in the diagram. Based on the substrate specificities of sulfotransferases, the biosynthetic pathways for CS-type disaccharide units can be classified into the initial "4-*O*-sulfation" or "6-*O*-sulfation" pathways. The disaccharide units that form the CS chains are denoted as O, A, C, D, or E units based on the individual sulfation patterns; 2S, 4S, and 6S represent 2-*O*-, 4-*O*-, and 6-*O*-sulfate groups, respectively

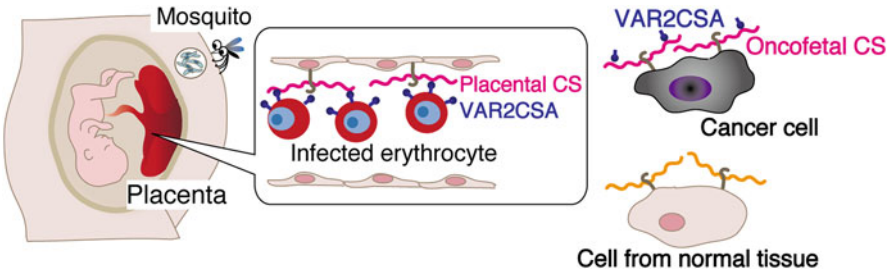


Fig. 2 Binding of malarial protein VAR2CSA to placenta-specific and cancer-associated CSs. Infected erythrocytes attach to the placenta through binding of VAR2CSA to the placental CS chains. Placental CS chains recognized by VAR2CSA are unique, as they comprise low-sulfated CS containing A units. Interestingly, CS chains expressed in various cancers are recognized by VAR2CSA. In contrast, VAR2CSA cannot bind to the CS chains expressed in noncancerous tissues, except for the placental tissue. Human cancer cells and placenta express a common and distinct form of CS, which can be specifically recognized by malarial VAR2CSA

HRAS gene, is characterized by multiple developmental abnormalities and tumor development. Activation of HRAS signaling reduces *C4ST-1* expression and causes sulfation, thereby leading to CS imbalance, which is associated with the pathogenesis of Costello syndrome (Kluppel et al. 2012). Furthermore, the expression level of *GalNAc4S-6ST* (CHST15) is negatively correlated with overall survival and disease-free survival of patients with ductal adenocarcinoma (Ito et al. 2017). These findings suggest that the expression of some glycosylation-associated genes correlates with cancer progression.

How does alteration of the expression of CS biosynthesis-associated genes contribute to the production of cancer-related CS structures? In this regard, the biosynthetic mechanism of oncofetal CS recognized by malarial VAR2CSA proteins has been extensively examined. VAR2CSA-recognizing oncofetal CS was originally discovered in placental malaria. Pregnant women are particularly susceptible to malarial infection because *Plasmodium falciparum* malaria-infected erythrocytes are sequestered in the placenta. The accumulation of infected erythrocytes in the placenta is caused by a parasite protein of the PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) family, known as VAR2CSA (Fig. 2). The VAR2CSA protein is implicated as an adhesion receptor for CS expressed only in the placenta, while CS chains expressed elsewhere in the body are not recognized by VAR2CSA (Fig. 2). Placental CS is a uniquely low-sulfated CS containing approximately 2–14% 4-*O*-sulfated CS disaccharides (A units) and 86–98% of non-sulfated CS disaccharides (O units) (Achur et al. 2000). Inconsistent with this result, a recent study showed that placental and tumor CS chains purified via affinity chromatography using recombinant VAR2 proteins contain a high content of A units (Spliid et al. 2021). This discrepancy may be due to the differences in CS preparation or the methods used for purifying the materials (Spliid et al. 2021). In a previous study on placental CS (Achur et al. 2000), membrane-bound CS-PGs were not analyzed. In addition, the

full-length VAR2CSA protein and the ID1-ID2a protein (CS-binding region of VAR2CSA) were used in previous studies (Achur et al. 2000) and recent studies (Spliid et al. 2021), respectively. Both proteins specifically bind to the CS present on placental syncytiotrophoblasts, although they might have slightly different specificities.

The function of low-sulfated placental CS chains is associated with the ability of trophoblasts to invade the uterine tissues and promote rapid cell proliferation during normal placental implantation. Proliferation and invasion are characteristics of cancer cells. In addition, GO and KEGG enrichment analyses have revealed that coexpression prognosis associated with genes in the placenta and colorectal cancer cells is mostly involved in "extracellular matrix organization," "epithelial–mesenchymal transition," and "cell adhesion," suggesting the connection between tumorigenesis and embryonic development in the placenta (Wu et al. 2021). Consistent with these findings, it has been shown that a similar type of placental CS is broadly expressed in malignant human cancer cells (Salanti et al. 2015). Thus, the CS recognized by VAR2CSA proteins can be used as a pan-cancer cell surface marker. Notably, VAR2CSA proteins show very limited binding to the CSs expressed in noncancerous cells or normal tissues, except for placental tissues (Salanti et al. 2015). A previous report demonstrated that the tumor CS chains recognized by VAR2CSA proteins upregulate tumor-associated integrin signaling through interactions with integrin (Clausen et al. 2016). In addition, targeting oncofetal CS with VAR2CSA could interfere with metastasis by inhibiting downstream integrin signaling (Clausen et al. 2016).

Biosynthesis of VAR2CSA-binding CS chains involves several enzymes. RNAi-mediated knockdown experiments have shown that CSGALNACT1 (ChGn-1) and CHST11 (C4ST-1) are important for VAR2CSA-binding CS chain synthesis (Salanti et al. 2015). In addition, the 4-*O*-sulfation level of CS chains is balanced by ARSB, which removes 4-*O*-sulfates from CS chains. A decreased ARSB activity increases the binding of VAR2CSA to CS chains (Salanti et al. 2015). This indicates that the A units in the CS chains constitute a critical component for VAR2CSA-binding. A recent study indicated that in addition to the low 4-*O*-sulfate levels, longer CS chains are important for VAR2CSA binding (Spliid et al. 2021). Recombinant VAR2 (ID1-ID2a) proteins predominantly bind to high-molecular weight CSs. In addition, CHPF and CHPF2 contribute to the synthesis of placental and oncofetal CS, which is recognized by recombinant VAR2. Inactivation of CHPF and, to a lesser extent, CHPF2 results in a reduction in CS chain length and loss of recombinant VAR2 binding. These enzymes, involved in the biosynthesis of VAR2CSA-recognizing CS, are upregulated in most types of tumors.

2 Binding Partners of CS: Soluble Factors

2.1 WNT Family Members

WNT signaling is one of the key cascades regulating development and stemness and is also tightly associated with cancer. The WNT/β-catenin pathway is activated when a WNT ligand binds to a seven-pass transmembrane Frizzled (Fz) receptor and its co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6) or its close relative LRP5 (Fig. 3). The formation of a likely Wnt-Fz-LRP6 complex, together with the recruitment of the scaffolding protein Disheveled (Dvl), results in LRP6 phosphorylation and activation as well as recruitment of the Axin complex to the receptors (MacDonald et al. 2009). These events lead to the inhibition of Axin-mediated β-catenin phosphorylation and thereby to the stabilization of β-catenin, which accumulates and travels to the nucleus to form complexes with T cell factor/

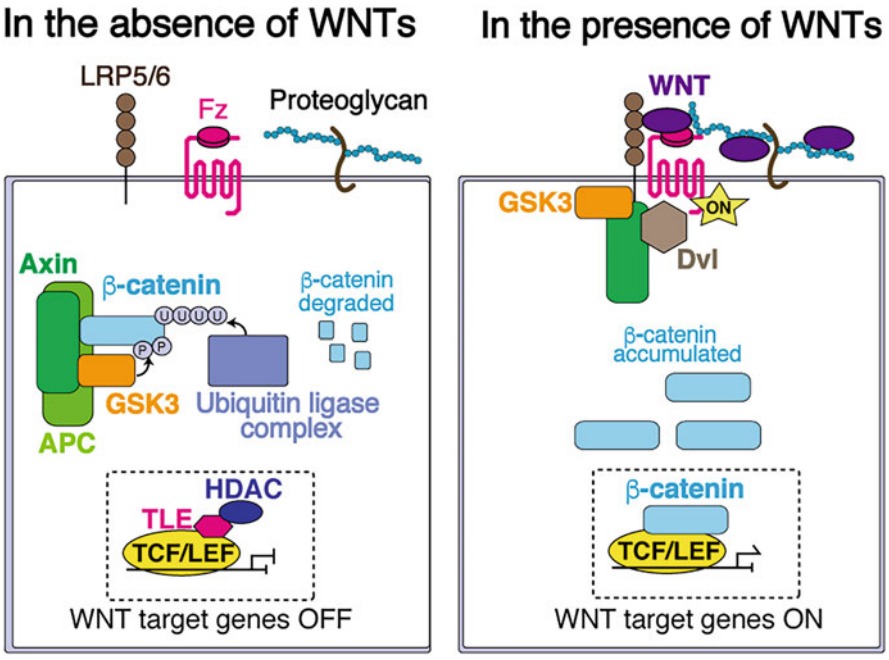


Fig. 3 Overview of WNT/β-catenin signaling. (**a**) In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, APC, and GSK3 and is phosphorylated by GSK3. Phosphorylated β-catenin is recognized by the ubiquitin ligase complex, which targets β-catenin for proteasomal degradation. WNT target genes are repressed by TCF-TLE/Groucho and histone deacetylases (HDAC). (**b**) In the presence of WNT ligand, a receptor complex forms between Fz and LRP5/6. Dvl recruitment by Fz leads to LRP5/6 phosphorylation and Axin recruitment. This disrupts the Axin-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it serves as a co-activator for TCF to activate the WNT responsive genes. CS chains of CS-PGs expressed on the cell surface might serve as binding sites for WNT3A molecules

lymphoid enhancer factor (TCF/LEF) and activate WNT target gene expression. WNTs are conserved in all metazoans. In mammals, the complexity and specificity of WNT signaling are in part achieved through 19 Wnt ligands. Among these WNT proteins, WNT3A can bind to CS chains. WNT3A promotes or suppresses tumor progression via the canonical WNT signaling pathway depending on the cancer type. Interestingly, WNT3A binds strongly to CS chains rich in the E unit (CS-E) (Nadanaka et al. 2008). In contrast, CS chains rich in the A unit (CS-A) or C unit (CS-C) show little binding to WNT3A. As shown in Fig. 2, the E unit is synthesized by C4ST-1 and GalNAc4S-6ST. A previous report showed that the expression levels of *C4ST-1* correlate with the activation levels of WNT3A signaling, and increased expression levels are accompanied by an increase in expression of the E unit of CS chains (Nadanaka et al. 2008). CS chains containing E units of CS-PGs expressed on the cell surface may serve as binding sites for WNT3A. Thus, CS chains containing E units of CS-PGs are expected to retain WNT3A near the cell surface so that WNT3A can interact with its receptor, thereby facilitating WNT3A signaling. Thus, CS chains containing the E units of CS-PGs expressed on the cell surface act as positive cofactors to increase WNT3A activity and have pro-tumorigenic functions. In contrast, exogenously added CS-E potently inhibits WNT3A signaling (Nadanaka et al. 2008). Consistent with this finding, it has been reported that the exogenously added CS-E is a negative regulator of pro-tumorigenic WNT/β-catenin signaling in two murine breast cancer cell lines, EMT6 and 4 T1 (Willis and Kluppel 2014). The exogenously added CS-E chains can suppress WNT/β-catenin signaling, most likely owing to competition with endogenous CS-PGs expressed in the cells for binding WNT3A. The effect of CS-E-mediated suppression of WNT/β-catenin signaling on EMT6 gene expression profiles has been investigated using microarray analysis. Microarray analysis revealed that the gene expression of type I collagen alpha 1 chains (*Col1a1*) is repressed by CS-E treatment. Because collagen is a positive regulator of cancer cell migration, it has been suggested that inhibition of WNT/β-catenin signaling by exogenously added CS-E causes anti-migratory effects (Willis and Kluppel 2014).

CS regulates WNT/β-catenin signaling by controlling the expression of Dickkopf WNT signaling pathway inhibitor 3 (DKK3) in prostate cancer (Bhattacharyya et al. 2017). The expression of ARSB, involved in the removal of the 4-*O*-sulfate group at the non-reducing end of CS chains, is reduced in malignant colon cancer, breast cancer, and prostate cancer (Feferman et al. 2013). ARSB silencing significantly increases 4-*O*-sulfated chondroitin levels in human prostate stem cells, whereas overexpression of ARSB reduces 4-*O*-sulfated chondroitin levels. The changes in the sulfation pattern of CS chains after alteration of the expression of ARSB control WNT/β-catenin signaling and the expression of the WNT target genes c-MYC and GATA-3. Suppression of WNT/β-catenin signaling and downregulation of WNT target genes by ARSB silencing are due to a reduction in DKK3 expression through promoter methylation. Promoter methylation of *DKK3* is mediated by inhibition of the ubiquitous non-receptor tyrosine phosphatase SHP2 (PTPN11) through its binding to 4-*O*-sulfated chondroitin. The 4-*O*-sulfate group of the GalNAc residue at the non-reducing end of the CS chains is thought to provide a binding site for

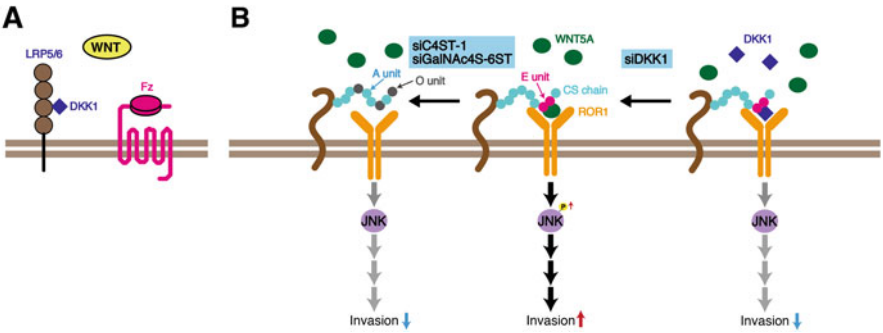


Fig. 4 Schematic of CS-E enhancement of invasive activity of the triple-negative breast cancer MDA-MB-231 cell line. (**a**) DKK proteins bind to LRP5/6 to prevent Fz-LRP5/6 complex formation and inhibit WNT/β-catenin signaling. (**b**) CS-E has an inherent ability to enhance the invasive activity of the human triple-negative breast cancer cell line MDA-MB-231. MDA-MB-231 cells express elevated levels of receptor tyrosine kinase-like orphan receptor 1 (ROR1), WNT5A, and DKK1 but LRP5/6 at very low levels. CS chains bind to WNT5A and ROR1 through E units, thereby signaling cancer cells to activate JNK1. Reducing the number of E units via knockdown of *C4ST-1* and *GalNAc4S-6ST* inhibits WNT5A-ROR1-JNK signaling. In addition, DKK1 binds to CS-E in a sulfation-dependent manner, and knockdown of *DKK1* increases invasive activity. ROR1-dependent invasion signaling is blocked by binding of the DKK1/CS-E complex to ROR1 in MDA-MB-231 cells. DKK1 exerts anti-tumorigenic effects by blocking the tumor invasion activity of CS-E as well as by inhibiting the β-catenin-dependent WNT signaling

SHP2. Incidentally, SHP2 is localized exclusively in the cytoplasm, where SHP2 has no chance of encountering CS chains on the cell surface. Thus, it remains unclear whether 4-*O*-sulfated chondroitin interacts with cytoplasmic SHP2. It is known, however, that a decline in SHP2 activity leads to sustained phosphorylation and activation of ERK1/2 and phospho-ERK1/2 increases the DNA methyltransferase activity (Bhattacharyya et al. 2017).

DKK1 was originally characterized as a secreted inhibitor of canonical WNT signaling. DKK1 binds to LRP5/6 co-receptors with high affinity and blocks β-catenin-dependent WNT signaling (Bafico et al. 2001). DKK1 disruption of the Wnt-induced Fz-LRP6 complex remains a likely mechanism for DKK1 action (Semenov et al. 2005). Recent studies have shown that elevated DKK1 expression contributes to tumor growth and poor prognosis in a range of cancers, suggesting its role in tumor aggressiveness independent of WNT signaling (Kagey and He 2017). The basal-like subtype breast cancer cell line MDA-MB-231 expresses elevated levels of DKK1 but low levels of LRP5/6 (Nadanaka et al. 2018). It has been shown that DKK1 binds to CS-E in a sulfation-dependent manner (Nadanaka et al. 2018). In addition, the invasive activity of MDA-MB-231 cells is enhanced by CS-E treatment. These findings suggest that DKK1 binds to an unidentified receptor in the presence of CS-E to enhance the invasive potential of MDA-MB-231 cells. Surprisingly, however, knockdown of *DKK1* increases the invasive activity (Fig. 4) (Nadanaka et al. 2022), and the invasiveness enhanced by the exogenously added

CS-E is further increased by knockdown of *DKK1*. These results suggest that DKK1 suppresses the tumor-promoting activity of CS-E by binding to CS-E.

2.2 Hedgehog

Mutations in the hedgehog (HH) pathway drive the formation of tumors in various organs. The HH pathway has several main components—(1) three HH homologs (sonic hedgehog (SHH), Indian hedgehog (IHH), and desert hedgehog (DHH)); (2) Patched1 (PTCH1); (3) a G protein-coupled receptor-like receptor Smoothened (SMO); and (4) three transcription factors (GLI1, GLI2, and GLI3) (Wu et al. 2017).

In contrast to the functional role of heparan sulfate proteoglycans (HS-PGs) as co-receptors for HH signaling, CS-PGs play an important role in modulating signaling pathways involving HH proteins (Cortes et al. 2009). Cortes et al. examined the role of CS-PGs in HH signaling using the brachymorphic (bm) mouse model (Sugahara and Schwartz 1979, 1982a,b) with a mutation in the gene *Papss2*, which encodes PAPS synthase 2 (PASS2), one of the two isoforms in mammals that catalyze synthesis of the universal sulfate donor PAPS (Kurima et al. 1998), thus resulting in severe undersulfation of CS-PGs. Although preferential reduction of CS sulfation occurs in the growth plate of bm mice, HS sulfation remains normal. The bm mouse model demonstrated that undersulfation of CS-PGs results in restricted IHH diffusion, which leads to reduced proliferation that significantly affects cartilage development and postnatal skeletal growth (Cortes et al. 2009). In contrast, a deficiency of HS enhances chondrocyte proliferation by increasing the range of HH signaling in the *Ext1* gene trap mutant (Koziel et al. 2004). HS-PGs expressed on the surface of target cells function as co-receptors for HH signaling and capture IHH molecules close to the plasma membrane for suitable interaction with its receptor. In contrast, soluble CS-PGs, such as aggrecan, transport IHH molecules from the IHH-producing cells to IHH target cells and are involved in the diffusion of IHH. CS is known to be a binding partner of HH morphogens in vitro and in vivo. A direct interaction of CS with IHH or SHH proteins has been observed, albeit with lower binding affinities than those for heparin or HS (Cortes et al. 2009; Whalen et al. 2013). IHH can directly bind to 4-*O*-sulfated chondroitin with a higher affinity than non-sulfated chondroitin (Cortes et al. 2009). In hepatocellular carcinoma, the CS chains synthesized by CHSY1 enhance HH signaling by enhancing SHH binding, and the SHH-induced *PTCH1* and *GLI2* expression is upregulated (Liu et al. 2017). In addition, cell migration and invasion, which are elevated by CHSY1, are significantly inhibited by vismodegib treatment of hepatocellular carcinoma cells to block the SHH pathway. Furthermore, the effect of CHSY1 on tumor metastasis in vivo has been analyzed using an animal model. CHSY1-overexpressing Hepa1-6 cells were injected into the tail veins of the mice. Overexpression of CHSY1 significantly enhanced the number of lung metastatic tumors, whereas blockage of HH signaling using vismodegib inhibited the CHSY1-mediated metastasis. These results suggest

that the CS synthesized by CHSY1 promotes malignancy via activation of the HH signaling pathway.

2.3 Midkine and Pleiotrophin

Midkine (MK) is a cytokine or heparin-binding growth factor that is part of a small protein family consisting of two members, with the second member being pleiotrophin (PTN) (Muramatsu 2010, 2014). MK is involved in various physiological processes, such as development, reproduction, and repair, and plays an important role in the pathogenesis of inflammatory and malignant diseases. Although several membrane proteins have been identified as MK receptors, the most well-established one is the receptor protein tyrosine phosphatase Z1 (PTPζ). PTPζ is a transmembrane tyrosine phosphatase with CS chains that are essential for high-affinity binding with MK (Maeda et al. 1999). The interaction between MK and PTPζ activates phosphoinositide 3-kinase (PI3-kinase) and extracellular signal-regulated kinase (ERK) for osteoblast cell migration and neural survival (Owada et al. 1999; Sakaguchi et al. 2003). Syndecan-3 is modified with CS chains (Chernousov and Carey 1993), and syndecan-3 is involved in the signal transduction through binding of MK to CS chains, thereby promoting cancer infiltration or migration and rapid development of peripheral invasion (Nakanishi et al. 1997). PTN is a multifunctional growth factor important for neural development, tissue regeneration, and cancer angiogenesis. NMR structural analysis showed that the C-terminal domain and hinge segment of PTN constitute the major CS-binding site in PTN (Ryan et al. 2016). The C-terminal tail of PTN is essential for maintaining stable interactions with CS rich in A units (CS-A), a type of CS commonly found on PTPζ. Although PTN can bind to both CS-A and oversulfated CS-E, the C-terminal tail of PTN is not necessary for CS-E binding. Downregulation of PTPζ abrogates the stimulatory effects of PTN on glioma cell proliferation and migration (Ulbricht et al. 2006). In addition, a reduction in the expression of GalNAc4S-6ST, which is the sulfotransferase responsible for the biosynthesis of E units, abrogates the stimulatory effects of PTN on glioma cell migration in U251MG cells, suggesting that binding of PTN to CS-E is required to enhance glioma cell mobility (Kobayashi et al. 2013).

2.4 Other Growth Factors

Malignant cancer cells produce a variety of soluble factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and tumor necrosis factor-α, which play major roles in tumor progression (Jayne et al. 2000). Among the variant forms of CS chains, CS-E strongly binds to a large variety of tumor-associated growth factors, including MK, PTN, FGF2,

FGF10, FGF16, FGF18, HGF, and HB-EGF (Deepa et al. 2002). Various commercial CS preparations, such as CS-A, CS-C, and CS-E, were individually pre-injected into mice 30 min before the intravenous injection of the murine osteosarcoma cell line LM8G7. CS-E treatment inhibited liver colonization by LM8G7 cells, whereas CS-A or CS-C treatment did not. Immunostaining analysis using a phage display antibody, GD3G7, specific to CS-E (Purushothaman et al. 2007), showed that the E units were expressed on the surface of LM8G7 cells at higher expression levels. The antibody GD3G7 strongly inhibited liver tumor focal formation, which suggested that the E units play a key role in the liver colonization of LM8G7 cells through the binding of CS-E with effector molecules such as tumor-associated growth factors.

As a member of the FGF family, FGF2 plays a significant role in proliferation, migration, invasion, and angiogenesis, thereby contributing to tumor pathogenesis. CS-PGs have been shown to modulate the binding of FGF2 to FGF receptors either autonomously, in the role of low-affinity binding sites (Smith et al. 2007), or in cooperation with HS chains (Deepa et al. 2004). In addition to heparin, CS controls FGF2-mediated proliferation of the human metastatic melanoma cell lines WM9 and M5 (Nikitovic et al. 2008).

There is a partial functional overlap between CS and HS during VEGF-induced sprouting angiogenesis (Le Jan et al. 2012). The key factors identified to regulate sprouting angiogenesis include VEGFA, FGF2, and platelet-derived growth factor B (PDGFB). Le Jan et al. reported that vascular sprouts induced by VEGF are normally formed in the absence of HS biosynthesis. An angiogenic sprouting model established using embryoid bodies (EBs) indicated that the reduced production of both HS and CS in $Ext1^{-/-}Ndst1^{-/-}$ EBs results in severely delayed angiogenesis. In contrast, the complete loss of HS production, as opposed to elevated CS production, is compatible with angiogenic sprouting in $Ext1^{-/-}$ EBs. These results suggest that CS can functionally compensate for the lack of HS, probably because increased CS levels can help in maintaining the normal levels of VEGF signaling. However, $Ext1^{-/-}$ EBs exhibit reduced adhesion of pericytes to nascent sprouts, which is associated with reduced stabilization of immature vessels. It is likely that the CS synthesis upregulated by a lack of HS production causes aberrant transforming growth factor β (TGFβ) and PDGFB signal transduction in $Ext1^{-/-}$ EBs. As proper TGFβ- and PDGF-signaling allows for pericyte differentiation and attachment, it is believed that the pericytes derived from $Ext1^{-/-}$ EBs detach from angiogenic sprouts. These results suggest that a balance between HS and CS production is required for proper TGF-β and PDGFB signaling.

HGF, produced by tumor and stromal cells, acts as a multifunctional cytokine and activates the c-MET receptor, which is expressed in different tumor cell types. The HGF/c-MET signaling pathway is associated with several cellular processes such as proliferation, survival, motility, angiogenesis, invasion, and metastasis (Noriega-Guerra and Freitas 2018). Dermatan sulfate (DS) can also stimulate the migration of cancer cells via the promotion of HGF activity. A unique feature that distinguishes DS from CS is the presence of iduronic acid (IdoA) in DS. As shown in Fig. 1, DSEs convert GlcA into its epimer, IdoA, to a variable degree. Increased DSE activity and proteins have also been found in biopsy samples from patients with esophageal

adenocarcinoma and gastric adenocarcinoma (Thelin et al. 2012). Downregulation of DSE suppresses cancer cell migration and invasion, probably through inhibition of the HGF-mediated ERK1/2 activation. These results suggest that IdoA in DS chains plays a significant role in cancer cell behavior. Endocan, previously known as endothelial cell-specific molecule-1 (ESM-1), is expressed in the capillaries of tumor tissues and secreted into the blood as soluble DS-PGs. Endocan can activate HGF signaling to promote tumor cell movement and proliferation (Delehedde et al. 2013). Endocan expression is elevated in lung, uterus, kidney, liver, brain, and breast cancers, among others. In addition, tumor prognosis, metastasis, and angiogenesis are positively associated with endocan expression. Thus, endocan could be used as a novel cancer marker and target for cancer therapy.

3 Binding Partners of CS: Membrane-Bound Type Proteins

3.1 Cadherin Family

The cadherin family is a transmembrane glycoprotein that mediates calcium-dependent cell–cell adhesion. A disturbance in cadherin-mediated cell–cell and cell–extracellular matrix adhesion and subsequent changes in adhesion-mediated signaling pathways induce tumor formation and progression. Based on ongoing research on the role of cadherin signaling in malignant tumors, cadherins are now being considered as potential targets for cancer therapies (Yu et al. 2019). Koike et al. reported that CS-E binds to *N*-cadherin and cadherin-11 (Koike et al. 2012). Interactions between CS-E and cadherins promote osteoblast differentiation by controlling ERK1/2 and SMAD signaling. In pre-osteoblastic MC3T3-E1 cells, ERK1/2 phosphorylation decreases in response to the *N*-cadherin-mediated adhesion (Hay et al. 2009). Moreover, reduction in ERK1/2 phosphorylation increases SMAD3 activity (Sowa et al. 2002). Furthermore, phosphorylated SMAD1/5/8 promotes the expression of osteoblastic differentiation markers such as alkaline phosphatase. This intracellular signaling is triggered by the direct binding of CS to cadherins, independent of homophilic interactions between cadherin molecules.

In the basal-like breast cancer cell line BT-549, the interaction between CS-E and *N*-cadherin enhances invasion activity through β-catenin-mediated intracellular signaling (Fig. 5) (Nadanaka et al. 2018). CSs interact with and induce the proteolytic cleavage of *N*-cadherin in BT-549 cells, yielding a C-terminal intracellular *N*-cadherin fragment that forms a complex with β-catenin. Classical cadherins are proteolytically cleaved by multiple proteases in a context-dependent manner (McCusker and Alfandari 2009). It is generally accepted that proteolysis of cadherins proceeds in two steps to liberate the cytoplasmic C-terminal fragment (CTF). CTFs of classical cadherins can also influence intracellular signaling events by interacting with molecules involved in multiple pathways. For example, the *N*-cadherin CTF interacts with β-catenin; this complex enters the nucleus, leading to β-catenin-dependent transcriptional activation, which can promote cell migration

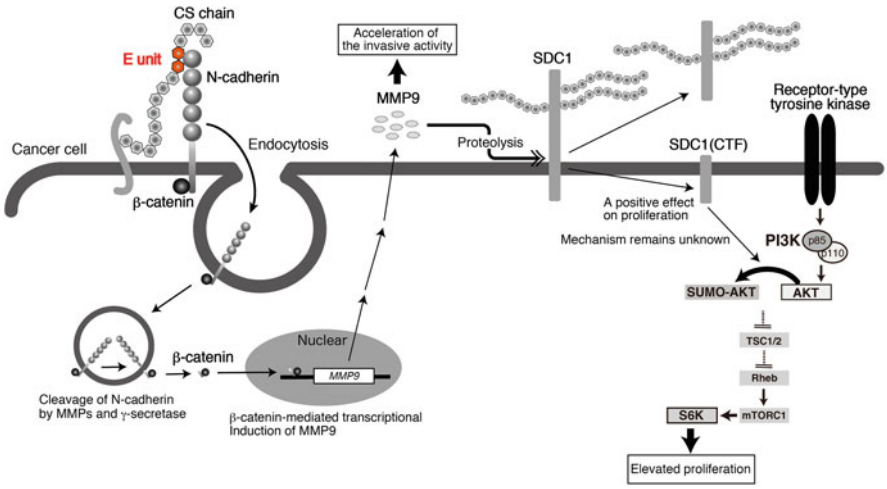


Fig. 5 Illustrated summary of how CS chains accelerate the invasive activity and proliferation of a basal-like breast cancer cell line BT-549. First, the CS chains expressed in BT-549 cells themselves bind to *N*-cadherin. The binding of CSs to *N*-cadherin triggers endocytosis, followed by cleavage of *N*-cadherin. As a result, the C-terminal domain of *N*-cadherin, which is associated with β-catenin, is released, translocated into the nucleus, and transcriptional induction of MMP9 by β-catenin is increased, which enhances cancer cell invasion. CS chains expressed in the BT-549 cells themselves are utilized for cell-autonomous enhancement of invasive potential, while BT-549 cells might take advantage of CS chains expressed in a CS-E-rich *N*-cadherin target organ for metastasis. SDC1 expressed at the cell surface is cleaved by MMPs, and N-terminal and C-terminal fragments (CTFs) of SDC1 are generated. SDC1(CTF) has a positive effect on cell proliferation. SDC1(CTF) is involved in SUMOylation of AKT1, although the mechanism underlying SUMOylation by SDC1 (CTF) remains unclear. SUMOylation of AKT1 enhances cell proliferation through the S6 kinase signaling pathway

and invasion (Reiss et al. 2005). In BT-549 cells, the matrix metalloproteinase 9 (*MMP9*) gene is induced by β-catenin. Thus, the proteolysis of *N*-cadherin is thought to be a regulatory step in *N*-cadherin/β-catenin nuclear signaling. However, it remains unclear as to how the proteolysis of *N*-cadherin itself is regulated. A recent study showed that the binding of CSs to *N*-cadherin triggers endocytosis-associated proteolysis of *N*-cadherin to activate β-catenin nuclear signaling. Of note, a particular sulfation pattern of CS is important for regulation of the invasion potential of BT-549 cells through the *N*-cadherin/β-catenin pathway, because *N*-cadherin specifically recognizes the E unit, a specific sulfate epitope synthesized by C4ST-1 and GalNAc4S-6ST.

Furthermore, induction of the expression of MMP9 through activation of the *N*-cadherin/β-catenin pathway enhances not only the invasion activity but also the proliferation of BT-549 cells. Syndecan-1 (SDC1), a cell-surface proteoglycan, is thought to serve as a promising substrate for MMPs (Manon-Jensen et al. 2013). In addition, the cleavage of SDC1 by MMPs is involved in tumor invasion and proliferation (Jang et al. 2020; Su et al. 2008; Szatmari et al. 2015; Wang et al.

2014), has been implicated in promoting breast cancer progression, and is highly expressed in basal-like breast cancers (Nguyen et al. 2013; Rousseau et al. 2011; Sayyad et al. 2019). A recent study showed that the CTF of SDC1 generated by MMPs enhances cell proliferation through SUMOylation of AKT1, although it remains unclear how the CTF of SDC1 controls the SUMOylation of AKT1 (Fig. 5) (Nadanaka et al. 2021).

3.2 Receptor Tyrosine Kinase-Like Orphan Receptor 1 (ROR1)

Particular attention has been focused on the functional and clinical significance of ROR1 in many types of malignant tumors, including breast cancer. ROR1 is positive in breast cancer specimens but not in normal breast tissues (Zhang et al. 2012), and high expression of ROR1 in breast cancer is associated with an aggressive phenotype (Fukuda et al. 2008). Several therapeutic strategies against ROR1 have been developed and evaluated in clinical trials (Zhao et al. 2021).

ROR1 is a transmembrane receptor that contains an extracellular region, transmembrane segment, and cytoplasmic region. The extracellular region includes an immunoglobulin-like domain, a cysteine-rich domain (CRD), and the Kringle domain. Although ligands for ROR1 have not been identified for many years, it is now known that ROR1 is a receptor for the WNT family signaling molecules WNT5A/B and WNT16, with WNT5A as the primary ligand (Karvonen et al. 2019; Sheikh et al. 2021; Zhao et al. 2021). The CRD of ROR1 is thought to interact with WNT5A (Masiakowski and Yancopoulos 1998; Saldanha et al. 1998). Although Fukuda et al. showed in vitro binding of ROR1 to WNT5A using immunoprecipitated WNT5A expressed in CHO cells (Fukuda et al. 2008), the direct binding between ROR1 and WNT5A has not been examined. A recent study showed that CS-E pre-mixed with WNT5A bound to ROR1, although neither WNT5A nor CS-E alone bound to ROR1. These results suggest that CS-E directly interacts with ROR1 in the presence of WNT5A (Fig. 4) (Nadanaka et al. 2022). Basal-like breast cancer MDA-MB-231 cells express elevated levels of ROR1, and their invasive activity predominantly depends on ROR1. In addition, the invasive potential of MDA-MB-231 cells is upregulated in response to exogenously added CS-E, whereas MDA-MB-231 cells become less responsive to CS-E after ROR1 knockdown. As WNT5A signals are transduced through RORs to activate the c-Jun N-terminal kinase (JNK) pathway (Klemm et al. 2011; Oishi et al. 2003; Pukrop et al. 2006), an inhibitor of JNK, SP600125, suppresses invasiveness in MDA-MB-231 cells. The exogenously added CS-E polymers activates JNK and chemically synthesize hexasaccharide sequences, including the E-E-E-containing sequence GalNAc(4-*O*-sulfate,6-*O*-sulfate)-GlcA-GalNAc(4-*O*-sulfate,6-*O*-sulfate)-GlcA-GalNAc(4-*O*-sulfate,6-*O*-sulfate)-GlcA-*O*-*p*-methoxyphenyl (E-E-E 6-mer) in MDA-MB-231 cells. This suggests that the invasion-promoting activity of CS-E is

mediated by WNT5A/ROR1/JNK signaling. To downregulate the expression of the A and E units, *C4ST-1* was knocked down, which strongly reduced the invasiveness of MDA-MB-231 cells. When only E units were reduced by *GalNAc4S-6ST* knockdown, the invasiveness is suppressed. These results suggest that a specific sulfation pattern, as observed on the E unit, is associated with the high invasive potential of MDA-MB-231 cells. However, it remains unclear whether the action mechanism of CS-E in ROR1-positive triple-negative cancers is universal.

3.3 Toll-Like Receptors

Certain proteoglycans, consisting of a core protein and glycosaminoglycan (GAG) chains, are among the many types of biomolecules that can function as damage-associated molecular pattern molecules (DAMPs) and directly input inflammatory signals into the cells through toll-like receptors (TLRs). The TLR-induced transcriptional activation of inflammatory and tumor-promoting cytokines by NF-κB contributes to injury- and inflammation-driven tumor promotion.

Biglycan (BGN) is one of the PGs that are reported to function as a DAMP. The release of BGNs, members of the family of small leucine-rich PGs, from extracellular matrices or macrophages can boost inflammation by enhancing the synthesis of tumor necrosis factor (TNF)-α and MIP-2 through TLR2 and TLR4 signaling (Schaefer et al. 2005). In addition, it has been reported that BGNs directly bind to either TLR2 or TLR4 and input inflammatory signals into cells (Babelova et al. 2009; Schaefer et al. 2005). However, it has been shown that the core protein itself cannot transduce signals and thus cannot increase the expression of TNF-α, MIP-2, or interleukin-1β (IL-1β) (Babelova et al. 2009; Schaefer et al. 2005). These findings suggest that the GAGs of BGNs are potential innate antigen analogs. BGN is a PG modified with CS/DS chains; surface plasmon resonance (SPR) analysis has indicated that DS can bind to the TLR4/myeloid differentiation factor 2 (MD2) complex with high affinity (Nadanaka et al. 2020). In addition, out of various commercial GAGs, only DS chains stimulate the TLR4-mediated NF-κB activation of gene expression in a dose-dependent manner. DS is thought to act as an endogenous inducer of inflammation and is thus closely associated with pathological conditions, as it activates TLR4 signaling. Reportedly, DS expression is increased in the esophageal squamous cell carcinoma cells obtained from human biopsy samples (Thelin et al. 2012). Increase in DS serves as an endogenous danger signal; thus, DS structures may be characteristic of inflammatory disease-related sugars.

We propose that exostosin (EXT)-like 2 (EXTL2) controls the biosynthesis of pro-inflammatory DS chains attached to a BGN core protein. Nadanaka et al. previously proposed the function of EXTL2 in GAG biosynthesis, demonstrating that EXTL2 can control in vivo GAG biosynthesis via an unconventional mechanism wherein EXTL2 transfers a GlcNAc residue to a phosphorylated tetrasaccharide linker, thereby blocking chain elongation (Nadanaka et al. 2013) (Fig. 6). The GAG chains purified from PGs secreted by wild-type ($Extl2^{+/+}$)

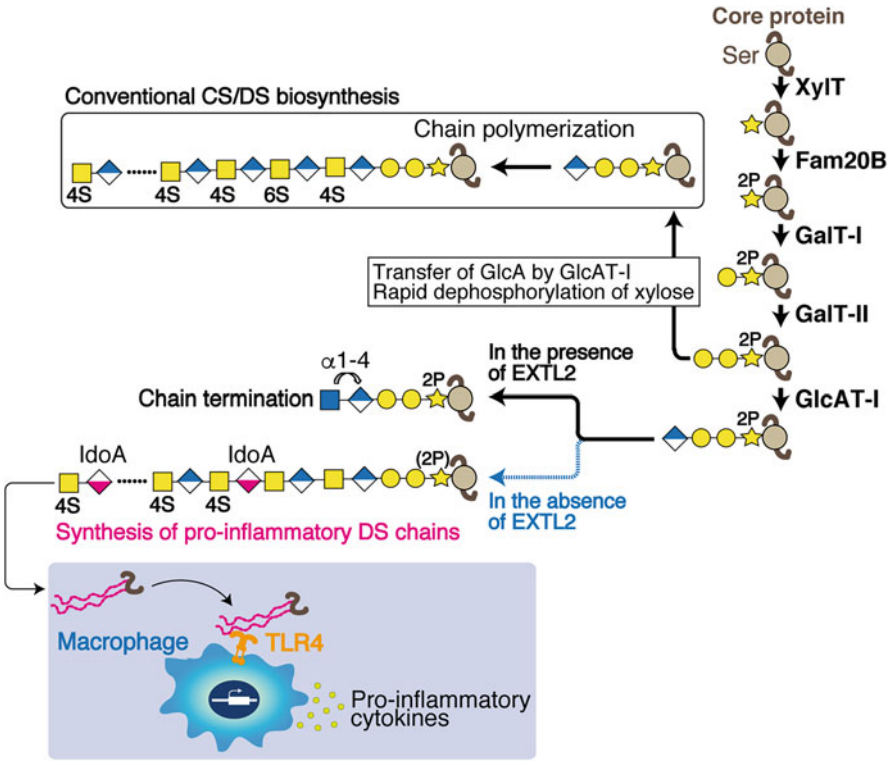


Fig. 6 Schematic representation of the biosynthesis of CS/DS in the presence or absence of EXTL2. After formation of the phosphorylated trisaccharide linkage Gal-Gal-Xyl(2P), GlcAT-I-mediated addition of GlcA can be accompanied by rapid dephosphorylation of xylose. Formation of the tetrasaccharide linkage region GlcA-Gal-Gal-Xyl is completed, followed by elongation of CS/DS chains by the CS/DS polymerase complex (conventional CS/DS biosynthesis). When a GlcA is transferred to the linkage region before dephosphorylation of xylose, EXTL2 adds a GlcNAc to the phosphorylated tetrasaccharide linkage to stop the elongation of CS/DS chains. Biosynthesis of CS/DS chains on a BGN core protein is regulated by EXTL2 in the liver. In the absence of EXTL2, the phosphorylated tetrasaccharide linkage can be used as an acceptor for polymerization, and CS/DS chains are elongated on a BGN core protein. BGN carrying CS/DS chains acts as a stronger DAMP than BGN synthesized in the presence of EXTL2 and, thus, highly activates TLR4/NF-κB signaling. Thus, loss of EXTL2 may be associated with chronic low-grade inflammation through the production of pro-inflammatory CSs

macrophages do not activate the TLR4 signaling pathway (Nadanaka et al. 2020). In contrast, the GAG chains obtained from *Extl2*$^{-/-}$ macrophages exhibit strong activity as TLR4 agonists. DS content is higher in the GAGs produced by *Extl2*$^{-/-}$macrophages than in the *Extl2*$^{+/+}$ macrophages. These results suggest that the loss of EXTL2 causes the production of DSs, which act as agonists for TLR4.

Furthermore, the pathophysiological significance of EXTL2 has been revealed, as mice deficient in EXTL2 (*EXTL2*$^{-/-}$) overproduce GAGs, which affects the

regeneration proceeding after liver injury, in addition to vascular calcification processes (Purnomo et al. 2013). Furthermore, the roles of EXTL2 in non-alcoholic steatohepatitis and hepatocarcinoma induced by dietary obesity and insulin resistance have been investigated (Nadanaka et al. 2020). Although *Extl2*$^{+/+}$ and *Extl2*$^{-/-}$ mice had a similar diabetic phenotype and suffered similar hepatocellular damage upon treatment with STZ and HFD, glutamine synthase (GS)-positive nodules, which are characteristic of hepatocarcinoma (Fujii et al. 2013), were detected in *Extl2*$^{-/-}$ mice (seven out of the nice mice) as early as 8 weeks of age. These findings suggest that pro-inflammatory DSs produced in the absence of EXTL2 promote tumorigenesis through the chronic low-grade inflammation induced by TLR4/NF-κB signaling (Fig. 6).

4 Future Perspectives

In this review, we describe the critical role of CS in malignant processes. Cancer cells utilize CS biosynthesis to enable malignant cancer features. Thus, the precise interference of aberrant CS biosynthetic pathways through small molecules or the elimination of cancer-associated CSs with specific CS sulfatases opens new avenues for therapy. In addition, the existence of CS receptors on cancer cell surfaces provides the new perspective that CS chains may serve as agonistic and/or antagonistic functional ligands for their respective receptor molecules. Therefore, the application of specific antibodies against cancer-associated CSs or their receptors holds promise in increasing cancer specificity. A deeper understanding of the molecular mechanisms underlying CS-associated malignant processes, with a focus on molecular partners, including CS-binding humoral factors and distinct CS receptors, will offer promising new therapeutic approaches for the treatment of CS-associated cancer progression.

References

Achur RN, Valiyaveettil M, Alkhalil A, Ockenhouse CF, Gowda DC (2000) Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillous spaces that mediate the adherence of Plasmodium falciparum-infected erythrocytes to the placenta. J Biol Chem 275(51):40344–40356. https://doi.org/10.1074/jbc.M006398200

Agnelli L, Forcato M, Ferrari F, Tuana G, Todoerti K, Walker BA, Morgan GJ, Lombardi L, Bicciato S, Neri A (2011) The reconstruction of transcriptional networks reveals critical genes with implications for clinical outcome of multiple myeloma. Clin Cancer Res 17(23):7402–7412. https://doi.org/10.1158/1078-0432.CCR-11-0596

Babelova A, Moreth K, Tsalastra-Greul W, Zeng-Brouwers J, Eickelberg O, Young MF, Bruckner P, Pfeilschifter J, Schaefer RM, Grone HJ, Schaefer L (2009) Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. J Biol Chem 284(36):24035–24048. https://doi.org/10.1074/jbc.M109.014266

Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA (2001) Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat Cell Biol 3(7):683–686. https://doi.org/10.1038/35083081

Bhattacharyya S, Feferman L, Tobacman JK (2017) Chondroitin sulfatases differentially regulate Wnt signaling in prostate stem cells through effects on SHP2, phospho-ERK1/2, and Dickkopf Wnt signaling pathway inhibitor (DKK3). Oncotarget 8(59):100242–100260. https://doi.org/10.18632/oncotarget.22152

Chernousov MA, Carey DJ (1993) N-syndecan (syndecan 3) from neonatal rat brain binds basic fibroblast growth factor. J Biol Chem 268(22):16810–16814

Clausen TM, Pereira MA, Al Nakouzi N, Oo HZ, Agerbaek MO, Lee S, Orum-Madsen MS, Kristensen AR, El-Naggar A, Grandgenett PM, Grem JL, Hollingsworth MA, Holst PJ, Theander T, Sorensen PH, Daugaard M, Salanti A (2016) Oncofetal chondroitin sulfate glycosaminoglycans are key players in integrin signaling and tumor cell motility. Mol Cancer Res 14(12):1288–1299. https://doi.org/10.1158/1541-7786.MCR-16-0103

Cooney CA, Jousheghany F, Yao-Borengasser A, Phanavanh B, Gomes T, Kieber-Emmons AM, Siegel ER, Suva LJ, Ferrone S, Kieber-Emmons T, Monzavi-Karbassi B (2011) Chondroitin sulfates play a major role in breast cancer metastasis: a role for CSPG4 and CHST11 gene expression in forming surface P-selectin ligands in aggressive breast cancer cells. Breast Cancer Res 13(3):R58. https://doi.org/10.1186/bcr2895

Cortes M, Baria AT, Schwartz NB (2009) Sulfation of chondroitin sulfate proteoglycans is necessary for proper Indian hedgehog signaling in the developing growth plate. Development 136(10):1697–1706. https://doi.org/10.1242/dev.030742

Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K (2002) Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. J Biol Chem 277(46):43707–43716. https://doi.org/10.1074/jbc.M207105200

Deepa SS, Yamada S, Zako M, Goldberger O, Sugahara K (2004) Chondroitin sulfate chains on syndecan-1 and syndecan-4 from normal murine mammary gland epithelial cells are structurally and functionally distinct and cooperate with heparan sulfate chains to bind growth factors. A novel function to control binding of midkine, pleiotrophin, and basic fibroblast growth factor. J Biol Chem 279(36):37368–37376. https://doi.org/10.1074/jbc.M403031200

Delehedde M, Devenyns L, Maurage CA, Vives RR (2013) Endocan in cancers: a lesson from a circulating dermatan sulfate proteoglycan. Int J Cell Biol 2013:705027. https://doi.org/10.1155/2013/705027

Feferman L, Bhattacharyya S, Deaton R, Gann P, Guzman G, Kajdacsy-Balla A, Tobacman JK (2013) Arylsulfatase B (N-acetylgalactosamine-4-sulfatase): potential role as a biomarker in prostate cancer. Prostate Cancer Prostatic Dis 16(3):277–284. https://doi.org/10.1038/pcan.2013.18

Fujii M, Shibazaki Y, Wakamatsu K, Honda Y, Kawauchi Y, Suzuki K, Arumugam S, Watanabe K, Ichida T, Asakura H, Yoneyama H (2013) A murine model for non-alcoholic steatohepatitis showing evidence of association between diabetes and hepatocellular carcinoma. Med Mol Morphol 46(3):141–152. https://doi.org/10.1007/s00795-013-0016-1

Fukuda T, Chen L, Endo T, Tang L, Lu D, Castro JE, Widhopf GF 2nd, Rassenti LZ, Cantwell MJ, Prussak CE, Carson DA, Kipps TJ (2008) Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. Proc Natl Acad Sci U S A 105(8):3047–3052. https://doi.org/10.1073/pnas.0712148105

Hay E, Nouraud A, Marie PJ (2009) N-cadherin negatively regulates osteoblast proliferation and survival by antagonizing Wnt, ERK and PI3K/Akt signalling. PLoS One 4(12):e8284. https://doi.org/10.1371/journal.pone.0008284

Herman D, Leakey TI, Behrens A, Yao-Borengasser A, Cooney CA, Jousheghany F, Phanavanh B, Siegel ER, Safar AM, Korourian S, Kieber-Emmons T, Monzavi-Karbassi B (2015) CHST11 gene expression and DNA methylation in breast cancer. Int J Oncol 46(3):1243–1251. https://doi.org/10.3892/ijo.2015.2828

Iida J, Dorchak J, Clancy R, Slavik J, Ellsworth R, Katagiri Y, Pugacheva EN, van Kuppevelt TH, Mural RJ, Cutler ML, Shriver CD (2015) Role for chondroitin sulfate glycosaminoglycan in NEDD9-mediated breast cancer cell growth. Exp Cell Res 330(2):358–370. https://doi.org/10.1016/j.yexcr.2014.11.002

Ito Z, Takakura K, Suka M, Kanai T, Saito R, Fujioka S, Kajihara M, Yanagisawa H, Misawa T, Akiba T, Koido S, Ohkusa T (2017) Prognostic impact of carbohydrate sulfotransferase 15 in patients with pancreatic ductal adenocarcinoma. Oncol Lett 13(6):4799–4805. https://doi.org/10.3892/ol.2017.6071

Jang B, Kim A, Hwang J, Song HK, Kim Y, Oh ES (2020) Emerging role of Syndecans in extracellular matrix remodeling in cancer. J Histochem Cytochem 68(12):863–870. https://doi.org/10.1369/0022155420930112

Jayne DG, Perry SL, Morrison E, Farmery SM, Guillou PJ (2000) Activated mesothelial cells produce heparin-binding growth factors: implications for tumour metastases. Br J Cancer 82(6):1233–1238. https://doi.org/10.1054/bjoc.1999.1068

Kagey MH, He X (2017) Rationale for targeting the Wnt signalling modulator Dickkopf-1 for oncology. Br J Pharmacol 174(24):4637–4650. https://doi.org/10.1111/bph.13894

Karvonen H, Perttila R, Niininen W, Hautanen V, Barker H, Murumagi A, Heckman CA, Ungureanu D (2019) Wnt5a and ROR1 activate non-canonical Wnt signaling via RhoA in TCF3-PBX1 acute lymphoblastic leukemia and highlight new treatment strategies via Bcl-2 co-targeting. Oncogene 38(17):3288–3300. https://doi.org/10.1038/s41388-018-0670-9

Klemm F, Bleckmann A, Siam L, Chuang HN, Rietkotter E, Behme D, Schulz M, Schaffrinski M, Schindler S, Trumper L, Kramer F, Beissbarth T, Stadelmann C, Binder C, Pukrop T (2011) Beta-catenin-independent WNT signaling in basal-like breast cancer and brain metastasis. Carcinogenesis 32(3):434–442. https://doi.org/10.1093/carcin/bgq269

Kluppel M, Samavarchi-Tehrani P, Liu K, Wrana JL, Hinek A (2012) C4ST-1/CHST11-controlled chondroitin sulfation interferes with oncogenic HRAS signaling in Costello syndrome. Eur J Hum Genet 20(8):870–877. https://doi.org/10.1038/ejhg.2012.12

Kobayashi T, Yan H, Kurahashi Y, Ito Y, Maeda H, Tada T, Hongo K, Nakayama J (2013) Role of GalNAc4S-6ST in astrocytic tumor progression. PLoS One 8(1):e54278. https://doi.org/10.1371/journal.pone.0054278

Koike T, Izumikawa T, Tamura J, Kitagawa H (2012) Chondroitin sulfate-E fine-tunes osteoblast differentiation via ERK1/2, Smad3 and Smad1/5/8 signaling by binding to N-cadherin and cadherin-11. Biochem Biophys Res Commun 420(3):523–529. https://doi.org/10.1016/j.bbrc.2012.03.024

Koziel L, Kunath M, Kelly OG, Vortkamp A (2004) Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. Dev Cell 6(6):801–813. https://doi.org/10.1016/j.devcel.2004.05.009

Kurima K, Warman ML, Krishnan S, Domowicz M, Krueger RC Jr, Deyrup A, Schwartz NB (1998) A member of a family of sulfate-activating enzymes causes murine brachymorphism. Proc Natl Acad Sci U S A 95(15):8681–8685. https://doi.org/10.1073/pnas.95.15.8681

Le Jan S, Hayashi M, Kasza Z, Eriksson I, Bishop JR, Weibrecht I, Heldin J, Holmborn K, Jakobsson L, Soderberg O, Spillmann D, Esko JD, Claesson-Welsh L, Kjellen L, Kreuger J (2012) Functional overlap between chondroitin and heparan sulfate proteoglycans during VEGF-induced sprouting angiogenesis. Arterioscler Thromb Vasc Biol 32(5):1255–1263. https://doi.org/10.1161/ATVBAHA.111.240622

Liu CH, Lan CT, Chou JF, Tseng TJ, Liao WC (2017) CHSY1 promotes aggressive phenotypes of hepatocellular carcinoma cells via activation of the hedgehog signaling pathway. Cancer Lett 403:280–288. https://doi.org/10.1016/j.canlet.2017.06.023

Liu LC, Wang YL, Lin PL, Zhang X, Cheng WC, Liu SH, Chen CJ, Hung Y, Jan CI, Chang LC, Qi X, Hsieh-Wilson LC, Wang SC (2019) Long noncoding RNA HOTAIR promotes invasion of breast cancer cells through chondroitin sulfotransferase CHST15. Int J Cancer 145(9):2478–2487. https://doi.org/10.1002/ijc.32319

MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 17(1):9–26. https://doi.org/10.1016/j.devcel.2009.06.016

Maeda N, Ichihara-Tanaka K, Kimura T, Kadomatsu K, Muramatsu T, Noda M (1999) A receptor-like protein-tyrosine phosphatase PTPzeta/RPTPbeta binds a heparin-binding growth factor midkine. Involvement of arginine 78 of midkine in the high affinity binding to PTPzeta. J Biol Chem 274(18):12474–12479. https://doi.org/10.1074/jbc.274.18.12474

Manon-Jensen T, Multhaupt HA, Couchman JR (2013) Mapping of matrix metalloproteinase cleavage sites on syndecan-1 and syndecan-4 ectodomains. FEBS J 280(10):2320–2331. https://doi.org/10.1111/febs.12174

Masiakowski P, Yancopoulos GD (1998) The Wnt receptor CRD domain is also found in MuSK and related orphan receptor tyrosine kinases. Curr Biol 8(12):R407. https://doi.org/10.1016/s0960-9822(98)70263-5

McCusker CD, Alfandari D (2009) Life after proteolysis: exploring the signaling capabilities of classical cadherin cleavage fragments. Commun Integr Biol 2(2):155–157

Mikami T, Kitagawa H (2013) Biosynthesis and function of chondroitin sulfate. Biochim Biophys Acta 1830(10):4719–4733. https://doi.org/10.1016/j.bbagen.2013.06.006

Muramatsu T (2010) Midkine, a heparin-binding cytokine with multiple roles in development, repair and diseases. Proc Jpn Acad Ser B Phys Biol Sci 86(4):410–425. https://doi.org/10.2183/pjab.86.410

Muramatsu T (2014) Structure and function of midkine as the basis of its pharmacological effects. Br J Pharmacol 171(4):814–826. https://doi.org/10.1111/bph.12353

Nadanaka S, Kitagawa H (2008) Heparan sulphate biosynthesis and disease. J Biochem 144(1):7–14. https://doi.org/10.1093/jb/mvn040

Nadanaka S, Ishida M, Ikegami M, Kitagawa H (2008) Chondroitin 4-O-sulfotransferase-1 modulates Wnt-3a signaling through control of E disaccharide expression of chondroitin sulfate. J Biol Chem 283(40):27333–27343. https://doi.org/10.1074/jbc.M802997200

Nadanaka S, Kinouchi H, Taniguchi-Morita K, Tamura J, Kitagawa H (2011) Down-regulation of chondroitin 4-O-sulfotransferase-1 by Wnt signaling triggers diffusion of Wnt-3a. J Biol Chem 286(6):4199–4208. https://doi.org/10.1074/jbc.M110.155093

Nadanaka S, Zhou S, Kagiyama S, Shoji N, Sugahara K, Sugihara K, Asano M, Kitagawa H (2013) EXTL2, a member of the EXT family of tumor suppressors, controls glycosaminoglycan biosynthesis in a xylose kinase-dependent manner. J Biol Chem 288(13):9321–9333. https://doi.org/10.1074/jbc.M112.416909

Nadanaka S, Kinouchi H, Kitagawa H (2016) Histone deacetylase-mediated regulation of chondroitin 4-O-sulfotransferase-1 (Chst11) gene expression by Wnt/beta-catenin signaling. Biochem Biophys Res Commun 480(2):234–240. https://doi.org/10.1016/j.bbrc.2016.10.035

Nadanaka S, Kinouchi H, Kitagawa H (2018) Chondroitin sulfate-mediated N-cadherin/beta-catenin signaling is associated with basal-like breast cancer cell invasion. J Biol Chem 293(2):444–465. https://doi.org/10.1074/jbc.M117.814509

Nadanaka S, Hashiguchi T, Kitagawa H (2020) Aberrant glycosaminoglycan biosynthesis by tumor suppressor EXTL2 deficiency promotes liver inflammation and tumorigenesis through toll-like 4 receptor signaling. FASEB J 34(6):8385–8401. https://doi.org/10.1096/fj.201902076R

Nadanaka S, Bai Y, Kitagawa H (2021) Cleavage of Syndecan-1 promotes the proliferation of the basal-like breast cancer cell line BT-549 via Akt SUMOylation. Front Cell Dev Biol 9:659428. https://doi.org/10.3389/fcell.2021.659428

Nadanaka S, Tamura JI, Kitagawa H (2022) Chondroitin sulfates control invasiveness of the basal-like breast cancer cell line MDA-MB-231 through ROR1. Front Oncol 12:914838. https://doi.org/10.3389/fonc.2022.914838

Nakanishi T, Kadomatsu K, Okamoto T, Ichihara-Tanaka K, Kojima T, Saito H, Tomoda Y, Muramatsu T (1997) Expression of syndecan-1 and -3 during embryogenesis of the central nervous system in relation to binding with midkine. J Biochem 121(2):197–205

Nguyen TL, Grizzle WE, Zhang K, Hameed O, Siegal GP, Wei S (2013) Syndecan-1 overexpression is associated with nonluminal subtypes and poor prognosis in advanced breast cancer. Am J Clin Pathol 140(4):468–474. https://doi.org/10.1309/AJCPZ1D8CALHDXCJ

Nikitovic D, Assouti M, Sifaki M, Katonis P, Krasagakis K, Karamanos NK, Tzanakakis GN (2008) Chondroitin sulfate and heparan sulfate-containing proteoglycans are both partners and targets of basic fibroblast growth factor-mediated proliferation in human metastatic melanoma cell lines. Int J Biochem Cell Biol 40(1):72–83. https://doi.org/10.1016/j.biocel.2007.06.019

Noriega-Guerra H, Freitas VM (2018) Extracellular matrix influencing HGF/c-MET signaling pathway: impact on cancer progression. Int J Mol Sci 19(11):3300. https://doi.org/10.3390/ijms19113300

Oishi I, Suzuki H, Onishi N, Takada R, Kani S, Ohkawara B, Koshida I, Suzuki K, Yamada G, Schwabe GC, Mundlos S, Shibuya H, Takada S, Minami Y (2003) The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. Genes Cells 8(7):645–654. https://doi.org/10.1046/j.1365-2443.2003.00662.x

Owada K, Sanjo N, Kobayashi T, Mizusawa H, Muramatsu H, Muramatsu T, Michikawa M (1999) Midkine inhibits caspase-dependent apoptosis via the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase in cultured neurons. J Neurochem 73(5):2084–2092

Prabhu SV, Bhattacharyya S, Guzman-Hartman G, Macias V, Kajdacsy-Balla A, Tobacman JK (2011) Extra-lysosomal localization of arylsulfatase B in human colonic epithelium. J Histochem Cytochem 59(3):328–335. https://doi.org/10.1369/0022155410395511

Pukrop T, Klemm F, Hagemann T, Gradl D, Schulz M, Siemes S, Trumper L, Binder C (2006) Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. Proc Natl Acad Sci U S A 103(14):5454–5459. https://doi.org/10.1073/pnas.0509703103

Purnomo E, Emoto N, Nugrahaningsih DA, Nakayama K, Yagi K, Heiden S, Nadanaka S, Kitagawa H, Hirata K (2013) Glycosaminoglycan overproduction in the aorta increases aortic calcification in murine chronic kidney disease. J Am Heart Assoc 2(5):e000405. https://doi.org/10.1161/JAHA.113.000405

Purushothaman A, Fukuda J, Mizumoto S, ten Dam GB, van Kuppevelt TH, Kitagawa H, Mikami T, Sugahara K (2007) Functions of chondroitin sulfate/dermatan sulfate chains in brain development. Critical roles of E and iE disaccharide units recognized by a single chain antibody GD3G7. J Biol Chem 282(27):19442–19452. https://doi.org/10.1074/jbc.M700630200

Reiss K, Maretzky T, Ludwig A, Tousseyn T, de Strooper B, Hartmann D, Saftig P (2005) ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. EMBO J 24(4):742–752. https://doi.org/10.1038/sj.emboj.7600548

Rousseau C, Ruellan AL, Bernardeau K, Kraeber-Bodere F, Gouard S, Loussouarn D, Sai-Maurel C, Faivre-Chauvet A, Wijdenes J, Barbet J, Gaschet J, Cherel M, Davodeau F (2011) Syndecan-1 antigen, a promising new target for triple-negative breast cancer immuno-PET and radioimmunotherapy. A preclinical study on MDA-MB-468 xenograft tumors. EJNMMI Res 1(1):20. https://doi.org/10.1186/2191-219X-1-20

Ryan E, Shen D, Wang X (2016) Structural studies reveal an important role for the pleiotrophin C-terminus in mediating interactions with chondroitin sulfate. FEBS J 283(8):1488–1503. https://doi.org/10.1111/febs.13686

Sakaguchi N, Muramatsu H, Ichihara-Tanaka K, Maeda N, Noda M, Yamamoto T, Michikawa M, Ikematsu S, Sakuma S, Muramatsu T (2003) Receptor-type protein tyrosine phosphatase zeta as a component of the signaling receptor complex for midkine-dependent survival of embryonic neurons. Neurosci Res 45(2):219–224. https://doi.org/10.1016/s0168-0102(02)00226-2

Salanti A, Clausen TM, Agerbaek MO, Al Nakouzi N, Dahlback M, Oo HZ, Lee S, Gustavsson T, Rich JR, Hedberg BJ, Mao Y, Barington L, Pereira MA, LoBello J, Endo M, Fazli L, Soden J, Wang CK, Sander AF, Dagil R, Thrane S, Holst PJ, Meng L, Favero F, Weiss GJ, Nielsen MA, Freeth J, Nielsen TO, Zaia J, Tran NL, Trent J, Babcook JS, Theander TG, Sorensen PH, Daugaard M (2015) Targeting human cancer by a glycosaminoglycan binding malaria protein. Cancer Cell 28(4):500–514. https://doi.org/10.1016/j.ccell.2015.09.003

Saldanha J, Singh J, Mahadevan D (1998) Identification of a frizzled-like cysteine rich domain in the extracellular region of developmental receptor tyrosine kinases. Protein Sci 7(8):1632–1635

Sayyad MR, Puchalapalli M, Vergara NG, Wangensteen SM, Moore M, Mu L, Edwards C, Anderson A, Kall S, Sullivan M, Dozmorov M, Singh J, Idowu MO, Koblinski JE (2019) Syndecan-1 facilitates breast cancer metastasis to the brain. Breast Cancer Res Treat 178(1): 35–49. https://doi.org/10.1007/s10549-019-05347-0

Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, Marsche G, Young MF, Mihalik D, Gotte M, Malle E, Schaefer RM, Grone HJ (2005) The matrix component biglycan is proinflammatory and signals through toll-like receptors 4 and 2 in macrophages. J Clin Invest 115(8):2223–2233. https://doi.org/10.1172/JCI23755

Schmidt HH, Dyomin VG, Palanisamy N, Itoyama T, Nanjangud G, Pirc-Danoewinata H, Haas OA, Chaganti RS (2004) Deregulation of the carbohydrate (chondroitin 4) sulfotransferase 11 (CHST11) gene in a B-cell chronic lymphocytic leukemia with a t(12;14)(q23;q32). Oncogene 23(41):6991–6996. https://doi.org/10.1038/sj.onc.1207934

Semenov M, Tamai K, He X (2005) SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J Biol Chem 280(29):26770–26775. https://doi.org/10.1074/jbc.M504308200

Sheikh MA, Kong X, Haymart B, Kaatz S, Krol G, Kozlowski J, Dahu M, Ali M, Almany S, Alexandris-Souphis T, Kline-Rogers E, Froehlich JB, Barnes GD (2021) Comparison of temporary interruption with continuation of direct oral anticoagulants for low bleeding risk procedures. Thromb Res 203:27–32. https://doi.org/10.1016/j.thromres.2021.04.006

Smith SM, West LA, Govindraj P, Zhang X, Ornitz DM, Hassell JR (2007) Heparan and chondroitin sulfate on growth plate perlecan mediate binding and delivery of FGF-2 to FGF receptors. Matrix Biol 26(3):175–184. https://doi.org/10.1016/j.matbio.2006.10.012

Sowa H, Kaji H, Yamaguchi T, Sugimoto T, Chihara K (2002) Activations of ERK1/2 and JNK by transforming growth factor beta negatively regulate Smad3-induced alkaline phosphatase activity and mineralization in mouse osteoblastic cells. J Biol Chem 277(39):36024–36031. https://doi.org/10.1074/jbc.M206030200

Spliid CB, Toledo AG, Sanderson P, Mao Y, Gatto F, Gustavsson T, Choudhary S, Saldanha AL, Vogelsang RP, Gogenur I, Theander TG, Leach FE 3rd, Amster IJ, Esko JD, Salanti A, Clausen TM (2021) The specificity of the malarial VAR2CSA protein for chondroitin sulfate depends on 4-O-sulfation and ligand accessibility. J Biol Chem 297(6):101391. https://doi.org/10.1016/j.jbc.2021.101391

Su G, Blaine SA, Qiao D, Friedl A (2008) Membrane type 1 matrix metalloproteinase-mediated stromal syndecan-1 shedding stimulates breast carcinoma cell proliferation. Cancer Res 68(22): 9558–9565. https://doi.org/10.1158/0008-5472.CAN-08-1645

Sugahara K, Schwartz NB (1979) Defect in 3′-phosphoadenosine 5′-phosphosulfate formation in brachymorphic mice. Proc Natl Acad Sci U S A 76(12):6615–6618. https://doi.org/10.1073/pnas.76.12.6615

Sugahara K, Schwartz NB (1982a) Defect in 3′-phosphoadenosine 5′-phosphosulfate synthesis in brachymorphic mice. I. Characterization of the defect. Arch Biochem Biophys 214(2):589–601. https://doi.org/10.1016/0003-9861(82)90064-9

Sugahara K, Schwartz NB (1982b) Defect in 3′-phosphoadenosine 5′-phosphosulfate synthesis in brachymorphic mice. II. Tissue distribution of the defect. Arch Biochem Biophys 214(2): 602–609. https://doi.org/10.1016/0003-9861(82)90065-0

Szatmari T, Otvos R, Hjerpe A, Dobra K (2015) Syndecan-1 in cancer: implications for cell signaling, differentiation, and prognostication. Dis Markers 2015:796052. https://doi.org/10.1155/2015/796052

Thelin MA, Svensson KJ, Shi X, Bagher M, Axelsson J, Isinger-Ekstrand A, van Kuppevelt TH, Johansson J, Nilbert M, Zaia J, Belting M, Maccarana M, Malmstrom A (2012) Dermatan sulfate is involved in the tumorigenic properties of esophagus squamous cell carcinoma. Cancer Res 72(8):1943–1952. https://doi.org/10.1158/0008-5472.CAN-11-1351

Ulbricht U, Eckerich C, Fillbrandt R, Westphal M, Lamszus K (2006) RNA interference targeting protein tyrosine phosphatase zeta/receptor-type protein tyrosine phosphatase beta suppresses

glioblastoma growth in vitro and in vivo. J Neurochem 98(5):1497–1506. https://doi.org/10.1111/j.1471-4159.2006.04022.x

Wang X, Zuo D, Chen Y, Li W, Liu R, He Y, Ren L, Zhou L, Deng T, Wang X, Ying G, Ba Y (2014) Shed Syndecan-1 is involved in chemotherapy resistance via the EGFR pathway in colorectal cancer. Br J Cancer 111(10):1965–1976. https://doi.org/10.1038/bjc.2014.493

Whalen DM, Malinauskas T, Gilbert RJ, Siebold C (2013) Structural insights into proteoglycan-shaped hedgehog signaling. Proc Natl Acad Sci U S A 110(41):16420–16425. https://doi.org/10.1073/pnas.1310097110

Willis CM, Kluppel M (2014) Chondroitin sulfate-E is a negative regulator of a pro-tumorigenic Wnt/beta-catenin-collagen 1 axis in breast cancer cells. PLoS One 9(8):e103966. https://doi.org/10.1371/journal.pone.0103966

Wu F, Zhang Y, Sun B, McMahon AP, Wang Y (2017) Hedgehog signaling: from basic biology to cancer therapy. Cell Chem Biol 24(3):252–280. https://doi.org/10.1016/j.chembiol.2017.02.010

Wu ZY, He YQ, Wang TM, Yang DW, Li DH, Deng CM, Cao LJ, Zhang JB, Xue WQ, Jia WH (2021) Glycogenes in oncofetal chondroitin sulfate biosynthesis are differently expressed and correlated with immune response in placenta and colorectal cancer. Front Cell Dev Biol 9:763875. https://doi.org/10.3389/fcell.2021.763875

Yu W, Yang L, Li T, Zhang Y (2019) Cadherin signaling in cancer: its functions and role as a therapeutic target. Front Oncol 9:989. https://doi.org/10.3389/fonc.2019.00989

Zhang S, Chen L, Cui B, Chuang HY, Yu J, Wang-Rodriguez J, Tang L, Chen G, Basak GW, Kipps TJ (2012) ROR1 is expressed in human breast cancer and associated with enhanced tumor-cell growth. PLoS One 7(3):e31127. https://doi.org/10.1371/journal.pone.0031127

Zhao Y, Zhang D, Guo Y, Lu B, Zhao ZJ, Xu X, Chen Y (2021) Tyrosine kinase ROR1 as a target for anti-cancer therapies. Front Oncol 11:680834. https://doi.org/10.3389/fonc.2021.680834

Part III
Signaling Pathways Modulated by Glycosylation

Significance of FUT8 in Pancreatic Cancer and Others

Caixia Liang, Wanli Song, and Jianguo Gu

Abstract Abnormal changes in glycosylation have been positively correlated with tumorigenesis and progression in cancer. In particular, an elevation in the level of core fucosylation has been observed. Core fucosylation is driven by fucosyltransferase 8 (FUT8), which catalyzes transfer of the fucosyl moiety from GDP-fucose to the innermost GlcNAc residue of *N*-glycans via an α1,6-linkage. Core fucosylation is known to be critical for various biological processes including inflammation and immune response, tumor initiation, and metastasis as well as in central nervous system diseases. FUT8 and/or core-fucosylated glycoproteins serve as important diagnosis biomarkers that provide a set of specific targets for therapeutic intervention. In this review, we summarize the existing evidence for the influence of core fucosylation in regulating cell surface protein stability and functional expression, as well as cancer cell signal transduction pathways in pancreatic cancer (PC) and in other cancers. In addition, we also discuss the relevance between FUT8 and cancer stemness, which is another aspect of FUT8 that could be applied to tumor therapy.

1 Introduction

Glycosylation is a commonly occurring posttranslational modification that occurs under the catalysis of a variety of glycosyltransferases to attach glycans to proteins or other organic molecules (Fuster and Esko 2005; Ohtsubo and Marth 2006). In eukaryotic cells, according to sugar-amino acid linkages, glycosylation is classified into one of two major forms: *N*-glycosylation (sequon: Asn-X-Ser/Thr, X can be any amino acid except proline) and O-glycosylation (sequon: Ser/Thr) (Eichler 2019; Christiansen et al. 2014). Protein glycosylation is involved in various pathophysiological processes of tumor development and progression such as regulating tumor

C. Liang (✉) · W. Song · J. Gu (✉)
Division of Regulatory Glycobiology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Medical and Pharmaceutical University, Sendai, Miyagi, Japan
e-mail: jgu@tohoku-mpu.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_6

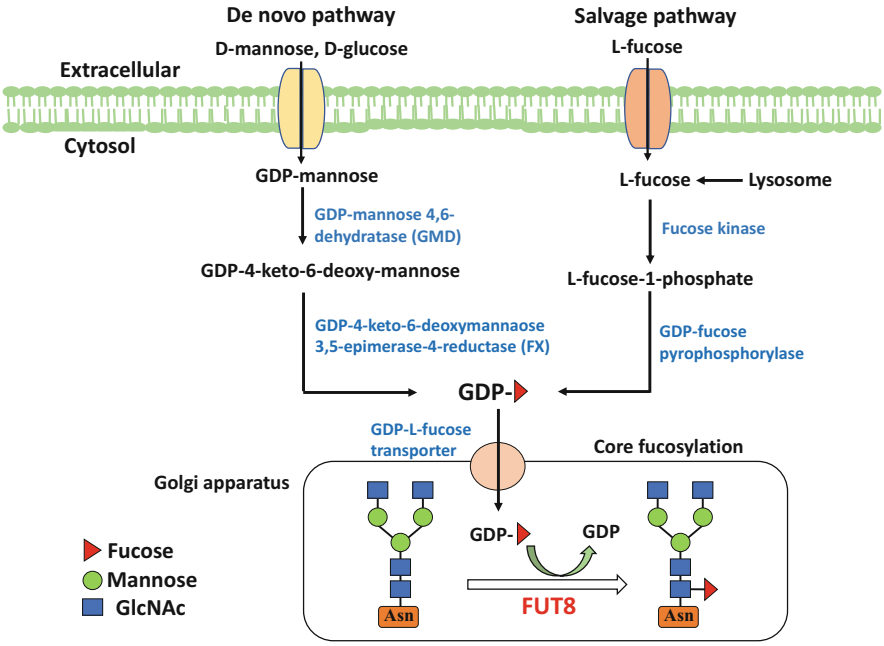


Fig. 1 GDP-fucose is synthesized through de novo and salvage pathways and is an essential component of core fucosylation. Core fucosylation of *N*-glycans is catalyzed by FUT8, which catalyzes the transfer of a fucose from GDP-L-fucose to the innermost GlcNAc residue via α-1,6 linkage

cell proliferation, invasion, metastasis, and angiogenesis (Ohtsubo and Marth 2006; Hakomori 2002; Chandler et al. 2019). Aberrant cancer-associated changes in protein glycosylation have been observed in cancers, including truncated O-glycans (Moreira et al. 2020), altered *N*-glycan branching (Kizuka and Taniguchi 2016), increased fucosylation (Miyoshi et al. 2008), and terminal sialylation (Vajaria et al. 2016), which serve as important tumor diagnostic biomarkers and/or provide a range of possible targets for therapeutic intervention.

Fucosylation is one of the most common modifications that take place when deoxyhexose fucose is transferred from GDP-fucose to glycoproteins or glycolipids under the catalysis of fucosyltransferases (FUTs) (Becker and Lowe 2003; Miyoshi et al. 2008). GDP-fucose is manufactured via de novo synthesis and salvage pathways (Fig. 1). In de novo synthesis, GDP-mannose is converted to GDP-fucose through enzymatic reactions catalyzed by GDP-mannose 4,6-dehydratase (GMD) and GDP-4-keto-6-deoxymannaose 3,5-epimerase-4-reductase (FX) (Tonetti et al. 1996; Niittymäki et al. 2006). In the salvage pathway, GDP-fucose is generated by the catalysis of fucose kinase and GDP-fucose pyrophosphorylase (Becker and Lowe 2003; Niittymäki et al. 2004). Subsequently, the GDP-fucose is transported into the Golgi complex via the membrane-anchored GDP-L-fucose transporter (Puglielli and Hirschberg 1999) where GDP-fucose

serves as a donor substrate for 11 fucosyltransferases (FUT1-11) to transfer the fucose to proper *N*-glycan substrates (Hirschberg 2001) (Fig. 1). Alterations in the expression of FUTs are closely related to malignant transformations of cancer such as invasion and metastasis (Lu et al. 2020; Park et al. 2020; Tu et al. 2013). In particular, FUT8 is considered to be directly related to tumors; it is upregulated in various cancers and has shown involvement in tumor characteristics (Liu et al. 2011; Zhao et al. 2008; Hutchinson et al. 1991).

2 Biological Functions and Implications of FUT8

Fucosyltransferase 8 (FUT8) is the only enzyme responsible for catalyzing core fucosylation, which is responsible for the transfer of fucose from GDP-L-fucose to the innermost GlcNAc residue of *N*-glycans via α-1,6 linkage (Norton and Mehta 2019; Bastian et al. 2021). Many pivotal glycoproteins in mammalian tissues are core fucosylated, and core fucosylation is essential to the functions of these proteins: E-cadherin, integrin, epidermal growth factor (EGF) receptor, transforming growth factor (TGF)-β receptor, c-mesenchymal-epithelial transition factor (c-Met), and Fms-like tyrosine kinase 3 (FLT3) (Zhao et al. 2006; Liu et al. 2019; Wang et al. 2005, 2015b; Duan et al. 2020). A deficiency in FUT8 can lead to disorders in normal organisms, causing cancers and a series of other diseases. Disruption of the FUT8 gene in mice ($FUT8^{-/-}$ mice) is known to cause severe growth retardation, death during postnatal development, emphysema-like changes (Wang et al. 2005), and neuronal disorders (Fukuda et al. 2011).

Core fucosylation is widely observed in mammalian tissues and is particularly abundant in brain tissue (Uozumi et al. 1996; Shimizu et al. 1993; Miyoshi et al. 1997). $FUT8^{-/-}$ mice exhibit increased locomotion that is particularly obvious as strenuous hopping behavior in a novel environment, which is consistent with a schizophrenia-like phenotype that is significantly reduced by treatment with haloperidol, an antipsychotic drug (Fukuda et al. 2011). Research has shown that $FUT8^{-/-}$ mice exhibit decreased performance in working memory and impaired prepulse inhibition through dysregulation of AMPA receptor, which is an ionotropic glutamate receptor (Gu et al. 2015). In addition, a deficiency of FUT8 promotes neuroinflammation by increasing the sensitivity of glial cells to inflammatory mediators, suggesting that the disorders of $FUT8^{-/-}$ mice are caused not only by neurons but also by glial cell dysfunction (Lu et al. 2019).

FUT8 also participates in immune response. Systemic lupus erythematosus (SLE) is an autoimmune multisystem disease characterized by loss of tolerance towards nuclear antigens, and the activation of $CD4^+$ T cells promotes the pathogenic process of SLE (Suárez-Fueyo et al. 2016; Abdirama et al. 2021). The recognition by T cell receptors (TCRs) of peptide-loaded major histocompatibility complex II (pMHC-II) on the B cell surface is important for the determination of $CD4^+$ T cell activation (Krogsgaard et al. 2005). Recent research has shown that SLE patients exhibit hyper core fucosylation in $CD4^+$ T cells, which is crucial for regulating TCR-pMHC-II

interaction in $CD4^+$ T cell activation (Liang et al. 2018; Sun et al. 2020). In addition, a lack of core fucosylation attenuates T-B cell communication via TCR-pMHC and suppresses IgG class switching by impairing $CD4^+$ T cell activation (Liang et al. 2018).

The importance of core fucosylation has also been established in individuals with FUT8-congenital disorder of glycosylation (FUT8-CDG), because these individuals carry pathogenic variants of FUT8. Individuals with FUT8-CDG typically experience intrauterine growth retardation, severe delays in growth and development, significantly shortened limbs, neurological impairments, and respiratory complications (Ng et al. 2020, 2018). These characteristics are consistent with some aspects observed in $FUT8^{-/-}$ mice. Taken together, these data clearly show that FUT8 is significantly involved in many important biological functions.

3 High Expression of FUT8 in Cancer

Altered FUT8 expression has been linked to clinical features of tumors and to the outcomes in extensive studies. Increased expression and enzymatic activity of FUT8 has been observed in cancers such as breast, lung, liver, colorectal, prostate, melanoma, and pancreatic (Table 1). Core fucosylation affects receptor activation, modulates cancer cell signal transduction pathways, and promotes tumor cell growth and metastasis (Liao et al. 2021; Bastian et al. 2021).

3.1 Core-Fucosylated Proteins as Tumor Diagnostic Biomarkers

Pancreatic cancer (PC) is one of the worlds' deadliest malignant diseases, and the overall 5-year survival rate among patients with PC is <5% (Jemal et al. 2008; Li et al. 2004). The poor survival rate is mainly due to the lack of validated, specific screening tests that reliably detect early-stage PC in people who have no symptoms. In most cases, patients with PC come to the hospital in an advanced stage when the cancer can no longer be removed with surgery or has spread from the pancreas to other parts of the body (Kamisawa et al. 2016). Therefore, there is an urgent clinical need to develop novel diagnostic methods for PC.

The detection of serum tumor markers is an effective diagnostic tool for pancreatic cancer, as well as for other cancers. FUT8 participates in α-fetoprotein (AFP) core fucosylation and can be used as a clinical marker for the early detection of hepatocellular carcinoma (HCC) (Nakagawa et al. 2006). Core-fucosylated prostate-specific antigen (PSA) is a diagnostic biomarker for prostate cancer (Lang et al. 2018). Chronic pancreatitis (CP) is a chronic inflammatory and fibrotic disease of the pancreas and a strong risk factor for the occurrence of pancreatic ductal

Table 1 Roles of FUT8 in various malignant tumors

Cancer type	Results in cancer	Reference
Breast cancer	FUT8-mediated TGF-β receptor core fucosylation promotes TGF-β signaling and EMT	Tu et al. (2017)
	Elevation of core-fucosylated *N*-glycans has been associated with lymph node metastasis and recurrent disease, as well as reduced rates of survival	Herrera et al. (2019)
Lung cancer	FUT8 is upregulated during EMT via the transactivation of β-catenin /LEF-1. Suppression of FUT8 inhibits tumor growth and metastasis	Chen et al. (2013)
	FUT8 regulates the cancer-promoting capacity of CAFs via the modification of EGFR fucosylation	Li et al. (2020)
Liver cancer	Deficiency of FUT8 attenuates the responses to EGF and HGF stimulation, downregulates intracellular signaling, and inhibits cell proliferation	Wang et al. (2015b)
	HCV-induced FUT8 promotes cell proliferation and causes 5-FU drug resistance	Li et al. (2019)
Colorectal cancer	FUT8 regulates E-cadherin expression and enhances E-cadherin-dependent cell-cell adhesion	Osumi et al. (2009)
Prostate cancer	FUT8 overexpression reduces the production of extracellular vesicles and increases the abundance of proteins associated with cell motility and metastasis resistance	Höti et al. (2020)
	Castration-induced FUT8 overexpression has resulted in the upregulation of EGFR and has led to an increase in drug resistance	Clark et al. (2020)
Melanoma	FUT8 overexpression facilitates invasion and tumor dissemination, due to an inhibition of the plasmin-mediated cleavage of L1CAM	Agrawal et al. (2017)
Pancreatic cancer	High levels of FUT8 expression have been associated with lymph node metastases and a worsening of relapse-free survival, while FUT8 knockdown has reduced tumor cell invasion	Tada et al. (2020)
	The silencing of FUT8 inhibits cell migration and proliferation and suppresses the features of cancer stemness	Liang et al. (2021)

adenocarcinoma (PDAC) (Malka et al. 2002; Talamini et al. 1999). In at least one study, serum core-fucosylated haptoglobin levels were significantly increased in CP patients compared with healthy volunteers, and these levels have since been used as an independent determinant for CP diagnosis (Ueda et al. 2016). Intraductal papillary mucinous neoplasm (IPMN) is a type of tumor that grows within the pancreatic ducts and has a high risk for the development of PDAC, which accounts for 90% of all pancreatic cancers (Muraki et al. 2021; Kleeff et al. 2016). FUT8 expression is upregulated in IPMN and correlates with the size of tumors. FUT8 levels in serum should be considered for evaluation in a future prospective study of IPMN (Watanabe et al. 2016). A report of remarkable increases (40%) in core-fucosylated biantennary glycans on ribonuclease 1 (RNase 1) in PC serum compared with healthy controls suggests that RNase 1 could be of clinical value for the detection of PC (Barrabés et al. 2007). In addition, aberrantly expressed mucin glycoproteins (MUC) are known to play important roles in PC. FUT8 could regulate the expression

of MUC4 and MUC1 at protein levels. Thus, core-fucosylated MUC1/4 could also serve as a diagnosis marker for PC (Kumar et al. 2015).

3.2 Roles of Core Fucosylation in Cell Adhesion and Cell Migration

Dysregulation of glycosylation is not only a consequence of cancer, it also is involved in fundamental molecular and cell biology processes, such as regulating cell signaling and cell adhesion, and eventually controlling tumor cell growth and metastatic and invasive behaviors (Pinho and Reis 2015). Several studies have shown that core fucosylation plays a critical role in cellular events through a variety of mechanisms. Abnormal expression of core fucosylation has been identified as a feature of metastatic cancer (Magalhães et al. 2017; Taniguchi et al. 2021; Shao et al. 2016). For example, core fucosylation of the L1 cell adhesion molecule (L1CAM) hinders its cleavage by protease plasmin, which facilitates L1CAM-mediated cell invasion and tumor dissemination in melanoma (Agrawal et al. 2017). FUT8 deficiency suppresses cell migration by interfering with the integrin/FAK pathway in breast cancer (Liu et al. 2019). In fact, FUT8-KO has been used to block α3β1 integrin-mediated cell migration and cell signaling, which was reinstated via reintroduction of the FUT8 gene (Zhao et al. 2006). FUT8 is upregulated in pancreatic cancer patients and correlates with cancer invasiveness, lymph node metastases, and a worsened state of relapse-free survival, whereas inhibition of FUT8 expression reduces tumor cell invasion and peritoneal metastasis (Tada et al. 2020). These results suggest that FUT8 plays an important role in both cell adhesion and cell migration.

3.3 Roles of FUT8 in Cellular Signaling

Altering the expression of core-fucosylated proteins interferes with cell signaling molecules that regulate the activation of tyrosine kinase protein receptors such as epidermal growth factor receptor (EGFR), IGF-1R, and FLT3 (Fig. 2). The EGFR signaling cascade is a key regulator in essential cellular functions that include proliferation, differentiation, survival, and migration (Sabbah et al. 2020). Core fucosylation plays an important role in the EGFR mediation of signaling pathways. A lack of core fucosylation in EGFR could change its conformation, which would block EGF binding, and subsequently reduce the phosphorylation levels of EGFR and EGFR-mediated ERK activation for cell proliferation, which may partially explain the growth retardation in FUT8-KO mice (Wang et al. 2006). In a similar manner, the functions of c-Met and HGF receptor were also downregulated by a lack of core fucosylation. In a recent study, FUT8-KO blocked DEN/PB-induced

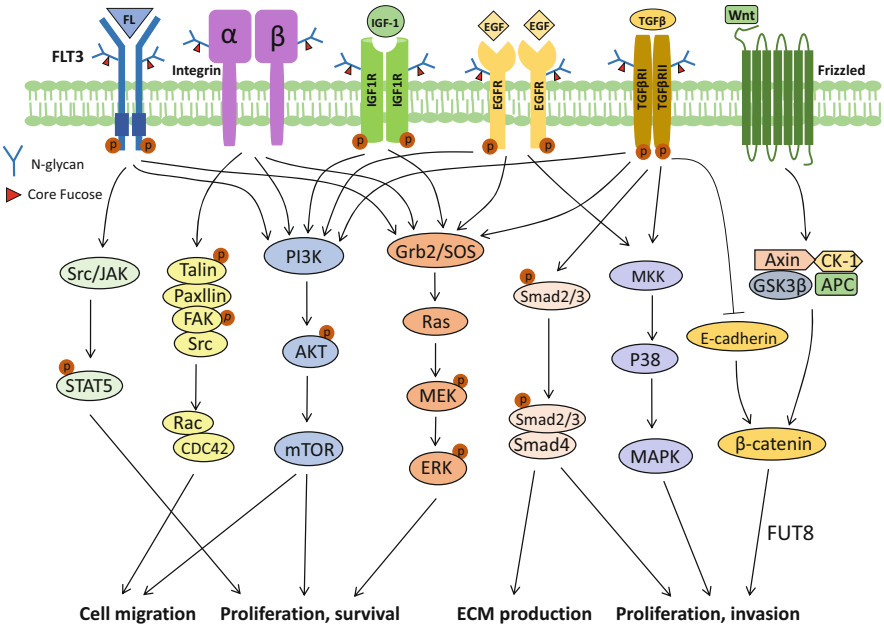


Fig. 2 Membrane receptors and intracellular signaling pathways regulated by core fucosylation

hepatocellular carcinoma formation (Wang et al. 2015b) and liver regeneration (Wang et al. 2015a). This particular inhibitory function was also confirmed using 2-fluoro-L-fucose (2FF), an analog of L-fucose, which inhibits core fucosylation by interfering with the normal synthesis of GDP-fucose (Fig. 1) in hepatoma cell lines (Zhou et al. 2017) and in pancreatic cancer cell lines (Liang et al. 2021). Interestingly, increasing the sialylation and fucosylation (catalyzed by FUT4 or FUT6) of EGFR suppresses its activation and function, but FUT8 is known to promote EGFR dimerization and phosphorylation in lung cancer cells (Liu et al. 2011). Further studies have indicated that the overexpression of FUT8 on EGFR can enhance EGF-mediated cell growth and increase the sensitivity to gefitinib (Matsumoto et al. 2008). Thus, the impact of FUT8-mediated core glycosylation on EGFR signaling may provide a therapeutic target for cancer.

Insulin-like growth factor receptor (IGF-1R) is a heterotetrameric receptor that consists of two α subunits located extracellularly and two β subunits that span the membrane. The α subunit is responsible for binding to the ligand IGF-1, and the β subunits exhibit tyrosine kinase activity and activate a variety of intracellular signaling pathways (MAPK, PI3K/AKT), which promotes cell survival and motility (Díez 1999; Vincent and Feldman 2002). IGF-1R requires core fucosylation for its cellular signaling, and the knockdown of FUT8 reduces IGF-1 signaling (Vanhooren et al. 2011). This phenomenon has also been observed in human trophoblast and choriocarcinoma cells (Yu et al. 2019). Recent studies have shown that impairment of the self-repair function of alveolar epithelial cells (AECs) is an important cause of

idiopathic pulmonary fibrosis (IPF). IGF-1 is a primary factor of increases in cell senescence (Kritschil et al. 2020). Suppressing core fucosylation in IGF-1R prevents its binding to IGF-1 and inhibits IGF-1/PI3K/AKT signaling, thus blocking the IPF process induced by AEC senescence (Sun et al. 2021).

Core fucosylation may also negatively regulate receptor functions. Fms-like tyrosine kinase 3 (FLT3) is a member of the tyrosine kinase receptor type III family, which is known to exert a significant effect on the expansion of hematopoietic progenitors in the pathogenesis of acute myeloid leukemia (AML) (Spiekermann et al. 2003). Oddly enough, in one study we found that a lack of core fucosylation in FLT3 induced ligand-independent dimerization on the cell surface, which then led to the aberrant activation of downstream signaling pathways such as p-STAT5, p-ERK, and p-AKT. This activation is known to induce IL-3-independent cell proliferation in Ba/F3 cells (Duan et al. 2020). Activation by deficient FUT8 was also observed in the signaling pathway for activin/P-Smad2, which is a member of the transforming growth factor (TGF)-β superfamily (Gu et al. 2013). Decreased core fucosylation of activin receptors, ACVR2A and ACVR1B, via knockdown of the FUT8 gene, enhanced activin binding and activin receptor-mediated signaling, which were cancelled by the restoration of FUT8 expression.

3.4 Roles of FUT8 in TGF-β-Induced EMT

Epithelial-mesenchymal transition (EMT) is a biological process that allows polarized epithelial cells to undergo multiple biochemical changes that enable them to acquire a mesenchymal phenotype and migrate to secondary sites (Thiery and Sleeman 2006). EMT is characterized by a loss of cell-cell adhesion and by the acquisition of cell motility. This process features a decreased expression of cell-cell adhesion molecules and epithelial markers such as E-cadherin and an increased expression of intermediate filament proteins and mesenchymal cell markers such as *N*-cadherin and integrins (Bhowmick et al. 2001; Maeda et al. 2005).

FUT8 is upregulated during TGF-β-induced EMT in breast cancer cells (Tu et al. 2017). The increase in FUT8 modifies TGF-β receptors I and II on a cell surface, which facilitates TGF-β binding and enhances downstream signaling. These developments stimulate breast cancer cell invasion and metastasis (Tu et al. 2017). The molecular mechanism for the induction of FUT8 during TGF-β-induced EMT has been explored in lung cancer cells (Chen et al. 2013). In that study, the expression of E-cadherin was suppressed during EMT, leading to the nuclear accumulation of β-catenin, which then formed a complex with lymphoid enhancer-binding factor-1 (LEF-1) to activate FUT8 expression (Chen et al. 2013). In addition, activation of the Wnt/β-catenin signaling pathway is also known to induce the expression of FUT8, which improves both cell invasion in hepatocellular carcinoma (HCC) (Zhang et al. 2020a) and EMT in breast cancer cells (Yang et al. 2017). Lin et al. found that blocking core fucosylation of TGF-β1 receptors by siRNA inhibits the phosphorylation of Smad2/3, which leads to the interruption of TGF-β/Smad2/3 signaling

activation and subsequently suppresses EMT development (Lin et al. 2011). These results strongly suggest that TGF-β signaling partially regulates EMT via FUT8.

4 Core Fucosylation Regulates the Stability of Glycoproteins Expressed on the Cell Surface

Many pivotal glycoproteins of tumors are highly core-fucosylated, and their functions are known to be regulated by core fucosylation (Okada et al. 2017; Matsumoto et al. 2008; Zhao et al. 2006; Geng et al. 2004). The latest research reports indicate that core fucosylation plays an important role in regulating the stability of cell surface glycoproteins. Programmed cell death protein-1 (PD-1) is an immune checkpoint, and binding with its ligand (PD-L1) plays a crucial role in T lymphocyte activation and tumor immune escape (Keir et al. 2008; Han et al. 2020). Blocking core fucosylation has reduced the PD-1 expression on the cell surface (Zhang et al. 2020b), which downregulated PD-1/PD-L1 interaction and enhanced T cell activation and led to more efficient tumor eradication (Okada et al. 2017). The underlying mechanism showed that suppression of core fucosylation in PD-1 promoted PD-1 ubiquitination and then increased the degradation of PD-1 by proteasomes (Zhang et al. 2020b; Okada et al. 2017). A similar result has been observed in the molecules of triple negative breast cancer (TNBC) patients who are not responsive to anti-PD1/PDL1 immunotherapy. B7 homolog 3 protein (B7H3) is also known as CD276 and is regarded as an immune checkpoint molecule for immunosuppressive activity. B7H3 is a highly *N*-glycosylated protein with core fucosylation, which makes it less prone to ubiquitin-proteasome degradation and allows it to be stably expressed on a cell surface, which inhibits the occurrence of a tumor immune response (Huang et al. 2021).

The lack of FUT8 is also known to reduce the expressions of E-cadherin and impaired E-cadherin-dependent cell-cell adhesion in colon carcinoma cells (Osumi et al. 2009). Our recent research has shown that FUT8-KO significantly suppresses the expression of EGFR on the surface of PC cells. Furthermore, treatment with cycloheximide, a protein synthesis inhibitor, showed that defective core fucosylation accelerates EGFR degradation, which suggests that core fucosylation plays an important role in maintaining the stability of the protein (Liang et al. 2021). However, the precise molecular mechanisms for how core fucosylation affects protein structure to regulate its stability remains to be elucidated in future study.

5 Glycosylation in Cancer Stem Cells

Cancer is a major cause of death worldwide. Despite significant progress in the diagnosis and treatment of cancer, the survival rate of progressive cancers remains insufficient. One of the most important reasons has been attributed to a distinct subpopulation of tumor cells that are referred to as cancer stem cells (CSCs). These cells have the capacity to self-renew and a facility for differentiation that drives tumor initiation, metastasis, relapse, and chemoresistance (Jordan et al. 2006; Clarke and Fuller 2006). Many well-known CSC markers such as CD133, CD44, EpCAM, and CD24 are heavily glycosylated, and alterations in glycosylation are associated with tumor development and tumor cell stemness (Barkeer et al. 2018). Bellis et al. revealed a significant upregulation of ST6Gal-I in human colon tumors, and high ST6Gal-I expression has been associated with the expression of the cancer stem cell markers CD133 and ALDH1, while the shRNA-mediated downregulation of ST6Gal-I reduces the percentage of CSCs in cancer cell populations (Swindall et al. 2013). Orospheres are CSC-rich oral squamous cell populations that are obtained via a spheroid culture technique. The expression levels of FUT3 and FUT6 (involved in SLex epitope synthesis) were more highly upregulated in orospheres compared with their adherent counterparts; also, orospheres expressed higher CSC markers and were more resistant to cisplatin/radiation in oral squamous cell carcinoma (Desiderio et al. 2015). FUT9 expression boosts the acquisition of stemness both in murine and human colon cancer cells towards a cancer stem-like transcriptional profile, phenotype, and function; other functional implications of CSCs also are promoted such as tumor growth and resistance to chemotherapy (Blanas et al. 2020). Breast cancer cells expressing high levels of CD44 and low levels of CD24 are resistant to chemotherapy and often lead to cancer relapse. In some studies FUT8 knockdown has reduced the $CD44^{+}/CD24^{-}$ population and increased the susceptibility to chemotherapy, indicating that FUT8 is essential for maintaining the stemness phenotype (Ma et al. 2021).

5.1 FUT8 Regulates Cancer Stemness

Pancreatic cancer has a high mortality rate, and one of the reasons could be the presence of pancreatic cancer stem cells, which were first identified by Simeone et al. in 2007 (Li et al. 2007). Terao et al. reported that increased cellular fucosylation is a common glycan change in pancreatic cancer stem cells (Terao et al. 2015), which is consistent with our recent study using PANC-1 and MIA PaCa-2 cell lines. Compared with MIA PaCa-2 cells, PANC-1 cells exhibit a greater degree of staining with *Aleuria aurantia* lectin (AAL), which specifically recognizes fucosylation. RT-PCR has shown that much higher expression levels of CSC biomarkers such as EpCAM, CD24, CD44, and CD133 are present in PANC-1 cells, which indicates that fucosylation modification could be related to cell stemness. Interestingly, a

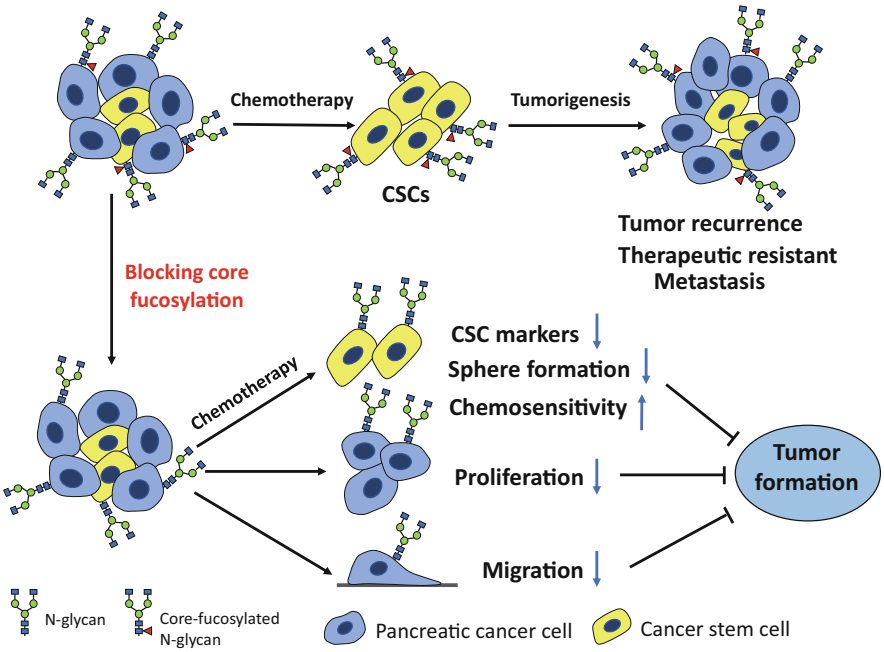


Fig. 3 Schematic diagram of core fucosylation and CSCs in PC. Tumor cells are heterogeneous and include CSC populations. CSCs are resistant to chemotherapy, which can lead to tumor recurrence and metastasis. PC cells express high levels of core fucosylation; blocking core fucosylation can inhibit cell proliferation, migration, and cancer stemness, which could be beneficial to prevent or inhibit tumor regrowth or recurrence

deficiency of FUT8 (FUT8-KO) greatly decreases the expression levels of these CSC biomarkers as well as sphere formation (Fig. 3). Furthermore, re-expression of FUT8 in FUT8-KO cells rescues the cell stemness features of these PC cells (Liang et al. 2021). In fact, this phenomenon is not restricted to PC; decreased or blocked core fucosylation has reduced stemness and EMT, which has also been observed in breast cancer cells (Yang et al. 2017). In addition, upregulated FUT8 has also been found in esophageal cancer stem-like cells, which were enriched using the sphere-formation method (Sadeghzadeh et al. 2020). These results strongly suggest that FUT8 could regulate cancer stemness.

5.2 FUT8 Increases Multidrug Resistance

Drug resistance is also a characteristic of cancer stemness and remains a major challenge to the efficiency of curative treatments (Gottesman 2002). Recent studies have revealed that changes in glycosylation regulate cancer stemness and interfere with tumor response to anticancer drugs, leading to the emergence of cancer cells

resistant to chemotherapy treatment (Mallard and Tiralongo 2017; Zhang et al. 2012; Ferreira et al. 2016). Aberrant glycosylation has proven to be related to chemotherapy resistance in malignant cells (Very et al. 2018). Fucosylated glycans are common modifications on the cell surface, and these participate in various biological processes such as differentiation, development, and tumor multidrug resistance (MDR). For example, compared with wild-type RMG-1 cells, the transfection of FUT1 and FUT2 genes into human ovarian carcinoma-derived RMG-I cells greatly promotes cell viability in the presence of 5-fluorouracil (5-FU) (Iwamori et al. 2005). The expression levels of FUT4 and its product, Lewis X antigen, are dramatically increased in patients with metastatic colorectal cancer (Blanas et al. 2019). Overexpression of FUT4 induces the RAF-MEK-ERK signaling pathway and exhibits significant resistance to anti-EGFR (cetuximab) and anti-VEGF (bevacizumab) chemotherapeutical agents (Giordano et al. 2015).

A growing body of evidence indicates that FUT8 is one of the main causes of cancer drug resistance (Cheng et al. 2013; Lv et al. 2019). Altered levels of FUT8 are responsible for resistance to 5-FU in human hepatocellular carcinoma via modulation of the PI3K/AKT signaling pathway with the involvement of MDR-related protein 1 (MRP1) expression (Cheng et al. 2013). HCV infection induces FUT8 expression in human hepatoma cells, which promotes expression of the drug-resistant proteins P-glycoprotein and MRP1 via activation of the PI3K/AKT/NF-κB signaling pathways; HCV also enhances 5-FU drug resistance (Li et al. 2019). On the other hand, FUT8-KO in a pro-B cell line has induced autodimerization of FLT3 and activated intracellular signaling such as the phosphorylations of STAT5, AKT, and ERK. An important development is that FUT8-KO increases the sensitivities for PKC412, a tyrosine kinase inhibitor, which suppresses cell proliferation (Duan et al. 2020). In a similar manner, an increase in core fucosylation in ovarian cancer cells has led to cisplatin resistance by suppressing copper transporter 1 (CTR1)-cisplatin interactions, while FUT8-KO weakens cisplatin resistance by promoting cellular cisplatin uptake (Lv et al. 2019). The effect of FUT8 on drug resistance has also been observed in pancreatic cancer cells. FUT8-KO has significantly increased the chemosensitivity for gemcitabine, which is a first-line therapy for advanced pancreatic cancer (Liang et al. 2021).

6 Conclusion and Perspectives

The prevalence of aberrant FUT8 and core-fucosylated glycoproteins, EGFR, TGFβR, PD-1, E-cadherin, and α3β1 integrin, have been reported in different types of cancers, as described above. The upregulation of core fucosylation is involved in regulating tumor malignance that results in promoting cell proliferation, metastasis, cancer stemness, and multidrug resistance (Fig. 3). Thus, targeting core fucosylation could be a feasible approach for the treatment of cancers.

Blocking core fucosylation via the use of an analog of L-fucose could be a useful method for the downregulation of cancer stemness and drug resistance. A lack of

specificity, however, could mean that other forms of fucosylation could be affected, as well as other forms of glycosylation via sugar metabolism. Therefore, an increase in specificity for inhibiting core fucosylation or directly targeting FUT8 genes or proteins could open new opportunities to develop novel therapeutic strategies to eradicate CSCs, which will help lower the rates of cancer recurrence and enhance the survival rates for cancer patients.

Acknowledgments This work was supported in part by a Grants-in-Aid for Scientific Research (19H03184 to J.G.) from the Japan Society for the Promotion of Science and by a Grants-in-Aid for Scientific Research on Innovative Areas (20H04909 to J.G.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Abdirama D, Tesch S, Grießbach AS, von Spee-Mayer C, Humrich JY, Stervbo U, Babel N, Meisel C, Alexander T, Biesen R, Bacher P, Scheffold A, Eckardt KU, Hiepe F, Radbruch A, Burmester GR, Riemekasten G, Enghard P (2021) Nuclear antigen-reactive CD4(+) T cells expand in active systemic lupus erythematosus, produce effector cytokines, and invade the kidneys. Kidney Int 99(1):238–246. https://doi.org/10.1016/j.kint.2020.05.051

Agrawal P, Fontanals-Cirera B, Sokolova E, Jacob S, Vaiana CA, Argibay D, Davalos V, McDermott M, Nayak S, Darvishian F, Castillo M, Ueberheide B, Osman I, Fenyo D, Mahal LK, Hernando E (2017) A systems biology approach identifies FUT8 as a driver of melanoma metastasis. Cancer Cell 31(6):804–819.e807. https://doi.org/10.1016/j.ccell.2017.05.007

Barkeer S, Chugh S, Batra SK, Ponnusamy MP (2018) Glycosylation of cancer stem cells: function in stemness, tumorigenesis, and metastasis. Neoplasia (New York, NY) 20(8):813–825. https://doi.org/10.1016/j.neo.2018.06.001

Barrabés S, Pagès-Pons L, Radcliffe CM, Tabarés G, Fort E, Royle L, Harvey DJ, Moenner M, Dwek RA, Rudd PM, De Llorens R, Peracaula R (2007) Glycosylation of serum ribonuclease 1 indicates a major endothelial origin and reveals an increase in core fucosylation in pancreatic cancer. Glycobiology 17(4):388–400. https://doi.org/10.1093/glycob/cwm002

Bastian K, Scott E, Elliott DJ, Munkley J (2021) FUT8 alpha-(1,6)-fucosyltransferase in cancer. Int J Mol Sci 22(1):455. https://doi.org/10.3390/ijms22010455

Becker DJ, Lowe JB (2003) Fucose: biosynthesis and biological function in mammals. Glycobiology 13(7):41r–53r. https://doi.org/10.1093/glycob/cwg054

Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL (2001) Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. J Biol Chem 276(50):46707–46713. https://doi.org/10.1074/jbc.M106176200

Blanas A, Cornelissen LAM, Kotsias M, van der Horst JC, van de Vrugt HJ, Kalay H, Spencer DIR, Kozak RP, van Vliet SJ (2019) Transcriptional activation of fucosyltransferase (FUT) genes using the CRISPR-dCas9-VPR technology reveals potent N-glycome alterations in colorectal cancer cells. Glycobiology 29(2):137–150. https://doi.org/10.1093/glycob/cwy096

Blanas A, Zaal A, van der Haar ÀI, Kempers M, Kruijssen L, de Kok M, Popovic MA, van der Horst JC, JvV S (2020) FUT9-driven programming of colon cancer cells towards a stem cell-like state. Cancers (Basel) 12(9):2580. https://doi.org/10.3390/cancers12092580

Chandler KB, Costello CE, Rahimi N (2019) Glycosylation in the tumor microenvironment: implications for tumor angiogenesis and metastasis. Cell 8(6):544. https://doi.org/10.3390/cells8060544

Chen CY, Jan YH, Juan YH, Yang CJ, Huang MS, Yu CJ, Yang PC, Hsiao M, Hsu TL, Wong CH (2013) Fucosyltransferase 8 as a functional regulator of nonsmall cell lung cancer. Proc Natl Acad Sci U S A 110(2):630–635. https://doi.org/10.1073/pnas.1220425110

Cheng L, Luo S, Jin C, Ma H, Zhou H, Jia L (2013) FUT family mediates the multidrug resistance of human hepatocellular carcinoma via the PI3K/Akt signaling pathway. Cell Death Dis 4(11): e923. https://doi.org/10.1038/cddis.2013.450

Christiansen MN, Chik J, Lee L, Anugraham M, Abrahams JL, Packer NH (2014) Cell surface protein glycosylation in cancer. Proteomics 14(4-5):525–546. https://doi.org/10.1002/pmic.201300387

Clark DJ, Schnaubelt M, Hoti N, Hu Y, Zhou Y, Gooya M, Zhang H (2020) Impact of increased FUT8 expression on the extracellular vesicle proteome in prostate cancer cells. J Proteome Res 19(6):2195–2205. https://doi.org/10.1021/acs.jproteome.9b00578

Clarke MF, Fuller M (2006) Stem cells and cancer: two faces of eve. Cell 124(6):1111–1115. https://doi.org/10.1016/j.cell.2006.03.011

Desiderio V, Papagerakis P, Tirino V, Zheng L, Matossian M, Prince ME, Paino F, Mele L, Papaccio F, Montella R, Papaccio G, Papagerakis S (2015) Increased fucosylation has a pivotal role in invasive and metastatic properties of head and neck cancer stem cells. Oncotarget 6(1): 71–84. https://doi.org/10.18632/oncotarget.2698

Díez J (1999) Insulin-like growth factor I in essential hypertension. Kidney Int 55(2):744–759. https://doi.org/10.1046/j.1523-1755.1999.00300.x

Duan C, Fukuda T, Isaji T, Qi F, Yang J, Wang Y, Takahashi S, Gu J (2020) Deficiency of core fucosylation activates cellular signaling dependent on FLT3 expression in a Ba/F3 cell system. FASEB J 34(2):3239–3252. https://doi.org/10.1096/fj.201902313RR

Eichler J (2019) Protein glycosylation. Curr Biol 29(7):R229–r231. https://doi.org/10.1016/j.cub.2019.01.003

Ferreira JA, Peixoto A, Neves M, Gaiteiro C, Reis CA, Assaraf YG, Santos LL (2016) Mechanisms of cisplatin resistance and targeting of cancer stem cells: adding glycosylation to the equation. Drug Resist Updat 24:34–54. https://doi.org/10.1016/j.drup.2015.11.003

Fukuda T, Hashimoto H, Okayasu N, Kameyama A, Onogi H, Nakagawasai O, Nakazawa T, Kurosawa T, Hao Y, Isaji T, Tadano T, Narimatsu H, Taniguchi N, Gu J (2011) Alpha1,6-fucosyltransferase-deficient mice exhibit multiple behavioral abnormalities associated with a schizophrenia-like phenotype: importance of the balance between the dopamine and serotonin systems. J Biol Chem 286(21):18434–18443. https://doi.org/10.1074/jbc.M110.172536

Fuster MM, Esko JD (2005) The sweet and sour of cancer: glycans as novel therapeutic targets. Nat Rev Cancer 5(7):526–542. https://doi.org/10.1038/nrc1649

Geng F, Shi BZ, Yuan YF, Wu XZ (2004) The expression of core fucosylated E-cadherin in cancer cells and lung cancer patients: prognostic implications. Cell Res 14(5):423–433. https://doi.org/10.1038/sj.cr.7290243

Giordano G, Febbraro A, Tomaselli E, Sarnicola ML, Parcesepe P, Parente D, Forte N, Fabozzi A, Remo A, Bonetti A, Manfrin E, Ghasemi S, Ceccarelli M, Cerulo L, Bazzoni F, Pancione M (2015) Cancer-related CD15/FUT4 overexpression decreases benefit to agents targeting EGFR or VEGF acting as a novel RAF-MEK-ERK kinase downstream regulator in metastatic colorectal cancer. J Exp Clin Cancer Res 34:108. https://doi.org/10.1186/s13046-015-0225-7

Gottesman MM (2002) Mechanisms of cancer drug resistance. Annu Rev Med 53:615–627. https://doi.org/10.1146/annurev.med.53.082901.103929

Gu W, Fukuda T, Isaji T, Hashimoto H, Wang Y, Gu J (2013) α1,6-fucosylation regulates neurite formation via the activin/phospho-Smad2 pathway in PC12 cells: the implicated dual effects of Fut8 for TGF-β/activin-mediated signaling. FASEB J 27(10):3947–3958. https://doi.org/10.1096/fj.12-225805

Gu W, Fukuda T, Isaji T, Hang Q, Lee HH, Sakai S, Morise J, Mitoma J, Higashi H, Taniguchi N, Yawo H, Oka S, Gu J (2015) Loss of α1,6-fucosyltransferase decreases hippocampal long term potentiation: implications for core fucosylation in the regulation of AMPA receptor

heteromerization and cellular signaling. J Biol Chem 290(28):17566–17575. https://doi.org/10.1074/jbc.M114.579938
Hakomori S (2002) Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci U S A 99(16):10231–10233. https://doi.org/10.1073/pnas.172380699
Han Y, Liu D, Li L (2020) PD-1/PD-L1 pathway: current researches in cancer. Am J Cancer Res 10(3):727–742
Herrera H, Dilday T, Uber A, Scott D, Zambrano JN, Wang M, Angel PM, Mehta AS, Drake RR, Hill EG, Yeh ES (2019) Core-fucosylated tetra-antennary N-glycan containing a single N-acetyllactosamine branch is associated with poor survival outcome in breast cancer. Int J Mol Sci 20(10):2528. https://doi.org/10.3390/ijms20102528
Hirschberg CB (2001) Golgi nucleotide sugar transport and leukocyte adhesion deficiency II. J Clin Invest 108(1):3–6. https://doi.org/10.1172/jci13480
Höti N, Lih TS, Pan J, Zhou Y, Yang G, Deng A, Chen L, Dong M, Yang RB, Tu CF, Haffner MC, Kay Li Q, Zhang H (2020) A comprehensive analysis of FUT8 overexpressing prostate cancer cells reveals the role of EGFR in castration resistance. Cancers (Basel) 12(2):468. https://doi.org/10.3390/cancers12020468
Huang Y, Zhang HL, Li ZL, Du T, Chen YH, Wang Y, Ni HH, Zhang KM, Mai J, Hu BX, Huang JH, Zhou LH, Yang D, Peng XD, Feng GK, Tang J, Zhu XF, Deng R (2021) FUT8-mediated aberrant N-glycosylation of B7H3 suppresses the immune response in triple-negative breast cancer. Nat Commun 12(1):2672. https://doi.org/10.1038/s41467-021-22618-x
Hutchinson WL, Du MQ, Johnson PJ, Williams R (1991) Fucosyltransferases: differential plasma and tissue alterations in hepatocellular carcinoma and cirrhosis. Hepatology 13(4):683–688
Iwamori M, Tanaka K, Kubushiro K, Lin B, Kiguchi K, Ishiwata I, Tsukazaki K, Nozawa S (2005) Alterations in the glycolipid composition and cellular properties of ovarian carcinoma-derived RMG-1 cells on transfection of the alpha1,2-fucosyltransferase gene. Cancer Sci 96(1):26–30. https://doi.org/10.1111/j.1349-7006.2005.00005.x
Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ (2008) Cancer statistics. CA Cancer J Clin 58(2):71–96. https://doi.org/10.3322/ca.2007.0010
Jordan CT, Guzman ML, Noble M (2006) Cancer stem cells. N Engl J Med 355(12):1253–1261. https://doi.org/10.1056/NEJMra061808
Kamisawa T, Wood LD, Itoi T, Takaori K (2016) Pancreatic cancer. Lancet 388(10039):73–85. https://doi.org/10.1016/s0140-6736(16)00141-0
Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26:677–704. https://doi.org/10.1146/annurev.immunol.26.021607.090331
Kizuka Y, Taniguchi N (2016) Enzymes for N-glycan branching and their genetic and nongenetic regulation in cancer. Biomol Ther 6(2):25. https://doi.org/10.3390/biom6020025
Kleeff J, Korc M, Apte M, La Vecchia C, Johnson CD, Biankin AV, Neale RE, Tempero M, Tuveson DA, Hruban RH, Neoptolemos JP (2016) Pancreatic cancer. Nat Rev Dis Primers 2:16022. https://doi.org/10.1038/nrdp.2016.22
Kritschil R, Zhang Z, Lei C, Zhong J, Dong Q, Lee J, Conover CA, Sowa G, Vallejo AN, Vo N (2020) Effects of suppressing bioavailability of insulin-like growth factor on age-associated intervertebral disc degeneration. JOR Spine 3(4):e1112. https://doi.org/10.1002/jsp2.1112
Krogsgaard M, Li QJ, Sumen C, Huppa JB, Huse M, Davis MM (2005) Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. Nature 434(7030):238–243. https://doi.org/10.1038/nature03391
Kumar S, Das S, Rachagani S, Kaur S, Joshi S, Johansson SL, Ponnusamy MP, Jain M, Batra SK (2015) NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 34(37):4879–4889. https://doi.org/10.1038/onc.2014.409
Lang R, Leinenbach A, Karl J, Swiatek-de Lange M, Kobold U, Vogeser M (2018) An endoglycosidase-assisted LC-MS/MS-based strategy for the analysis of site-specific

core-fucosylation of low-concentrated glycoproteins in human serum using prostate-specific antigen (PSA) as example. Clin Chim Acta 480:1–8. https://doi.org/10.1016/j.cca.2018.01.040
Li D, Xie K, Wolff R, Abbruzzese JL (2004) Pancreatic cancer. Lancet 363(9414):1049–1057. https://doi.org/10.1016/s0140-6736(04)15841-8
Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM (2007) Identification of pancreatic cancer stem cells. Cancer Res 67(3):1030–1037. https://doi.org/10.1158/0008-5472.can-06-2030
Li S, Liu XY, Pan Q, Wu J, Liu ZH, Wang Y, Liu M, Zhang XL (2019) Hepatitis C virus-induced FUT8 causes 5-FU drug resistance in human hepatoma Huh7.5.1 cells. Viruses 11(4):378. https://doi.org/10.3390/v11040378
Li F, Zhao S, Cui Y, Guo T, Qiang J, Xie Q, Yu W, Guo W, Deng W, Gu C, Wu T (2020) α1,6-fucosyltransferase (FUT8) regulates the cancer-promoting capacity of cancer-associated fibroblasts (CAFs) by modifying EGFR core fucosylation (CF) in non-small cell lung cancer (NSCLC). Am J Cancer Res 10(3):816–837
Liang W, Mao S, Sun S, Li M, Li Z, Yu R, Ma T, Gu J, Zhang J, Taniguchi N, Li W (2018) Core fucosylation of the T cell receptor is required for T cell activation. Front Immunol 9:78. https://doi.org/10.3389/fimmu.2018.00078
Liang C, Fukuda T, Isaji T, Duan C, Song W, Wang Y, Gu J (2021) α1,6-Fucosyltransferase contributes to cell migration and proliferation as well as to cancer stemness features in pancreatic carcinoma. Biochim Biophys Acta Gen Sub 1865(6):129870. https://doi.org/10.1016/j.bbagen.2021.129870
Liao C, An J, Yi S, Tan Z, Wang H, Li H, Guan X, Liu J, Wang Q (2021) FUT8 and protein core fucosylation in tumours: from diagnosis to treatment. J Cancer 12(13):4109–4120. https://doi.org/10.7150/jca.58268
Lin H, Wang D, Wu T, Dong C, Shen N, Sun Y, Sun Y, Xie H, Wang N, Shan L (2011) Blocking core fucosylation of TGF-β1 receptors downregulates their functions and attenuates the epithelial-mesenchymal transition of renal tubular cells. Am J Physiol Renal Physiol 300(4):F1017–F1025. https://doi.org/10.1152/ajprenal.00426.2010
Liu YC, Yen HY, Chen CY, Chen CH, Cheng PF, Juan YH, Chen CH, Khoo KH, Yu CJ, Yang PC, Hsu TL, Wong CH (2011) Sialylation and fucosylation of epidermal growth factor receptor suppress its dimerization and activation in lung cancer cells. Proc Natl Acad Sci U S A 108(28):11332–11337. https://doi.org/10.1073/pnas.1107385108
Liu D, Gao Z, Yue L (2019) Fucosyltransferase 8 deficiency suppresses breast cancer cell migration by interference of the FAK/integrin pathway. Cancer Biomark 25(4):303–311. https://doi.org/10.3233/cbm-190209
Lu X, Zhang D, Shoji H, Duan C, Zhang G, Isaji T, Wang Y, Fukuda T, Gu J (2019) Deficiency of α1,6-fucosyltransferase promotes neuroinflammation by increasing the sensitivity of glial cells to inflammatory mediators. Biochim Biophys Acta Gen Subj 1863(3):598–608. https://doi.org/10.1016/j.bbagen.2018.12.008
Lu HH, Lin SY, Weng RR, Juan YH, Chen YW, Hou HH, Hung ZC, Oswita GA, Huang YJ, Guu SY, Khoo KH, Shih JY, Yu CJ, Tsai HC (2020) Fucosyltransferase 4 shapes oncogenic glycoproteome to drive metastasis of lung adenocarcinoma. EBioMedicine 57:102846. https://doi.org/10.1016/j.ebiom.2020.102846
Lv X, Song J, Xue K, Li Z, Li M, Zahid D, Cao H, Wang L, Song W, Ma T, Gu J, Li W (2019) Core fucosylation of copper transporter 1 plays a crucial role in cisplatin-resistance of epithelial ovarian cancer by regulating drug uptake. Mol Carcinog 58(5):794–807. https://doi.org/10.1002/mc.22971
Ma M, Guo D, Tan Z, Du J, Guan F, Li X (2021) Fucosyltransferase 8 regulation and breast cancer suppression by transcription factor activator protein 2γ. Cancer Sci 112(8):3190–3204. https://doi.org/10.1111/cas.14987
Maeda M, Johnson KR, Wheelock MJ (2005) Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. J Cell Sci 118(Pt 5):873–887. https://doi.org/10.1242/jcs.01634

Magalhães A, Duarte HO, Reis CA (2017) Aberrant glycosylation in cancer: a novel molecular mechanism controlling metastasis. Cancer Cell 31(6):733–735. https://doi.org/10.1016/j.ccell.2017.05.012

Malka D, Hammel P, Maire F, Rufat P, Madeira I, Pessione F, Lévy P, Ruszniewski P (2002) Risk of pancreatic adenocarcinoma in chronic pancreatitis. Gut 51(6):849–852. https://doi.org/10.1136/gut.51.6.849

Mallard BW, Tiralongo J (2017) Cancer stem cell marker glycosylation: nature, function and significance. Glycoconj J 34(4):441–452. https://doi.org/10.1007/s10719-017-9780-9

Matsumoto K, Yokote H, Arao T, Maegawa M, Tanaka K, Fujita Y, Shimizu C, Hanafusa T, Fujiwara Y, Nishio K (2008) N-glycan fucosylation of epidermal growth factor receptor modulates receptor activity and sensitivity to epidermal growth factor receptor tyrosine kinase inhibitor. Cancer Sci 99(8):1611–1617. https://doi.org/10.1111/j.1349-7006.2008.00847.x

Miyoshi E, Uozumi N, Noda K, Hayashi N, Hori M, Taniguchi N (1997) Expression of alpha1-6 fucosyltransferase in rat tissues and human cancer cell lines. Int J Cancer 72(6):1117–1121. https://doi.org/10.1002/(sici)1097-0215(19970917)72:6<1117::aid-ijc29>3.0.co;2-#

Miyoshi E, Moriwaki K, Nakagawa T (2008) Biological function of fucosylation in cancer biology. J Biochem 143(6):725–729. https://doi.org/10.1093/jb/mvn011

Moreira IB, Pinto F, Gomes C, Campos D, Reis CA (2020) Impact of truncated O-glycans in gastric-cancer-associated CD44v9 detection. Cell 9(2):264. https://doi.org/10.3390/cells9020264

Muraki T, Jang K-T, Reid MD, Pehlivanoglu B, Memis B, Basturk O, Mittal P, Kooby D, Maithel SK, Sarmiento JM, Christians K, Tsai S, Evans D, Adsay V (2021) Pancreatic ductal adenocarcinomas associated with intraductal papillary mucinous neoplasms (IPMNs) versus pseudo-IPMNs: relative frequency, clinicopathologic characteristics and differential diagnosis. Mod Pathol 35(1):96–105. https://doi.org/10.1038/s41379-021-00902-x

Nakagawa T, Uozumi N, Nakano M, Mizuno-Horikawa Y, Okuyama N, Taguchi T, Gu J, Kondo A, Taniguchi N, Miyoshi E (2006) Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. J Biol Chem 281(40):29797–29806. https://doi.org/10.1074/jbc.M605697200

Ng BG, Xu G, Chandy N, Steyermark J, Shinde DN, Radtke K, Raymond K, Lebrilla CB, AlAsmari A, Suchy SF, Powis Z, Faqeih EA, Berry SA, Kronn DF, Freeze HH (2018) Biallelic mutations in FUT8 cause a congenital disorder of glycosylation with defective fucosylation. Am J Hum Genet 102(1):188–195. https://doi.org/10.1016/j.ajhg.2017.12.009

Ng BG, Dastsooz H, Silawi M, Habibzadeh P, Jahan SB, Fard MAF, Halliday BJ, Raymond K, Ruzhnikov MRZ, Tabatabaei Z, Taghipour-Sheshdeh A, Brimble E, Robertson SP, Faghihi MA, Freeze HH (2020) Expanding the molecular and clinical phenotypes of FUT8-CDG. J Inherit Metab Dis 43(4):871–879. https://doi.org/10.1002/jimd.12221

Niittymäki J, Mattila P, Roos C, Huopaniemi L, Sjöblom S, Renkonen R (2004) Cloning and expression of murine enzymes involved in the salvage pathway of GDP-L-fucose. Eur J Biochem 271(1):78–86. https://doi.org/10.1046/j.1432-1033.2003.03904.x

Niittymäki J, Mattila P, Renkonen R (2006) Differential gene expression of GDP-L-fucose-synthesizing enzymes, GDP-fucose transporter and fucosyltransferase VII. APMIS 114(7-8):539–548. https://doi.org/10.1111/j.1600-0463.2006.apm_461.x

Norton PA, Mehta AS (2019) Expression of genes that control core fucosylation in hepatocellular carcinoma: systematic review. World J Gastroenterol 25(23):2947–2960. https://doi.org/10.3748/wjg.v25.i23.2947

Ohtsubo K, Marth JD (2006) Glycosylation in cellular mechanisms of health and disease. Cell 126(5):855–867. https://doi.org/10.1016/j.cell.2006.08.019

Okada M, Chikuma S, Kondo T, Hibino S, Machiyama H, Yokosuka T, Nakano M, Yoshimura A (2017) Blockage of core fucosylation reduces cell-surface expression of PD-1 and promotes anti-tumor immune responses of T cells. Cell Rep 20(5):1017–1028. https://doi.org/10.1016/j.celrep.2017.07.027

Osumi D, Takahashi M, Miyoshi E, Yokoe S, Lee SH, Noda K, Nakamori S, Gu J, Ikeda Y, Kuroki Y, Sengoku K, Ishikawa M, Taniguchi N (2009) Core fucosylation of E-cadherin enhances cell-cell adhesion in human colon carcinoma WiDr cells. Cancer Sci 100(5): 888–895. https://doi.org/10.1111/j.1349-7006.2009.01125.x

Park S, Lim JM, Chun JN, Lee S, Kim TM, Kim DW, Kim SY, Bae DJ, Bae SM, So I, Kim HG, Choi JY, Jeon JH (2020) Altered expression of fucosylation pathway genes is associated with poor prognosis and tumor metastasis in non-small cell lung cancer. Int J Oncol 56(2):559–567. https://doi.org/10.3892/ijo.2019.4953

Pinho SS, Reis CA (2015) Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer 15(9):540–555. https://doi.org/10.1038/nrc3982

Puglielli L, Hirschberg CB (1999) Reconstitution, identification, and purification of the rat liver golgi membrane GDP-fucose transporter. J Biol Chem 274(50):35596–35600. https://doi.org/10.1074/jbc.274.50.35596

Sabbah DA, Hajjo R, Sweidan K (2020) Review on epidermal growth factor receptor (EGFR) structure, signaling pathways, interactions, and recent updates of EGFR inhibitors. Curr Top Med Chem 20(10):815–834. https://doi.org/10.2174/1568026620666200303123102

Sadeghzadeh Z, Khosravi A, Jazi MS, Asadi J (2020) Upregulation of fucosyltransferase 3, 8 and protein O-fucosyltransferase 1, 2 genes in esophageal cancer stem-like cells (CSLCs). Glycoconj J 37(3):319–327. https://doi.org/10.1007/s10719-020-09917-z

Shao K, Chen ZY, Gautam S, Deng NH, Zhou Y, Wu XZ (2016) Posttranslational modification of E-cadherin by core fucosylation regulates Src activation and induces epithelial-mesenchymal transition-like process in lung cancer cells. Glycobiology 26(2):142–154. https://doi.org/10.1093/glycob/cwv089

Shimizu H, Ochiai K, Ikenaka K, Mikoshiba K, Hase S (1993) Structures of N-linked sugar chains expressed mainly in mouse brain. J Biochem 114(3):334–338. https://doi.org/10.1093/oxfordjournals.jbchem.a124177

Spiekermann K, Bagrintseva K, Schwab R, Schmieja K, Hiddemann W (2003) Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. Clin Cancer Res 9(6):2140–2150

Suárez-Fueyo A, Bradley SJ, Tsokos GC (2016) T cells in systemic lupus erythematosus. Curr Opin Immunol 43:32–38. https://doi.org/10.1016/j.coi.2016.09.001

Sun Y, Li Z, Liang W, Zhang Y, Song W, Song J, Xue K, Wang M, Sun W, Gu J, Li M, Li W (2020) A novel immunochromatographic strips assay for rapid and simple detection of systemic lupus erythematosus. Sci Rep 10(1):14178. https://doi.org/10.1038/s41598-020-71137-0

Sun W, Jing X, Yang X, Huang H, Luo Q, Xia S, Wang P, Wang N, Zhang Q, Guo J, Xu Z (2021) Regulation of the IGF1 signaling pathway is involved in idiopathic pulmonary fibrosis induced by alveolar epithelial cell senescence and core fucosylation. Aging (Albany NY) 13(14): 18852–18869. https://doi.org/10.18632/aging.203335

Swindall AF, Londoño-Joshi AI, Schultz MJ, Fineberg N, Buchsbaum DJ, Bellis SL (2013) ST6Gal-I protein expression is upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines. Cancer Res 73(7):2368–2378. https://doi.org/10.1158/0008-5472.can-12-3424

Tada K, Ohta M, Hidano S, Watanabe K, Hirashita T, Oshima Y, Fujnaga A, Nakanuma H, Masuda T, Endo Y, Takeuchi Y, Iwashita Y, Kobayashi T, Inomata M (2020) Fucosyltransferase 8 plays a crucial role in the invasion and metastasis of pancreatic ductal adenocarcinoma. Surg Today 50(7):767–777. https://doi.org/10.1007/s00595-019-01953-z

Talamini G, Falconi M, Bassi C, Sartori N, Salvia R, Caldiron E, Frulloni L, Di Francesco V, Vaona B, Bovo P, Vantini I, Pederzoli P, Cavallini G (1999) Incidence of cancer in the course of chronic pancreatitis. Am J Gastroenterol 94(5):1253–1260. https://doi.org/10.1111/j.1572-0241.1999.01075.x

Taniguchi N, Ohkawa Y, Maeda K, Harada Y, Nagae M, Kizuka Y, Ihara H, Ikeda Y (2021) True significance of N-acetylglucosaminyltransferases GnT-III, V and α1,6 fucosyltransferase in

epithelial-mesenchymal transition and cancer. Mol Asp Med 79:100905. https://doi.org/10.1016/j.mam.2020.100905

Terao N, Takamatsu S, Minehira T, Sobajima T, Nakayama K, Kamada Y, Miyoshi E (2015) Fucosylation is a common glycosylation type in pancreatic cancer stem cell-like phenotypes. World J Gastroenterol 21(13):3876–3887. https://doi.org/10.3748/wjg.v21.i13.3876

Thiery JP, Sleeman JP (2006) Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7(2):131–142. https://doi.org/10.1038/nrm1835

Tonetti M, Sturla L, Bisso A, Benatti U, De Flora A (1996) Synthesis of GDP-L-fucose by the human FX protein. J Biol Chem 271(44):27274–27279. https://doi.org/10.1074/jbc.271.44.27274

Tu Z, Lin YN, Lin CH (2013) Development of fucosyltransferase and fucosidase inhibitors. Chem Soc Rev 42(10):4459–4475. https://doi.org/10.1039/c3cs60056d

Tu CF, Wu MY, Lin YC, Kannagi R, Yang RB (2017) FUT8 promotes breast cancer cell invasiveness by remodeling TGF-β receptor core fucosylation. Breast Cancer Res 19(1):111. https://doi.org/10.1186/s13058-017-0904-8

Ueda M, Kamada Y, Takamatsu S, Shimomura M, Maekawa T, Sobajima T, Fujii H, Nakayama K, Nishino K, Yamada M, Kobayashi Y, Kumada T, Ito T, Eguchi H, Nagano H, Miyoshi E (2016) Specific increase in serum core-fucosylated haptoglobin in patients with chronic pancreatitis. Pancreatology 16(2):238–243. https://doi.org/10.1016/j.pan.2016.01.004

Uozumi N, Yanagidani S, Miyoshi E, Ihara Y, Sakuma T, Gao CX, Teshima T, Fujii S, Shiba T, Taniguchi N (1996) Purification and cDNA cloning of porcine brain GDP-L-Fuc:N-acetyl-beta-D-glucosaminide alpha1—>6fucosyltransferase. J Biol Chem 271(44):27810–27817. https://doi.org/10.1074/jbc.271.44.27810

Vajaria BN, Patel KR, Begum R, Patel PS (2016) Sialylation: an avenue to target cancer cells. Pathol Oncol Res 22(3):443–447. https://doi.org/10.1007/s12253-015-0033-6

Vanhooren V, Dewaele S, Kuro OM, Taniguchi N, Dollé L, van Grunsven LA, Makrantonaki E, Zouboulis CC, Chen CC, Libert C (2011) Alteration in N-glycomics during mouse aging: a role for FUT8. Aging Cell 10(6):1056–1066. https://doi.org/10.1111/j.1474-9726.2011.00749.x

Very N, Lefebvre T, El Yazidi-Belkoura I (2018) Drug resistance related to aberrant glycosylation in colorectal cancer. Oncotarget 9(1):1380–1402. https://doi.org/10.18632/oncotarget.22377

Vincent AM, Feldman EL (2002) Control of cell survival by IGF signaling pathways. Growth Hormon IGF Res 12(4):193–197. https://doi.org/10.1016/s1096-6374(02)00017-5

Wang X, Inoue S, Gu J, Miyoshi E, Noda K, Li W, Mizuno-Horikawa Y, Nakano M, Asahi M, Takahashi M, Uozumi N, Ihara S, Lee SH, Ikeda Y, Yamaguchi Y, Aze Y, Tomiyama Y, Fujii J, Suzuki K, Kondo A, Shapiro SD, Lopez-Otin C, Kuwaki T, Okabe M, Honke K, Taniguchi N (2005) Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. Proc Natl Acad Sci U S A 102(44):15791–15796. https://doi.org/10.1073/pnas.0507375102

Wang X, Gu J, Miyoshi E, Honke K, Taniguchi N (2006) Phenotype changes of Fut8 knockout mouse: core fucosylation is crucial for the function of growth factor receptor(s). Methods Enzymol 417:11–22. https://doi.org/10.1016/s0076-6879(06)17002-0

Wang Y, Fukuda T, Isaji T, Lu J, Gu W, Lee HH, Ohkubo Y, Kamada Y, Taniguchi N, Miyoshi E, Gu J (2015a) Loss of α1,6-fucosyltransferase suppressed liver regeneration: implication of core fucose in the regulation of growth factor receptor-mediated cellular signaling. Sci Rep 5:8264. https://doi.org/10.1038/srep08264

Wang Y, Fukuda T, Isaji T, Lu J, Im S, Hang Q, Gu W, Hou S, Ohtsubo K, Gu J (2015b) Loss of α1,6-fucosyltransferase inhibits chemical-induced hepatocellular carcinoma and tumorigenesis by down-regulating several cell signaling pathways. FASEB J 29(8):3217–3227. https://doi.org/10.1096/fj.15-270710

Watanabe K, Ohta M, Yada K, Komori Y, Iwashita Y, Kashima K, Inomata M (2016) Fucosylation is associated with the malignant transformation of intraductal papillary mucinous neoplasms: a lectin microarray-based study. Surg Today 46(10):1217–1223. https://doi.org/10.1007/s00595-015-1299-8

Yang HF, Yu M, Jin HD, Yao JQ, Lu ZL, Yabasin IB, Yan Q, Wen QP (2017) Fentanyl promotes breast cancer cell stemness and epithelial-mesenchymal transition by upregulating α1, 6-fucosylation via Wnt/β-catenin signaling pathway. Front Physiol 8:510. https://doi.org/10.3389/fphys.2017.00510

Yu M, Cui X, Wang H, Liu J, Qin H, Liu S, Yan Q (2019) FUT8 drives the proliferation and invasion of trophoblastic cells via IGF-1/IGF-1R signaling pathway. Placenta 75:45–53. https://doi.org/10.1016/j.placenta.2018.11.005

Zhang Z, Zhao Y, Jiang L, Miao X, Zhou H, Jia L (2012) Glycomic alterations are associated with multidrug resistance in human leukemia. Int J Biochem Cell Biol 44(8):1244–1253. https://doi.org/10.1016/j.biocel.2012.04.026

Zhang C, Wu Q, Huang H, Chen X, Huang T, Li W, Zhang J, Liu Y (2020a) Caveolin-1 upregulates Fut8 expression by activating the Wnt/β-catenin pathway to enhance HCC cell proliferative and invasive ability. Cell Biol Int 44(11):2202–2212. https://doi.org/10.1002/cbin.11426

Zhang N, Li M, Xu X, Zhang Y, Liu Y, Zhao M, Li P, Chen J, Fukuda T, Gu J, Jin X, Li W (2020b) Loss of core fucosylation enhances the anticancer activity of cytotoxic T lymphocytes by increasing PD-1 degradation. Eur J Immunol 50(11):1820–1833. https://doi.org/10.1002/eji.202048543

Zhao Y, Itoh S, Wang X, Isaji T, Miyoshi E, Kariya Y, Miyazaki K, Kawasaki N, Taniguchi N, Gu J (2006) Deletion of core fucosylation on alpha3beta1 integrin down-regulates its functions. J Biol Chem 281(50):38343–38350. https://doi.org/10.1074/jbc.M608764200

Zhao YY, Takahashi M, Gu JG, Miyoshi E, Matsumoto A, Kitazume S, Taniguchi N (2008) Functional roles of N-glycans in cell signaling and cell adhesion in cancer. Cancer Sci 99(7):1304–1310. https://doi.org/10.1111/j.1349-7006.2008.00839.x

Zhou Y, Fukuda T, Hang Q, Hou S, Isaji T, Kameyama A, Gu J (2017) Inhibition of fucosylation by 2-fluorofucose suppresses human liver cancer HepG2 cell proliferation and migration as well as tumor formation. Sci Rep 7(1):11563. https://doi.org/10.1038/s41598-017-11911-9

Effects of Bullfrog Sialic Acid–Binding Lectin in Cancer Cells

Takeo Tatsuta and Masahiro Hosono

Abstract A cancer cell agglutinin isolated from bullfrog (*Rana catesbeiana*) eggs was named *R. catesbeiana* sialic acid–binding lectin (cSBL), because its agglutinative activity was inhibited by sialic acid–containing glycoconjugates. The lectin was subsequently found to belong to the vertebrate-secreted ribonuclease family, and cSBL exhibits ribonuclease activity as well as remarkable antitumor effects. Recent studies have shown that cSBL exerts antitumor effects in vitro and in vivo under conditions where cSBL cancer-selective apoptosis-inducing effects and no undesired side effects are observed. Furthermore, cSBL exhibited a preferred synergistic effect with other drugs. Here, we describe the unique antitumor signal revealed in the latest cSBL studies and the possibility of developing a cancer treatment strategy using cSBL.

1 Introduction

Advances in modern medicine have reduced the mortality rate of infectious diseases such as HIV and tuberculosis, as well as improving general health worldwide. As of 2021, the development of newly approved RNA vaccines has shown remarkable efficacy in the prevention of new infectious diseases, and modern medical care is still evolving day by day. However, global health is still threatened by diseases such as cancer, heart disease, and cerebrovascular disease (WHO 2020). Despite the remarkable advances in cancer chemotherapy in recent years, for example, immune checkpoint inhibitors (Robert 2020), cancer is still the leading cause of death in many countries. It is estimated that as many as ten million cancer-related deaths occurred in 2020 (Sung et al. 2021). Thus, therapeutic advances in cancer prevention, early detection, and treatment will improve survival and quality of life for many people worldwide.

T. Tatsuta · M. Hosono (✉)
Division of Cell Recognition, Institute of Molecular Biomembrane and Glycobiology, Tohoku Medical and Pharmaceutical University, Sendai, Japan
e-mail: mhosono@tohoku-mpu.ac.jp; t-takeo@tohoku-mpu.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_7

Sugar chains (glycans) are termed third life chains, coming after nucleic acids and proteins to play essential roles in living bodies via their various biochemical interactions. Such as binding with functional molecules (Reily et al. 2019). The surfaces of cells are covered with innumerable glycans, and the activities of such glycoproteins or glycolipids can be controlled via glycosylation. Cell-surface glycans can significantly alter their sequence structure during cell differentiation, maturation, and activation, and function as discriminating molecules (Marth and Grewal 2008). For example, differentiated cells recognize glycan structures on other cells and cooperate with each other (Varki 2017). Microorganisms such as bacteria and viruses use differences in surface glycans to bind to and infect specific cells (Van Breedam et al. 2014). Furthermore, surface glycan structure also changes significantly in cancer cells (Costa et al. 2020). Changes in glycan structure have been shown to alter the location of cell surface receptors and their susceptibility to ligands, affecting cell proliferation and invasion (Rodrigues et al. 2018). It also affects the cancer-surveillance activity of immune cells (Mereiter et al. 2019). Therefore, glycans are very useful for identifying cells and are thus expected to be utilizable biomarkers for identifying cancer cells. In fact, many biomarkers currently in clinical use recognize glycans (Tikhonov et al. 2020). Hence, glycans are currently attracting much attention, but research on them is somewhat difficult. The structures of sugar chains are complicated and liable to change due to their sensitivity to environmental factors, and can even be decomposed. Furthermore, the structure cannot be directly amplified unlike nucleic acids. Therefore, it is difficult to recognize, analyze, and quantify certain disease-related glycans, and development of tools for recognizing glycans is required for their use in diagnosis and treatment.

Lectins are useful tools for targeting difficult-to-handle glycans. Our laboratory has focused on the identification and analysis of naturally derived lectins for many years, and has purified and reported some lectins from fish and amphibians. Specifically, sialic acid–binding lectin purified from bullfrog (*Rana catesbeiana*) eggs (cSBL) has potential as a cancer treatment due to its antitumor activity via a unique RNA-degradation mechanism. In this section, we describe in detail the functions of cSBL that have been clarified thus far, including background of the discovery of this lectin and the possibility of its application in cancer treatment.

2 Discovery of cSBL as a Lectin

2.1 Lectins

Lectins are proteins that bind to specific glycan structures and are universally present in species from animals and plants to microorganisms, and have greatly contributed to glycan analysis research. Historically, the first lectin identified is considered to be ricin from castor tree (*Ricinus communis*) seeds by Stillmark in 1888. Ricin is a plant-derived protein capable of agglutinating red blood cells, and the name hemagglutinins or phytoagglutinins was used at the time (Sharon and Lis 2004). Sumner

and Howell reported that concanavalin A, a lectin isolated from jack bean (*Canavalia ensiformis*), agglutinates cells such as erythrocytes, and this agglutination was inhibited by sucrose, the first demonstration of the sugar specificity of lectins (Sumner and Howell 1936). It was predicted that agglutination was caused by the reaction of concanavalin A with carbohydrates on the cell surface. Since the 1940s, there have been many discoveries of blood type-specific hemagglutinins (Mäkelä 1957; Sharon and Lis 2004). These findings demonstrated the ability of plant agglutinins to distinguish between erythrocytes of different blood types, and thus, Boyd and Shapleigh proposed the name "lectins," from the Latin *legere*, meaning to pick out or choose (Boyd and Shapleigh 1954). Subsequently, the term "lectin" was generalized by Sharon et al. to all sugar-specific agglutinins of nonimmune origin, regardless of source and blood group specificity (Sharon and Lis 1972). Goldstein et al. (Goldstein et al. 1980) further defined lectins in detail: "A lectin is a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates. Lectins bear at least two sugar-binding sites, and agglutinate animal and/or plant cells. The specificity of a lectin is usually defined in terms of the monosaccharide(s) or oligosaccharides that inhibit lectin-induced agglutination reactions." Generally, lectin is used as a general term for substances that have specific binding activity for sugars among proteins or glycoproteins present in living organisms. With the subsequent research into numerous lectins, numerous pivotal biological functions of lectins have been revealed, such as immunomodulating effects (Clement et al. 2010), modulation of the intestinal transport system (Yamamoto et al. 2013), and antimicrobial and insecticidal activities (Dias et al. 2015), among others. Furthermore, many lectins are currently used as important biological and diagnostic tools. Recently, a lectin array technology that acquires sugar chain profiles of various biological samples via their reactivity with multiple lectins has been developed (Hirabayashi et al. 2013), and a new therapeutic approach that uses lectins to target specific cells using lectin-drug conjugates (LDC) has also been reported (Shimomura et al. 2018). Thus, novel research and development using lectins has drawn attention in a wide range of fields, from technological development to diagnosis and medical treatment.

2.2 *Sialic Acid–Binding Lectin from Rana Catesbeiana Oocytes*

In our laboratory, research on frog egg agglutinin was started by Kawauchi et al. in the mid-1970s (Kawauchi et al. 1975). Initially, crude egg fractions were used, and samples derived from Japanese brown frog (*Rana japonica*) and dark-spotted frog (*Rana nigromaculata*) eggs showed agglutinative activity with rat ascites cancer cells (AH109A) (Yokota et al. 1975). Later, it was found that bullfrog (*R. catesbeiana*) egg extract, which belongs to the same genus *Rana*, contains two lectins that show different agglutinative activities, one with human blood group A

erythrocytes, and the other with mouse Ehrlich ascites carcinoma cells and AH109A. These two lectins were separated by column chromatography, and their binding specificity, agglutinative activity, and physical and chemical properties were studied. One of the lectins showed preferential agglutination of cancer cells and displayed specific binding to sialyl glycoproteins (Sakakibara et al. 1977), and was consequently designated sialic acid–binding lectin, cSBL. In 1987, Nitta et al. isolated cSBL and characterized its lectin activity (Nitta et al. 1987). cSBL preferentially agglutinates a large variety of tumor cells, but not normal red blood cells, lymphocytes, or fibroblasts. This phenomenon correlates with a higher binding activity of cSBL with tumor cells. The binding reactivity of cSBL was tested in 20 human and animal cancer cells and 10 normal cells, and it was observed that the majority of cancer cells showed high reactivity to cSBL, while normal cells (fibroblasts, lymphocytes, and erythrocytes) exhibited low reactivity (10–50% reactivity compared to S-180 mouse ascites cells used as a standard). Tumor cell agglutination induced by cSBL was inhibited by mucin, fetuin, and keratan sulfate, but not by less-sialylated glycoproteins, such as transferrin. Inhibition by mucin or fetuin was greatly reduced by the desialylation of the glycoproteins with sialidase. Treatment of tumor cells with sialidase reduced cSBL-induced agglutination, and sialidase-dependent reduction of tumor cell agglutination was inhibited by the sialidase inhibitor. However, tumor cell agglutination was not inhibited by chondroitin sulfate or hyaluronic acid. Thus, lectin-dependent tumor cell agglutination was considered to be due to the high density of sialic acid on the cell surface. When the effects of glycosaminoglycans on the agglutinative activity of cSBL were tested, heparin showed strong inhibitory effects, and it was speculated that heparin interfered with the binding of cSBL to cells. Polyamines also inhibit cell agglutination, and it has been proposed that they interact with negatively charged cell surface components, including sialosyl residues (i.e., the lectin-binding region), resulting in inhibition of agglutination. These results suggest that tumor cell agglutination occurs because of the recognition of sialic acid–containing molecules on the cell surface by cSBL. The position of the sialic acid–binding site of cSBL remains unknown, but Irie et al. reported two putative sialic acid–binding sites on the cSBL structure. One is a site near the N-terminus, where sialic acid binds mainly via hydrogen bonds, and the other is a loop consisting of amino acid residues 57–75, where sialic acid binds via hydrophobic interactions. The binding of sialic acid to the loop was thought to be very weak, and the weakly bound sialic acid was considered to be easily washed out by soaking in buffer without sialic acid (Irie et al. 1998). Due to this weak binding to the loop site, and no reports of cSBL-homologous lectins so far, the lectin activity of cSBL remains unclear, especially regarding the structure of the binding target. Nevertheless, the complete amino acid sequence of cSBL was presented by Titani et al. in 1987 (Titani et al. 1987). The 111-residue sequence was determined by peptide sequencing. Initially, this protein was considered to be a new type of lectin, as its sequence was unique and no homology to known protein sequences was found in the database at the time. However, 3 years later, the sequence of a homolog, *Rana japonica* egg lectin (jSBL), was determined, and a homology search with the updated protein sequence data bank revealed that these amino acid sequences were homologous to

those of the pancreatic ribonuclease family (vertebrate-secreted RNase family) (Kamiya et al. 1990). The sequence identities with human angiogenin and pancreatic RNase, members of the vertebrate-secreted RNase family, were 35% and 37%, respectively. Similarities were especially apparent in the four proteins (cSBL, jSBL, human angiogenin, and pancreatic ribonuclease) around His-12, Lys-41, and His-119, the major active site residues conserved in the ribonucleases, and around three of the four disulfide bonds. Thus, research created new insights into cSBL as a ribonuclease.

3 cSBL as an RNase

RNase is a ribonucleic-acid-degrading enzyme that is ubiquitous in all cells, and is a pioneer of enzyme research due to its discovery over 100 years ago and its subsequent thorough study. Enzymatically, it is roughly divided into endo- and exo-types, but there are numerous types present in all species from animals and plants to microorganisms. Since the number of reports on RNases is still increasing, their classification method, including naming, has changed frequently. RNase A, which is reportedly the most studied enzyme in the history of science, is purified from the bovine pancreas. Although a structural family consisting of RNase A and its homologs (more than 100 species) has commonly been called the RNase A superfamily (Beintema et al. 1986, 1997), in recent years, the renaming of this superfamily as the vertebrate-secreted RNase superfamily has been proposed by D'Alessio, because most are secretory proteins produced by vertebrates (D'Alessio 2011). RNases can be classified into the vertebrate-secreted (RNase A), T1, T2, H, L, and P families, among others (Deshpande and Shankar 2002; Fang and Ng 2011). Vertebrate-secreted RNases are relatively small, containing approximately 130 amino acids. They preferentially cleaved pyrimidines at an optimal pH of 7–8. This group of RNases has a characteristic peptide motif (CKXXNTF) in the middle of the sequence and is encoded by a single exon (D'Alessio 2011). They contain two His and one Lys as catalytic active centers and 6–8 cysteines, forming 3–4 disulfide bonds (Cho et al. 2005). Sequence identity varies from 20% to nearly 100%, and their three-dimensional structures are very similar, consisting mainly of three α-helices and four antiparallel β-sheet structures. This structure is also termed kidney-shape, as it is reminiscent of a kidney (Raines 1998).

Immediately after cSBL was found to be a vertebrate-secreted RNase, its RNase activity was investigated (Okabe et al. 1991). The base specificity of cSBL was studied using eight dinucleotide phosphates as substrates and eight nucleotides as inhibitors. The base specificities of the B1 and B2 sites of cSBL were U > C and G > U > A, C, respectively. cSBL was more resistant than RNase A to heat treatment, guanidine-HCl, and pH-induced denaturation; retaining its native conformation up to at least 70 °C at pH 7.5. Agglutination of AH109A cells by cSBL was inhibited by nucleotides, indicating that the agglutination sites are related to the catalytic sites for RNase activity of cSBL. In 1992, Liao et al. purified a

pyrimidine-guanine sequence-specific RNase (termed RC-RNase) from *R. catesbeiana* oocytes, which was found to be identical to cSBL (Liao 1992). Further study clarified that although cSBL lacked a Cys-65–Cys-72 disulfide bond, the locations of the other three disulfide bonds were similar to those of the RNase A family. cSBL was found to exhibit RNase activity and catalytic properties resembling those of bovine RNase A. For example, cSBL hydrolyzed poly-uridylic acid and poly-cytidylic acid and preferred the former (Nitta et al. 1993). Nitta et al. thus proposed the name catalytic lectin (leczyme) as a multifunctional molecule with lectin and enzymatic activity (Nitta et al. 1996). It is also noteworthy that even though members of human vertebrate-secreted RNase were strongly inhibited by the human placental RNase inhibitor, the RNase and tumor cell agglutinative activity of cSBL were not affected by the inhibitor (Nitta et al. 1993). This RNase inhibitor-refractory property has a decisive effect on the antitumor activity of cSBL, described in detail in Sect. 5.

4 cSBL as an Antitumor RNase

4.1 Cytotoxic RNases: Evasion of RNase Inhibitors

To date, many antitumor RNases have been identified in a variety of species, including plants, bacteria, fungi, and animals, across RNase family types. Well-known and representative antitumor RNases mainly belong to the vertebrate-secreted RNase family (D'Alessio 2011). Bovine pancreatic RNase A has been thoroughly studied from all aspects, including physicochemical, enzymatic, biochemical, and molecular biology. Since RNase A exhibits RNA-degrading activity, its antitumor activity was investigated more than half a century ago. Although cytotoxicity was observed at high doses (on the order of mg) (Section et al. 1955; LeDoux 1955), subsequent studies revealed that no significant activity is observed at practically low concentrations (de Lamirande 1961; Roth 1963). The major reason for the restricted antitumor effect of RNase A is the neutralization of its ribonucleolytic activity by the endogenous cytosolic RNase inhibitor.

Human RNase inhibitor is a 450 residues, 49 kDa protein with an isoelectric point of 4.7. It contains leucine-rich repeats, forms strong complexes with mammalian ribonucleases, and plays an important role in controlling the life span of RNA (Vicentini et al. 1990; Kobe and Deisenhofer 1995). It is the major intracellular protein making up approximately 0.1% of the total cytosolic protein and is highly conserved across various mammalian species (Lee and Vallee 1993; D'Alessio and Riordan 1997). RNase inhibitors form a 1:1 noncovalent complex with mammalian vertebrate-secreted RNase family members, and the dissociation constant is of the order of fM, indicating strong affinity (Vicentini et al. 1990; Kobe and Deisenhofer 1996). Therefore, the presence of this inhibitor is inevitably a barrier for RNase-mediated cytotoxicity. Consequently, cytotoxic RNases must satisfy one of the following three conditions: (1) insensitive to the RNase inhibitor (Dickson et al.

2005), (2) present at sufficient levels to saturate the RNase inhibitor thus access RNA (Leich et al. 2007), or (3) binds to other molecules or localizes in organelles where RNase inhibitor is not present, such as the nucleus, so binding to RNase inhibitor is reduced (Gaur et al. 2001; Bosch et al. 2004). Indeed, when RNase inhibitor-sensitive noncytotoxic RNases such as RNase A are injected directly into *Xenopus* oocytes, which lack strong inhibitors of mammalian RNases, they display cytotoxic activity (Saxena et al. 1991). Currently, onconase, BS-RNase, and PE5 are representatives of known cytotoxic RNases that show antitumor activity while avoiding inhibition by RNase inhibitors.

4.2 Antitumor RNases

Onconase

Onconase (ONC) is a polypeptide containing104 amino acid residues, isolated from leopard frog (*Rana pipiens*) eggs by Ardelt et al. (1991) belonging to the vertebrate-secreted RNase family (Ardelt et al. 1991). The enzyme activity of ONC is only $1/10^2$ to $1/10^5$ of that of RNase A (Boix et al. 1996). However, the cytotoxicity of ONC is stronger than RNase A, despite its low enzyme activity, due to its mechanism of RNase inhibitor avoidance (Rutkoski and Raines 2008). The antitumor effect of ONC has been verified in many cancer cells, such as hepatoma, colon cancer, and leukemia (Wu et al. 1993; Iordanov et al. 2000). ONC has been studied in clinical trials including patients with malignant mesothelioma, nonsmall cell lung cancer, renal cell cancer, and others (Mikulski et al. 1995; Vogelzang and Stadler 1999; Costanzi et al. 2005). Unfortunately, due to the establishment of other drugs as first-line treatment for malignant mesothelioma (combination of pemetrexed and cisplatin) and the rise of cancer immunotherapy, interest in ONC has diminished, and it has not been approved as a therapeutic drug until now. However, in the previously mentioned clinical trials, ONC was confirmed to prolong the survival period while showing favorable safety, and even now, attempts are being made to use it in combination with other drugs or to utilize an ONC fusion drug (Smolewski et al. 2020).

BS-RNase

BS-RNase, a bovine seminal ribonuclease discovered by Hosokawa and Irie, and Dostal and Matousek in 1972 (Hosokawa and Irie 1971; Dostál and Matoušek 1972; D'Alessio et al. 1972a), is the sole native dimeric member of the vertebrate-secreted RNase family (D'Alessio et al. 1972b; Sorrentino and Libonati 1994). RNase inhibitor binds tightly to monomeric BS-RNase but not to dimeric BS-RNase; thus, it shows remarkable antitumor activity (Murthy and Sirdeshmukh 1992). The cancer-selective antitumor effect of BS-RNase has been confirmed in vitro as well as

in vivo in several cancers, including thyroid cancer, melanoma, and seminoma (Poucková et al. 1998; Kotchetkov et al. 2001). Furthermore, Fiorini et al. demonstrated that BS-RNase exhibits strong antiproliferative and proapoptotic effects in pancreatic adenocarcinoma cell lines, and that it triggers Beclin1-mediated autophagy in cancer cells that is ineffective in normal cells (Fiorini et al. 2014). Although it is not completely understood how BS-RNase binds to cancer cells, adsorption to negative charges present on the cell surface has been proposed (Bracale et al. 2002), and BS-RNase has been found to bind to the extracellular matrix, suggesting that the interaction with the extracellular matrix is important for cytotoxicity (Mastronicola et al. 1995; Bracale et al. 2002).

PE5

In humans, the vertebrate-secreted RNase family contains eight members involved in RNA clearance and maturation, and immune defense, while some members have unique physiological effects such as angiogenesis (Koczera et al. 2016). One of the control mechanisms for these functional RNases is RNase inhibitor interference (Dickson et al. 2005). Modification of human RNases has also been attempted to overcome RNase inhibitor obstruction, resulting in cytotoxicity. PE5 is a cytotoxic artificial RNase 1 (human pancreatic RNase, HP-RNase) variant with five mutated residues in the N-terminal domain (Arg4Ala, Lys6Ala, Gln9Glu, Asp16Gly, and Ser17Asn). These mutations result in the RNase having a discontinuous, unconventional nuclear localization signal (NLS) (Rodríguez et al. 2006). The NLS of PE5 is recognized by α-importin, which imports the protein to the nucleus (Bosch et al. 2004). Once inside the nucleus, it degrades nuclear RNA and displays cytotoxicity, although cytoplasmic RNA is unaffected (Tubert et al. 2011). Despite being sensitive to the RNase inhibitor, PE5 demonstrates cytotoxicity against several cancer cell lines (Castro et al. 2011, 2012). PE5 exhibits cytotoxicity via apoptosis associated with p21WAF1/CIP1 pathway induction and the inactivation of JNK in the doxorubicin-resistant ovarian cancer cell line, NCI/ADR-RES. Furthermore, it has cytostatic effects, as evidenced by the increase in the number of cells in S and G2/M cell cycle phases in PE5-treated cells (Castro et al. 2011), and the synergistic effects with doxorubicin were also revealed (Castro et al. 2012). The mechanism observed in the NCI/ADR-RES cells was found to be different from that observed for ONC. The effect of PE5 on NCI/ADR-RES cells has also been studied via transcriptome analysis using microarray technology, revealing that PE5 induces changes in pleiotropic gene expression, especially reducing the expression of genes involved in cancer cell metabolism (Vert et al. 2016). Moreover, studies have been conducted to further modify PE5 to improve its therapeutic potential (Vert et al. 2012) and very recently, one of these variants (NLSPE5) demonstrated cancer-selective cytotoxicity. NLSPE5 reduced migration and invasion of highly invasive breast cancer cells, accompanied by downregulation of N-cadherin expression, and inhibition of cancer stem cell (CSC) development, as well as diminishing the self-renewal capacity of CSCs, suggesting their possible targeting by cytotoxic RNase (Castro et al. 2021).

4.3 cSBL

Since the agglutination activity of cSBL is considered cancer cell-selective and does not interact with human RNase inhibitors, research on cSBL as an anticancer drug candidate has been conducted since its discovery. The substantial antitumor effect of cSBL was first reported by Nitta et al. in 1994 (Nitta et al. 1994a). cSBL significantly inhibited the proliferation of mouse leukemia P388 and L1210 cells in vitro as well as sarcoma 180 and Mep II ascites cells in vivo, while 50 mg/kg injection of cSBL, one-third of the lethal dose for normal mice, did not show any undesired side effects in the in vivo experiment. Meanwhile, the cSBL-resistant P388 cell variant, RC-150, was established (Nitta et al. 1994b). The doubling time, tumorigenicity, and lethality of RC-150 cells were similar to those of parental P388 cells. cSBL agglutinated both P388 and RC-150 cells, and no difference was observed between the sialidase-labile sialic acid levels in RC-150 and P388 cells. The 50% inhibitory concentration of cSBL for growth of P388 cells was approximately 3.1–6.2 μM, whereas concentrations as high as 100 μM were ineffective on the growth of RC-150 cells. Analysis using dansylcadaverine-labeled cSBL showed that the internalization of cSBL was observed in parent P388 cells, but not in resistant RC-150 cells, indicating the importance of not only cell-surface binding but also cell internalization. The addition of benzyl-α-N-acetylgalactosamine (GalNAc) to the culture medium diminished the effect of cSBL, suggesting that the internalization of cSBL may be mediated by O-linked carbohydrate chain(s) of the glycoconjugates (Irie et al. 1998). Thus, the antitumor activity of cSBL is thought to be a result of the coordination between lectin and RNase activity, i.e., recognition of glycoconjugates containing sialic acids on tumor cell surfaces and decomposition of RNA required for cell survival, respectively (Nitta 2001). A few years after the first report of the antitumor effect of cSBL, Liao et al. reported the cancer-selective cytotoxic effect of cSBL: cSBL inhibited the growth of several carcinoma cell lines but did not affect healthy human and mouse fibroblasts (Liao et al. 1996). Subsequently, Hu et al. reported that cSBL was cytotoxic to human hepatoma cell lines at different degrees of differentiation. Interestingly, the cytotoxicity of cSBL in different hepatoma cells correlated with the extent of differentiation, but not the proliferation rate of the cells (Hu et al. 2001a). Wei et al. reported a similar phenomenon in which retinoic acid (RA) or dimethyl sulfoxide (DMSO)-induced differentiation resulted in HL-60 cells becoming resistant to cSBL (Wei et al. 2002). These results indicate that differentiation is a significant factor in the selective cytotoxicity of cSBL. Only a few studies have compared the effects of ONC and cSBL; however, Tang et al. reported that compared with ONC, cSBL harbors more cancer-selective antitumor activity, as ONC was toxic to healthy human HS-68 foreskin fibroblasts, whereas cSBL was not (Tang et al. 2005). Furthermore, Lee et al. showed that cSBL internalization into baby hamster kidney BHK-21 cells was enhanced by Japanese encephalitis virus (JEV) infection, which causes enhanced apoptosis in JEV-infected BHK-21 cells, indicating that cSBL also possesses antiviral activity (Lee et al. 2011). Although there are some unclear or controversial reported mechanisms of cSBL antitumor activity even

today, we summarize the signal transduction pathways and related mechanisms of antitumor activity in Sect. 5.

4.4 Benefits of Antitumor RNase in Cancer Treatment

Vertebrate-secreted RNases are expected to be minimally immunogenic because of their compact structure and homology with human pancreatic RNase (Costanzi et al. 2005). They also exhibit high thermal stability and strong resistance to protein denaturants and proteases (Okabe et al. 1991; Notomista et al. 2000; Rosenberg et al. 2001). These features are considered to be one reason for their potent antitumor activity in vivo and provide benefits for commercialization. Furthermore, in current cancer therapies, undesired side effects due to the low cancer cell selectivity of conventional drugs and the emergence of resistance to these drugs have become major issues. Recently, attempts have been made to overcome these issues using molecular-targeted and antibody drugs, but both are still developing fields, and currently, many conventional drugs are used that target DNA. Antitumor RNases as anticancer agents is a new cancer treatment strategy that targets RNA. There are several advantages to this: (1) Cell toxicity of antitumor RNAase has already been demonstrated for many types of cancer. (2) Many cytotoxic RNases have been reported to have high cancer cell selectivity, although the mechanisms have not yet been completely elucidated. (3) They may show synergistic antitumor effects with other drugs in combination treatment. (4) They have no genetic toxicity, unlike conventional DNA-damaging drugs or radiotherapy. (5) There are no reports showing cross-resistance with small molecule compounds, but rather strong cell-death-inducing effects on multidrug-resistant cells. (6) Antitumor RNases can exert pleiotropic effects, because they affect multiple RNA substrates, whereas drugs that target a specific protein have the advantage of being highly specific, but are often inadequate to adapt to the multifactorial complexity of the cancer phenotype, and are often accompanied by the emergence of resistance. There is little information about resistance acquisition in cancer cells against antitumor RNases; thus, it is unclear whether resistance will appear frequently. However, since RNA is targeted, it may be possible to overcome resistance through abnormal gene expression. The advantages of using antitumor RNases for cancer therapy are summarized based on the central dogma in Fig. 1.

5 Antitumor Mechanism of cSBL

5.1 cSBL-Induced Apoptosis

The initial antitumor mechanism of cSBL was thought to be inhibition of protein synthesis via RNA degradation. In fact, reduced incorporation of [^{3}H] leucine was

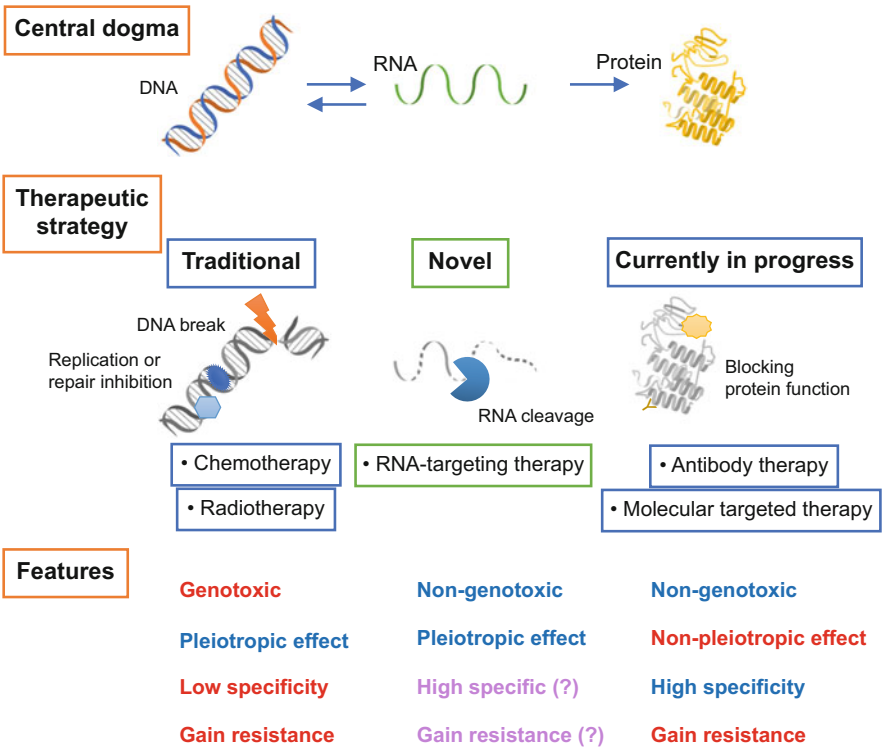


Fig. 1 Features of therapeutic strategies. Traditional DNA-targeting therapies, including chemotherapy and radiotherapy, are mainly selective for quickly proliferating cells, but are therefore often also toxic to healthy cells. In addition, since they have genotoxicity, it is necessary to pay attention to the development of secondary cancers. In many cases, tolerance is induced through abnormal expression of proteins such as drug efflux pumps. Therapies that target specific molecules, such as antibody therapy and molecular targeted therapy, have the advantage of being nongenotoxic and highly selective. However, the nature of cancer is highly diverse, and targeting only a single protein may be ineffective in some patients. Furthermore, resistance appears due to the acquisition of mutations in target genes and overexpression of abnormal proteins. RNA-targeting therapy is a new approach with several advantages, including nongenotoxicity and pleiotropy. It induces cell death signals by degrading RNA. Although there is little information on resistance and selectivity, it inhibits gene expression, so it may prevent resistance acquisition by abnormal genetic expression, including those for concomitant drugs

demonstrated in cSBL-treated cells (Nitta et al. 1994a), and treatment with cSBL leads to alterations in signal transduction and intracellular protein kinase cascades, such as decreased intracellular Ca^{2+} concentration, decreased protein kinase A activity, and increased protein kinase G activity (Nitta et al. 1996). However, subsequent analysis of the antitumor effects revealed that cSBL induced apoptosis. In early experiments with mouse leukemia p388 cells, it was found that cSBL induces apoptotic morphological changes, such as nuclear condensation, the disappearance of microvilli, and biochemical changes, such as caspase-8/−3 activation,

phosphatidylserine externalization, and DNA fragmentation. In addition, increased expression of the Fas antigen and tumor necrosis factor (TNF) receptor was observed in cSBL-treated cells (Nitta 2001). Subsequently, cSBL was reported to cause caspase-7 activation in human breast cancer MCF-7 cells that lack caspase-3, and apoptosis is prevented by the overexpression of Bcl-XL in the cells (Hu et al. 2001b).

After 2013, our group reported the validity of cSBL in human leukemia cell lines and the detailed mechanism of cSBL-induced cell death (Tatsuta et al. 2013b). cSBL degrades cellular RNA and manifests significant cytotoxic effects in several types of human leukemia cell lines, including MDR cells, when conventional DNA-damaging clinical agents, such as etoposide and doxorubicin, are not cytotoxic to these cells. cSBL-induced DNA fragmentation was completely blocked by the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl- [O-methyl]-fluoromethylketone (z-VAD), suggesting that apoptosis is induced in a caspase-dependent manner. Analysis to identify the cSBL-induced signaling pathway was performed using a combination of specific caspase inhibitors and mitochondrial membrane depolarization–detecting reagents. cSBL-induced mitochondrial depolarization was not diminished by z-VAD, whereas TNF-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial depolarization was completely inhibited by z-VAD. This indicated that the cytotoxicity of cSBL was induced through caspase-dependent apoptosis, whereby mitochondrial perturbation occurs as an upstream event.

Endoplasmic reticulum (ER) stress is also reportedly involved in cSBL-induced apoptosis (Tatsuta et al. 2013a). cSBL induces the endoplasmic unfolded protein response (UPR). Additionally, inhibition of caspase-4, the initiator caspase in ER stress-mediated apoptosis, reduces cSBL-induced apoptosis. In further experiments with specific caspase inhibitors, caspase-9 activation was found to be involved in caspase-4 activation, at least to some extent; however, because cSBL-induced mitochondrial membrane depolarization or UPR is unaffected by caspase-9 or caspase-4 inhibition, respectively, we concluded that mitochondrial perturbations and ER stress occurred independently in cSBL-treated cells. These results suggest that cSBL may induce apoptosis via multiple apoptotic pathways, including ER stress and the mitochondrial pathway. A comparative study using thapsigargin, an inducer of endoplasmic reticulum stress apoptosis, revealed that the mitochondrial pathway contributes significantly to cSBL-induced apoptosis (Tatsuta et al. 2013a).

RNase activity has also been shown to be required for cSBL cytotoxicity; however, it remains unclear how cSBL-induced RNA degradation leads to apoptosis. In our study conducted on human leukemia (Tatsuta et al. 2013b), malignant mesothelioma (Tatsuta et al. 2014b), and breast cancer cell lines (Kariya et al. 2016; Tatsuta et al. 2018a), JNK and p38 mitogen-activated protein kinases (MAPK) were strongly activated by cSBL treatment, suggesting that these proteins may be involved in cSBL-induced apoptosis. p38 inhibitors and knockdown of p38 attenuates cSBL-induced cell death (Kariya et al. 2016), indicating that RNA degradation by cSBL activates these stress kinases, which leads to apoptosis. However, similarly to inhibition of caspase activation by z-VAD, the inhibition of p38 MAPK did not completely rescue cSBL-induced cell death. Thus, it is possible that cSBL might be

involved in a p38- and caspase-independent cell death-inducing pathway, or when the action of stress kinases and caspases is prevented, RNA breakdown by cSBL may lead to cell death via a different signal.

5.2 *In Vivo Antitumor Activity of cSBL*

The in vivo antitumor effect of cSBL was initially investigated using mouse cell lines. The survival of mice inoculated with cSBL-treated sarcoma-180 cells was prolonged compared to untreated controls (Nitta et al. 1994a). After inoculation with sarcoma-180 cells (1×10^6 cells/200 μL), the control group had 0% survival at 16 days, whereas the group treated with cSBL (1 mg) had 100% survival at 45 days. Intraperitoneal administration of cSBL to mice with sarcoma-180 and Mep II ascites cancer was also performed, and again, continuous administration of cSBL for 10 days achieved 100% survival within the experimental period (45 days) without any undesired side effects. Although there are only a few in vivo studies that evaluate human cells, Chen et al. investigated the inhibitory ability of cSBL on the growth of glioblastoma DBTRG cells in a mouse model (Chen et al. 2015). Single subcutaneous administration of cSBL on the opposite side of the lower abdominal region to the tumor significantly inhibited DBTRG cell growth compared to the control under conditions where no changes in body weight were observed. Furthermore, we recently established malignant mesothelioma xenografts with H2452 and MSTO cells (Tatsuta et al. 2018b). In in vivo studies with these grafts on the effect of cSBL, no obvious toxicities or body weight changes were observed during the experimental period; however, in both types of xenografts, significant tumor growth suppression was observed after twice-weekly cSBL (2.5 mg/kg) intratumoral injection for 4 weeks. The effect of cSBL was also superior to that of the current therapeutic agent, pemetrexed, administered by a previously reported method based on the maximum tolerated dose (Tonkinson et al. 1999; Kawabata et al. 2014). In H2452 xenografted groups, cSBL showed a tumor-suppressive effect earlier than in the pemetrexed-treated group, and antitumor effects of pemetrexed were not observed in the MSTO xenografts.

5.3 *Cancer Selectivity*

The antitumor effect of cSBL has been examined in several different cancer cell lines and primary or immortalized nonmalignant cells derived from normal tissues (Tatsuta et al. 2014c). In general, cSBL suppresses the growth of various types of cancer cells (carcinoma [cervical, hepatocellular, oral, and breast], sarcoma, glioma, mesothelioma, leukemia [T-cell, promyelotic, and erythro], and lymphoma), but not healthy cells (fibroblasts, melanocytes, keratinocytes, mesothelial cells, and mammary epithelial cells). The sensitivities of some cancer cell lines are contradictory

between reports, but such contradictions may be caused by different experimental conditions. There is still no clear evidence for why cSBL is selective for cancer cells, but some hypotheses have been proposed. The binding of cSBL is selective to cancer cells and abolished by sialic acid–containing molecules, but not by monosialic acids (Nitta et al. 1987). Since changes in the glycans on cancer cell surfaces can occur during tumorigenesis (Dennis et al. 1999; Hakomori 2002), and the anion content (such as sialic acid) on cancer cells may increase (Wang et al. 2009), the particular sialic acid–containing structure recognized by cSBL may only exist on cancer cell membranes. It has also been shown that cSBL accumulates in the peripheral region of the nucleus after being taken up by cancer cells, although the detailed mechanism is not clear (Kariya et al. 2016). There may be differences in incorporation into cells, intracellular transportation, and/or localization between cancer cells and healthy cells. Furthermore, there are various factors that affect the selective cytotoxicity. As mentioned in Sect. 4.3, it has been suggested that differentiation status (Hu et al. 2001a; Wei et al. 2002) or JEV infection (Lee et al. 2011) are significant factors in the selective cytotoxicity. These may influence the effect of cSBL through changes such as the binding and uptake of cSBL. Tseng et al. suggested that the estrogen receptor is also an essential factor that enables cSBL cytotoxicity, since cSBL selectively induced cell death in estrogen-receptor-positive breast cancer cell lines (MCF7 and ZR-75-1), but not in estrogen-receptor-negative breast cancer cell lines (MDA-MB-231 and ZR-75-30) in their experiment. However, our recent comprehensive investigation focusing on breast cancer cell lines with different phenotypes, such as estrogen receptor-positive, progesterone receptor (PgR)-positive, human epidermal growth factor receptor type 2 (HER2)-positive, and triple-negative, revealed that cSBL induces apoptosis in all cancer cell lines tested, including estrogen-receptor-negative cells, even though it was less effective to healthy cells (Kariya et al. 2016; Tatsuta et al. 2018a). Therefore, we concluded that the estrogen receptor is not a significant factor in cSBL selectivity. Investigation of breast cancer revealed other aspects of the effect of cSBL on the expression of cancer-related proteins such as epidermal growth factor receptor (EGFR), which we discuss in Sect. 5.4.

5.4 Effect of cSBL in Cancer-Related Molecules

By investigating breast cancers, we found that cSBL decreases the expression levels of estrogen receptor, progesterone receptor (PgR), and human EGFR type 2 (HER2) (Tatsuta et al. 2018a). Furthermore, treatment with cSBL resulted in decreased protein levels of all ErbB proteins in each breast cancer cell line tested in the experiment (six cell lines), including EGFR in triple-negative MDA-MB231 and MDA-MB-468 cells. These results are interesting, because therapies targeting the hormone receptor or ErbB family could be novel efficient breast cancer treatments (Masoud and Pagès 2017). The cause of estrogen receptor, PgR, and ErbB family downregulation is currently under investigation. Degradation by proteasomes and/or

stabilization by heat shock proteins (HSPs) is implicated in the turnover of these molecules. cSBL causes strong activation of p38, and it has also been reported to change the localization of HSP70 and HSC70 (Ogawa et al. 2014; Tatsuta et al. 2014a). We speculate that the reduced expression levels of estrogen receptor, PgR, and Erb B family in cSBL-treated cells may be associated with p38 activation and/or HSPs.

Most recently, we aimed to identify the key molecules whose expression was affected by cSBL to further understand the mechanism of action of cSBL using microarrays. Since cSBL degrades intracellular RNA, it is difficult to analyze gene expression in cSBL-treated cells. We exposed malignant mesothelioma H28 cells to low concentrations of cSBL for extended periods of time to obtain cloned cell lines with altered cell properties. Two clones were selected, and the RNA obtained from them was used for transcriptome analysis by microarray (Tatsuta et al. 2021). Microarray analysis detected 927 differentially expressed genes (DEGs). Bioinformatic analysis of these DEGs indicated that there were significant pleiotropic changes in the expression profiles of several genes, including multiple genes involved in metabolic pathways in cSBL-resistant cells. Interestingly, all DEGs involved in metabolic pathways were related to lipid and carbohydrate metabolism. At present, the significance of this change in carbohydrate and lipid metabolism is unknown, but these findings are novel insights into cSBL, and it may inform new methodologies of anticancer strategies using cSBL. In particular, glucose metabolism and the importance of the Warburg effect in cancer has been refocused on (Liberti and Locasale 2016). Regarding the DEGs, the expressions of some members of the aldo-keto reductase (AKR) family and the ATP-binding cassette (ABC) transporter superfamily were markedly downregulated. Among these, it was particularly interesting that cSBL action reduced the level of AKR1B10, which has been reported as a candidate biomarker for malignant pleural mesothelioma prognosis. These findings reveal novel aspects of the effect of cSBL, which may contribute to the development of new therapeutic strategies for malignant mesothelioma.

5.5 Synergistic Effects with Other Drugs

It has been reported that cSBL has a highly synergistic antitumor effect with other reagents. Combination treatment of cSBL with interferon γ (IFN-γ) enhances apoptosis in MCF-7 and SK-Hep-1 cells (Hue et al. 2001a; Tang et al. 2005). Hu et al. proposed that binding and entry of cSBL may be facilitated by IFN-γ, resulting in severe cell death. Tang et al. found that synergistic cytotoxicity with IFN-γ was not observed in HL-60 cells, and suggested that this is because of the differentiation status of HL-60. As cSBL is thought to be ineffective to the differentiated cells, differentiation by IFN-γ could make HL-60 cells insusceptible in that case.

Combination treatment of cSBL with TRAIL induced synergistic apoptosis. The synergistic effects were caused by the amplification of an apoptotic signal, which enhanced the truncation of Bid, a proapoptotic member of the Bcl-2 family, and

caspase activation, resulting in drastic mitochondrial perturbations (Tatsuta et al. 2014b). Furthermore, the combined effect of cSBL was evaluated with conventional drugs for malignant mesothelioma, pemetrexed and cisplatin. The combination of cSBL and pemetrexed exhibited a strong synergistic effect that was comparable or even superior to the standard regimen of pemetrexed and cisplatin. The cytostatic effect of pemetrexed and the cytotoxic effect of cSBL cooperated without any repulsion, while the effects of pemetrexed and cisplatin on p21 expression were counteractive when used in combination (Satoh et al. 2017a). We further analyzed the combined effect of cSBL using a larger number of drugs, including EGFR-tyrosine kinase inhibitors. In a multiple drug combination study, pemetrexed + cSBL and pemetrexed+cisplatin+cSBL were more effective than the other regimens in terms of antiproliferative and synergistic effects. The pemetrexed + cSBL+TRAIL combination also showed strong synergy by playing additive roles without interference; thus, these combinations may serve as a rational regimen with antiproliferative mechanisms (Satoh et al. 2017b).

Additionally, as cSBL reduces the expression of EGFR, PgR, and estrogen receptors, combining cSBL with drugs that target the signaling of these receptors may provide favorable synergistic effects. Furthermore, since cSBL suppresses the expression of AKR1B10 and some ABC transporters, which are related to malignancy and resistance of cancers, multidrug combinations including cSBL may enhance the effects of other drugs and prevent drug resistance.

6 Conclusions and Future Problems

cSBL is a multifunctional protein with both lectin and ribonuclease activities, showing cancer cell-selective antitumor effects via a unique mechanism on various cancer cells, including multidrug-resistant cells. Furthermore, it has favorable properties as an anticancer drug, such as minimal immunogenicity and high stability in blood flow because of its compact structure, homology to human analogs, and resistance to heat, denaturants, and proteases. cSBL also has synergistic effects in combination with other drugs and has the potential to suppress the expression of molecules involved in cancer cell malignancy. A schematic illustration describing the antitumor effect of cSBL, including the signal induced by cSBL, and its valuable features for developing new therapeutic strategies are summarized in Fig. 2. The use of cSBL opens up new possibilities for treatment strategies in addition to currently limited chemotherapies. It is necessary in future research to further elucidate the mechanism of antitumor action, especially the factors of cancer cell selectivity of cSBL, as well as in vivo safety and pharmacokinetic studies for clinical application. Furthermore, as with other protein-based drugs, the development of stronger cSBL variants and cSBL fusion drugs using genetic modification techniques may lead to new therapies. We hope that a more detailed understanding of the antitumor mechanism of cSBL will contribute to the development of cancer therapeutics in the future.

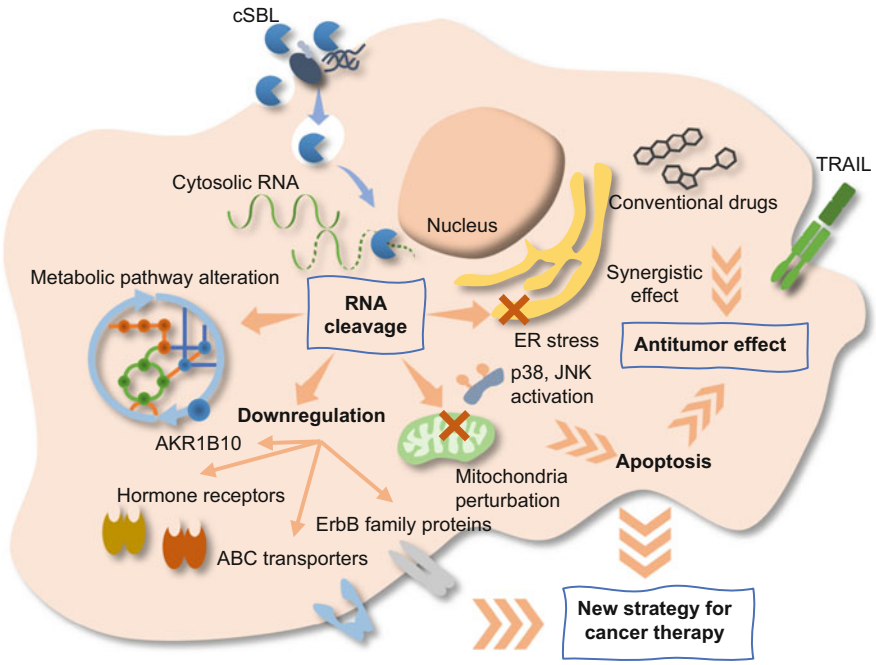


Fig. 2 Schematic illustration of antitumor effects of cSBL. cSBL somehow binds (or adsorbs) to the cell membrane. Once cSBL internalizes into cells, it localizes near the nucleus and degrades RNA. The RNA cleavage induces pleiotropic signals including apoptosis signaling. There are some favorable features of its antitumor activity, such as synergistic effect with other drugs and downregulation of cancer-related molecules

References

Ardelt W, Mikulski SM, Shogen K (1991) Amino acid sequence of an anti-tumor protein from Rana pipiens oocytes and early embryos. Homology to pancreatic ribonucleases. J Biol Chem 266: 245–251. https://doi.org/10.1016/S0021-9258(18)52427-3

Beintema JJ, Fitch WM, Carsana A (1986) Molecular evolution of pancreatic-type ribonucleases. Mol Biol Evol 3:262–275. https://doi.org/10.1093/oxfordjournals.molbev.a040393

Beintema JJ, Breukelman HJ, Carsana A, Furiat A (1997) 8 - evolution of vertebrate ribonucleases: ribonuclease A superfamily. In: D'Alessio G, Riordan JF (eds) Ribonucleases. Academic Press, New York, pp 245–269

Boix E, Wu Y, Vasandani VM et al (1996) Role of the N terminus in RNase A homologues: differences in catalytic activity, ribonuclease inhibitor interaction and cytotoxicity. J Mol Biol 257:992–1007. https://doi.org/10.1006/jmbi.1996.0218

Bosch M, Benito A, Ribó M et al (2004) A nuclear localization sequence endows human pancreatic ribonuclease with cytotoxic activity. Biochemistry 43:2167–2177. https://doi.org/10.1021/bi035729+

Boyd WC, Shapleigh E (1954) Specific precipitating activity of plant agglutinins (lectins). Science 119:419. https://doi.org/10.1126/science.119.3091.419

Bracale A, Spalletti-Cernia D, Mastronicola M et al (2002) Essential stations in the intracellular pathway of cytotoxic bovine seminal ribonuclease. Biochem J 362:553–560. https://doi.org/10.1042/bj3620553

Castro J, Ribó M, Navarro S et al (2011) A human ribonuclease induces apoptosis associated with p21WAF1/CIP1 induction and JNK inactivation. BMC Cancer 11:9. https://doi.org/10.1186/1471-2407-11-9

Castro J, Ribó M, Puig T et al (2012) A cytotoxic ribonuclease reduces the expression level of P-glycoprotein in multidrug-resistant cell lines. Investig New Drugs 30:880–888. https://doi.org/10.1007/s10637-011-9636-2

Castro J, Tornillo G, Ceada G et al (2021) A nuclear-directed ribonuclease variant targets cancer stem cells and inhibits migration and invasion of breast cancer cells. Cancers (Basel) 13(17):4350. https://doi.org/10.3390/cancers13174350

Chen JN, Yiang GT, Lin YF et al (2015) Rana catesbeiana ribonuclease induces cell apoptosis via the caspase-9/−3 signaling pathway in human glioblastoma DBTRG, GBM8901 and GBM8401 cell lines. Oncol Lett 9:2471–2476. https://doi.org/10.3892/ol.2015.3117

Cho S, Beintema JJ, Zhang J (2005) The ribonuclease A superfamily of mammals and birds: identifying new members and tracing evolutionary histories. Genomics 85:208–220. https://doi.org/10.1016/j.ygeno.2004.10.008

Clement F, Pramod SN, Venkatesh YP (2010) Identity of the immunomodulatory proteins from garlic (Allium sativum) with the major garlic lectins or agglutinins. Int Immunopharmacol 10:316–324. https://doi.org/10.1016/j.intimp.2009.12.002

Costa AF, Campos D, Reis CA, Gomes C (2020) Targeting glycosylation: a new road for cancer drug discovery. Trends Cancer 6:757–766. https://doi.org/10.1016/j.trecan.2020.04.002

Costanzi J, Sidransky D, Navon A, Goldsweig H (2005) Ribonucleases as a novel pro-apoptotic anticancer strategy: review of the preclinical and clinical data for ranpirnase. Cancer Investig 23:643–650. https://doi.org/10.1080/07357900500283143

D'Alessio G (2011) The superfamily of vertebrate-secreted ribonucleases. In: Ribonucleases. Springer, Berlin, pp 1–34

D'Alessio G, Riordan JF (1997) Ribonucleases: structures and functions. Elsevier Science

D'Alessio G, Floridi A, De Prisco R et al (1972a) Bull semen ribonucleases. 1. Purification and physico-chemical properties of the major component. Eur J Biochem 26:153–161. https://doi.org/10.1111/j.1432-1033.1972.tb01751.x

D'Alessio G, Parente A, Guida C, Leone E (1972b) Dimeric structure of seminal ribonuclease. FEBS Lett 27:285–288. https://doi.org/10.1016/0014-5793(72)80642-2

de Lamirande G (1961) Action of deoxyribonuclease and ribonuclease on the growth of Ehrlich ascites carcinoma in mice. Nature 192:52–54. https://doi.org/10.1038/192052a0

Dennis JW, Granovsky M, Warren CE (1999) Glycoprotein glycosylation and cancer progression. Biochim Biophys Acta 1473:21–34. https://doi.org/10.1016/s0304-4165(99)00167-1

Deshpande RA, Shankar V (2002) Ribonucleases from T2 family. Crit Rev Microbiol 28:79–122. https://doi.org/10.1080/1040-840291046704

Dias RD, Machado LDS, Migliolo L, Franco OL (2015) Insights into animal and plant lectins with antimicrobial activities. Molecules 20:519–541. https://doi.org/10.3390/molecules20010519

Dickson KA, Haigis MC, Raines RT (2005) Ribonuclease inhibitor: structure and function. Prog Nucleic Acid Res Mol Biol 80:349–374. https://doi.org/10.1016/S0079-6603(05)80009-1

Dostál J, Matoušek J (1972) Purification of aspermatogenic substance in bull seminal vesicle fluid. Reproduction 31:273–274. https://doi.org/10.1530/jrf.0.0310273

Fang EF, Ng TB (2011) Ribonucleases of different origins with a wide spectrum of medicinal applications. Biochim Biophys Acta Rev Cancer 1815:65–74. https://doi.org/10.1016/j.bbcan.2010.09.001

Fiorini C, Gotte G, Donnarumma F et al (2014) Bovine seminal ribonuclease triggers Beclin1-mediated autophagic cell death in pancreatic cancer cells. Biochim Biophys Acta, Mol Cell Res 1843:976–984. https://doi.org/10.1016/j.bbamcr.2014.01.025

Gaur D, Swaminathan S, Batra JK (2001) Interaction of human pancreatic ribonuclease with human ribonuclease inhibitor: generation of inhibitor-resistant cytotoxic variants. J Biol Chem 276: 24978–24984. https://doi.org/10.1074/jbc.M102440200

Goldstein I, Hughes R, Monsigny M et al (1980) What should be called a lectin? Nature 285:66. https://doi.org/10.1038/285066b0

Hakomori S (2002) Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci 99:10231–10233. https://doi.org/10.1073/pnas.172380699

Hirabayashi J, Yamada M, Kuno A, Tateno H (2013) Lectin microarrays: concept, principle and applications. Chem Soc Rev 42:4443–4458. https://doi.org/10.1039/c3cs35419a

Hosokawa S, Irie M (1971) Purification and properties of seminal vesicle ribonucleases. J Biochem 69:683–697. https://doi.org/10.1093/oxfordjournals.jbchem.a129518

Hu C-CACA, Lee Y-HH, Tang C-HAHA et al (2001a) Synergistic cytotoxicity of Rana catesbeiana ribonuclease and IFN-γ, on hepatoma cells. Biochem Biophys Res Commun 280:1229–1236. https://doi.org/10.1006/bbrc.2001.4272

Hu CCA, Tang CHA, Wang JJ (2001b) Caspase activation in response to cytotoxic Rana catesbeiana ribonuclease in MCF-7 cells. FEBS Lett 503:65–68. https://doi.org/10.1016/S0014-5793(01)02691-6

Iordanov MS, Ryabinina OP, Wong J et al (2000) Molecular determinants of apoptosis induced by the cytotoxic ribonuclease onconase: evidence for cytotoxic mechanisms different from inhibition of protein synthesis. Cancer Res 60:1983–1994

Irie M, Nitta K, Nonaka T (1998) Biochemistry of frog ribonucleases. Cell Mol Life Sci 54:775–784. https://doi.org/10.1007/s000180050206

Kamiya Y, Oyama F, Oyama R et al (1990) Amino acid sequence of a lectin from japanese frog (Rana japonica) eggs. J Biochem 108:139–143. https://doi.org/10.1093/oxfordjournals.jbchem.a123153

Kariya Y, Tatsuta T, Sugawara S et al (2016) RNase activity of sialic acid-binding lectin from bullfrog eggs drives antitumor effect via the activation of p38 MAPK to caspase-3/7 signaling pathway in human breast cancer cells. Int J Oncol 49:1334–1342. https://doi.org/10.3892/ijo.2016.3656

Kawabata S, Chiang C-T, Tsurutani J et al (2014) Rapamycin downregulates thymidylate synthase and potentiates the activity of pemetrexed in non-small cell lung cancer. Oncotarget 5:1062–1070. https://doi.org/10.18632/oncotarget.1760

Kawauchi H, Sakakibara F, Watanabe K (1975) Agglutinins of frog eggs: a new class of proteins causing preferential agglutination of tumor cells. Experientia 31:364–365. https://doi.org/10.1007/BF01922588

Kobe B, Deisenhofer J (1995) A structural basis of the interactions between leucine-rich repeats and protein ligands. Nature 374:183–186. https://doi.org/10.1038/374183a0

Kobe B, Deisenhofer J (1996) Mechanism of ribonuclease inhibition by ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A. J Mol Biol 264:1028–1043. https://doi.org/10.1006/jmbi.1996.0694

Koczera P, Martin L, Marx G, Schuerholz T (2016) The ribonuclease a superfamily in humans: canonical RNases as the buttress of innate immunity. Int J Mol Sci 17:1278. https://doi.org/10.3390/ijms17081278

Kotchetkov R, Cinatl J, Krivtchik AA et al (2001) Selective activity of BS-RNase against anaplastic thyroid cancer. Anticancer Res 21:1035–1042

LeDoux L (1955) Action of ribonuclease on certain ascites tumours. Nature 175:258–259. https://doi.org/10.1038/175258b0

Lee FS, Vallee BL (1993) Structure and action of mammalian ribonuclease (angiogenin) inhibitor. Prog Nucleic Acid Res Mol Biol 44:1–30. https://doi.org/10.1016/s0079-6603(08)60215-9

Lee YH, Wei CW, Wang JJ, Chiou CT (2011) Rana catesbeiana ribonuclease inhibits Japanese encephalitis virus (JEV) replication and enhances apoptosis of JEV-infected BHK-21 cells. Antivir Res 89:193–198. https://doi.org/10.1016/j.antiviral.2011.01.002

Leich F, Stöhr N, Rietz A et al (2007) Endocytotic internalization as a crucial factor for the cytotoxicity of ribonucleases. J Biol Chem 282:27640–27646. https://doi.org/10.1074/jbc.M702240200

Liao YD (1992) A pyrimidine-guanine sequence-specific ribonuclease from Rana catesbeiana (bullfrog) oocytes. Nucleic Acids Res 20:1371–1377. https://doi.org/10.1093/nar/20.6.1371

Liao Y-D, Huang H-C, Chan H-J, Kuo S-J (1996) Large-scale preparation of a ribonuclease from Rana catesbeiana (bullfrog) oocytes and characterization of its specific cytotoxic activity against tumor cells. Protein Expr Purif 7:194–202. https://doi.org/10.1006/prep.1996.0027

Liberti MV, Locasale JW (2016) The Warburg effect: how does it benefit cancer cells? Trends Biochem Sci 41:211–218. https://doi.org/10.1016/j.tibs.2015.12.001

Mäkelä O (1957) Studies in hemagglutinins of leguminosae seeds. Ann Med Exp Biol Fenn 35:1–133

Marth JD, Grewal PK (2008) Mammalian glycosylation in immunity. Nat Rev Immunol 8:874–887. https://doi.org/10.1038/nri2417

Masoud V, Pagès G (2017) Targeted therapies in breast cancer: new challenges to fight against resistance. World J Clin Oncol 8:120–134. https://doi.org/10.5306/wjco.v8.i2.120

Mastronicola MR, Piccoli R, D'Alessio G (1995) Key extracellular and intracellular steps in the antitumor action of seminal ribonuclease. Eur J Biochem 230:242–249. https://doi.org/10.1111/j.1432-1033.1995.tb20557.x

Mereiter S, Balmaña M, Campos D et al (2019) Glycosylation in the era of cancer-targeted therapy: where are we heading? Cancer Cell 36:6–16. https://doi.org/10.1016/j.ccell.2019.06.006

Mikulski S, Chun H, Mittelman A et al (1995) Relationship between response rate and median survival in patients with advanced nonsmall cell lung-cancer - comparison of onconase(r) with other anticancer agents. Int J Oncol 6:889–897. https://doi.org/10.3892/ijo.6.4.889

Murthy BS, Sirdeshmukh R (1992) Sensitivity of monomeric and dimeric forms of bovine seminal ribonuclease to human placental ribonuclease inhibitor. Biochem J 281(2):343–348. https://doi.org/10.1042/bj2810343

Nitta K (2001) Leczyme. Methods Enzymol 341:368–374

Nitta K, Takayanagi G, Kawauchi H, Hakomori S (1987) Isolation and characterization of Rana catesbeiana lectin and demonstration of the lectin-binding glycoprotein of rodent and human tumor cell membranes. Cancer Res 47:4877–4883

Nitta K, Oyama F, Oyama R et al (1993) Ribonuclease activity of sialic acid-binding lectin from Rana catesbeiana eggs. Glycobiology 3:37–45. https://doi.org/10.1093/glycob/3.1.37

Nitta K, Ozaki K, Ishikawa M et al (1994a) Inhibition of cell proliferation by Rana catesbeiana and Rana japonica lectins belonging to the ribonuclease superfamily. Cancer Res 54:920–927

Nitta K, Ozaki K, Tsukamoto Y et al (1994b) Characterization of a Rana catesbeiana lectin-resistant mutant of leukemia P388 cells. Cancer Res 54:928–934. https://doi.org/10.3892/ijo.9.1.19

Nitta K, Ozaki K, Tsukamoto Y et al (1996) Catalytic lectin (leczyme) from bullfrog (Rana catesbeiana) eggs. Int J Oncol 9:19–23. https://doi.org/10.3892/ijo.9.1.19

Notomista E, Catanzano F, Graziano G et al (2000) Onconase: an unusually stable protein. Biochemistry 39:8711–8718. https://doi.org/10.1021/bi000415x

Ogawa Y, Sugawara S, Tatsuta T et al (2014) Sialyl-glycoconjugates in cholesterol-rich microdomains of P388 cells are the triggers for apoptosis induced by Rana catesbeiana oocyte ribonuclease. Glycoconj J 31:171–184. https://doi.org/10.1007/s10719-013-9513-7

Okabe Y, Katayama N, Iwama M et al (1991) Comparative base specificity, stability, and lectin activity of two lectins from eggs of Rana catesbeiana and R. japonica and liver ribonuclease from R. catesbeiana. J Biochem 109:786–790. https://doi.org/10.1093/oxfordjournals.jbchem.a123457

Poucková P, Soucek J, Jelínek J et al (1998) Antitumor action of bovine seminal ribonuclease. Cytostatic effect on human melanoma and mouse seminoma. Neoplasma 45:30–34

Raines RT (1998) Ribonuclease A. Chem Rev 98:1045–1065. https://doi.org/10.1021/cr960427h

Reily C, Stewart TJ, Renfrow MB, Novak J (2019) Glycosylation in health and disease. Nat Rev Nephrol 15:346–366. https://doi.org/10.1038/s41581-019-0129-4

Robert C (2020) A decade of immune-checkpoint inhibitors in cancer therapy. Nat Commun 11:10–12. https://doi.org/10.1038/s41467-020-17670-y

Rodrigues JG, Balmaña M, Macedo JA et al (2018) Glycosylation in cancer: selected roles in tumour progression, immune modulation and metastasis. Cell Immunol 333:46–57. https://doi.org/10.1016/j.cellimm.2018.03.007

Rodríguez M, Benito A, Tubert P et al (2006) A cytotoxic ribonuclease variant with a discontinuous nuclear localization signal constituted by basic residues scattered over three areas of the molecule. J Mol Biol 360:548–557. https://doi.org/10.1016/j.jmb.2006.05.048

Rosenberg HF, Zhang J, Di LY, Dyer KD (2001) Rapid diversification of RNase A superfamily ribonucleases from the bullfrog, Rana catesbeiana. J Mol Evol 53:31–38. https://doi.org/10.1007/s002390010188

Roth JS (1963) Ribonuclease activity and cancer: a review. Cancer Res 23:657–666

Rutkoski TJ, Raines RT (2008) Evasion of ribonuclease inhibitor as a determinant of ribonuclease cytotoxicity. Curr Pharm Biotechnol 9:185–199. https://doi.org/10.2174/138920108784567344

Sakakibara F, Takayanagi G, Ise H, Kawauchi H (1977) Isolation of two agglutinins with different biological properties from the eggs of Rana catesbiana (author's transl). Yakugaku Zasshi 97:855–862. https://doi.org/10.1248/yakushi1947.97.8_855

Satoh T, Tatsuta T, Sugawara S et al (2017a) Synergistic anti-tumor effect of bullfrog sialic acid-binding lectin and pemetrexed in malignant mesothelioma. Oncotarget 8:42466–42477. https://doi.org/10.18632/oncotarget.17198

Satoh T, Tatsuta T, Sugawara S et al (2017b) Combinatorial treatment using Leczyme as a new candidate of anti-cancer drug for malignant mesothelioma cell lines. 東北医科薬科大学研究誌 = J Tohoku Med Pharm Univ 64:65–76

Saxena SKK, Rybak SMM, Winkler G et al (1991) Comparison of RNases and toxins upon injection into xenopus oocytes. J Biol Chem 266:21208–21214. https://doi.org/10.1016/s0021-9258(18)54842-0

Section C, Ledoux L, Section C (1955) Action of ribonuclease on two solid tumours in vivo. Nature 176:36–37. https://doi.org/10.1038/176036a0

Sharon N, Lis H (1972) Lectins: cell-agglutinating and sugar-specific proteins. Science 177:949–959. https://doi.org/10.1126/science.177.4053.949

Sharon N, Lis H (2004) History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology 14:53–62. https://doi.org/10.1093/glycob/cwh122

Shimomura O, Oda T, Tateno H et al (2018) A novel therapeutic strategy for pancreatic cancer: targeting cell surface glycan using rBC2LC-N lectin-drug conjugate (LDC). Mol Cancer Ther 17:183–195. https://doi.org/10.1158/1535-7163.MCT-17-0232

Smolewski P, Kowalik M, Witkowska M, Smolewski P (2020) Ribonucleases and immunoribonucleases as potential modalities of anticancer therapy. Drug Drug Abus:1–8. https://doi.org/10.31487/j.dda.2020.01.04

Sorrentino S, Libonati M (1994) Human pancreatic-type and nonpancreatic-type ribonucleases: a direct side-by-side comparison of their catalytic properties. Arch Biochem Biophys 312:340–348. https://doi.org/10.1006/abbi.1994.1318

Sumner JB, Howell SF (1936) Identification of hemagglutinin of Jack Bean with concanavalin A. J Bacteriol 32:227–237. https://doi.org/10.1128/jb.32.2.227-237.1936

Sung H, Ferlay J, Siegel RL et al (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71:209–249. https://doi.org/10.3322/caac.21660

Tang C-HA, Hu C-CA, Wei C-W, Wang J-J (2005) Synergism of Rana catesbeiana ribonuclease and IFN-gamma triggers distinct death machineries in different human cancer cells. FEBS Lett 579:265–270. https://doi.org/10.1016/j.febslet.2004.11.086

Tatsuta T, Hosono M, Miura Y et al (2013a) Involvement of ER stress in apoptosis induced by sialic acid-binding lectin (leczyme) from bullfrog eggs. Int J Oncol 43:1799–1808. https://doi.org/10.3892/ijo.2013.2128

Tatsuta T, Hosono M, Sugawara S et al (2013b) Sialic acid-binding lectin (leczyme) induces caspase-dependent apoptosis-mediated mitochondrial perturbation in Jurkat cells. Int J Oncol 43:1402–1412. https://doi.org/10.3892/ijo.2013.2092

Tatsuta T, Hosono M, Ogawa Y et al (2014a) Downregulation of Hsp70 inhibits apoptosis induced by sialic acid-binding lectin (leczyme). Oncol Rep 31:13–18. https://doi.org/10.3892/or.2013.2814

Tatsuta T, Hosono M, Takahashi K et al (2014b) Sialic acid-binding lectin (leczyme) induces apoptosis to malignant mesothelioma and exerts synergistic antitumor effects with TRAIL. Int J Oncol 44:377–384. https://doi.org/10.3892/ijo.2013.2192

Tatsuta T, Sugawara S, Takahashi K et al (2014c) Cancer-selective induction of apoptosis by leczyme. Front Oncologia 4:1–6. https://doi.org/10.3389/fonc.2014.00139

Tatsuta T, Sato S, Sato T et al (2018a) Sialic acid-binding lectin from bullfrog eggs exhibits an anti-tumor effect against breast cancer cells including triple-negative phenotype cells. Molecules 23(10):2714. https://doi.org/10.3390/molecules23102714

Tatsuta T, Satoh T, Sugawara S et al (2018b) Sialic acid-binding lectin from bullfrog eggs inhibits human malignant mesothelioma cell growth in vitro and in vivo. PLoS One 13:1–15. https://doi.org/10.1371/journal.pone.0190653

Tatsuta T, Nakasato A, Sugawara S, Hosono M (2021) Transcriptomic alterations in malignant pleural mesothelioma cells in response to long-term treatment with bullfrog sialic acid-binding lectin. Mol Med Rep 23(6):467. https://doi.org/10.3892/mmr.2021.12106

Tikhonov A, Smoldovskaya O, Feyzkhanova G et al (2020) Glycan-specific antibodies as potential cancer biomarkers: a focus on microarray applications. Clin Chem Lab Med 58:1611–1622. https://doi.org/10.1515/cclm-2019-1161

Titani K, Takio K, Kuwada M et al (1987) Amino acid sequence of sialic acid binding lectin from frog (Rana catesbeiana) eggs. Biochemistry 26:2189–2194. https://doi.org/10.1021/bi00382a018

Tonkinson JL, Worzalla JF, Teng CH, Mendelsohn LG (1999) Cell cycle modulation by a multitargeted antifolate, LY231514, increases the cytotoxicity and antitumor activity of gemcitabine in HT29 colon carcinoma. Cancer Res 59:3671–3676

Tubert P, Rodríguez M, Ribó M et al (2011) The nuclear transport capacity of a human-pancreatic ribonuclease variant is critical for its cytotoxicity. Investig New Drugs 29:811–817. https://doi.org/10.1007/s10637-010-9426-2

Van Breedam W, Pöhlmann S, Favoreel HW et al (2014) Bitter-sweet symphony: glycan-lectin interactions in virus biology. FEMS Microbiol Rev 38:598–632. https://doi.org/10.1111/1574-6976.12052

Varki A (2017) Biological roles of glycans. Glycobiology 27:3–49. https://doi.org/10.1093/glycob/cww086

Vert A, Castro J, Ruiz-Martínez S et al (2012) Generation of new cytotoxic human ribonuclease variants directed to the nucleus. Mol Pharm 9:2894–2902. https://doi.org/10.1021/mp300217b

Vert A, Castro J, Ribó M et al (2016) A nuclear-directed human pancreatic ribonuclease (PE5) targets the metabolic phenotype of cancer cells. Oncotarget 7:18309–18324. https://doi.org/10.18632/oncotarget.7579

Vicentini AM, Kieffer B, Matthies R et al (1990) Protein chemical and kinetic characterization of recombinant porcine ribonuclease inhibitor expressed in Saccharomyces cerevisiae. Biochemistry 29:8827–8834. https://doi.org/10.1021/bi00489a046

Vogelzang NJ, Stadler WM (1999) Gemcitabine and other new chemotherapeutic agents for the treatment of metastatic bladder cancer. Urology 53:243–250. https://doi.org/10.1016/s0090-4295(98)00501-9

Wang F-L, Cui S-X, Sun L-P et al (2009) High expression of alpha 2, 3-linked sialic acid residues is associated with the metastatic potential of human gastric cancer. Cancer Detect Prev 32:437–443. https://doi.org/10.1016/j.cdp.2009.01.001

Wei CWW, Hu CCACA, Tang CHAHA et al (2002) Induction of differentiation rescues HL-60 cells from Rana catesbeiana ribonuclease-induced cell death. FEBS Lett 531:421–426. https://doi.org/10.1016/S0014-5793(02)03577-9

WHO (WHO) (2020) Global Health estimates 2020: deaths by cause, age, sex, by country and by region, 2000–2019. World Health Organization, Geneva

Wu Y, Mikulski SM, Ardelt W et al (1993) A cytotoxic ribonuclease: study of the mechanism of onconase cytotoxicity. J Biol Chem 268:10686–10693. https://doi.org/10.1016/s0021-9258(18)82252-9

Yamamoto S, Tomiyama M, Nemoto R et al (2013) Effects of food lectins on the transport system of human intestinal CaCO-2 cell monolayers. Biosci Biotechnol Biochem 77:1917–1924. https://doi.org/10.1271/bbb.130367

Yokota M, Sakakibara H, Kawauchi H (1975) Separation of agglutinin from eggs of Rana nigromaculata nigromaculata Hallowell and its interaction with ascites hepatoma (author's transl). Yakugaku Zasshi 95:50–55. https://doi.org/10.1248/yakushi1947.95.1_50

Part IV
Interaction Between Carbohydrate and Carbohydrate-Recognizing Molecules

Opposite Functions of Mono- and Disialylated Glycosphingo-Lipids on the Membrane of Cancer Cells

Koichi Furukawa, Yuhsuke Ohmi, Farhana Yesmin, Kazunori Hamamura, Yuji Kondo, Yuki Ohkawa, Noboru Hashimoto, Robiul H. Bhuiyan, Kei Kaneko, Orie Tajima, and Keiko Furukawa

Abstract Since cDNAs of glycosyltransferase genes were isolated, and become applicable for genetic engineering of glycosylation patterns, biological functions of glycolsphingolipids have been largely elucidated via glyco-remodeling cells and animals. The progress in these glycobiology techniques has enabled us to understand the roles of "tumor-specific" carbohydrates during these 3 decades. Tumor antigens recognized by host immune systems of cancer patients were classified into three

K. Furukawa (✉) · F. Yesmin · R. H. Bhuiyan · K. Kaneko · O. Tajima · K. Furukawa
Department of Biomedical Sciences, Chubu University College of Life and Health Sciences, Kasugai, Aichi, Japan
e-mail: koichi@isc.chubu.ac.jp; ikekoneka@isc.chubu.ac.jp; oriet@isc.chubu.ac.jp; keikofu@isc.chubu.ac.jp

Y. Ohmi
Department of Medical Engineering, Chubu University College of Life and Health Sciences, Kasugai, Aichi, Japan
e-mail: ooumi82@isc.chubu.ac.jp

K. Hamamura
Department of Pharmacology, Aichi Gakuin University School of Dentistry, Nagoya, Aichi, Japan
e-mail: hamak@dpc.agu.ac.jp

Y. Kondo
Department of Biochemistry II, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan
e-mail: kondoy@med.nagoya-u.ac.jp

Y. Ohkawa
Department of Glyco-Oncology and Medical Biochemistry, Osaka International Institute, Osaka, Japan
e-mail: yuki.ohkawa@oici.jp

N. Hashimoto
Department of Tissue Regeneration, Tokushima University Graduate School Institute of Biomedical Sciences, Tokushima, Japan
e-mail: nhashimoto@tokushima-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_8

classes based on “autologous typing”, that is, class 1: individual antigens present only in the patient’s tumors, class 2: shared antigens among some group of cancers, but not in normal cells, class 3: universal antigens that are present in not only some cancers but also in normal cells. Many of class 2 antigens have been elucidated to be carbohydrate antigens, and are considered to be differentiation antigens. Many of them have been used for cancer diagnosis and treatment. Functional analyses of those cancer-associated glycosphingolipids based on the genetic engineering of glyco-genes have revealed that disialylated glycolipids generally enhance malignant properties such as cell growth, invasion, and motility. On the other hand, monosialylated glycolipids rather suppress those phenotypes. As a regulatory platform for cell signaling, cell surface microdomains, glycolipid-enriched microdomain (GEM)/rafts have been proposed. Interestingly, cancer-associated glycosphingolipids play critical roles in the composition of GEM/rafts, and in the regulation of signal transduction. Molecular complex formation of glycolipids with membrane molecules that are defined by enzyme-mediated activation of radical sources/mass spectrometry should be a key factor to regulate cell signals and to determine the cell fates. They are also expected to be targets of the cancer treatment.

1 Biological Functions of Glycosphingolipids Have Been Further Understood Due to Glycoengineering

Since cDNAs of glycosyltransferases responsible for the synthesis of glycosphingolipids were isolated (Nagata et al. 1992; Haraguchi et al. 1994; Nara et al. 1994; Sasaki et al. 1994; Miyazaki et al. 1997) and became available for the genetic engineering of cultured cells and experimental animals, a number of experiments using those glyco-remodeling materials have been done, leading to the big progress in the understanding of their function and significance in the cells and mice (Furukawa et al. 2004). Main glycolipid synthetic pathways are shown in Fig. 1. Actually, many trials of genetic engineering of glycosyltransferase genes have been performed, and brought about various novel findings in the phenotypes of cultured cells and animals (Stanley 2016). Many other degradation enzymes and modifying enzymes of carbohydrates have been genetically engineered, and intriguing results have been reported (Miyagi et al. 2018; Cavdarli et al. 2020). Although the modified genes and resulting outputs are clear, mechanisms for observed abnormal phenotypes are frequently ambiguous. This is because changes of some glycosyltransferase gene generally affect all structures present at the downstream of the synthetic pathways in which the modified gene exists. Therefore, we have to clarify which glycosphingolipids in the pathway are responsible for the resulting phenotypes. In particular, remaining structures in the knockout mice/cells often compensate for the function of deleted structures, suggesting importance to carefully interpret the results (Furukawa et al. 2020).

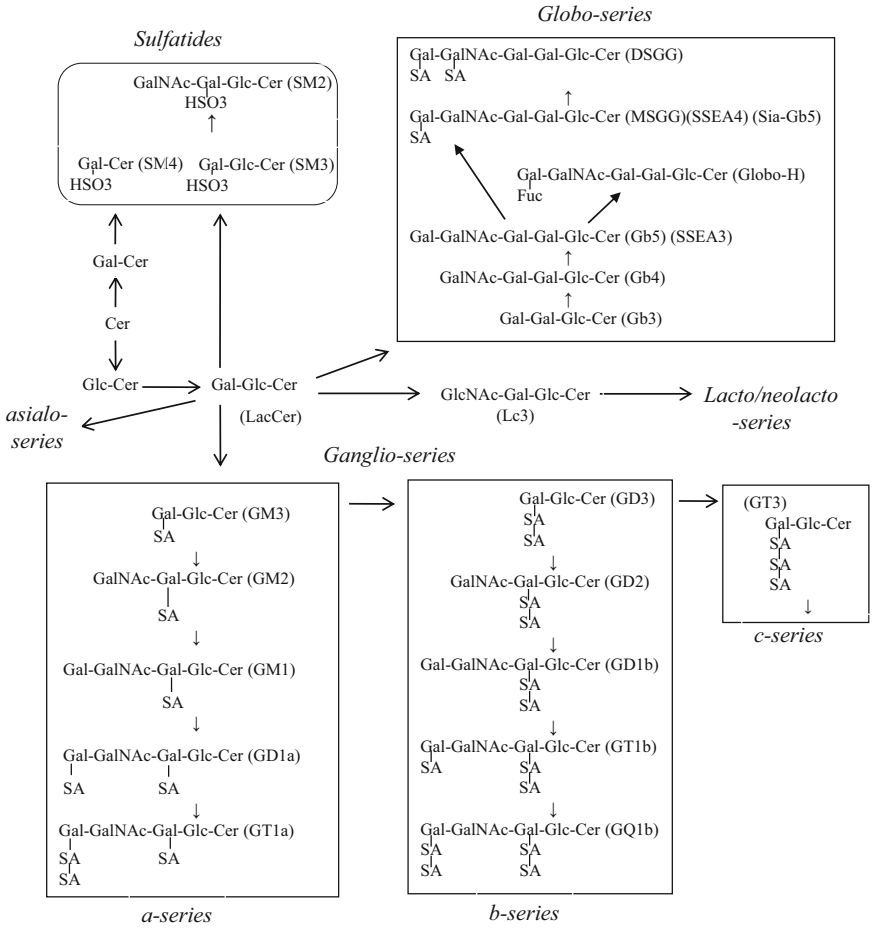


Fig. 1 Synthetic pathway of glycosphingolipids. Starting from lactosylceramide (LacCer), majority of glycosphingolipids are synthesized by the actions of the individual key enzymes

2 Cancer-Associated Glycolipids Often Increase Malignant Properties

During a long-term search of cancer specific antigens, Old's group proposed 3 classes of tumor antigens based on "autologous typing" (Old 1981). They are as follows: class 1: individual antigens present only in the patient's tumor sample, class 2: shared antigens among some group of cancers in some patients, but not in normal cells, class 3: universal antigens that are present in some cancers but also in normal cells. There were many reports on carbohydrate antigens, which could be found only in cancer cells, but not in normal cells and tissues (Hakomori 1986; Lloyd 1991). Thus, these antigens have been considered as cancer-associated carbohydrates, and

Table 1 Three class of tumor antigens based on the distribution and derivation

Classification	Distribution	Derivation
1. Neoantigen (individual antigen)	Only tumor of the patient	Genomic mutation
2. Group-shared antigen	Some cancer groups and patients not normal cells (exception[a])	Differentiation developmental overexpression
3. Universal antigen	Some cancers of some patients and normal cells	Broadly expressed with various density

[a]Exception: expressed at fetal stage, testis, very restricted sites, etc.

to be class 2 tumor antigens, being used in the diagnosis and therapy of cancers (Lloyd and Old 1989; Indellicato et al. 2020). Those carbohydrate antigens can be detected on proteins (mucine) or lipids (ceramide). Some of them are found in both proteins and lipids. Among them, sialic-acid-containing glycosphingolipids are named as gangliosides, and have been utilized as tumor markers of neuroectoderm-derived cancers. For example, ganglioside GD3 in malignant melanomas (Furukawa and Lloyd 1990; Dippold et al. 1980; Pukel et al. 1982; Portoukalian et al. 1979; Carubia et al. 1984), GD2 in neuroblastomas (Saito and Yu 1985; Schultz et al. 1984), and GD3/GD2 in gliomas (Fredman et al. 1986; Wikstrand et al. 1994; Kawai et al. 1999; Vukelić et al. 2007; Iwasawa et al. 2018). In addition to neuroectoderm-derived tumors, some other cancers also express these gangliosides. For example, T-cell leukemias (Siddiqui et al. 1984; Merritt et al. 1987; Furukawa et al. 1993; Okada et al. 1996), osteosarcomas (Shibuya et al. 2012; Azuma et al. 2005), small cell lung carcinomas (Cheresh et al. 1986; Yoshida et al. 2001), and breast cancers (Cazet et al. 2012; De Giorgi et al. 2011). Recently, many other cancers have also been reported to express these gangliosides (Sato et al. 2020; Ravindranath et al. 2004; Vantaku et al. 2017). Thus, these ganglioside antigens belong to "class 2" tumor antigens, and have been investigated on their roles in tumor cells, showing differential significances for various carbohydrate structures (Ohmi et al. 2018). On the other hand, some neutral glycolipids have also been considered to be cancer-associated antigens (Yu et al. 2020; Ragupathi 1996). Brief classification of cancer-associated antigens is shown in Table 1.

3 Membrane Microdomains GEM/Rafts as Platforms of Signal Transduction

Simons proposed a membrane microdomains, lipid rafts containing cholesterol, sphingolipids, and GPI-anchored proteins (Simons and Ikonen 1997). Majority of glycosphingolipids are enriched in this microdomain. These microdomains are resistant to nonionic detergent, being called detergent-resistant microdomain (DRM). Hakomori proposed to call this microdomain as glycolipid-enriched microdomain (GEM), and showed that GEM/rafts play crucial roles as a platform

of cell signal transduction (Hakomori 2003). In particular, ganglioside GM3 is localized in GEM/rafts, and forms complexes with membrane molecules such as tetraspanins (Hakomori 2010). Although molecular basis for molecular clustering in GEM/rafts is not clear at this moment, dynamic processes of raft formation from nanoscale to microscale were proposed by Simons et al. (Simons and Toomre 2000; Simons and Gerl 2010). Kohmura et al. demonstrated that dimer formation of same ganglioside species might be an initial step for the formation of GEM/rafts using ultrahigh-resolution imaging technique (Komura et al. 2016).

Biochemical and imaging analyses of intracellular distribution of glycolipid-associated molecules demonstrated that sugar moiety of glycosphingolipids on the cell surface and cellular conditions (resting or activated) regulates their localization inside/outside of GEM/rafts. It is of great interest that fine structures of carbohydrate portion in glycolipids might control the intracellular localization of membrane molecules inside/outside of GEM/rafts as if they are regulators of the composition of GEM/rafts (Ohkawa et al. 2010; Yesmin et al. 2021), and resultant cell signals (Furukawa et al. 2014) as reported by Sonnino (Sonnino and Prinetti 2010).

4 Disialyl Glycosphingolipids Generally Enhance Malignant Phenotypes and Signals in Cancer

During analyses of phenotypic changes in cancer cells expressing cancer-associated glycosphingolipids, it has been shown that disialylated gangliosides with a tandem mode linkage 2 sialic acids often enhance malignant properties (Furukawa et al. 2012a, b) in various kinds of tumors. Namely, gangliosides GD3 and GD2 enhance malignant properties such as increased proliferation, invasion, and cell adhesion to extracellular matrices. However, these effects of gangliosides on the tumor phenotypes are not identical. The modes of action were diverse among cancer lineages (Furukawa et al. 2012a, b). This is deeply related to the expression of membrane molecules in the vicinity of cancer-associated gangliosides at membrane microdomains, GEM/rafts (Hakomori 1998; Hakomori et al. 1998). The profiles of intracytoplasmic proteins primarily expressed in the individual cells are also critical for how those gangliosides regulate the feature of cells, signals, and cell fates (Furukawa et al. 2012a).

In the study of melanomas, we clarified that various adaptor molecules such as p130Cas, paxillin, and focal adhesion kinase (FAK) were activated due to tyrosine phosphorylation, leading to the enhanced cell proliferation and invasion under GD3 expression (Hamamura et al. 2005, 2008). Then, we also revealed that integrin was involved in the regulation of cell signaling via FAK and Src family kinase Yes during cell adhesion (Ohkawa et al. 2008). Strong physical and functional association of integrins and FAK was previously shown in small cell lung cancer (SCLC) cells. Anti-GD2 monoclonal antibody induced apoptosis of SCLC cells via anoikis (Yoshida et al. 2001). During the cell detachment from culture dishes, SCLC cells

showed reduced phosphorylation levels of FAK (Aixinjueluo et al. 2005). Interestingly, osteosarcoma cells showed increased cell invasion and motility under expression of GD3 and GD2 (Shibuya et al. 2012). Here, cell adhesion to extracellular matrix such as collagen 1 was markedly reduced in GD3/GD2-expressing cells compared to GD3/GD2-negative control cells. GD3/GD2-positive cells showed much lower adhesion than the controls. These findings were quite opposite compared with results observed in melanoma transfectant cells with high GD3 expression (Furukawa et al. 2012a; Hamamura et al. 2005). These differences between melanomas and osteosarcomas seemed to be due to different signaling pathways basically working in the individual cell lineages (Shibuya et al. 2012). Activated Lyn (p-Lyn) was detected in osteosarcoma cells instead of p-Yes in the case of melanoma cells (Hamamura et al. 2011).

5 Monosialyl Glycosphingolipids Often Suppress Cancer Phenotypes and Signals in Animal Cancer Cells

In contrast with disialylated gangliosides, monosialylated glycosphingolipids (or a-series gangliosides) often showed suppressive effects on the cell phenotypes. Accordingly, they also suppress cell signals in various tumor cells (Furukawa et al. 2012b). For example, a rat pheochromocytoma line PC12 showed distinct effects between GM1 and GD3 on the signaling via nerve growth factor (NGF) and Trk A receptor (Nishio et al. 2004; Fukumoto et al. 2000). NGF-TrkA signaling between GM1-expressing and GD3-expressing PC12 was quite contrastive, that is, poor phosphorylation of TrkA and subsequent lowered activation of Erk1/2 upon NGF stimulation in the former and enhanced TrkA phosphorylation and highly activated Erk1/2 in the latter. In a mouse fibroblast cell line, SWISS-3 T3, overexpression of GM1/GD1b synthase induced suppressed cell growth and lowered activation levels of PDGF receptor based on its altered localization in GEM/rafts (Mitsuda et al. 2002).

In a mouse Lewis lung cancer (LLC) cells, transfectants of GM2/GD2 synthase cDNA with high expression of GM2 showed lowered malignant properties and reduced activation levels of focal adhesion kinase (FAK) (Chen et al. 2003). On the other hand, Zhang et al. established high-metastatic and low-metastatic LLC sublines by repeated injection into mice, and compared their surface molecules. Consequently, high-metastatic sublines expressed lower levels of GM1/GD1a than the low-metastatic parent line (Zhang et al. 2006). Roles of GM1 in the suppression of metastasis were directly shown using GM1 synthase-suppressed transfectants of an RNAi-expression vector. Suppression of GM1 synthase resulted in the increased malignant phenotypes including metastatic property due to the shift of MMP-9 and integrins to GEM/rafts. The secretion and activation levels of MMP-9 were consequently elevated.

In a B16 mouse melanoma subline B78, transfectant cells of GM2/GD2 synthase cDNA showed high GM2 expression and reduced cell proliferation (Tsurifune et al. 2000).

6 GM1/GD1b Synthase and Caveolin-1 Suppressed Malignant Phenotypes of Human Cancers

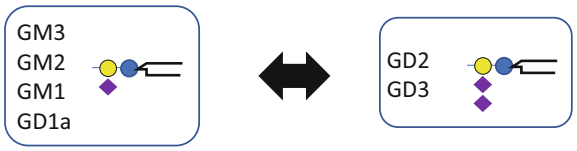
In human melanoma cells, SK-MEL-73 melanoma cell line expressing GD3 and GD2 was transfected with GM1/GD1b synthase cDNA (Dong et al. 2012). GM1-high transfectant cells showed reduction of cell growth and invasion. They also showed dispersed ganglioside distribution compared with the control line. Consequently, tyrosine-phosphorylation levels of p130Cas and paxillin were reduced in the transfectant cells. Furthermore, gangliosides shifted to the non-GEM/raft fraction dominantly contained unsaturated fatty acids compared with the control cells, suggesting that glyco-remodeling affected ceramide composition of gangliosides. Interestingly, similar reduced malignant phenotypes were observed in the melanoma transfectant cells with Caveolin-1 cDNA in SK-MEL-28 (Nakashima et al. 2007). Accordingly, reduced phosphorylation of p130Cas and paxillin was also observed. Furthermore, GD3 was dispersed from GEM/rafts, losing the leading edges that could be found in the control cells with immunocytochemistry. Thus, Caveolin-1 should have regulatory functions of signaling, although it has been used as a GEM/rafts marker as well as GM1.

All these results commonly observed among various cancers indicated that the expression of monosialylated (or a-series) gangliosides results in the attenuation of malignant phenotypes and malignant signals (Furukawa et al. 2012a, b) in contrast with disialylated gangliosides as summarized in Fig. 2. These results were recently confirmed with genetically engineered mice of GD3 synthase gene (Ohkawa et al. 2021; Zhang et al. 2021).

7 A Novel Approach to Elucidate Mechanisms for Functioning of Cancer-Associated Gangliosides

To investigate molecular mechanisms for biological functions of glycosphingolipids on the cell surface, it seems critical to identify membrane molecules that physically associate with glycosphingolipids in the vicinity of cell membrane of living cells. This is because glycosphingolipids are embedded on the outer layer of lipid bilayer membrane, and don't contain intracytoplasmic domains (Groux-Degroote et al. 2017).

Cluster formation of cancer-associated glycosphingolipids with various membrane molecules on the cell surface in a horizontal manner, and also in vertical



GM2 in Lewis LC: metastasis ↓ p-FAK ↓

GM1 in melanoma: growth ↓ migration ↓
GM1 in Lewis LC: metastasis ↓
MMP-9 secretion/ activation ↓
GM1 in Swiss3T3: PDGF signals ↓
GM1 in PC12: NGF signals ↓

GM3 in sq. cell ca.: EGF signals ↓
GM3 in adipocytes: insulin signals ↓

GD1a in FBJ-L1: migration ↓ adhesion ↓ metastasis ↓

GD3 in melanoma: growth signal ↑
integrin signal ↑
p-p130Cas ↑ p-paxillin ↑
p-FAK ↑ p-Yes ↑
GD3 in PC12: growth signal ↑

GD2 in SCLC: growth signal ↑ invasion ↑
anti-GD2 mAb pFAK ↓ p-p38 ↑
GD2 in breast ca.: c-Met activation
GD2 in osteosarcoma: migration ↑ invasion ↑
p-p130Cas ↑ p-paxillin ↑ p-Lyn↑
GD2 in glioma: adhesion ↑ invasion ↑
GD2 in melanoma: growth ↑ invasion ↑
adhesion ↑ integrin signal ↑

Fig. 2 Contrastive functions between monosialyl and disialyl gangliosides. Disialylated gangliosides with tandem linked 2 sialic acids often enhance malignant properties in various kind of tumors, while monosialylated (or a-series) gangliosides reduce malignant phenotypes and malignant signals

connection with intracellular molecules, has been demonstrated to date. But, enzyme-mediated activation of radical sources (EMARS) combined with mass spectrometry (MS) developed by Honke and Kotani (Kotani et al. 2008), might be a markedly useful technique to identify membrane molecules that are present around cancer-associated glycosphingolipids within 100–300 nm on the cell surface without any special apparatus (Furukawa et al. 2019). From the candidate molecules detected in EMAR/MS results, we can identify physically associated molecules with cancer-associated glycolipids on the living cell membrane (Hashimoto et al. 2012). Thus, this approach enables us to identify molecular profiles of clustering molecules with particular targets (Kotani et al. 2008).

Using anti-GD3 monoclonal antibody, EMARS/MS was utilized to identify GD3-associated molecules in melanoma cells. Among molecules found both in GEM/rafts fraction and GD3-targeted EMARS-labeled molecules, Neogenin was exclusively detected only in GD3-positive cells (Hashimoto et al. 2012). This molecule was found in GEM/raft fraction of only GD3-positive melanoma cells, but not of GD3-negative cells. GD3 expression induced recruitment of Neogenin and γ-secretase to GEM/rafts, leading to the increased levels of intracytoplasmic domain (ICD) of Neogenin based on the action of γ-secretase (Kaneko et al. 2016). Consequently, Neogenin ICD promotes expression of various genes as a transcription factor (Kaneko et al. 2016), resulting in the enhanced expression of various molecules. ChIP-seq analysis revealed many target genes such as GPR126, STXBP5,

MMP16, SPATA31A1, and S6K should be targets of Ne-ICD. These genes were actually upregulated by the expression of Ne-ICD. These results demonstrated, for the first time, how cancer-associated GD3 enhances malignant properties of melanomas.

In mouse glioma cells, PDGF-Rα was identified as a GD3-associated membrane molecule using EMARS-MS technique (Ohkawa et al. 2015). Physical association and clustering of GD3 and PDGF-Rα resulted in the activation of a Src family kinase, Yes and also activation of downstream paxillin (Ohkawa et al. 2015). Immunoprecipitation/immunoblotting revealed ternary complex formation among GD3, PDGFRα, and Yes, being colocalized in lamellipodia. Furthermore, in SCLC cells, ASCT2, a glutamine transporter, was identified as a GD2-associated molecule and shown to be localized in GEM/rafts. Co-operation of GD2 and ASCT2 resulted in the enhancement of cell proliferation and migration via increased phosphorylation of the mTOR complex 1 signaling axis (Esaki et al. 2018).

These results indicated that EMARS/MS technique is highly effective to elucidate the molecular complex formation around cancer-associated glycolipids on the living cell surface. It can also give us clues to reveal vertical molecular sequences originating from cancer-associated glycolipids. Thus, heterogeneity in GEM/rafts according to each "core" glycolipid might be clarified using this technique for the foresighted finding with morphological studies (Fujita et al. 2007).

8 *trans*-Interaction of Glycosphingolipids with Their Ligand Molecules Also Affects the Nature of Cells on Both Sides

Considering the meaning of GEM/rafts in the regulation of cell phenotypes and cell signals, their significance as a platform for the signal transduction might be the most critical and intriguing. The results elucidated by EMARS/MS approaches also support its importance in the generation of malignant signals and cancer phenotypes. Majority of molecules identified by this approach using glycolipids as the target molecules belong to *cis*-acting membrane molecules present around the cancer-associated glycolipids as shown in Fig. 3.

On the other hand, molecular clustering on the cell membrane consisting of glycosphingolipids and associated molecules is also involved in the recognition and binding of the carbohydrate structures by carbohydrate-recognizing molecules from outside of cells (*trans*-acting). Binding of many bacterial toxins occurs in this manner (Fig. 3). Recently, we reported that recognition of ganglioside GD3 on the cell membrane by a sialic acid-recognizing lectin, Siglec-7, is dependent on by the clustering mode of GD3 in lipid rafts (Hashimoto et al. 2019). Here, recruitment of GD3 to lipid rafts was determined by the presence/absence of hydroxyl group in ceramide moiety. Thus, the cluster formation of GD3 in lipid rafts confers its binding with Siglec-7, and finally sensitivity to the killing with NK cells (Hashimoto et al.

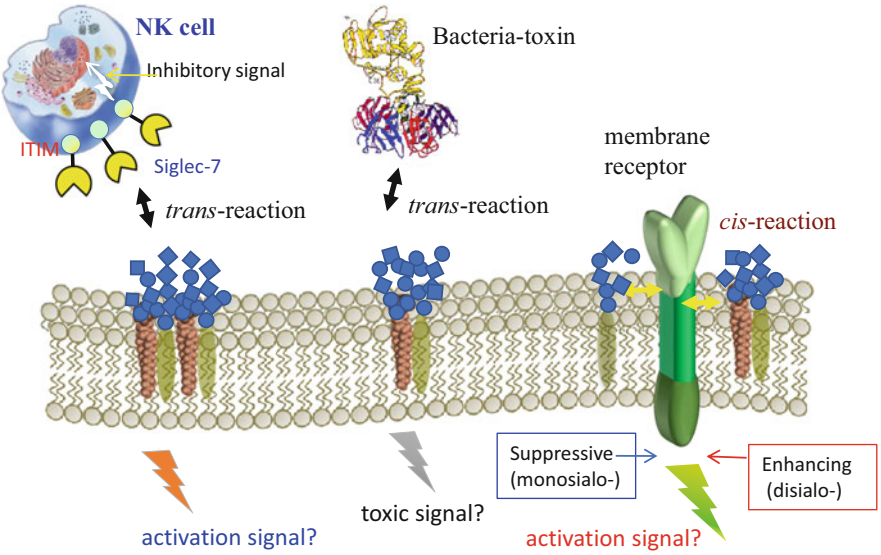


Fig. 3 *cic*- and *trans*-interaction of glycosphingolipids and ligand molecules. As representative examples, interaction between Siglec-7 on NK cells and sialylated membrane molecules is shown. Bacterial toxins such as Cholera-toxin or Vero-toxin are also ligands of glycolipids with *trans*-interaction. Membrane receptors such as growth factor receptors and integrins interact with glycolipids on the same membrane with *cis*-interaction

2019). How Siglec-7 recognizes only clustered GD3 in GEM/rafts remains to be investigated. Consequently, *trans*-interaction of cancer-associated glycosphingolipids and carbohydrate-recognizing molecules (lectins) should determine the survival intensity of the cancer cells in our bodies based on the modulation of the escape of cancer cells from immune surveillance of host immune systems.

Not only Siglecs, but other glycolipid-recognizing molecules such as soluble lectins and monoclonal antibodies also interact with cancer-associated glycosphingolipids on the cell surface in a *trans*-binding manner. In particular, an anti-GM3 monoclonal antibody, M2590, reacted only with GM3 with "high density" or "topographical distribution" (Sakiyama et al. 1987). This binding mode might indicate that mAb M2590 can recognize only GM3 concentrated in lipid rafts, while precise mechanisms remain to be clarified.

9 Application of Signal Studies for Novel Cancer Treatment

A number of trials have been performed to apply mouse and/or human monoclonal antibodies reactive with cancer-associated gangliosides for cancer treatment (Houghton et al. 1985; Irie and Morton 1986; Yamaguchi et al. 1987). Anti-ganglioside mAbs have been rigorously studied for their effects in the suppression

of mouse melanomas, human neuroblastomas, and human melanomas (Harel et al. 1990). Vaccine therapy with gangliosides or neutral glycolipids and various adjuvants have also been challenged (Yu et al. 2020; Ragupathi 1996; Livingston 1995; Sigal et al. 2022). They showed markedly nice results in some cases, but not so good in some cases. Anti-GD2 mAbs showed significant effects in extending remission intervals of neuroblastoma patients when used during remission phase (Kushner et al. 2015).

Generally, immune therapy of cancers toward cancer-associated glycosphingolipids can be successful by the definitely specific expression patterns, for example, "class 2" group in Old's proposal (Old 1981) (Table 1). Thus, the specificity in the expression of carbohydrate structures on malignant cells has strictly been required, while those with relatively high levels in malignant cells compared to normal cells and tissues have been also accepted as Rituximab (anti-CD20) used in B-cell lymphomas (McLaughlin et al. 1998).

Various novel strategies have been constructed to eradicate malignant tumors based on the expression/function analyses of cancer-associated glycosphingolipids, and been applied in clinical. GD3 and GD2 are synthesized via the action of GD3 synthase, and specifically activate several signals that are essential for the malignant phenotypes of cancers. Therefore, in addition to these gangliosides and ganglioside synthases, signaling molecules, which are located at the downstream of signaling pathway activated by those cancer-associated gangliosides and play a crucial role in the promotion of malignant phenotypes, can be targets for cancer therapy (Furukawa et al. 2006) (Fig. 4). As fundamental studies for the substantial basis for cancer treatment toward glycosylation machinery, sh-RNA- expressing plasmids of GD3 synthase were tried (Ko et al. 2006), showing excellent effects on the gene expression and cell phenotypes. Repeated transfection of anti-GD3 synthase siRNA into lung cancer cells resulted in the suppression of GD2 expression and also reduction in the cell growth and invasion activity. Moreover, cell apoptosis could be induced by the repeated siRNA transfection. Stable introduction of RNAi expression vector (sh-RNA) in human SCLC cell lines showed slower cell growth than the control cells in severe combined immunodeficiency mice (Ko et al. 2006).

Small interfering RNA(s) (siRNA(s)) mixed with atello-collagen was tried to suppress human malignant melanomas grafted on nu/nu mice (Makino et al. 2016). Combined siRNAs against two adaptor molecules, that is, p130Cas and paxillin, showed most efficient suppression activity of the tumor growth in mice compared with that of either siRNA (Makino et al. 2016). In agreement with the tumor suppression effects, reduction in Ki-67-positive cell number was demonstrated, suggesting that blockade of GD3-mediated growth signaling pathway by siRNAs might be a novel and promising therapeutic strategy against malignant melanomas.

As one of important signaling molecules located at the downstream of membrane gangliosides, FAK has been also considered as a promising target of cancer treatment (van Nimwegen and van de Water 2007; Sulzmaier et al. 2014; Zimmer and Steeg 2015; Chauhan and Khan 2021; Dawson et al. 2021). FAK plays an important role in signaling pathways, which are triggered by integrin-mediated cell adhesions and by growth factor receptors (Fig. 4) (van Nimwegen and van de Water 2007).

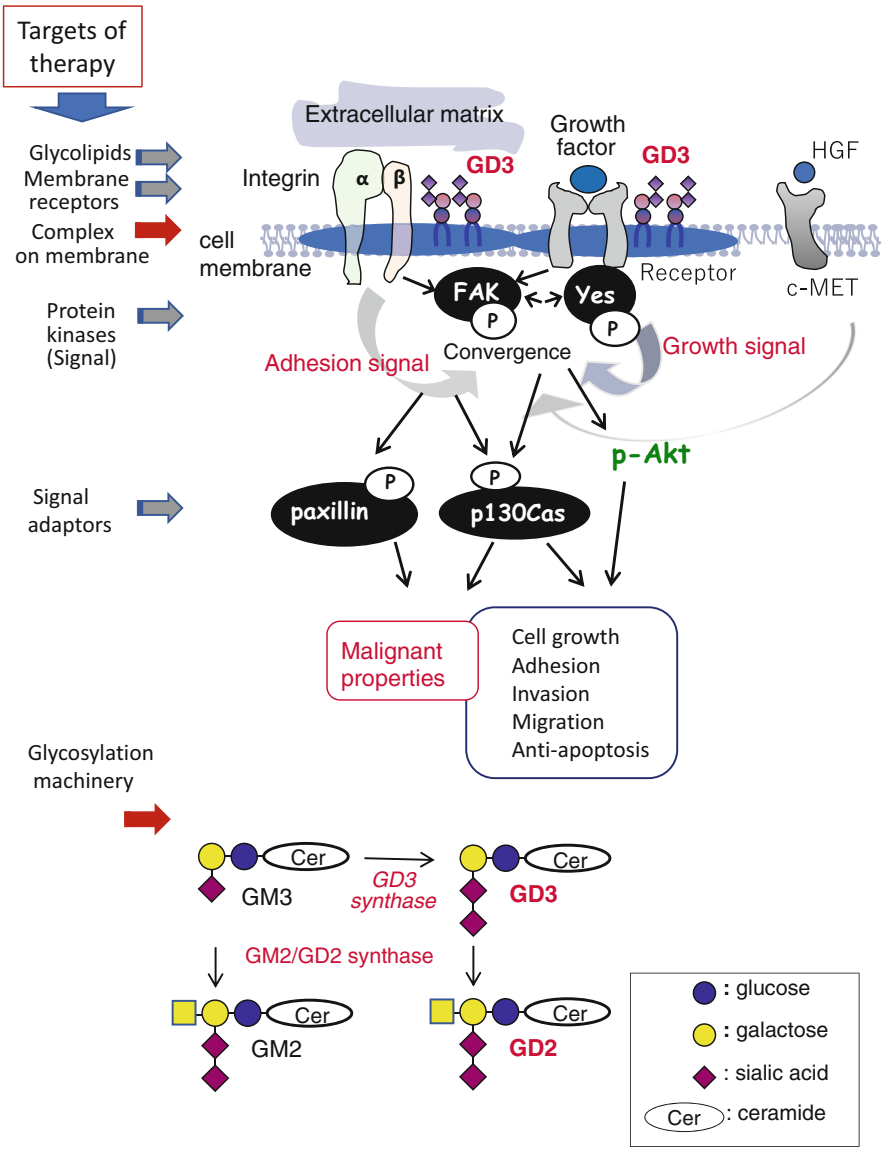


Fig. 4 Strategies for cancer treatment by targeting molecules activated by ganglioside expression including GSL-membrane molecular complexes. In addition to cancer-associated glycolipids on the membrane, their associating molecules on the cell surface and activated signaling molecules at the downstream of the signaling pathway can be targets of therapeutics for cancers (Furukawa et al. 2006)

FAK is highly expressed in some solid tumors and also in stromal cells of the tumor microenvironments (Sulzmaier et al. 2014), and considered to promote tumor progression and metastasis. Therefore, small molecular weight FAK inhibitors

have been developed, and been used in preclinical trials (Chauhan and Khan 2021). In particular, FAK is considered to be promising target of metastasis prevention trials (Zimmer and Steeg 2015), and targeting FAK in anticancer combination therapies is now being expected (Dawson et al. 2021). These studies well correspond with our past reports on GD3- or GD2-mediated signal activation in melanomas (Hamamura et al. 2008; Yesmin et al. 2021) or small cell lung cancers (Aixinjueluo et al. 2005).

10 Conclusion

Among 3 classes of tumor antigens listed in Table 1 (Old 1981), cancer-associated carbohydrate antigens should be included in "class 2" (shared) tumor antigens. Compared with "neoantigens" originating from gene mutations occurred in cancer cells, "class 2" antigens are much better targets for cancer immunotherapy. First of all, it is easy to construct therapeutic strategy because of common presence among similar cancers compared with class 1 (unique) antigens or neo-antigens. Secondly, it is possible to depend on relative abundance of antigens on cancers and to consider as targets of therapy, while they are expressed in some sites, some developmental stages of life with much lower levels. Finally, many successful examples have been reported so far and many trials are ongoing. Consequently, we are now challenging novel cancer treatment targeting molecules forming molecular complexes with cancer-associated glycosphingolipids on the membrane and/or in the cytoplasm (Kotani et al. 2008; Furukawa et al. 2008).

References

Aixinjueluo W, Furukawa K, Zhang Q, Hamamura K, Tokuda N, Yoshida S, Ueda R, Furukawa K (2005) Mechanisms for the apoptosis of small cell lung cancer cells induced by anti-GD2 monoclonal antibodies: roles of anoikis. J Biol Chem 280:29828–29836. https://doi.org/10.1074/jbc.M414041200

Azuma K, Tanaka M, Uekita T, Inoue S, Yokota J, Ouchi Y, Sakai R (2005) Tyrosine phosphorylation of paxillin affects the metastatic potential of human osteosarcoma. Oncogene 24:4754–4764. https://doi.org/10.1038/sj.onc.1208654

Carubia JM, Yu RK, Macala LJ, Kirkwood JM, Varga JM (1984) Gangliosides of normal and neoplastic human melanocytes. Biochem Biophys Res Commun 120(2):500–504. https://doi.org/10.1016/0006-291x(84)91282-8

Cavdarli S, Delannoy P, Groux-Degroote S (2020) O-acetylated gangliosides as targets for cancer immunotherapy. Cell 9(3):741. https://doi.org/10.3390/cells9030741

Cazet A, Bobowski M, Rombouts Y, Lefebvre J, Steenackers A, Popa I, Guérardel Y, Le Bourhis X, Tulasne D, Delannoy P (2012) The ganglioside G(D2) induces the constitutive activation of c-Met in MDA-MB-231 breast cancer cells expressing the G(D3) synthase. Glycobiology 22:806–816. https://doi.org/10.1093/glycob/cws049

Chauhan A, Khan T (2021) Focal adhesion kinase-An emerging viable target in cancer and development of focal adhesion kinase inhibitors. Chem Biol Drug Des 97(3):774–794. https://doi.org/10.1111/cbdd.13808

Chen HH, Fukumoto S, Furukawa K, Nakao A, Akiyama S, Urano T, Furukawa K (2003) Suppression of lung metastasis of mouse Lewis lung cancer P29 with transfection of the ganglioside GM2/GD2 synthase gene. Int J Cancer 103:169–176. https://doi.org/10.1002/ijc.10797

Cheresh DA, Rosenberg J, Mujoo K, Hirschowitz L, Reisfeld RA (1986) Biosynthesis and expression of the disialoganglioside GD2, a relevant target antigen on small cell lung carcinoma for monoclonal antibody-mediated cytolysis. Cancer Res 46:5112–5118

Dawson JC, Serrels A, Stupack DG, Schlaepfer DD, Frame MC (2021) Targeting FAK in anticancer combination therapies. Nat Rev Cancer 21(5):313–324. https://doi.org/10.1038/s41568-021-00340-6

De Giorgi U, Cohen EN, Gao H, Mego M, Lee BN, Lodhi A, Cristofanilli M, Lucci A, Reuben JM (2011) Mesenchymal stem cells expressing GD2 and CD271 correlate with breastcancer-initiating cells in bone marrow. Cancer Biol Ther 11:812–815. https://doi.org/10.4161/cbt.11.9.15178

Dippold WG, Lloyd KO, Li LT, Ikeda H, Oettgen HF, Old LJ (1980) Cell surface antigens of human malignant melanoma: definition of six antigenic systems with mouse monoclonal antibodies. Proc Natl Acad Sci U S A 77:6114–6118. https://doi.org/10.1073/pnas.77.10.6114

Dong Y, Ikeda K, Hamamura K, Zhang Q, Kondo Y, Matsumoto Y, Ohmi Y, Yamauchi Y, Furukawa K, Taguchi R, Furukawa K (2012) GM1 / GD1b / GA1 synthase expression results in the reduced cancer phenotypes with modulation of composition and raft-localization of gangliosides in a melanoma cell line. Cancer Sci 101:2039–2047. https://doi.org/10.1111/j.1349-7006.2010.01613.x

Esaki N, Ohkawa Y, Hashimoto N, Tsuda Y, Ohmi Y, Bhuiyan RH, Kotani N, Honke K, Enomoto A, Takahashi M, Furukawa K, Furukawa K (2018) ASC amino acid transporter 2, defined by enzyme-mediated activation of radical sources, enhances malignancy of GD2-positive small-cell lung cancer. Cancer Sci 109:141–153. https://doi.org/10.1111/cas.13448

Fredman P, von Holst H, Collins VP, Ammar A, Dellheden B, Wahren B, Granholm L, Svennerholm L (1986) Potential ganglioside antigens associated with human gliomas. Neurol Res 8:123–126. https://doi.org/10.1080/01616412.1986.11739744

Fujita A, Cheng J, Hirakawa M, Furukawa K, Kusunoki S, Fujimoto T (2007) Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. Mol Biol Cell 18:2112–2122. https://doi.org/10.1091/mbc.e07-01-0071

Fukumoto S, Mutoh T, Hasegawa T, Miyazaki H, Okada M, Goto G, Furukawa K, Urano T (2000) GD3 synthase gene expression in PC12 cells results in the continuous activation of TrkA and ERK1/2 and enhanced proliferation. J Biol Chem 275:5832–5838. https://doi.org/10.1074/jbc.275.8.5832

Furukawa K, Lloyd KO (1990) In: Ferrone S (ed) Human melanoma: from basic research to clinical application. Springer, Heidelburg, pp 15–30

Furukawa K, Akagi T, Nagata Y, Yamada Y, Shimotohno K, Cheung N-K, Shiku H, Furukawa K (1993) GD2 ganglioside on human T-lymphotropic virus type I-infected T cells: possible activation of beta-1,4-N-acetylgalactosaminyltransferase gene by p40tax. Proc Natl Acad Sci U S A 90:1972–1976. https://doi.org/10.1073/pnas.90.5.1972

Furukawa K, Tokuda N, Okuda T, Tajima O, Furukawa K (2004) Glycosphingolipids in engineered mice: insights into function. Semin Cell Dev Biol 15:389–396. https://doi.org/10.1016/j.semcdb.2004.03.006

Furukawa K, Hamamura K, Aixinjueluo W, Furukawa K (2006) Biosignals modulated by tumor-associated carbohydrate antigens: novel targets for cancer therapy. Ann N Y Acad Sci 1086:185–198. https://doi.org/10.1196/annals.1377.017

Furukawa K, Hamamura K, Nakashima H, Furukawa K (2008) Molecules in the signaling pathway activated by gangliosides can be targets of therapeutics for malignant melanomas. Proteomics 8:3312–3316. https://doi.org/10.1002/pmic.200800228

Furukawa K, Hamamura K, Ohkawa Y, Ohmi Y, Furukawa K (2012a) Disialyl ganglio-sides enhance tumor phenotypes with differential modalities. Glycoconj J 29:579–584. https://doi.org/10.1007/s10719-012-9423-0

Furukawa K, Ohkawa Y, Yamauchi Y, Hamamura K, Ohmi Y, Furukawa K (2012b) Fine tuning of cell signals by glycosylation. J Biochem 151:573–578. https://doi.org/10.1093/jb/mvs043

Furukawa K, Ohmi Y, Kondo Y, Ohkawa Y, Hashimoto N, Tajima O, Furukawa K (2014) The role of glycosphingolipifds. Lessons from knockout mice. In: Sillence D (ed) Lipid rafts. Properties, controversies and roles in signal transduction. Nova Publishers, New York, pp 1–20

Furukawa K, Ohmi Y, Ohkawa Y, Bhuiyan RH, Zhang P, Tajima O, Hashimoto N, Hamamura K, Furukawa K (2019) New era of research on cancer-associated glycosphingolipids. Cancer Sci 110:1544–1551. https://doi.org/10.1111/cas.14005

Furukawa K, Ohmi Y, Yesmin F, Tajima O, Kondo Y, Pu Zhang P, Hashimoto N, Ohkawa Y, Bhuiyan RH, Furukawa K (2020) Novel molecular mechanisms for roles of gangliosides in the nervous system elucidated by genetic engineering. Int J Mol Sci 11(21):E1906. https://doi.org/10.3390/ijms21061906

Groux-Degroote S, Guérardel Y, Delannoy P (2017) Gangliosides: structures, biosynthesis, analysis, and roles in cancer. Chembiochem 18:1146–1154. https://doi.org/10.1002/cbic.201600705

Hakomori S (1986) Tumor-associated glycolipid antigens, their metabolism and organization. Chem Phys Lipids 42:209–233

Hakomori S (1998) Cancer-associated glycosphingolipid antigens: their structure, organization, and function. Acta Anat (Basel) 161(1-4):79–90. https://doi.org/10.1159/000046451

Hakomori S (2003) Structure, organization, and function of glycosphingolipids in membrane. Curr Opin Hematol 10:16–24. https://doi.org/10.1097/00062752-200301000-00004

Hakomori SI (2010) Glycosynaptic microdomains controlling tumor cell phenotype through alteration of cell growth, adhesion, and motility. FEBS Lett 584:1901–1906. https://doi.org/10.1016/j.febslet.2009.10.065

Hakomori S, Yamamura S, Handa AK (1998) Signal transduction through glyco(sphingo)lipids. Introduction and recent studies on glyco(sphingo)lipid-enriched micro-domains. Ann N Y Acad Sci 845:1–10. https://doi.org/10.1111/j.1749-6632.1998.tb09657.x

Hamamura K, Furukawa K, Hayashi T, Hattori T, Nakano J, Nakashima H, Okuda T, Mizutani H, Hattori H, Ueda M, Urano T, Lloyd KO, Furukawa K (2005) Ganglioside GD3 promotes cell growth and invasion through p130Cas and paxillin in malignant melanoma cells. Proc Natl Acad Sci U S A 102:11041–11046. https://doi.org/10.1073/pnas.0503658102

Hamamura K, Tsuji M, Ohkawa Y, Nakashima H, Miyazaki S, Urano T, Yamamoto N, Ueda M, Furukawa K, Furukawa K (2008) Focal adhesion kinase as well as p130Cas and paxillin is crucially involved in the enhanced malignant properties under expression of ganglioside GD3 in melanoma cells. Biochim Biophys Acta 1780(3):513–519. https://doi.org/10.1016/j.bbagen.2007.11.002

Hamamura K, Tsuji M, Hotta H, Ohkawa Y, Takahashi M, Shibuya H, Nakashima H, Yamauchi Y, Hashimoto N, Hattori H, Ueda M, Furukawa K, Furukawa K (2011) Functional activation of Src family kinase yes protein is essential for the enhanced malignant properties of human melanoma cells expressing ganglioside GD3. J Biol Chem 286:18526–18537. https://doi.org/10.1074/jbc.M110.164798

Haraguchi M, Yamashiro S, Yamamoto A, Furukawa K, Takamiya K, Lloyd KO, Shiku H, Furukawa K (1994) Isolation of GD3 synthase gene by expression cloning of GM3 alpha-2,8-sialyltransferase cDNA using anti-GD2 monoclonal antibody. Proc Natl Acad Sci U S A 91:10455–10459. https://doi.org/10.1073/pnas.91.22.10455

Harel W, Shau H, Hadley CG, Morgan AC Jr, Reisfeld RA, Cheresh DA, Mitchell MS (1990) Increased lysis of melanoma by in vivo-elicited human lymphokine-activated killer cells after addition of antiganglioside antibodies in vitro. Cancer Res 50:6311–6315

Hashimoto N, Hamamura K, Kotani N, Furukawa K, Kaneko K, Honke K, Furukawa K (2012) Proteomic analysis of ganglioside-associated membrane molecules: substantial basis for molecular clustering. Proteomics 12(21):3154-3163 aa. https://doi.org/10.1002/pmic.201200279

Hashimoto N, Ito S, Tsuchida A, Bhuiyan RH, Okajima T, Yamamoto A, Furukawa K, Ohmi Y, Furukawa K (2019) The ceramide moiety of disialoganglioside (GD3) is essential for GD3 recognition by the sialic acid-binding lectin SIGLEC7 on the cell surface. J Biol Chem 294(28): 10833–10845. https://doi.org/10.1074/jbc.RA118.007083

Houghton AN, Mintzer D, Cordon-Cardo C, Welt S, Fliegel B, Vadhan S, Carswell E, Melamed MR, Oettgen HF, Old LJ (1985) Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma. Proc Natl Acad Sci U S A 82:1242–1246. https://doi.org/10.1073/pnas.82.4.1242

Indellicato R, Zulueta A, Caretti A, Trinchera M (2020) Complementary use of carbohydrate antigens Lewis a, Lewis b, and Sialyl-Lewis a (CA19.9 epitope) in gastrointestinal cancers: biological rationale towards a personalized clinical application. Cancers 12:1509. https://doi.org/10.3390/cancers12061509

Irie RF, Morton DL (1986) Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside GD2. Proc Natl Acad Sci U S A 83:8694–8698. https://doi.org/10.1073/pnas.83.22.8694

Iwasawa T, Zhang P, Ohkawa Y, Momota H, Wakabayashi T, Ohmi Y, Bhuiyan RH, Furukawa K, Furukawa K (2018) Enhancement of malignant properties of human glioma cells by ganglioside GD3/GD2. Int J Oncol 52:1255–1266. https://doi.org/10.3892/ijo.2018.4266

Kaneko K, Ohkawa Y, Hashimoto N, Ohmi Y, Kotani N, Honke K, Ogawa M, Okajima T, Furukawa K, Furukawa K (2016) Neogenin, defined as a GD3-associated molecule by enzyme-mediated activation of radical sources, confers malignant properties via intracytoplasmic domain in melanoma cells. J Biol Chem 291:16630–16643. https://doi.org/10.1074/jbc.M115.708834

Kawai K, Takahashi H, Watarai S, Ishizu H, Fukai K, Tanabe Y, Nose S, Kuroda S (1999) Occurrence of ganglioside GD3 in neoplastic astrocytes. An immunocytochemical study in humans. Virchows Arch 434:201–205. https://doi.org/10.1007/s004280050328

Ko K, Furukawa K, Takahashi T, Urano T, Sanai Y, Nagino M, Nimura Y, Furukawa K (2006) Fundamental study of small interfering RNAs for ganglioside GD3 synthase gene as a therapeutic target of lung cancers. Oncogene 25:6924–6935. https://doi.org/10.1038/sj.onc.1209683

Komura N, Suzuki KG, Ando H, Konishi M, Koikeda M, Imamura A, Chadda R, Fujiwara TK, Tsuboi H, Sheng R, Cho W, Furukawa K, Furukawa K, Yamauchi Y, Ishida H, Kusumi A, Kiso M (2016) Raft-based interactions of gangliosides with a GPI-anchored receptor. Nat Chem Biol 12:402–410. https://doi.org/10.1038/nchembio.2059

Kotani N, Gu J, Isaji T, Udaka K, Taniguchi N, Honke K (2008) Biochemical visualization of cell surface molecular clustering in living cells. Proc Natl Acad Sci U S A 105:7405–7409. https://doi.org/10.1073/pnas.0710346105

Kushner BH, Ostrovnaya I, Cheung IY, Kuk D, Kramer K, Modak S, Yataghene K, Cheung NK (2015) Prolonged progression-free survival after consolidating second or later remissions of neuroblastoma with Anti-G(D2) immunotherapy and isotretinoin: a prospective Phase II study. Onco Targets Ther 4:e1016704. https://doi.org/10.1080/2162402X.2015.1016704

Livingston PO (1995) Augmenting the immunogenicity of carbohydrate tumor antigens. Semin Cancer Biol 6(6):357–366. https://doi.org/10.1016/1044-579x(95)90005-5

Lloyd KO (1991) Humoral immune responses to tumor-associated carbohydrate antigens. Semin Cancer Biol 2:421–431

Lloyd KO, Old LJ (1989) Human monoclonal antibodies to glycolipids and other carbohydrate antigens: dissection of the humoral immune response in cancer patients. Cancer Res 49:3445–3451

Makino Y, Hamamura K, Takei Y, Buiyan RH, Ohkawa Y, Ohmi Y, Nakashima H, Furukawa K, Furukawa K (2016) A therapeutic trial of human melanomas with combined small interfering RNAs targeting adaptor molecules p130Cas and paxillin activated under expression of ganglioside GD3. Biochim Biophys Acta 1860:1753–1763. https://doi.org/10.1016/j.bbagen.2016.04.005

McLaughlin P, Grillo-López AJ, Link BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, Jain V, Ho AD, Lister J, Wey K, Shen D, Dallaire BK (1998) Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. J Clin Oncol 16:2825–2833. https://doi.org/10.1200/JCO.1998.16.8.2825

Merritt WD, Casper JT, Lauer SJ, Reaman GH (1987) Expression of GD3 ganglioside in childhood T-cell lymphoblastic malignancies. Cancer Res 47:1724–1730

Mitsuda T, Furukawa K, Fukumoto S, Miyazaki H, Urano T, Furukawa K (2002) Overexpression of ganglioside GM1 results in the dispersion of platelet-derived growth factor receptor from glycolipid-enriched microdomains and in the suppression of cell growth signals. J Biol Chem 277:11239–11246. https://doi.org/10.1074/jbc.M107756200

Miyagi T, Takahashi K, Yamamoto K, Shiozaki K, Yamaguchi K (2018) Biological and pathological roles of ganglioside sialidases. Prog Mol Biol Transl Sci 156:121–150. https://doi.org/10.1016/bs.pmbts.2017.12.005

Miyazaki H, Fukumoto S, Okada M, Hasegawa T, Furukawa K (1997) Expression cloning of rat cDNA encoding UDP-galactose:GD2 beta1,3-galactosyl-transferase that determines the expression of GD1b/GM1/GA1. J Biol Chem 272:24794–24799. https://doi.org/10.1074/jbc.272.40.24794

Nagata Y, Nagata Y, Yamashiro S, Yodoi J, Lloyd KO, Shiku H, Furukawa K (1992) Expression cloning of beta 1,4 N-acetylgalactosaminyltransferase cDNAs that determine the expression of GM2 and GD2 gangliosides. J Biol Chem 267:12082–12089

Nakashima H, Hamamura K, Houjou T, Taguchi R, Yamamoto N, Mitsudo K, Tohnai I, Ueda M, Urano T, Furukawa K, Furukawa K (2007) Overexpression of caveolin-1 in a human melanoma cell line results in dispersion of ganglioside GD3 from lipid rafts and alteration of leading edges, leading to attenuation of malignant properties. Cancer Sci 98:512–520. https://doi.org/10.1111/j.1349-7006.2007.00419.x

Nara K, Watanabe Y, Maruyama K, Kasahara K, Nagai Y, Sanai Y (1994) Expression cloning of a CMP-NeuAc:NeuAc alpha 2-3Gal beta 1-4Glc beta 1-1'Cer alpha 2,8-sialyltransferase (GD3 synthase) from human melanoma cells. Proc Natl Acad Sci U S A 91:7952–7956. https://doi.org/10.1073/pnas.91.17.7952

Nishio M, Fukumoto S, Furukawa K, Ichimura A, Miyazaki H, Kusunoki S, Urano T, Furukawa K (2004) Overexpressed GM1 suppresses nerve growth factor (NGF) signals by modulating the intracellular localization of NGF receptors and membrane fluidity in PC12 cells. J Biol Chem 279(32):33368–33378. https://doi.org/10.1074/jbc.M403816200

Ohkawa Y, Miyazaki S, Miyata M, Hamamura K, Furukawa K, Furukawa K (2008) Essential roles of integrin-mediated signaling for the enhancement of malignant properties of melanomas based on the expression of GD3. Biochem Biophys Res Commun 373:14–19. https://doi.org/10.1016/j.bbrc.2008.05.149

Ohkawa Y, Miyazaki S, Hamamura K, Kambe M, Miyata M, Tajima O, Ohmi Y, Yamauchi Y, Furukawa K, Furukawa K (2010) Ganglioside GD3 enhances adhesion signals and augments malignant properties of melanoma cells by recruiting integrins to glycolipid-enriched microdomains. J Biol Chem 285:27213–27223. https://doi.org/10.1074/jbc.M109.087791

Ohkawa Y, Momota H, Kato A, Hashimoto N, Tsuda Y, Kotani N, Honke K, Suzumura A, Furukawa K, Ohmi Y, Natsume A, Wakabayashi T, Furukawa K (2015) Ganglioside GD3 enhances invasiveness of gliomas by forming a complex with platelet-derived growth factor receptor α and yes kinase. J Biol Chem 290:16043–16058. https://doi.org/10.1074/jbc.M114.635755

Ohkawa Y, Zhang P, Momota H, Kato A, Hashimoto N, Ohmi Y, Bhuiyan RH, Farhana Y, Natsume A, Wakabayashi T, Furukawa K, Furukawam K (2021) Lack of GD3 synthase (St8sia1) attenuates malignant properties of gliomas in genetically engineered mouse model. Cancer Sci 112:3756–3768. https://doi.org/10.1111/cas.15032

Ohmi Y, Kambe M, Ohkawa Y, Hamamura K, Tajima O, Takeuchi R, Furukawa K, Furukawa K (2018) Differential roles of gangliosides in malignant properties of melanomas. PLoS One 13: e0206881. https://doi.org/10.1371/journal.pone.0206881

Okada M, Furukawa K, Yamashiro S, Yamada Y, Haraguchi M, Horibe K, Kato K, Tsuji Y, Furukawa K (1996) High expression of ganglioside alpha-2,8-sialyltransferase (GD3 synthase) gene in adult T-cell leukemia cells unrelated to the gene expression of human T-lymphotropic virus type I. Cancer Res 56:2844–2848

Old LJ (1981) Cancer immunology: the search for specificity – G.H.A. Clows Memorial Lecture. Cancer Res 41:361–375

Portoukalian J, Zwingelstein G, Doré JF (1979) Lipid composition of human malignant melanoma tumors at various levels of malignant growth. Eur J Biochem 94:19–23. https://doi.org/10.1111/j.1432-1033.1979.tb12866.x

Pukel CS, Lloyd KO, Travassos LR, Dippold WG, Oettgen HF, Old LJ (1982) GD3, a prominent ganglioside of human melanoma. Detection and characterisation by mouse monoclonal antibody. J Exp Med 155:1133–1147. https://doi.org/10.1084/jem.155.4.1133

Ragupathi G (1996) Carbohydrate antigens as targets for active specific immunotherapy. Cancer Immunol Immunother 43(3):152–157. https://doi.org/10.1007/s002620050316

Ravindranath MH, Muthugounder S, Presser N, Selvan SR, Portoukalian J, Brosman S, Morton DL (2004) Gangliosides of organ-confined versus metastatic androgen-receptor-negative prostate cancer. Biochem Biophys Res Commun 324(1):154–165. https://doi.org/10.1016/j.bbrc.2004.09.029

Saito M, Yu RK, Cheung N-K (1985) Ganglioside GD2 specificity of monoclonal antibodies to human neuroblastoma cell. Biochem Biophys Res Commun 127:1–7. https://doi.org/10.1016/s0006-291x(85)80117-0

Sakiyama H, Takahashi T, Hirabayashi Y, Taniguchi M (1987) Change in the topographical distribution of GM3 during cell spreading and growth: immunostaining with monoclonal antibody against GM3. Cell Struct Funct 12:93–105. https://doi.org/10.1247/csf.12.93

Sasaki K, Kurata K, Kojima N, Kurosawa N, Ohta S, Hanai N, Tsuji S, Nishi T (1994) Expression cloning of a GM3-specific alpha-2,8-sialyltransferase (GD3synthase). J Biol Chem 269:15950–15956

Sato M, Shimada S, Watanabe M, Kawasaki Y, Sato T, Morozumi K, Mitsuzuka K, Ito A (2020) Expression of ganglioside disialosyl globopentaosyl ceramide in prostate biopsy specimens as a predictive marker for recurrence after radical prostatectomy. Tohoku J Exp Med 252(1):1–8. https://doi.org/10.1620/tjem.252.1

Schultz G, Cheresh DA, Varki NM, Yu A, Staffileno LK, Reisfeld RA (1984) Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. Cancer Res 44:5914–5920

Shibuya H, Hamamura K, Hotta H, Matsumoto Y, Nishida Y, Hattori H, Furukawa K, Ueda M, Furukawa K (2012) Enhancement of malignant properties of human osteosarcoma cells with disialyl gangliosides GD2/GD3. Cancer Sci 103(9):1656–1664. https://doi.org/10.1111/j.1349-7006.2012.02344.x

Siddiqui B, Buehler J, DeGregorio MW, Macher BA (1984) Differential expression of ganglioside GD3 by human leukocytes and leukemia cells. Cancer Res 44:5262–5265

Sigal DS, Hermel DJ, Hsu P, Pearce T (2022) The role of Globo H and SSEA-4 in the development and progression of cancer, and their potential as therapeutic targets. Future Oncol 18(1): 117–134. https://doi.org/10.2217/fon-2021-1110

Simons K, Gerl MJ (2010) Revitalizing membrane rafts: new tools and insights. Nat Rev Mol Cell Biol 11:688–699. https://doi.org/10.1038/nrm2977

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569–572. https://doi.org/10.1038/42408

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31–39. https://doi.org/10.1038/35036052

Sonnino S, Prinetti A (2010) Gangliosides as regulators of cell membrane organization and functions. Adv Exp Med Biol 688:165–184. https://doi.org/10.1007/978-1-4419-6741-1_12

Stanley P (2016) What have we learned from glycosyltransferase knockouts in mice? J Mol Biol 428:3166–3182. https://doi.org/10.1016/j.jmb.2016.03.025

Sulzmaier FJ, Jean C, Schlaepfer DD (2014) FAK in cancer: mechanistic findings and clinical applications. Nat Rev Cancer 14(9):598–610. https://doi.org/10.1038/nrc3792

Tsurifune T, Ito T, Li XJ, Yamashiro S, Okada M, Kanematsu T, Shiku H, Furukawa K (2000) Alteration of tumor phenotypes of B16 melanoma after genetic remodeling of the ganglioside profile. Int J Oncol 17:159–165

van Nimwegen MJ, van de Water B (2007) Focal adhesion kinase: a potential target in cancer therapy. Biochem Pharmacol 73(5):597–609. https://doi.org/10.1016/j.bcp.2006.08.011

Vantaku V, Donepudi SR, Ambati CR, Jin F, Putluri V, Nguyen K, Rajapakshe K, Coarfa C, Battula VL, Lotan Y, Putluri N (2017) Expression of ganglioside GD2, reprogram the lipid metabolism and EMT phenotype in bladder cancer. Oncotarget 8(56):95620–95631. https://doi.org/10.18632/oncotarget.21038. eCollection

Vukelić Z, Kalanj-Bognar S, Froesch M, Bîndila L, Radić B, Allen M, Peter-Katalinić J, Zamfir AD (2007) Human gliosarcoma-associated ganglioside composition is complex and distinctive as evidenced by high-performance mass spectrometric determination and structural characterization. Glycobiology 17:504–515. https://doi.org/10.1093/glycob/cwm012

Wikstrand CJ, Fredman P, Svennerholm L, Bigner DD (1994) Detection of glioma-associated gangliosides GM2, GD2, GD3, 3′-isoLM1 3′,6′-isoLD1 in central nervous system tumors in vitro and in vivo using epitope-defined monoclonal antibodies. Prog Brain Res 101:213–223. https://doi.org/10.1016/s0079-6123(08)61951-2

Yamaguchi H, Furukawa K, Fortunato SR, Livingston PO, Lloyd KO, Oettgen HF, Old LJ (1987) Cell-surface antigens of melanoma recognized by human monoclonal antibodies. Proc Natl Acad Sci U S A 84:2416–2420. https://doi.org/10.1073/pnas.84.8.2416

Yesmin F, Bhuiyan RH, Ohmi Y, Yamamoto S, Kaneko K, Ohkawa Y, Zhang P, Hamamura K, Nai-Kong CV, Kotani N, Honke K, Okajima T, Kambe M, Tajima O, Furukawa K, Furukawa K (2021) Ganglioside GD2 enhances malignant phenotypes of melanoma cells by co-operating with integrins. Int J Mol Sci 23:423. https://doi.org/10.3390/ijms23010423

Yoshida S, Fukumoto S, Kawaguchi H, Sato S, Ueda R, Furukawa K (2001) Ganglioside G(D2) in small cell lung cancer cell lines: enhancement of cell proliferation and mediation of apoptosis. Cancer Res 61:4244–4252

Yu J, Hung JT, Wang SH, Cheng JY, Yu AL (2020) Targeting glycosphingolipids for cancer immunotherapy. FEBS Lett 594(22):3602–3618. https://doi.org/10.1002/1873-3468.13917

Zhang Q, Furukawa K, Chen HH, Sakakibara T, Urano T, Furukawa K (2006) Metastatic potential of mouse Lewis lung cancer cells is regulated via ganglioside GM1 by modulating the matrix metalloprotease-9 localization in lipid rafts. J Biol Chem 281:18145–18155. https://doi.org/10.1074/jbc.M512566200

Zhang P, Ohkawa Y, Yamamoto S, Momota H, Kato A, Kaneko K, Natsume A, Yesmin F, Ohmi Y, Okajima T, Wakabayashi T, Furukawa K, Furukawa K (2021) St8sia1-deficiency in mice alters tumor environments of gliomas, leading to reduced disease severity. Nagoya J Med Sci 83(3):535–549. https://doi.org/10.18999/nagjms.83.3.535

Zimmer AS, Steeg PS (2015) Meaningful prevention of breast cancer metastasis: candidate therapeutics, preclinical validation, and clinical trial concerns. J Mol Med (Berl) 93(1):13–29. https://doi.org/10.1007/s00109-014-1226-2

Tumor Progression through Interaction of Mucins with Lectins and Subsequent Signal Transduction

Shungo Iwamoto, Naoki Itano, and Hiroshi Nakada

Abstract Glycans bearing on cell surface glycoproteins are often altered in tumor cells. A variety of aberrant glycans, tumor-associated carbohydrate antigens, are typically expressed on mucins. Some lectins recognizing these antigens have been identified mainly on immune cells and play a role as inhibitory receptors.

Binding of soluble lectins and/or membrane-bound lectins (lectin receptors) to membrane-bound mucins such as MUC1 triggers signaling in tumor cells. On the other hand, membrane-bound and/or soluble mucins play a role as ligands for lectin receptors, initiating signal transduction in immune cells. These mucin-lectin networks contribute to both tumor progression and immune suppression. In this chapter, first, we mainly describe our current data on MUC1-mediated signaling through interactions with galectin-3 and Siglec-9, and then review lectin receptor-mediated signaling triggered by binding of mucins and its biological significance.

1 Introduction

1.1 General

Aberration of the cell surface carbohydrate moieties has been established as a universal characteristic of malignant cells. However, the functional implications of the changes in cell surface glycoconjugates in malignancy are not completely understood yet. Mucins are heavily *O*-glycosylated glycoproteins and are present on luminal epithelial surfaces. They are involved in protection and lubrication of the cell surface. Several lines of evidence have demonstrated that membrane-bound mucins are also involved in more complex biological events such as cell signaling, cell adhesion, and cell differentiation. Of the membrane-bound mucins, MUC1 has been extensively studied and is viewed as a tumor-associated molecule because of its

S. Iwamoto · N. Itano · H. Nakada (✉)
Department of Molecular Bioscience, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan
e-mail: hnakada@cc.kyoto-su.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_9

frequent overexpression and aberrant glycosylation in various cancers (Gendler and Spicer 1995; Kudelka et al. 2015). Generally, mucins carry a variety of tumor-associated carbohydrate antigens, some of which are referred to as tumor glycocodes. Mucins readily come into contact with not only soluble lectins but also lectin receptors expressed on infiltrating cells in a tumor microenvironment and lectins could play a role as plausible, physiological ligands for membrane-bound mucins such as MUC1. Necessarily, lectin receptors initiate signal transduction through interaction with soluble and/or membrane-bound mucins. It has been demonstrated that lectins also play critical roles in cell trafficking, signaling, and inflammation.

1.2 Mucin Family

Mucins, a large family of extremely *O*-glycosylated glycoproteins, are produced by the epithelia of the respiratory, gastrointestinal, and reproductive tracts (Hollingsworth and Swanson 2004). Deregulation of mucin expression has been demonstrated to be one of the most prominent characteristics of numerous types of cancers (Gendler and Spicer 1995; Hollingsworth and Swanson 2004). Based on their structural characteristics and physiological fates, mucins have been classified into two groups: membrane-bound mucins (MUC1, MUC3A, MUC3B, MUC4, MUC12-18, MUC20, MUC21) and secreted mucins (MUC2, MUC5AC, MUC5B, MUC6-8, MUC19). Membrane-bound mucins are localized to the apical surface of normal epithelial cells, but in malignant cells, they are distributed on the entire cell surface due to a loss of apical-basal polarity. Secretory mucins cover the epithelial surface and play a role as a protective barrier. The protein backbone of mucins is characterized by tandemly repeated motifs (tandem repeats). Due to a variable number of tandem repeats, mucins frequently exhibit polymorphism both within and between individuals (Gendler and Spicer 1995; Moniaux et al. 2001). The tandem repeat regions are especially rich in serine and threonine, which are possible *O*-glycosylation sites (Strous and Dekker 1992).

1.3 Expression of Tumor-Associated Carbohydrate Antigens Due to Aberrant Glycosylation

Among tumor-associated carbohydrate antigens, the most common alterations occur in the mucin-type *O*-glycans. Typical alterations are under-glycosylation and addition of aberrant terminal sugars. They are synthesized in tumor cells, generating novel oligosaccharides that are highly expressed in various cancers, whereas they are weakly expressed in normal tissues. Figure 1 shows the biosynthesis of four major core structures and tumor-associated carbohydrate antigens including tumor glycocodes. A large family of polypeptide GalNAc-transferases (ppGalNAc-T) initiates

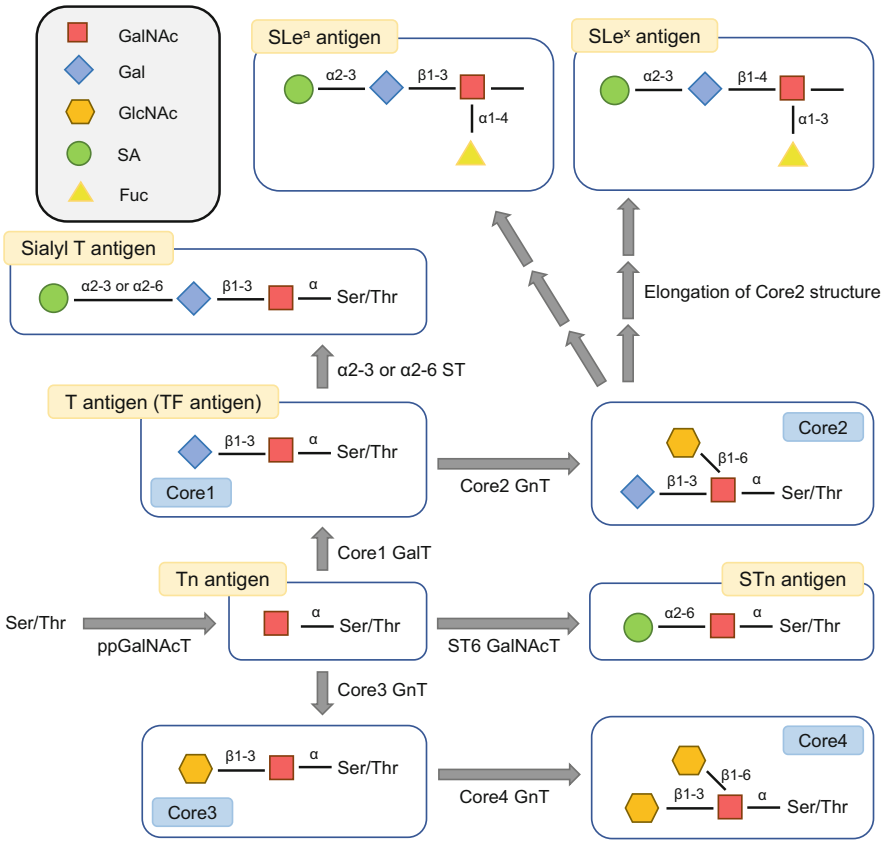


Fig. 1 Biosynthesis of *O*-glycan and cancer-associated carbohydrate antigens. Every sugar of *O*-linked mucin-type oligosaccharides is transferred enzymatically from a specific nucleotide sugar. The biosynthesis of mucin-type *O*-glycans is initiated by the addition of *N*-acetylgalactosamine (GalNAc) to Ser or Thr residues, and then extended into Core 1, Core 2, Core 3, and Core 4 structures through the transfer of the indicated sugar. The arrows indicate the enzyme involved in each reaction, and linkage lines indicate the attachment for each sugar. *ppGalNAcT* polypeptide *N*-acetylgalactosamine transferase; *Core 1 GalT* Core 1 galactose transferase; *Core 2 GnT* Core 2 *N*-acetylglucosamine transferase; *Core 3 GnT* Core 3 *N*-acetylglucosamine transferase; *Core 4 GnT* Core 4 *N*-acetylglucosamine transferase; *GalNAcT N*-acetylgalactosamine transferase; *ST* sialyltransferase; *SLe*^{a} sialylLewis^a; *SLe*^{x} sialylLewis^x

mucin-type *O*-glycosylation and synthesizes GalNAcα–Ser/Thr (Bennett et al. 2012). It appears that each member of the multiple ppGalNAc-T has a slightly different but overlapping substrate specificity, and the repertoire of ppGalNAc-T expressed in a cell controls the *O*-glycosylation pattern. Specific glycosyltransferases then add sugar residues to yield four main *O*-glycan core structures containing GlcNAc, Gal, or GalNAc substitutions of GalNAcα–Ser/Thr. This elongation often does not occur in cancer cells, generating GalNAcα–Ser/Thr, which is referred to as Tn antigen (Dahr et al. 1974). Core 1 is synthesized by core

1 β1,3 Gal transferase (Core 1 β3 Gal-T) (Ju et al. 2002). Core1, as a terminal structure often referred to as the T antigen (TF antigen), is prevalent in cancer cells (Thomas and Winzler 1969). Core 1β3 Gal-T requires a specific chaperon, Core 1 β3 Gal-T-specific molecular chaperon (Cosmic), for proper folding and functional activity (Ju and Cummings 2002; Ju et al. 2008; Sun et al. 2011). Eventually, a lack of Cosmic expression increases the levels of Tn and sialylTn antigens (Ju et al. 2008; Hofmann et al. 2015). It has also been demonstrated that ppGalNAc-T1, which is one of the glycosyltransferases involved in the initiation of the mucin-type *O*-glycosylation and normally restricted to the Golgi apparatus, can be relocalized to the endoplasmic reticulum, leading to an increase of truncated *O*-glycans (Gill et al. 2010, 2011). Core 2 β1,6 GlcNAc transferase (GlcNAc-T) converts the T antigen to the core 2 structure. A number of related β1,6 GlcNAc-Ts appear to exist with distinct substrate specificities. Core 2 β1,6 GlcNAc-T2 (mucin-type) activity is highly detected in normal colon tissues. Core 2 β1,6 GlcNAc-T1 (leukocyte-type) seems to be elevated in colon cancer tissues. Core 3 is synthesized by core 3 β1,3 GlcNAc-T (Iwai et al. 2002). This enzyme activity is found only in mucin-secreting tissues and is low in colon cancer tissues. Core 4 β1,6 GlcNAc-T activity is found in colonic tissues and cancer cell lines. Because synthesis of core 3 precedes that of core 4, synthesis of core 4 is limited by low activity and the tissue distribution of core 3 β1,3 GlcNAc-T. Some truncated *O*-glycans are sialylated mainly due to the overexpression of α2,6 and α2,3 sialyltransferases (SA-T) (Lu and Gu 2015). Thus, sialyTn and sialyT antigens are commonly found in tumor tissues. Tn antigen is found in more than 90% of breast cancers, and expression of sialyTn antigen is also detected in more than 80% of epithelial cancers and associated with a poor prognosis (Munkley 2016). Tn and T antigens are coexpressed on approximately 85% of adenocarcinomas (Karanikas et al. 1997). Tn and sialylTn antigens are also coexpressed in various malignant tissues including colon, breast, prostate, respiratory, pancreas, ovarian, and gastric cancers (Julien et al. 2012; Freitas et al. 2019). T antigen is found in 90% of various cancers, and is prevalent in gastric, colon, pancreas, ovary, and prostate cancers (Cazet et al. 2010). The four main *O*-glycan core structures can be extended, branched, and terminated by many different families of glycosyltransferases. The Core 2 structure is extended by the addition of β1,4 Gal to GlcNAc (type 2 chain) by β1,4 Gal-T or by the addition of β1,3 Gal to GlcNAc (type 1 chain) by β1,3 Gal-T. SialylLewis^{x} (SLe^{x}) antigen is synthesized by further elongation of the type 2 chain by the addition of α 2,3 SA to Gal by α2,3 SA-T and of α1,3 Fuc to GlcNAc by α1,3 Fuc-T. SialylLewis^{a} (SLe^{a}) antigen is synthesized by elongation of the type 1 chain by the addition of α2,3 SA to Gal by α2,3 SA-T and of α1,4 Fuc to GlcNAc by α1,4 Fuc-T. SLe^{a} antigen is detected in more than 50% of lung, liver, breast, pancreas, stomach, and colon cancers. SLe^{x} antigen is found in more than 90% of colon, esophagus, ovary, breast, pancreas, and stomach cancers.

1.4 Lectins

Three major lectin families, namely, S-type, C-type and I-type lectins, are expressed in our body. Galectins belong to S-type lectins and share conserved carbohydrate recognition domains (CRD) of ~130 amino acids with affinity for β-galactoside (Sindrewicz et al. 2016). So far, 15 galectins have been identified in mammalian and classified into three groups; The proto-type group with one CRD (Galectin-1,-2,-5,-7,-10,-13,-14, and -15), the tandem-repeat type with two CRDs connected by a nonconserved linker (Galectin-4, -6, −8, -9, and -12), and the chimeric type with one CRD and a nonlectin N-terminal domain (Galectin-3) (Cooper and Barondes 1999; Ruvolo 2016; Sciacchitano et al. 2018). Galectins are synthesized on free ribosomes in the cytosol. In spite of lacking a signal peptide, they are secreted outside the cell via a nonclassical pathway and thus are found on the cell surface and/or in the extracellular space (Sato and Hughes 1994). Galectins possess a variety of functions depending on their intra- or extracellular distributions. Intracellular galectins may have an effect on cell signaling by interacting with signaling proteins in the cytosol such as RAS proteins and β-catenin, and RNA splicing through binding of components of the spliceosome complex in the nuclei (Liu et al. 2002; Elad-Sfadia et al. 2004; Shimura et al. 2004; Fritsch et al. 2016). Extracellular galectins are exposed to a variety of glycoconjugates. Some galectins are capable of forming lattices through their cross-linking. The formation of a cell surface glycoprotein lattice has been demonstrated to influence cell signaling, cell migration, and cell adherence (Hönig et al. 2018). Galectin-3 was originally identified in murine peritoneal macrophages and named Mac-2 (Ho and Springer 1982). Later, it was detected in a variety of tumor tissues. The expression level and distribution of galectin-3 are different in various human tumors and are correlated with tumor progression (Liu and Rabinovich 2005). The concentration of circulating galectin-3 in the serum of cancer patients is higher than that in healthy individuals (Iurisci et al. 2000; Saussez et al. 2008). Because galectin-3 can act as a bivalent or multivalent ligand (Yang et al. 1998; Ahmad et al. 2004), it can cross-link cell surface glycoconjugates, which, like many other receptor-ligand systems, can trigger a cascade of transmembrane signaling (Nieminen et al. 2007; Gao et al. 2014). C-type lectin receptors such as dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (Geijtenbeek et al. 2000; Kwon et al. 2002) and macrophage galactose lectin (MGL) (Higashi et al. 2002; van Vliet et al. 2013) play critical roles in immune regulation. Although C-type lectin receptors were initially considered to function as scavenger receptors that bind and take up pathogens upon recognition of particular carbohydrate structures, it has been revealed that some C-type lectin receptors may play a role in cell adhesion, signaling, or uptake of antigen. Thus, they are considered to be involved in the recognition and uptake of aberrantly glycosylated tumor antigens. Some lectin receptors could also play a role as signaling molecules, the signaling being initiated through ligation of the glyco-code expressing mucins and/or mucin-type *O*-glycan-carrying glycoproteins. The uptake of the glyco-code carrying antigens through these lectin receptors primarily results in exhibiting an

immunosuppressive or tolerogenic phenotype. Although many lectin-receptor-mediated signaling pathways have not fully resolved, it is notable that glycan-lectin interactions downmodulate immune responses, contributing to tumor progression through immune evasion.

Siglecs (sialic acid binding Ig-like lectins) belong to I-type lectins and are mostly expressed on immune cells (Macauley et al. 2014; van de Wall et al. 2020). In human, 14 different functional Siglecs have been identified. The conserved Siglecs, that is, Siglec-1 (sialoadhesin), Siglec-2 (CD22), Siglec-4 (MAG), and Siglec-15, have orthologs in different mammalian species. On the other hand, CD33 (Siglec-3)-related Siglecs have undergone rapid evolutionary adaptation. This family includes Siglec-3, Siglec-5, Siglec-6, Siglec-7, Siglec-8, Siglec-9, Siglec-10, and Siglec-11 (Angata 2018). In mice, no orthologs of human CD33-related Siglecs have been found, but functional paralogues have been defined (Läubli et al. 2014). Although Siglecs recognize sialoglycans commonly, the range of binding specificity varies with different Siglecs. For instance, Siglec-9 exhibits a broad binding specificity (Padler-Karavani et al. 2014), whereas Siglec-8 recognizes rather limited number of structures of sialoglycans including sulfate groups (Pröpster et al. 2016). An increased density of sialoglycans facilitates interaction with inhibitory Siglec receptors on immune cells and modulates the immune response to cancer. Because mucins possess a variety of oligosaccharides including sialoglycans, they are expected to act as a scaffold for the binding of lectins.

2 Mucin-Mediated Tumor Progression

2.1 Expression of MUC1

MUC1 is a membrane-bound and high molecular weight (>400 kDa) glycoprotein that is expressed at the basal level in most epithelial cells. Epithelial cells lose their polarity associated with transformation, and thereby, MUC1 is expressed throughout the entire cell membrane of tumor cells, allowing it to interact with receptors or ligands that are restricted to the lateral and basal borders in normal cells (Nath and Mukherjee 2014; Chugh et al. 2015). In malignant tumors, MUC1 is overexpressed (Chugh et al. 2015), and its expression level is correlated with a poor prognosis (Kufe et al. 1984; Kufe 2009). Actually, more than 90% of breast carcinomas overexpress MUC1 (Gendler 2001). Using pancreas, breast and myeloma cell lines, rat fibroblasts, and a MUC1 transgenic model of human breast cancer, overexpression of MUC1 has shown to induce transformation and increase tumorigenicity (Li et al. 2003; Schroeder et al. 2004; Singh et al. 2007).

2.2 *Structure of MUC1*

A schematic structure of MUC1 is shown in Fig. 2. After synthesis as a single polypeptide, it is autocleaved in the endoplasmic reticulum into two subunits (N-and C-terminal subunits) that form a stable heterodimeric complex held together through a noncovalent interaction on the cell surface (Levitin et al. 2005; Macao et al. 2006). The N-terminal domain (MUCI-ND), which is a large extracellular domain, contains variable numbers of 20 amino acid tandem repeats including five serine/threonine residues (Gendler et al. 1988; Siddiqui et al. 1988). MUC1 is an extremely huge membrane-bound molecule and is easily accessible to lectins because of its rod-like structure, which is longer (200–500 nm) than typical cell surface molecules (~30 nm) (Becker et al. 1989). Thus, cell-cell and cell-extracellular matrix interactions are sterically hindered by masking of adhesion molecules such as E-cadherin (Wesseling et al. 1996). The C-terminal domain (MUC1-CD) is comprised of a short extracellular domain, a transmembrane domain, and a cytoplasmic tail. MUC1-CD is involved in not only anchoring of the MUC1-ND to the membrane but also various signaling events. MUC1-CD has several serine, threonine, and tyrosine phosphorylation sites that can interact with some key signaling molecules such as β-catenin, p53, and NF-κB (Singh and Hollingsworth 2006). These signaling molecules are translocated with MUC1-CD to the nucleus. The Cys-Gln-Cys (CQC) motif in

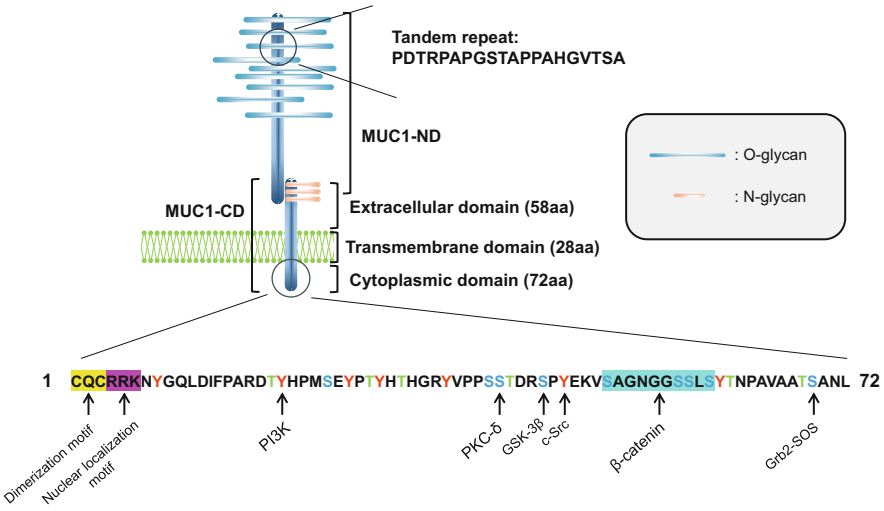


Fig. 2 Schematic structure of MUC1. MUC1 is a high molecular weight membrane glycoprotein. The N-terminal ectodomain (MUCI-ND) consists of variable numbers (25–100) of 20-amino acid (AA) tandem repeats that are extremely *O*-glycosylated. The C-terminal domain (MUC1-CD) is composed of a 58-AA extracellular domain, a 28-AA transmembrane domain, and a 72-AA cytoplasmic domain. MUC1-CD interacts with cytoplasmic proteins related to signal transduction such as β-catenin. Several signalings also occur in a MUC1-CD phosphorylation–dependent manner

MUC1-CD is required for its homodimerization and interaction with nuclear translocation–related proteins such as importin and nucleoporin (Raina et al. 2012; Leng et al. 2007).

2.3 *Growth Factor Receptor–Mediated Phosphorylation of MUC1 and Subsequent Signaling*

It has been extensively demonstrated that phosphorylation occurs through the interaction of MUC1 with specific receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (MET), and platelet-derived growth factor receptor β (PDGFR β) (Schroeder et al. 2001; Singh and Hollingsworth 2006; Singh et al. 2007, 2008; Kufe 2009, 2013; Bafna et al. 2010; Roy et al. 2011; Apostolopoulos et al. 2015; Hanson and Hollingsworth 2016). The cooperation of MUC1 with growth factor receptors is shown schematically in Fig. 3a. The ErbB family consists of four transmembrane receptors: ErbB1 (EGFR), ErbB2 (Her2), ErbB3 (Her3), and ErbB4 (Her4). MUC1 interacts with all four members of the ErbB family of growth factor receptor tyrosine kinases through MUC1-CD and potentiates ErbB-dependent signal transduction (Li et al. 2001; Schroeder et al. 2001). Ramasamy et al. reported that galectin-3 is responsible for the interaction between MUC1 and EGFR (Ramasamy et al. 2007). Binding of a ligand to ErbB1 triggers phosphorylation of MUC1-CD at tyrosine in the YEKV motif (Li et al. 2001). Phosphorylation of MUC1-CD enhances its affinity for the c-Src SH2 domain and β-catenin, and facilitates nuclear translocation. Interaction of

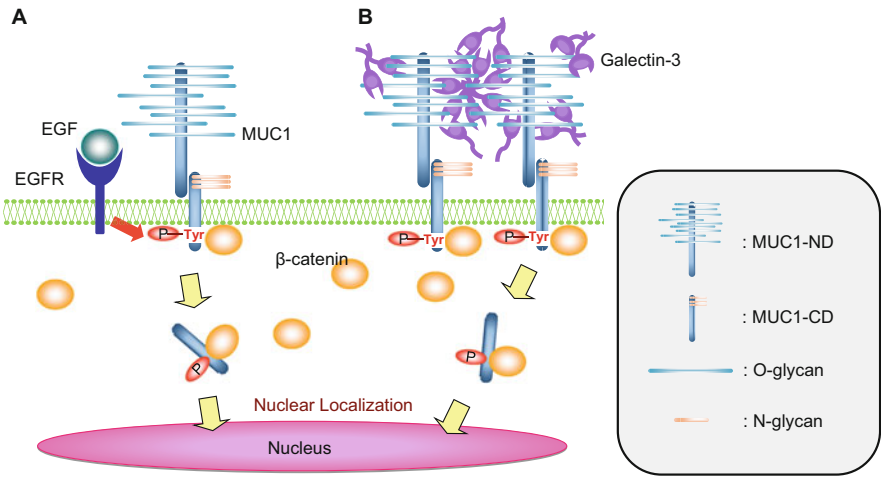


Fig. 3 MUC1-mediated signaling. (**a**) MUC1-CD is phosphorylated through the interaction with a receptor tyrosine kinase such as epidermal growth factor receptor, leading to various signaling pathways. (**b**) MUC1-mediated signaling also occurs through the binding of galectin-3 to MUC1

the c-Src-SH2 domain with the YEKV motif inhibits the binding of glycogen synthase kinase 3β (GSK3β), which can also phosphorylate MUC1-CD and reduce its interaction with β-catenin. The tyrosine in the YEKV motif is also phosphorylated by c-Src, which can facilitate binding with β-catenin directly and prevent interaction with GSK3β. Thus, MUC1-CD functions by integrating signals from the EGFR and Wnt pathways. ErbB2-ErbB3 heterodimerization, which is stimulated by the ErbB3 ligand, heregulin, enhances the interaction of MUC1-CD with γ-catenin and targeting of the MUC1-CD/γ-catenin complex to the nucleus (Li et al. 2003). MUC1 also interacts with fibroblast growth factor receptor 3 (FGFR3). On stimulation with FGF in breast cancer cells, tyrosine of MUCI-CD is phosphorylated, followed by an increase in the binding of MUC1 to β-catenin and targeting of the MUC1/β-catenin complex to the nucleus (Ren et al. 2006). MUC1-CD binds directly to estrogen receptor α (ERα) DNA-binding domain, and thereby stabilizes ERα by blocking its ubiquitination and degradation. Thus, MUC1 also controls cell proliferation through interaction with ERα (Wei et al. 2006). MUC1-CD contains 22 potential phosphorylation sites, giving rise to a wide array of potential phosphorylation patterns. The specific pattern of phosphorylated sites may designate association of MUC1-CD with different downstream effectors, such as growth factor receptor-bound protein-2 (GRB2)/son of sevenless (SOS), to initiate downstream signaling cascades (Pandey et al. 1995; Bitler et al. 2010). Studies on MUC1-related events that promote tumor progression are still accumulating. Shukla et al. reported that MUC1 stabilizes and activates hypoxia-inducible factor-1α to regulate metabolism in pancreatic cancer (Shukla et al. 2017).

2.4 MUC1-Mediated Signaling Through the Binding of Galectin-3 to MUC1

As described above, it has been reported that MUC1 associates with several growth factor receptors and potentiates growth factor–dependent signaling through MUC1-CD. However, in these studies, MUC1-CD but not MUC1-ND has been focused. A few studies have demonstrated endogenous ligands for MUC1. Binding of intracellular adhesion molecule can initiate calcium signaling (Rahn et al. 2004). Another study showed that MUC1 signaling could be triggered by binding of *Pseudomonas aeruginosa* or its flagellin protein, leading to activation of MAP kinase (Lillehoj et al. 2004). As described above, MUC1-ND has an extended structure and truncated short oligosaccharides such as T antigen. Thus, MUC1 is expected to act as a particularly effective scaffold for the presentation of oligosaccharide chains to lectins.

Binding of Galectin-3 to MUC1

It has been reported that MUC1 is a natural ligand of endogenous galectin-3 and T antigen carried on MUC1 interacts with galectin-3 (Yu et al. 2007). These studies have focused on the effect of galectin-3 on cell behavior such as cell adhesion. The interaction of MUC1 with galectin-3 induces the polarization of MUC1 on the cell surface, leading to the exposure of cell adhesion molecules. The exposed adhesion molecules facilitate hemolytic aggregation of tumor cells and adhesion of tumor cells to endothelial cells (Yu et al. 2007; Zhao et al. 2009, 2010). Overexpression of MUC1 in A375 melanoma cells inhibits adhesion to endothelial cells, but addition of galectin-3 increases adhesion (Zhao et al. 2009). Adhesion molecules such as CD44, which are normally masked by MUC1, are exposed on treatment with galectin-3 (Zhao et al. 2009, 2010). Piyush et al. reported that binding of galectin-3 to MUC1 increases MUC1-EGFR association, leading to enhanced and prolonged EGFR activation (Piyush et al. 2017). As described above, MUC1-mediated out-side-in signaling has been limited. We tried to elucidate the effect of galectin-3 on MUC1-mediated signaling mainly using human MUC1 gene transfectants (Mori et al. 2015). With respect to the binding site for galectin-3, different results have been reported. Galectin-3 can interact with either *O*-glycans branches on MUC1-ND (Yu et al. 2007) or *N*-glycans on MUC1-ND (Ramasamy et al. 2007). 3 T3/MUC1 cells were established by introducing human MUC1 cDNA into mouse 3 T3 cells. MUC1 was prepared from 3 T3/MUC1 cells and denatured with 4 M guanidine-HCl, followed by separation of MUC1-ND and -CD by CsCl centrifugation. It was revealed that galectin-3 could bind to MUC1-ND but not to MUC1-CD, this being consistent with the finding that MUC1 binds to to galectin-3 even after *N*-glycanase treatment (Tanida et al. 2014). Because HCT116 cells (a human colorectal cancer cell line) express negligible levels of mucins including MUC1, MUC1 cDNA-transfected HCT116 cells (HCT116/MUC1 cell) were also prepared (Mori et al. 2015). Among the galectin family, galectin-1 and -3 were endogenously expressed in HCT116 cells and their expression levels were not affected by MUC1 cDNA transfection. Binding of galectin-3 to MUC1 was confirmed by a coimmunoprecipitation assay using antigalectin-3 antibodies and anti-MUC1-ND antibodies. Expression of T antigen on MUC1 was also demonstrated by a pull-down assay using peanut agglutinin-conjugated agarose. Extracellular galectins bound to the cell surface glycoconjugates were labeled with biotin, and biotin-labeled galectin-1 and -3 were detected by immunoblotting. About twofold of biotin-labeled galectin-3 was detected in HCT116/MUC1 cells compared with in HCT116/Mock cells, whereas the level of biotin-labeled galectin-1 on the surface of HCT116/MUC1 cells was similar to that on HCT116/Mock cells. These results suggest that galectin-3 but not galectin-1 is retained on the cell surface through its binding to MUC1. In this context, it has been demonstrated that although the galectin family exhibits high affinity for β-galactoside-containing carbohydrate chains such as T antigen, its affinity for each β-galactoside-containing carbohydrate chain differs among galectins (Hirabayashi et al. 2002). In the case of T antigen, galectin-3

exhibits higher affinity than galectin-1 does (Bian et al. 2011). Therefore, although both galectin-1 and -3 in HCT116 cells may be secreted extracellularly, galectin-3 is likely to preferentially bind to T antigen carried on MUC1-ND. Immunochemical observation of MUC1 and galectin-3 on the cell surface demonstrated that the uneven distribution of galectin-3 on the cell surface seems to parallel that of MUC1, suggesting the association of galectin-3 with MUC1 on the cell surface. The distribution of MUC1 in tumor tissues also almost completely coincided with that of galectin-3. In contrast, in nonmalignant tissues, MUC1 was detected slightly, and its distribution seemed to be different from that of galectin-3, probably due to limited distribution of MUC1 on the apical surface and/or nonexpression of T antigen carried on MUC1 in nonmalignant tissues.

Enhanced Phosphorylation of ERK1/2 and Akt and Tumor Progression

It has been extensively demonstrated that MUC1 potentiates growth factor-triggered signal transduction and multiple signal transduction pathways are initiated by phosphorylation of tyrosine residues. First, phosphorylation of MUC1-CD on treatment with galectin-3 was investigated (Mori et al. 2015). To exclude the possible effects of endogenous galectin-3 and growth factors included in serum and/or produced by the cells, cells were preincubated in serum-free lactose-containing medium and extensively washed with the same medium before an experiment. Treatment of HCT116/MUC1 cells with galectin-3 for 4 min enhanced the phosphorylation of MUC1-CD about 2.3 fold. Furthermore, because A549 cells (a human lung cancer cell line) and SKOV3 cells (a human ovary cancer cell line) expressed negligible and considerable levels of MUC1, respectively, a human *MUC1* gene transfectant (A549/MUC1) and control cell (A549/Mock), and human *MUC1* gene knockdown cells (SKOV3/Si) and control cells (SKOV3/Scr) have also been established. These cells were treated with or without galectin-3 for 10 min, followed by detection of phosphorylated ERK1/2 and Ark by immunoblotting. Except for phosphorylated ERK1, which was under a detectable level, phosphorylation of ERK2 and Akt was enhanced more prominently in MUC1-expressing cells as compared with the respective control cells. Phosphorylation of Akt was enhanced more prominently on treatment with galectin-3 in HCT116/MUC1 cells (~fourfold) than in HCT116/Mock (~twofold), suggesting that although galectin-3 triggered MUC1-mediated signaling mainly contributes to enhanced phosphorylation of Akt, other unknown cell surface glycoproteins may also be involved in Akt phosphorylation. Although HCT116/MUC1 cells express EGFR, this galectin-3-dependent phosphorylation of ERK1/2 and Akt was detected even in the presence of an EGFR inhibitor, indicating that enhancement of ERK1/2 and Akt phosphorylation is involved in the galectin-3-triggered signaling pathway but not in EGFR-related events.

On treatment with monovalent galectin-3, which was prepared by digestion with collagenase type VII (Morris et al. 2004; Dam et al. 2005), phosphorylation of ERK1/2 and Akt was markedly reduced, indicating that the multivalency of

galectin-3 is critical for initiation of MUC1-mediated signaling. This finding is consistent with the reports that galectin-3 can cross-link cell surface glycoconjugates, initiating a cascade of transmembrane signaling events (Yang et al. 1998; Ahmad et al. 2004; Nieminen et al. 2007). When galectin-3 is present freely or bound to a monovalent binding site, the major form of galectin-3 is monomeric (Morris et al. 2004). In contrast, when galectin-3 binds to multivalent binding sites, galectin-3 forms oligomers through association with the galectin-3 N-terminal domain and behaves as a multivalent lectin (Yang et al. 1998; Ahmad et al. 2004). Because *O*-glycans expressed on MUC1, which are the binding sites for galectin-3, may be carried repeatedly on the tandem repeats of the MUC1 core protein, MUC1 may be one of the most preferable ligands for oligomerization of galectin-3. It is generally agreed that the binding affinity of galectin-3 for oligosaccharides carried on proteins including MUC1 increases dramatically by clustering of its protein (Dam et al. 2005). The significance of the MUC1 extracellular domain has been also demonstrated that MUC1-mediated signaling is dependent on the number of tandem repeats (Cascio et al. 2011). In addition, homooligomerization of MUC1-CD through interaction of a juxtamembrane region, the CQC motif, in MUC1-CD is required for MUC1-mediated signal transduction (Kufe 2009; Raina et al. 2012; Kharband et al. 2014). Clustering of MUC1 through binding of galectin-3 to MUC1 may facilitate oligomerization of MUC1-CD. It is well known that activation of the MAPK and PI3K/Akt signaling pathways leads to a variety of cellular functions such as proliferation and motility (Engelman 2009). Analyses using MTT assays demonstrated that proliferation of HCT116/MUC1 and A549/MUC1 cells was enhanced by forced expression of MUC1 as compared with that of the respective control cells, whereas proliferation of SKOV3/Si cells was downmodulated by silencing of MUC1 expression as compared with that of SKOV3/Scr cells. Similar results were also obtained using anchorage-dependent clonogenic assays. Wound healing assays revealed that cell motility was also facilitated by forced expression of MUC1 in HCT116 cells and decreased by silencing of MUC1 in SKOV3 cells. These results indicate that cell motility is also correlated with the expression level of MUC1. To further examine whether or not endogenous galectin-3 contributes to promotion of malignancy in MUC1-expressing cells, HCT116/MUC1 cells expressing a lower level of galectin-3 by treatment of HCT116/MUC1 cells with galectin-3 shRNA (HCT116/MUC1-Gal-3/Si) were established. Expectedly, silencing of galectin-3 in HCT116/MUC1 cells also downregulated the proliferation and motility. Furthermore, downregulation of the cell motility of HCT116/MUC1-Gal-3/Si cells recovered to the level in control cells on treatment with exogenous galectin-3. These results suggest that cooperation of MUC1 and galectin-3 may be necessary for promotion of the tumor malignancy, this being consistent with the fact that tumor malignant phenotypes such as cell growth and motility change with the levels of MUC1 and galectin-3 expression. With these data as a background, galectin-3 antagonists have been searched for. It has been reported that galectin-3 antagonists, GCS-100 and lactulose-L-leucin, which are oligosaccharides prepared from citrus pectin and modified, and synthetic glucosamine, respectively, inhibit several malignant progression in multiple myeloma and prostate cancer cells (Chauhan et al. 2005;

Streetly et al. 2010). Thus, we synthesized glycopolymers carrying several repeats of *N*-acetyllactosamine (LacNAc), which exhibited high affinity for galectin-3. Because the binding affinity of LacNAc for galectin-3 was dependent on the number of repetitive LacNAcs (Hirabayashi et al. 2002), artificial glycopolymers carrying single, tandem, and triplet LacNAc repeats were prepared. HCT116/MUC1 and HCT116/Mock cells were cultured for 72 h in the presence or absence of these glycopolymers, and then the effects of the glycopolymers were examined. The glycopolymers carrying triplet LacNAc repeats inhibited cell proliferation most significantly. Proliferation of HCT116/MUC1 cells was more markedly inhibited than that of HCT116/Mock cells. These results suggest that galectin-3 bound to MUC1 was excluded by glycopolymers, and thereby, cell proliferation was inhibited.

Enhanced Recruitment of β-Catenin to MUC1 on Treatment with Galectin-3

As described in Sect. 2.3, growth factor receptor–mediated phosphorylation of MUC1 enhances the recruitment and subsequent translocation of β-catenin to the nucleus. The effect of galectin-3-engagement on the recruitment of β-catenin to MUC1 was also examined (Tanida et al. 2014). After complete exclusion of growth factors and galectin-3 contained in the serum and produced by 3 T3/MUC1 cells, the cells were treated with galectin-3 and then coimmunoprecipitation assay was performed. β-Catenin and MUC1-CD were detected in the immunoprecipitates with anti-MUC1-CD and anti-β-catenin antibodies, respectively.

The level of recruited β-catenin peaked at 30–40 min after treatment with galectin-3 and then decreased to the control level by 50 min. Thus, it is considered that the constitutive activation of MUC1-mediated signaling in an autocrine/paracrine manner caused by ligation of galectin-3 promotes uncontrolled tumor cell malignancy in a tumor microenvironment. As shown schematically in Fig. 3b, we consider that this may be an alternative pathway that promotes tumor cell malignancy even though growth factors such as EGF and FGF and/or their receptors are absent and/or nonfunctional.

With respect to the biological roles of the two pathways, we speculate that whether or not each pathway works depends on the distribution of related proteins on the cell surface as shown schematically in Fig. 4. In polarized epithelial tissues at the initial stage of carcinogenesis, growth factors are still secreted to the apical side, but their receptors are localized basolaterally, which prevents ligation of the growth factors (Vermeer et al. 2003; Tanos and Rodriguez-Boulan 2008). From this special-temporal view, derangement of the epithelial sheet structure due to loss of organized cell-cell adhesion may be a prerequisite for growth-factor-triggered tumor progression. In contrast, MUC1 is localized apically in the premalignant phase before dissemination of epithelial cells, which also prevents the association of growth factor receptors with MUC1. In addition, galectin-3 is expressed in a variety of epithelial cells and secreted predominantly to the apical side. Moreover, this lectin is

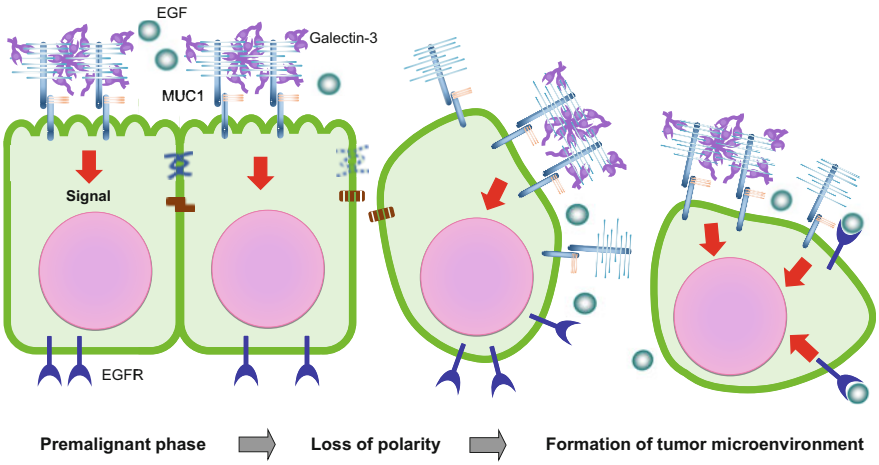


Fig. 4 Special-temporal change of signaling pathways with tumor progression. In polarized epithelia, MUC1 and EGF are transported to the apical side, while EGFR is transported basolaterally. Thus, neither ligation of EGF with EGFR nor association of MUC1 with EGFR could be performed. Loss of cell polarity due to derangement of cell-cell interaction is prerequisite for EGFR/MUC1 cooperation and ligation of EGF with EGFR

recycled by the cells and plays a role in apical protein trafficking (Delacour et al. 2006; Hönig et al. 2015). Thus, cooperation of galectin-3 with MUC1 may be functional temporally and specially even in the early phase of tumor progression. In a tumor-microenvironment formed after dissemination of epithelial cells, MUC1 may be able to cooperate with growth factor receptors in addition to the cooperation of MUC1 with galectin-3.

2.5 MUC1-Mediated Induction of Urokinase Type Plasminogen Activator (uPA)

It has been reported that high levels of MUC1 are detected in invasive and metastatic carcinomas among various types of tumors (Kufe et al. 1984; Qu et al. 2004), suggesting that MUC1 contributes to cell invasion and metastasis as well as cell proliferation. In addition to the function of MUC1 as a physical barrier for cell-cell and cell-extracellular matrix (ECM) interactions, which enhance cell invasion, MUC1 may be involved in cell invasion more directly through the signaling associated with MUC1. Degradation of the ECM by proteolytic enzymes is a prerequisite for cancer cell invasion and metastasis. Among proteolytic enzymes, uPA and matrix metalloproteinases (MMPs) play critical roles in degradation of the ECM, leading to tumor cell invasion and metastasis (Danø et al. 2005). The level of uPA expression is correlated with the invasive potential and a poor prognosis in various tumor cells including breast, stomach, and colon cancer (Sidenius and Blasi

2003). uPA is produced by cancer cells and/or stromal cells as a proenzyme uPA (pro-uPA). uPA secreted into the tumor microenvironment is converted to an active form after its binding to the uPA receptor (uPAR). uPA is involved in activation of plasminogen and MMPs. Plasminogen is cleaved by uPA to form plasmin. The plasmin can degrade ECM components directly and/or indirectly through MMP-2 and -9, thereby facilitating cell invasiveness (Danø et al. 2005). NF-κB is one of the transcription factors associated with MUC1-CD, and uPA is included among the NF-κB target genes (Ahmad et al. 2007). The promoter of uPA is highly conserved and contains multiple functional elements including the NF-κB element (Irigoyen et al. 1999; Cicek et al. 2009). Thus, it was speculated that the underlying molecular mechanism of MUC1-induced invasiveness could be explained by uPA, the expression of which may be promoted by association of MUC1-CD with NF-κB. NF-κB is composed of two subunits (RelA/p65 and p50), and a complex with an inhibitor of IκB is formed in the cytoplasm. Degradation of IκB by the IκB kinase (IKK) complex induces NF-κB activation, followed by translocation of the activated NF-κB p65 subunit to the nucleus (Hayden and Ghosh 2008).

Correlated Expression of uPA with MUC1

Based on the background described above, we investigated MUC1-promoted cancer cell invasion (Mori et al. 2014). To detect some mRNAs induced by forced expression of MUC1 in HCT116 cells, we performed microarray analysis of mRNA using HCT116/MUC1 and HCT116/Mock cells. Among some genes that were differentially expressed in the two types of cells, it was revealed that uPA was elevated 2.4 fold in HCT116/MUC1 cells as compared with in HCT116/Mock cells. Elevated expression of uPA protein in HCT116/MUC1 cells was also demonstrated by immunoblotting and observed immunochemically. Furthermore, in MUC1-overexpressing A549 cells (A549/MUC1) and MUC1 knockdown SKOV3 cells (SKOV3/Si), and the respective control cells, the level of uPA changed in parallel with the level of MUC1. Furthermore, immunostaining of uPA and MUC1 in human stomach, colon, pancreas, and breast tumor tissues revealed that the distribution of uPA coincided with that of MUC1 in tumor tissues, but not in nonmalignant tissues.

Formation of the MUC1-CD/NF-κB p65 Complex and Translocation of the Complex to the Nucleus

It has been reported that the promoter of uPA contains multiple functional elements including NF-κB ones (Irigoyen et al. 1999; Cicek et al. 2009), and that MUC1-CD interacts with the NF-κB p65 subunit and modulates the activity of the NF-κB pathway in breast cancers (Ahmad et al. 2007). Formation of the MUC1-CD/NF-κB p65 complex in HCT116/MUC1 cells was confirmed by a coimmunoprecipitation assay. As described in the previous section, we considered that binding of endogenous galectin-3 to MUC1 induces the recruitment of NF-κB p65 to MUC1-CD. After

exclusion of cell surface galectin-3 as described above, HCT116/MUC1 cells were treated with recombinant galectin-3. The level of NF-κB p65 immunoprecipitated with anti-MUC1-CD antibodies increased clearly, peaking at 20 min after the incubation with galectin-3. Cytoplasmic and nuclear fractions were prepared from HCT116 and SKOV3, and the respective MUC1-overexpressing (HCT116/MUC1) and MUC-knockdown (SKOV3/Si) cells, and MUC1-CD and NF-κB p65 were detected by immunoblotting. In addition to the cytoplasmic fraction, MUC1-CD was also detected in the nuclear fractions of HCT116/MUC1 and SKOV3/Scr cells. Although similar levels of NF-κB p65 were detected in the cytoplasmic fractions of these cells, irrespective of the different levels of MUC1, the levels of NF-κB p65 were increased and decreased in the nuclear fractions of HCT116/MUC1 and SKOV3/Si cells, respectively, suggesting that NF-κB p65 transported to the nucleus increased in parallel with the level of MUC1. Furthermore, colocalization of MUC1-CD with NF-κB p65 in the nucleus was observed on confocal imaging.

Recruitment of the MUC1-CD/NF-κB p65 Complex to the uPA Promoter

In order to determine whether or not the MUC1-CD/NF-κB p65 complex is responsible for transcriptional regulation of uPA, a ChIP assay was performed. The uPA promoter contains two potential NF-κB p65 binding sites. Chromatin was immunoprecipitated from cells expressing different levels of MUC1 with anti-NF-κB p65 antibodies. The uPA promoter region containing two consensus NF-κB p65 binding sites and a control other region were amplified by PCR, and the intensities of the bands were determined. Occupancy of NF-κB p65 at the NF-κB p65 binding sites was enhanced ~fourfold in HCT116/MUC1 cells as compared with in HCT116/Mock cells. Similarly, silencing of MUC1 in SKOV3 cells markedly decreased the occupancy of NF-κB p65 in SKOV3/Si cells. Furthermore, re-ChIP assays revealed that the uPA promoter was co-occupied by NF-κB p65 and MUC1-CD in HCT116/MUC1 and SKOV3/Scr cells, suggesting that MUC1-CD promotes recruitment of NF-κB p65 to the uPA promoter.

Enhancement of uPA Transcriptional Activity Through Binding of the MUC1-CD/NF-κB p65 Complex to the uPA Promoter

Regulation of uPA transcriptional activity by the MUC1-CD/NF-κB p65 complex was analyzed by means of luciferase assays. The uPA transcriptional activity was increased (~1.8 fold) in HCT116/MUC1 cells and decreased by about 50% in SKOV3/Si cells, as compared with in the respective control cells. To further confirm that this transcriptional activation was induced through the formation of the MUC1-CD/NF-κB p65 complex on the uPA promoter, plasmids in which mutations were introduced into the two NF-κB binding consensus sequences were constructed, and then the plasmids were transfected into cells. Expectedly, the uPA transcriptional activity was reduced to the level in control cells by the mutations of the NF-κB

binding sites in the uPA promoter region in HCT116/MUC1 and SKOV3/Scr cells, indicating that binding of the MUC1-CD/NF-κB p65 complex to the uPA promoter actually induces transcription of uPA. In this context, it has been reported that expression of uPA is regulated by multiple elements, among which β-catenin is also included. As described above, MUC1-CD also forms a complex with β-catenin, β-catenin thereby being stabilized (Yamamoto et al. 1997; Li et al. 2001; Huang et al. 2005; Ren et al. 2006; Tanida et al. 2013). Thus, there is a possibility that the MUC1-CD/β-catenin complex regulates the uPA expression. It is also well known that the TCF/LEF (T cell factor/lymphoid enhancer factor) transcription factor is necessary for the MUC1-CD/β-catenin complex to bind to the promoters of β-catenin/TCF signaling target genes. Thus, mutation of the TCF-binding sites on the promoter may alter uPA transcriptional activity. To examine this possibility, a plasmid in which mutations were introduced into the TCF4-binding consensus sequence was constructed, and then the plasmid was transfected into MUC1-expressing cells (HCT116/MUC1 and SKOV3/Scr). Using this system, it was demonstrated that uPA transcriptional activity was not altered by mutation of TCF4-binding sites in the uPA promoter region. Thus, it was revealed that transcription of uPA was regulated by the MUC1-CD/NF-κB p65 complex but not by the MUC1-CD/β-catenin complex.

MUC1-Dependent Cell Invasion Through Elevation of uPA Expression

uPA is secreted into the extracellular space and plays a crucial role directly and/or indirectly in cancer cell invasion. The amount of secreted uPA in the conditioned medium and the activity of uPA and MMPs were determined by means of ELISA and zymography assays, respectively. The level of uPA increased ~tenfold in HCT116/MUC1 cells as compared with in HCT116/Mock cells and decreased partially in SKOV3 cells on the knockdown of MUC1. Expectedly, higher levels of uPA and MMP-2/9 activities were detected in MUC1-expressing cells (HCT116/MUC1 and SKOV3/Scr) as compared with in the respective control cells. Next, invasion and migration assays were performed using Matrigel- and fibronectin-coated chambers, respectively, and the ratio of invading cells versus migrating cells was calculated to exclude the effect of other cell properties such as proliferation. The ratio increased (~twofold) in HCT116/MUC1 cells and decreased by about 70% in SKOV3/Si cells as compared with in the respective control cells. Furthermore, invasion assays using cells treated with GM6001, amiloride and JSH-23, which are inhibitors of MMPs, uPA and NF-κB, were performed, respectively. Treatment with all three inhibitors decreased the invasion of HCT116/MUC1 cells by about 40%. It was noted that NF-κB inhibitor JSH-23 effectively inhibited the cell invasion. To examine the effect of JSH-23 on the NF-κB/MUC1-CD complex formation, a coimmunoprecipitation assay was performed using JSH-23-treated HCT116/MUC1 cells. Expectedly, NF-κB p65 and MUC1-CD co-immunoprecipitated with anti-MUC1-CD antibodies and anti-NF-κB p65 antibodies, respectively were clearly decreased. Eventually, the level of NF-κB p65/

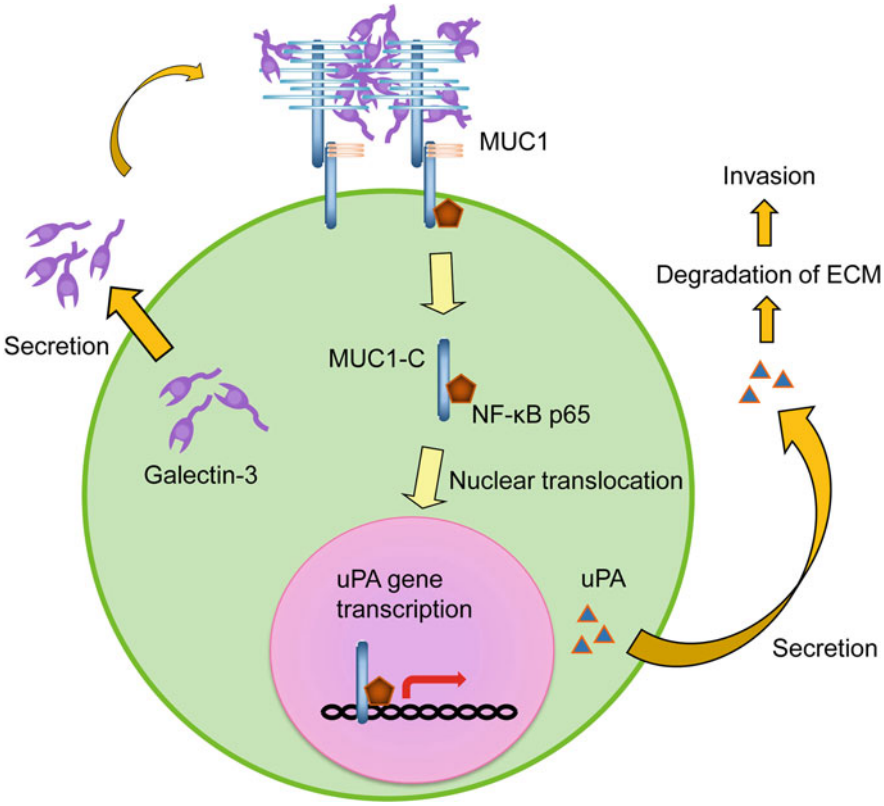


Fig. 5 MUC1-mediated induction of uPA. Binding of galectin-3 to MUC1 triggers formation of the MUC1-CD/NF-κB p65 complex and subsequent activation of NF-κB signaling pathways, leading to enhancement of the uPA production

MUC1-CD complex in the nucleus decreased on the treatment with JSH-23. Taken together, these results are summarized schematically in Fig. 5. Galectin-3-triggered formation of the MUC1-CD/NF-κB p65 complex activates the NF-κB signaling pathway, and subsequently promotes the production of uPA and activation of MMP2/9, leading to enhanced invasiveness of cancer cells. This cascade is a novel mechanism by which MUC1 promotes cell invasion, and inhibition of this cascade may be one of the useful targets for treating cancer.

2.6 MUC1-Mediated Induction of Trophoblast Cell Surface Antigen 2 (Trop-2)

As described in Sect. 2.5.1, we tried to detect some genes induced in HCT116 cells by forced expression of MUC1. The level of Trop-2 mRNA was enhanced as well as

that of uPA mRNA in HCT116/MUC1 cells as compared with HCT116/Mock cells. Trop-2 is a 36-kDa transmembrane glycoprotein and is highly expressed in a variety of epithelial cancer cells (Cubas et al. 2009; Trerotola et al. 2013a; Xie et al. 2016). Enhanced expression of Trop-2 in tumor tissues is correlated with tumor malignancy and poor survival of patients with various cancers (Cubas et al. 2009; Trerotola et al. 2013b; Zeng et al. 2016). Thus, Trop-2 has been identified as an oncogene leading to tumorigenesis and invasiveness (Wang et al. 2008).

MUC1-Dependent Expression of Trop-2

As described above, we searched for differentially expressed mRNAs in HCT116/MUC1 and HCT116/Mock cells. In addition to that of uPA mRNA, the level of Trop-2 mRNA was enhanced markedly about 47-fold in HCT116/MUC1 cells as compared with in HCT116/Mock cells (Yamashita et al. 2019). Trop-2 promoter activity was elevated ~twofold in HCT116/MUC1 cells in comparison with in HCT116/Mock cells. Immunochemical staining also demonstrated the induction of Trop-2 in HCT116/MUC1 cells. Furthermore, immunostaining of tumor tissues showed that MUC1 and Trop-2 exhibited similar distributions. MCF7 cells, a human breast cancer cell line, expressed detectable levels of MUC1 and Trop-2. Expression of Trop-2 was down-modulated on treatment of MCF7 cells with MUC1 siRNA, suggesting the MUC1-dependent induction of Trop-2 expression.

Involvement of Transcriptional Factor Sp1 in Trop-2 Expression

Among various transcriptional inhibitors such as JSH-23 (NF-κB inhibitor) and PNU-74654 (β-catenin inhibitor), the level of Trop-2 mRNA was down-modulated only by mithramycin A (Sp1 inhibitor). It has been demonstrated that Sp1 plays a critical role by upregulating genes that induce tumor progression, and that the binding of Sp1 to a GC-rich region of DNA is inhibited by mithramycin A, resulting in down-regulation of multiple genes that promote tumor progression (Blume et al. 1991; Rao et al. 2016). Downmodulation of Trop-2 mRNA on treatment with mithramycin A is consistent with the fact that Trop-2 promoter contains the GC-rich region (Lin et al. 2012). The effect of mithramycin A on Trop-2 promoter activity was also determined by means of a luciferase assay. About 60% of Trop-2 transcriptional activity decreased on the treatment with mithramycin A in HCT116/MUC1 cells, but no effect was detected in that of HCT116/Mock cells. In addition to mithramycin A, expression of Trop-2 was also decreased in MCF-7 cells through the knockdown of Sp1 on treatment with Sp1 siRNA. With respect to the correlation between MUC1 and Sp1, a coimmunoprecipitation assay demonstrated the formation of a MUC1 and Sp1 complex, suggesting that the MUC1/Sp1 complex may be involved in the induction of Trop-2.

Induction of Trop-2 Through the Binding of Galectin-3 to MUC1

As described above, binding of galectin-3 to MUC1 initiated multiple signaling pathways. After excluding cell surface galectin-3 as described above, MCF-7 cells were incubated with recombinant galectin-3, and then Sp1 coimmunoprecipitated with MUC1 was detected by immunoblotting. The level of coimmunoprecipitated Sp1 increased by about 50%, peaking at 30 min after the ligation of galectin-3 to MUC1, suggesting that engagement of galectin-3 induces the recruitment of Sp1 to MUC1-CD, initiating MUC1-mediated signaling. Galectin-3-knockdown cell (HCT116/MUC1-Gal-3/Si) was also generated by introducing human galectin-3 shRNA into HCT116/MUC1 cells. Reduction of Trop-2 mRNA and protein in HCT116/MUC1-Gal-3/Si cells was confirmed by means of microarray analyses and immunoblotting, respectively, suggesting that endogenous galectin-3 up-regulates the expression of Trop-2. Furthermore, the expression of Trop-2 was increased by ~twofold on the treatment of HCT116/MUC1-Gal-3/Si cells with exogenous galectin-3, suggesting that galectin-3 upregulates Trop-2 expression through its binding to MUC1. Because MUC1 and galectin-3 are transported to the apical side of epithelial cells, this Trop-2 induction may occur even in the early phase before the dissemination of epithelial cells.

Enhancement of Cell Motility Through Trop-2 Phosphorylation

At the initial stage of epithelial tumors, dysfunction of tight junction is one of the critical steps of tumor progression. Trop-2 and its closest homolog, epithelial cell adhesion molecule (EpCAM), belong to the tumor-associated calcium signal transducer (*TACSTD*) gene family (Cubas et al. 2009), and directly interact with claudin-1 and claudin-7 (Nakatsukasa et al. 2010; Lei et al. 2012), suggesting the involvement of these molecules in the function of tight junctions. In order to investigate the tumor-promoting activity of Trop-2, human FLAG-tagged wild-type Trop-2 cDNA was introduced into HCT116 cells (HCT116/WT cells). The cytoplasmic region of Trop-2 has three possible phosphorylation sites (Ser-303, Tyr-306, Ser-322). Phosphorylation of Tyr-306 was not detected by immunoblotting using anti-phosphotyrosine antibodies. In addition to HCT116/WT cells, HCT116 cells expressing Trop-2 mutated with Ala substituted at Ser-303 (HCT116/S303A cells) or at Ser-322 (HCT116/S322A cells) were established (Mori et al. 2019). Trop-2 in HCT116/WT cells and HCT116/S-303A cells exhibited two bands on SDS-PAGE using Phos-tag-containing acrylamide gel, but Trop-2 in HCT116/S322A cells gave a single band, indicating that the Ser-322 residue was phosphorylated. Furthermore, to investigate the biological significance of Trop-2 phosphorylation, HCT116 cells expressing phosphomimetic Trop-2 mutated with Glu instead of Ser-322 were also established (HCT116/S322E cells). With respect to the function of Trop-2, it has been reported that Trop-2 directly interacts with claudin-7 and that the interaction is required for claudin-7 to be stabilized or to prevent its

degradation (Nakatsukasa et al. 2010). The levels of Trop-2 coimmunoprecipitated with anticlaudin-7 antibodies from lysates of wild-type Trop-2 (HCT116/WT), phosphorylation-blocked Trop-2 (HCT116/S322A), and phophomimetic Trop-2 (HCT116/S322E)-expressing cells were compared by immunoblotting. It was noted that phosphorylation-blocked Trop-2 (S322A) interacted with claudin-7 the strongest and a phosphomimetic variant (S322E) the weakest, suggesting that phosphorylation of Trop-2 leads to a decrease in the interaction with claudin-7. Furthermore, expression of claudin-7 in wild-type and mutated Trop-2-expressing HCT116 cells was compared. Although the level of claudin-7 mRNA was not different substantially among these cells, the level of claudin-7 protein in HCT116/S322E cells was markedly decreased compared with that in the other cells, indicating that the reduction in the amount of claudin-7 protein seems to occur posttranscriptionally. Immunochemical observation revealed that claudin-7 in HCT116/Mock cells was clearly localized to cell-cell borders in the same manner as that in the normal epithelial cells, whereas diffuse membrane staining of claudin-7 was observed in HCT116/WT cells. Its diffuse distribution was observed more prominently in phophomimetic HCT116/S322E cells. It has also been noted that cell surface claudin-7 decreased, and a part of claudin-7 was dispersed in the cytoplasm. In contrast, claudin-7 was closely concentrated at the cell membrane in phosphorylation-blocked HCT116/S322A cells. These results suggest that the cell-cell interaction may be regulated by downstream events after phosphorylation of the Ser322 residue of Trop-2. Migration assays using Transwells also demonstrated that HCT116/S322A cells were significantly less motile than other Trop-2-expressing cells, which to a greater or lesser extent possess negative charges at amino acid 322, suggesting that phosphorylation of Trop-2 may be critical for acquisition of motility potential. During oncogenic transformation, epithelial tumor cells typically lose tight junctions, leading to derangement of the tissue architecture and loss of cell polarity. In fact, it has been shown that expression of some claudins is either deregulated or lost in cancers, and that the level of claudin-7 is reduced as an early event in colorectal carcinogenesis (Moustafa et al. 2002; Kominsky et al. 2003; Usami et al. 2006; Lu et al. 2011). In contrast, it is known that Trop-2 is overexpressed in various cancer cells. Trop-2 is also widely expressed in normal tissues (Lipinski et al. 1981), suggesting that it may play a certain biological role in epithelial tissues. One possibility is that Trop-2 is responsible for the maintenance of tight junction, which is demonstrated by analysis of corneal epithelial cell disorder, GDLD (Takaoka et al. 2007; Nakatsukasa et al. 2010). Thus, these results suggest that Trop-2 phosphorylation may be responsible for the decrease or mislocalization of claudin-7, leading to dysfunction of claudin-7. Taken together, we speculate that ligation of galectin-3 with MUC1 on the apical side of premalignant epithelial cells induces the expression of Trop-2 and that phosphorylation of Trop-2 leads to a decrease in the stability of claudin-7, favoring the derangement of tight junction, as shown in Fig. 6 schematically.

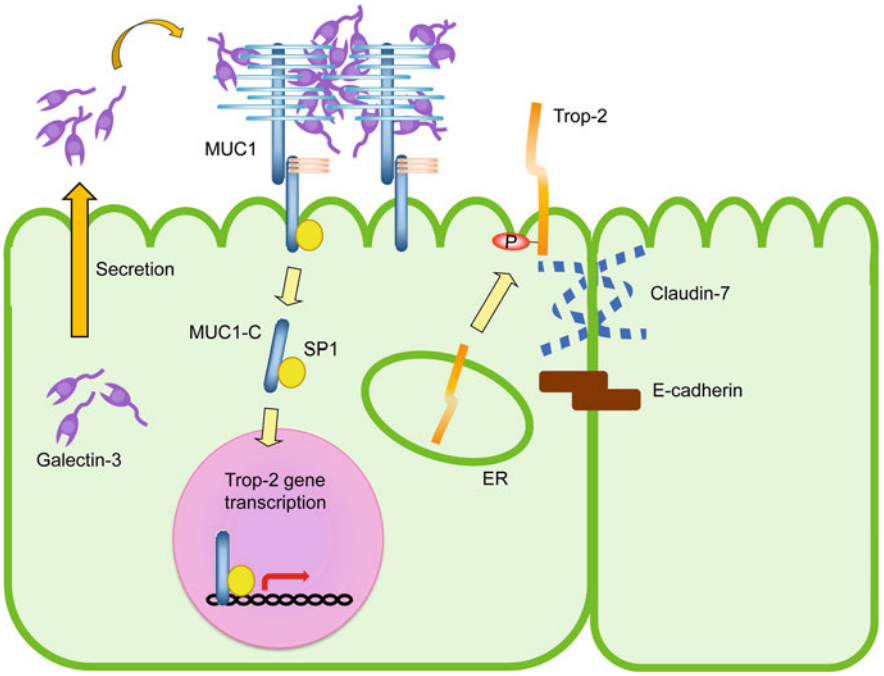


Fig. 6 MUC1-mediated induction of Trop-2. Binding of galectin-3 to MUC1 triggers formation and subsequent transportation of MUC1-CD/SP-1 complex, leading to enhancement of Trop-2 transcription. Phosphorylation of Trop-2 leads to decrease and mislocalization of claudin-7, suggesting the derangement of tight junction. Because MUC1 and galectin-3 are transported to the apical side of epithelial cells, Trop-2 induction may occur even in the premalignant phase before the dissemination of epithelial cells

2.7 MUC1-Mediated Signaling Triggered by Ligation of Siglec-9

Because mucins carry a variety of sialoglycans including tumor glyco-codes, they are expected to bind to Siglec family. Previously we demonstrated that tumor-produced soluble mucins bind to Siglec-2 (Toda et al. 2009) and Siglec-9 (Ohta et al. 2010), which are expressed on B cells and dendritic cells, respectively, leading to negative immunomodulation in these cells. Similarly, many reports demonstrated that lectin receptors mediate immune suppression by responding to the tumor glyco-codes (RodrÍguez et al. 2018). Although it is generally agreed that most Siglecs are masked by endogenous *cis* ligands, they are able to interact with *trans* ligands that are structurally accessible and carry a high level of appropriately linked sialic acids. Mucins such as MUC1 seem to be some of the most preferential *trans* ligands for Siglecs, because MUC1 is an extremely high molecular glycoprotein with a large number of glyco-codes due to its tandem repeat and is easily accessible to Siglecs on the cell surface due to its protruding rod-like structure. When lectin receptors play a

role as ligands for signaling molecules carrying glyco-codes such as MUC1, it is expected that the interaction between lectin receptors and glyco-code-carrying molecules triggers mutual signal transduction in both types of cells (Tanida et al. 2013; Beatson et al. 2016).

Binding of Soluble Siglec-9 (sSiglec-9) to MUC1

Human MUC1 cDNA transfectants (mouse 3 T3/MUC1 and HCT116/MUC1 cells) were used in this experiment (Tanida et al. 2013). Soluble tagged Siglec-1, -3, -5, and -9 were added to cell lysates, and proteins associated with these Siglecs were pulled down, followed by analyses by immunoblotting. Although all sSiglecs associated with MUC1, sSiglec-9 preferentially bound to MUC1. This may be consistent with the fact that Siglec-9 exhibits a relatively wide sugar specificity range including α2–3 and α2–6 sialic acid–linked *O*-glycans (Angata and Varki 2000; Varki and Angata 2006). Immunostaining of human tumor tissues demonstrated that Sigalec-9-positive cells were frequently present in MUC1-positive colon, breast, and pancreatic tissues.

MUC1-Mediated Signaling Triggered by Binding of sSiglec-9

As described in Sect. 2.4.3, recruitment of β-catenin to MUC1-CD was enhanced on engagement of galectin-3. Similarly, β-catenin coimmunoprecipitating with anti-MUC1-CD antibodies was detected by immunoblotting only when the cells were treated with sSiglec-9. Recruitment of β-catenin to MUC1-CD was time-dependent, peaking at 20 min after treatment with sSiglec-9. It has been reported that c-Src, Lyn, and Lck can bind and phosphorylate MUC1-CD, facilitating the recruitment of β-catenin (Masri and Gendler 2005; Mukherjee et al. 2005). Coimmunoprecipitation assay also revealed that cSrc was recruited to MUC1-CD and the level of coimmunoprecipitated cSrc increased through ligation with sSiglec-9, maybe leading to enhance recruitment of β-catenin to MUC1-CD. These results indicate that recruitment of β-catenin was triggered by the binding of Siglec-9 to MUC1.

MUC1-Mediated Signaling Triggered by Membrane- Bound Siglec-9

In order to mimic the interaction between MUC1-expressing tumor cells and Siglec-9-expressing immune cells, Siglec-9-expressing cells (HEK293/Siglec-9 cells) were established by introducing human Siglec-9 cDNA into HEK293 cells. In addition, to demonstrate that MUC1-mediated signaling is sialic acid dependent, 3 T3/MUC1 and HCT116/MUC1 cells were treated with neuraminidase. Removal of sialic acid residues from the cell surface was confirmed by the binding of *Maackia amurensis* mitogen and sSiglec-9 to the cells. HEK293/Siglec-9 cells or HEK293/Mock cells were mixed with MUC1-expressing cells, followed by gentle rotation at 37 °C for

20 min. β-Catenin coimmunoprecipitating with anti-MUC1-CD antibodies was detected by immunoblotting. Recruitment of β-catenin to MUC1-CD significantly increased in 3 T3/MUC1 (~sevenfold) and HCT116/MUC1 cells (~sixfold) on coincubation with HEK293/Siglec-9 cells compared with coincubation with HEK293/Mock cells, indicating that membrane-bound Siglec-9 could also interact with MUC1 and initiate MUC1-mediated signaling. When neuraminidase-treated MUC1-expressing cells were used, recruitment of β-catenin to MUC1-CD was substantially reduced to the basal level, indicating that binding of Siglec-9 to sialoglycans expressed on MUC1 is responsible for the signaling.

Downmodulation of β-Catenin Phosphorylation and Enhanced Nuclear Transport of β-Catenin

With respect to the stability of β-catenin, GSK-3 phosphorylates β-catenin and thereby targets it for proteosomal degradation (Orford et al. 1997). After treatment of cells with or without sSiglec-9 for 40 min, phosphorylated β-catenin was detected by immunoblotting with antiphosphorylated β-catenin antibodies. The level of phosphorylated β-catenin decreased by about 40% on treatment with sSiglec-9, suggesting that ligation of sSiglec-9 with MUC1 elevated the recruitment of β-catenin and thereby inhibited the phosphorylation of β-catenin by GSK-3, leading to stabilization of β-catenin. Immunochemical observation revealed that β-catenin was accumulated in the nucleus on treatment with sSiglec-9 for 1 h. The number of cells containing β-catenin in the nucleus increased about threefold on the treatment with sSiglec-9. Colocalization of MUC1-CD and β-catenin in the nucleus was also observed in sSiglec-9-treated cells but not in nontreated cells. These results suggest that ligation of MUC1 with sSiglec-9 decreased phosphorylated β-catenin, maybe resulting in an increased nuclear level of β-catenin. β-Catenin functions as a transcriptional coactivator that increases the expression of cell cycle progression genes cyclin-D1 and *c-myc*, thus leading to the progression of diverse tumor malignancies. In fact, the level of *c-myc* was also elevated on treatment with sSiglec-9, and consistently, the proliferation of Siglec-9-traeted cells increased about 1.5 fold at 72 h after the treatment with sSiglec-9 compared with that of sSigelc-9-nontreated cells. In this experiment, using a coculture model of MUC1-expressing cells and Siglec-9-expressing cells mimicking the interaction between MUC1-expressing tumor cells and Siglec-9-expressing immune cells in a tumor microenvironment, the recruitment of β-catenin to MUC1-CD was clearly enhanced. This effect was almost completely abolished by pretreatment of MUC1-expressing cells with neuraminidase. The recruited β-catenin was thereafter transported to the nucleus, leading to the enhancement of cell growth. These findings suggest that Siglec-9 expressed on immune cells may play a role as a potential counterreceptor for MUC1. Eventually, it is expected that MUC1 also plays a role as a ligand for Siglec-9. Beatson et al. reported that MUC1 modulates the tumor immunological environment through the engagement of Siglec-9 (Beatson et al. 2016), as discussed later.

2.8 MUC4-Mediated Tumor Progression (Bafna et al. 2010; Kozloski et al. 2010; Hanson and Hollingsworth 2016)

MUC4 is widely expressed on the epithelial surface of the eyes, oral cavity, middle ears, respiratory tract, female reproductive tract, gastrointestinal tract, and mammary glands. Aberrant expression of MUC4 has been demonstrated in a variety of carcinomas where MUC4 is implicated in affecting various cellular phenotypes that promote tumor progression, including cell adhesion (Komatsu et al. 1997), promotion of oncogenesis (Ponnusamy et al. 2008), and cell signaling through modulation of the ErbB2 receptor activity (Funes et al. 2006). MUC4 is synthesized as a single polypeptide chain and cleaved early after synthesis, yielding MUC4α and MUC4β, which reassociate to form a noncovalently associated heterodimer. MUC4α contains a tandem repeat domain of 145–395 repeats of 16 amino acids, a cysteine-rich domain, a nitrogen homology domain, an adhesion-associated domain, and a von Willebrand factor type D domain (Duraisamy et al. 2006). MUC4β has three EGF-like domains and a short cytoplasmic region of 22 amino acids (Duraisamy et al. 2006). Similar to MUC1, MUC4 has been demonstrated to play a role in the regulation of cell growth signaling through interaction with the ErbB family of growth factor receptors (Chaturvedi et al. 2008; Lakshmanan et al. 2015). The interaction of MUC4 with receptor tyrosine-protein kinase erbB-2 (ErbB2/HER2) stabilizes its expression and activity (Chaturvedi et al. 2008). HER2 can regulate cell proliferation and metastasis by activating downstream MAPK, Akt, FAK, and c-Src family kinase pathways (Holbro and Hynes 2004). Stabilization of the MUC4/HER2 complex also enhanced the activation of the ERK1/2 and MAPK signaling pathway, leading to mediating of cancer cell proliferation (Chaturvedi et al. 2008). In addition, the stabilized MUC4/HER2 complex can protect cancer cells from trastuzumab, a target therapy against HER2 (Nagy et al. 2005; Chaturvedi et al. 2008). Only the EGF-like domains, that is, not the tandem repeat domain or cytoplasmic domain, are necessary for the interaction of MUC4 with HER2 (Workman et al. 2009). Thus, the *O*-glycosylated extracellular domain has no relation to signaling; however, exposure of the EGF-like domain due to decreased *O*-glycosylation associated with tumors may enhance the signaling.

2.9 MUC16-Mediated Tumor Progression (Haridas et al. 2014; Hanson and Hollingsworth 2016)

MUC16 is the largest membrane-bound mucin with a huge molecular weight of about 20 MDa (Yin and Lloyd 2001; Haridas et al. 2014), and is normally expressed on the ocular surface, and in the upper respiratory tract, mesothelium lining body cavities, internal organs, and reproductive organs. The extracellular portion of MUC16, which is produced through cleavage from the cell surface of ovarian cancer cell, is known as CA125, and is used as a biomarker for ovarian cancer (Bast Jr et al.

1998). The N-terminal region of MUC16 is a heavily *O*-glycosylated nontandem repeat domain that consists of approximately 12,000 amino acids. The following tandem repeat region is composed of 60 repeats of 156 amino acids (Maeda et al. 2004). MUC16 contains 56 sea urchin sperm protein, enterokinase, and agrin (SEA) domains, many of which are interspersed among tandem repeats. A large number of cysteine residues are present in the SEA domains and are able to form disulfide bonds in inter- and intramolecular manners (O'Brien et al. 2001). MUC16 has a short cytoplasmic region of 32 amino acids with several potential phosphorylation sites (Hattrup and Gendler 2008). It has been reported that MUC16 interacts with Src and tyrosine protein kinase Yes of the Src family of kinases, and with β-catenin and E-cadherin (Akita et al. 2013). MUC16 is phosphorylated at tyrosine 22142 in the cytoplasmic region, leading to promotion of shedding of the extracellular domain of MUC16 and to degradation of β-catenin and E-cadherin (Thériault et al. 2011). Rao et al. reported that forced expression of the 114 amino acid C-terminal portion of MUC16 could induce transformation and tumor invasion (Rao et al. 2015), suggesting a critical role of the C-terminal portion in the signaling. The cytoplasmic region of MUC16 also interacts with Janus kinase 2 (JAK2), leading to promotion of proliferation and enhancement of STAT3 activity (Lakshmanan et al. 2012). Consistently, expression of the 114 amino acid polypeptide in pancreatic cancer cells elevates the nuclear localization of JAK2, promoting metastatic and stem-like properties (Das et al. 2015). The poly-basic sequence (RRRKK) in the cytoplasmic region of MUC16 is known to interact with the ezrin/radixin/moesin family of proteins (ERM). The ERM domain present in JAK2 is considered to be responsible for the interaction with MUC16. The MUC16 ectodomain carrying carbohydrate moieties seems to have no relation to the signaling. However, hypoglycosylation associated with tumors may enhance proteolytic cleavage of the extracellular domain, leading to potential signaling of the cytoplasmic region (Bruney et al. 2014). From another point of view, various tumor-associated carbohydrate antigens including tumor glyco-codes, which are mainly expressed in mucins, can play a role as ligands for lectin receptor expressed mainly in immune cells. MUC16 produced by an ovarian cancer cell line, OVCAR-3 cells, carries various carbohydrate antigens such as Tn, sialylTn, and sialylLewis^{a} antigens (Akita et al. 2012). In fact, Siglec-9 was identified as the receptor for MUC16 (Belisle et al. 2010), and MUC16 has been shown to bind both E- and L-selectin (Chen et al. 2012). This is described in next section.

3 Lectin-Receptor-Mediated Immune Regulation

As we described above, MUC1-mediated signaling occurs through the binding of lectins such as galectin-3 and Siglec-9, leading to tumor progression. The glyco-code-expressing mucins are the main binding partners or counterreceptors for lectin receptors (RodrÍguez et al. 2018). In this case, lectin and mucins carrying the glyco-codes play roles as a receptor and a ligand, respectively. DC-SIGN, MGL, and

Siglecs are involved in the recognition and uptake of aberrantly glycosylated tumor antigens. It has been considered that DC-SIGN and MGL facilitate uptake of antigens for antigen presentation and modulate subsequent immune responses (Sancho and e Sousa 2012). It has been reported that both circulating and tumor-infiltrating DCs are functionally impaired in tumor-bearing animals (Chaux et al. 1997) and in cancer patients (Gabrilovich et al. 1997). As described above, mucins produced by cancer cells are found in the sera of cancer patients and easily come into contact with various circulating cells in the bloodstream of cancer patients or with infiltrating cells in cancer tissues. Some lectin receptors could also play a role as signaling molecules, which are initiated through ligation of glyco-code-expressing mucins and/or mucin-type glycoproteins. Although many lectin-receptor-mediated signaling pathways have not fully resolved, glycan-lectin interaction downmodulates immune responses, contributing to immune evasion.

3.1 DC-SIGN-Mediated Immune Regulation (RodrÍguez et al. 2018)

DC-SIGN, a type II transmembrane protein, is a C-type lectin-receptor (CLR) and is expressed by macrophages and immature DCs (van Gisbergen et al. 2005). DC-SIGN is composed of a carbohydrate recognition domain (CRD), a neck region composed of 7.5 repeats containing 23 amino acid residue repeats, and a transmembrane region, followed by a cytoplasmic region containing recycling and internalization motifs (Kwon et al. 2002). Formation of the DC-SIGN tetramer occurs through the neck-repeat domain (Mitchell et al. 2001) and increases the binding affinity of ligands containing repetitive sugar moieties. In addition to adhesive and antigen-recognition properties, engagement of DC-SIGN on DCs initiates signal transduction, leading to extensive modulation of immune responses, especially when coactivated with Toll-like receptor (TLR)-mediated signaling (Gringhuis et al. 2007). During TLR-mediated signaling on engagement of pathogens, simultaneous DC-SIGN-mediated signaling results in tolerance by default (Hawiger et al. 2001). Thus, DC-SIGN may play a crucial role in induction of tolerance for homeostatic control (Geijtenbeek et al. 2004). Although DC-SIGN is known to recognize N-linked high mannose oligosaccharides, whose interaction plays a role in internalization of pathogens (Ding et al. 2017), it also binds to fucose-containing structures such as Lewis antigens (Unger et al. 2012).

Mannose- and fucose-carrying pathogens induce a distinct DC-SIGN signaling pathway. The proximal DC-SIGN signaling complex (signalosome) consisting of LSP1, KSR1, and CNK plays a critical role in the constitutive recruitment of Raf1 to DC-SIGN. On engagement of mannose-carrying pathogens, Raf-1 is activated through recruitment of upstream effectors LARG and RhoA to the DC-SIGN signalosome. This Raf-1-mediated signaling leads to enhanced production of proinflammatory cytokines and T helper 1 (Th1) responses (Gringhuis et al. 2009).

In contrast, DC-SIGN-mediated signaling triggered by fucose-carrying pathogens depends on crosstalk with TLR signaling and leads to activation of kinase IKKε in a LSP1-dependent but CNK-KSR1-Raf-1-independent manner. IKKε-mediated phosphorylation inactivates deubiquitinase CYLD, and thereby results in accumulation of ubiquitinated Bcl3 in the nucleus. This pathway modulates production of cytokines and chemokines to drive T helper 2 (Th2) polarization (Gringhuis et al. 2014a). Because tumor-produced mucins frequently carry fucose-containing *O*-glycans such as Lewis antigens, DC-SIGN is expected to bind to mucins such as MUC1. In fact, it has been reported that tumor-derived MUC1 mucins interact with differentiating monocytes and induce IL-10 high, IL-12 low regulatory DCs (Monti et al. 2004). Similarly, Lewis antigens carried on CEA bind to DC-SIGN, which leads to upregulation of the anti-inflammatory cytokines IL-10 and IL-27, and induction of Th2, T follicular helper (T_{FH}), or regulatory T (T_{reg}) cells (García-Vallejo et al. 2014; Gringhuis et al. 2014b). These reports indicate that tumor-derived MUC1 induces DCs to interact with IL-10-producing, IL-12-deficient antigen-presenting cells (APCs), which have a limited capacity to trigger protective Th1 responses, and the ability to promote T cell anergy and regulatory activity.

3.2 *MGL-Mediated Immune Regulation (van Vliet et al. 2005, 2008)*

MGL is a type II transmembrane protein and is exclusively expressed on macrophages and DCs (Jégouzo et al. 2013; van Kooyk et al. 2015). Because MGL recognizes LacdiNAc (GalNAc β1,4 GlcNAc β1-), and terminal α and β GalNAc residues with exposed C3- and C4-hydroxy groups (Ozaki et al. 1995), it could bind to the tumor glyco-codes, Tn antigen (Beatson et al. 2015) and sialylTn antigen (Mantovani et al. 2017), which are frequently carried on MUC1. Thus, it has been reported that MGL bound specifically to MUC1 carrying Tn antigen in primary colon carcinoma, but not to MUC1 from normal epithelial cells (Saeland et al. 2007). This binding specificity is shared by the mouse MGL2, whereas the mouse MGL1 orthologue exhibits exclusive specificity for the $Lewis^{a/x}$ antigen (Singh et al. 2009). MGL signaling also synergizes with TLR-mediated pathways, leading to modulation of the signaling of TLR2, TLR4, and TLR7/8 in DCs (van Kooyk et al. 2015). The binding of Tn antigens to MGL expressed on DCs through conjunction with simultaneous TLR2-mediated signaling leads to a tolerogenic phenotype with increased production of IL-10 and TNF-α (Higashi et al. 2002). Ligation of human MGL with mucins carrying Tn antigen induces phosphorylation of ERK1/2, followed by activation of p90RSK and CREB, leading to elevation of IL-10 and TNF-α production (Higashi et al. 2002; Li et al. 2012). MGL engagement also activates p50 NF-κB subunits, leading to elevation of IL-10 production (Higashi et al. 2002; Napoletano et al. 2012). Costa et al. also reported that expression of Tn antigen in tumor cells and its interaction with MGL2-expressing CD11cF4/

80-positive cells promote immunosuppression (da Costa et al. 2021). Related with this glyco-code-MGL interaction, CD45, mucin-type cell surface glycoprotein expressed on effector T cells, has been identified as a counterreceptor for MGL. CD45 is a prototypic tyrosine phosphatase expressed on T cells. Glycosylation-dependent interactions of MGL with CD45 on effector T cells downmodulate T cell receptor–mediated signaling and subsequent cytokine responses, leading to decrease of T cell proliferation and increase of T cell death (van Vliet et al. 2006). Because MGL is expressed on M2-like tumor-associated macrophages (TAMs), which are able to promote tumor growth and metastasis (Saeland et al. 2007), it is speculated that interaction of MGL with Tn and/or sialyTn antigens carried on MUC1 may modulate the TAM activity. Furthermore, it has been reported that MGLs are preferentially expressed on tolerogenic APCs located at tumor sites (Zizzari et al. 2015). Truncated *O*-glycans produced by glioblastomas and colon cancers also contribute to immune suppression (Cornelissen et al. 2020; Dusoswa et al. 2020). Taken together, MGLs may play a role as an immunomodulator in a tumor microenvironment, leading to tumor-induced immune tolerance. In fact, a glycopeptide comprising MUC1-three tandem repeats carrying Tn antigens could modulate DC maturation and initiation of a strong CD8 T cell immune response (Zizzari et al. 2015). In this context, this MUC1 glycopeptide is expected to be used for antitumor vaccines targeting DCs via MGL (Artigas et al. 2017).

3.3 Siglec-Mediated Immune Regulation (Adams et al. 2018; van de Wall et al. 2020)

Many cell surface glycans of mammalian cells are commonly terminated by a sialic acid. These sialoglycans can engage various endogenous lectin-receptors including Siglecs. The extracellular part of Siglecs is composed of an N-terminal carbohydrate recognition domain (CRD) and a variable number of C2 domains. Activating Siglecs contain a positively charged amino acid in the transmembrane domain that mediates interaction with DAP12 upon Siglec engagement. The intracellular part of inhibitory Siglecs is characterized by immunoreceptor tyrosine–based inhibition-motif (ITIM) or ITIM-like structures mediating immune inhibition through the recruitment of SHP1 and SHP2 phosphatase on binding to sialoglycans. Consistently, hypersialylation in tumor cells often correlates with tumor invasion and a poor prognosis (Swindall et al. 2013). Furthermore, it has been reported that although healthy peripheral blood T cells express no CD33-related Siglecs, tumor-infiltrating lymphocytes upregulate inhibitory CD33-related Siglecs, predominantly Siglec-9 in patients with non-small cell lung cancer, colorectal cancer, epithelial ovarian cancer, and melanomas (Stanczak et al. 2018; Haas et al. 2019). Because mucins commonly carry a large number of sialoglycans, there is a possibility that Siglecs could bind to various mucins. It has been reported that MUC1 is a natural ligand of endogenous Siglec-1 (Nath et al. 1999) and Siglec-4 (Swanson et al. 2007).

Siglec-9-Mediated Immune Regulation

Siglec-9 is expressed on human NK cells, B cells, and monocytes (Belisle et al. 2010). The effects of artificial glycopolymers carrying α2,3 and α2,6 linked sialic acids, bovine submaxillary mucin (BSM), and MUC2 mucin produced by a human colon cancer cell line, LS180 cells, on immature DCs were investigated. Because BSM and MUC2 mucin express a sialylTn antigen, these mucins and artificial glycopolymers bound to recombinant Siglec-9. Immature DCs were prepared from monocytes of human peripheral blood. Using labeled BSM and MUC2 mucin, their binding to the surface of immature DCs was confirmed with a flow cytometer. When immature DCs were treated with LPS in the presence of these mucins or artificial glycopolymers, the production of IL-12 was significantly reduced, but that of IL-10 was not. Desialylated mucins failed to induce these effects. A similar level of IL-12 was decreased on treatment with anti-Siglec-9 antibodies (Ohta et al. 2010). It is generally agreed that IL-12 is responsible for driving the development of the Th1 immune response, suggesting that mucins could promote the induction of immune regulation in synergy with other immunosuppressive factors. As described in Sect. 2.7, we demonstrated that MUC1-mediated signaling occurred through the engagement of Siglec-9. Beastson et al. reported that sialylT antigen carried on MUC1 (MUC1-ST) played a role as a ligand for Siglec-9 expressed in myeloid cells, and that on treatment with MUC1-ST, macrophages phenotypically changed to TAM with increased expression of PD-L1. With respect to Siglec-mediated signaling, it is generally agreed that inhibitory Siglecs downmodulate the signaling through the phosphorylation of ITIMs and subsequent recruitment of SHP-1 and/or SHP-2, as described above. In this experiment, unexpectedly, binding of MUC1-ST to Siglec-9 did not activate SHP-1/2 but induced calcium flux, leading to MEK-ERK activation. Eventually, MUC1-ST induced monocytes to secrete several factors associated with inflammation and tumor progression such as IL-6, M-CSF, and plasminogen activator inhibitor (Beatson et al. 2016). In addition, ligation of Siglec-9 with sialoglycans carried on MUC1 led to polarization to M2 macrophages in vitro, leading to modulation of the tumor immunological environment (Beatson et al. 2016). Jandus et al. reported that interaction of Siglec-7/9 with sialoglycans directly reduced NK cell activity (Jandus et al. 2014). Sialylated antigens imposed a regulatory program on DCs via Siglec E. DCs loaded with sialylated antigens induced de novo Treg cells and inhibited the generation of new effector T cells (Perdicchio et al. 2016a). Sialoglycans expressed on melanoma cells were correlated with increased tumor growth in vivo and associated with increased accumulation of Treg cells, a low influx of effector T cells, and reduced NK cell activity (Perdicchio et al. 2016b). Because endogenous ligands such as mucins usually carry a variety of glyco-codes, there is a possibility that multiple lectin receptors may be involved. On the other hand, because the same glyco-codes are expressed on various mucins, not only MUC1 but also other mucins may be ligands for the lectin receptor. As described above, MUC16 has also been identified as a ligand for Siglec-9 (Belisle et al. 2010). Thus, analyses of signaling may be complicated. Actually, Siglec-mediated

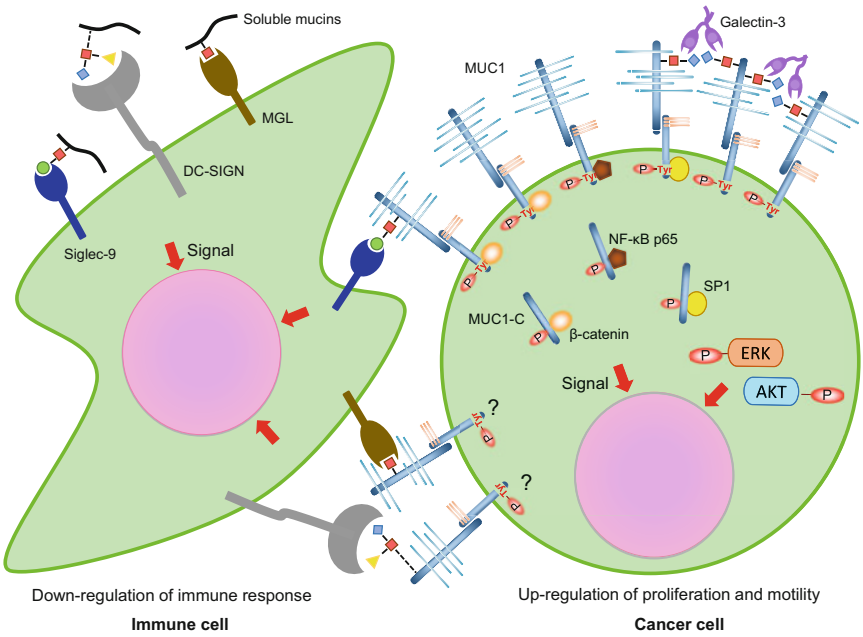


Fig. 7 Promotion of tumor malignancy and immune evasion by mucin-lectin network. MUC1-mediated signaling through binding of soluble and membrane-bound lectins promotes tumor progression in cancer cells. Inhibitory lectin receptor–mediated signaling through binding of mucins downmodulates immune responses in immune cells

downstream events after ligation with sialoglycans have not been fully understood; however, these Siglec-mediated phenotypic changes seem to have established that interaction of inhibitory Siglecs with sialylated glyco-codes on MUC1 likely inhibits the immune response. Based on these data, some practical experiments have been performed. A sialic acid mimetic, which inhibits intratumoral biosynthesis of sialoglycan, enhanced T-cell-mediated antitumor activity (Büll et al. 2018). An anti-HER-2 antibody conjugated with a bacterial sialidase was demonstrated to increase tumor cell killing (Xiao et al. 2016). Figure 7 shows the interactions of mucins with lectins in a tumor environment schematically. It should be noted that the interaction of membrane-bound mucins with lectin receptors may be able to initiate mutual signaling, leading to tumor progression and immune evasion in tumor cells and infiltrated immune cells, respectively. Soluble mucins include soluble MUC1 and CA125, which are produced through shedding from membrane-bound MUC1 and MUC16, respectively. Because mucins generally express multiple tumor glyco-codes such as Tn, T, and sialylTn antigens, we consider that mucins may ligate to multiple lectin receptors, initiating multiple signaling pathways.

References

Adams OJ, Stanczak MA, von Gunten S et al (2018) Targeting sialic acid-Siglec interactions to reverse immune suppression in cancer. Glycobiology 28:640–647

Ahmad N, Gabius HJ, André S et al (2004) Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. J Biol Chem 279: 10841–10847

Ahmad R, Raina D, Trivedi V et al (2007) MUC1 oncoprotein activates the IkappaB kinase beta complex and constitutive NF-kappaB signalling. Nat Cell Biol 9:1419–1427

Akita K, Tanaka M, Tanida S et al (2013) CA125/MUC16 interacts with Src family kinases, and over-expression of its C-terminal fragment in human epithelial cancer cells reduces cell-cell adhesion. Eur J Cell Biol 92:257–263

Akita K, Yoshida S, Ikehara Y et al (2012) Different levels of sialyl-Tn antigen expressed on MUC16 in patients with endometriosis and ovarian cancer. Int J Gynecol Cancer 22:531–538

Angata T (2018) Possible influences of endogenous and exogenous ligands on the evolution of human Siglecs. Front Immunol 9:2885

Angata T, Varki A (2000) Cloning, characterization, and phylogenetic analysis of siglec-9, a new member of the CD33-related group of siglecs. Evidence for co-evolution with sialic acid synthesis pathways. J Biol Chem 275:22127–22135

Apostolopoulos V, Stojanovska L, Gargosky SE (2015) MUC1 (CD227): a multi-tasked molecule. Cell Mol Life Sci 72:4475–4500

Artigas G, Monteiro JT, Hinou H et al (2017) Glycopeptides as targets for dendritic cells: exploring MUC1 glycopeptides binding profile toward macrophage galactose-type lectin (MGL) orthologs. J Med Chem 60:9012–9021

Bafna S, Kaur S, Batra SK (2010) Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. Oncogene 29:2893–2904

Bast RC Jr, Xu FJ, Yu YH et al (1998) CA 125: the past and the future. Int J Biol Marker 13:179–187

Beatson R, Maurstad G, Picco G et al (2015) The breast cancer-associated glycoforms of MUC1, MUC1-Tn and sialyl-Tn, are expressed in COSMC wild-type cells and bind the C-type lectin MGL. PLoS One 10:e0125994

Beatson R, Tajadura-Ortega V, Achkova D et al (2016) The mucin MUC1 modulates the tumor immunological microenvironment through engagement of the lectin Siglec-9. Nat Immunol 17: 1273–1281

Becker JW, Erickson HP, Hoffman S et al (1989) Topology of cell adhesion molecules. Proc Natl Acad Sci U S A 86:1088–1092

Belisle JA, Horibata S, Jennifer GAA et al (2010) Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes. Mol Cancer 9:118

Bennett EP, Mandel U, Clausen H et al (2012) Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. Glycobiology 22:736–756

Bian CF, Zhang Y, Sun H et al (2011) Structural basis for distinct binding properties of the human galectins to Thomsen-Friedenreich antigen. PLoS One 6:e25007

Bitler BG, Goverdhan A, Schroeder JA (2010) MUC1 regulates nuclear localization and function of the epidermal growth factor receptor. J Cell Sci 123:1716–1723

Blume SW, Snyder RC, Ray R et al (1991) Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. J Clin Invest 88:1613–1621

Bruney L, Conley KC, Moss NM et al (2014) Membrane-type I matrix metalloproteinase-dependent ectodomain shedding of mucin16/CA-125 on ovarian cancer cells modulates adhesion and invasion of peritoneal mesothelium. Biol Chem 395:1221–1231

Büll C, Boltje TJ, Balneger N et al (2018) Sialic acid blockade suppresses tumor growth by enhancing T-cell-mediated tumor immunity. Cancer Res 78:3574–3588

Cascio S, Zhang L, Finn OJ et al (2011) MUC1 protein expression in tumor cells regulates transcription of proinflammatory cytokines by forming a complex with nuclear factor-κB p65 and binding to cytokine promoters: importance of extracellular domain. J Biol Chem 286: 42248–42256

Cazet A, Julien S, Bobowski M et al (2010) Tumour-associated carbohydrate antigens in breast cancer. Breast Cancer Res 12:204

Chaturvedi P, Singh AP, Chakraborty S et al (2008) MUC4 mucin interacts with and stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res 68:2065–2070

Chauhan D, Li G, Podar K et al (2005) A novel carbohydrate-based therapeutic GCS-100 overcomes bortezomib resistance and enhances dexamethasone-induced apoptosis in multiple myeloma cells. Cancer Res 65:8350–8358

Chaux P, Favre N, Martin M et al (1997) Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. Int J Cancer 72:619–624

Chen SH, Dallas MR, Balzer EM et al (2012) Mucin 16 is a functional selectin ligand on pancreatic cancer cells. FASEB J 26:1349–1359

Chugh S, Gnanapragassam VS, Jain M et al (2015) Pathobiological implications of mucin glycans in cancer: sweet poison and novel targets. Biochim Biophys Acta 1856:211–225

Cicek M, Fukuyama R, Cicek MS et al (2009) BRMS1 contributes to the negative regulation of uPA gene expression through recruitment of HDAC1 to the NF-kappaB binding site of the uPA promoter. Clin Exp Metastasis 26:229–237

Cooper DN, Barondes SH (1999) God must love galectins; he made so many of them. Glycobiology 9:979–984

Cornelissen LAM, Blanas A, Zaal A et al (2020) Tn antigen expression contributes to an immune suppressive microenvironment and drives tumor growth in colorectal cancer. Front Oncol 10: 1622

Cubas R, Li M, Chen C et al (2009) Trop2: a possible therapeutic target for late stage epithelial carcinomas. Biochim Biophys Acta 1796:309–314

da Costa V, van Vliet SJ, Carasi P et al (2021) The Tn antigen promotes lung tumor growth by fostering immunosuppression and angiogenesis via interaction with macrophage galactose-type lectin 2 (MGL2). Cancer Lett 518:72–81

Dahr W, Uhlenbruck G, Bird GW (1974) Cryptic A-like receptor sites in human erythrocyte glycoproteins: proposed nature of Tn-antigen. Vox Sang 27:29–42

Dam TK, Gabius HJ, André S et al (2005) Galectins bind to the multivalent glycoprotein asialofetuin with enhanced affinities and a gradient of decreasing binding constants. Biochemistry 44:12564–12571

Danø K, Behrendt N, Høyer-Hansen G et al (2005) Plasminogen activation and cancer. Thromb Haemost 93:676–681

Das S, Rachagani S, Torres-Gonzalez MP et al (2015) Carboxyl-terminal domain of MUC16 imparts tumorigenic and metastatic functions through nuclear translocation of JAK2 to pancreatic cancer cells. Oncotarget 6:5772–5787

Delacour D, Cramm-Behrens CI, Drobecq H et al (2006) Requirement for galectin-3 in apical protein sorting. Curr Biol 16:408–414

Ding D, Yao Y, Zhang S et al (2017) C-type lectins facilitate tumor metastasis. Oncol Lett 13:13–21

Duraisamy S, Ramasamy S, Kharbanda S et al (2006) Distinct evolution of the human carcinoma-associated transmembrane mucins, MUC1, MUC4 AND MUC16. Gene 373:28–34

Dusoswa SA, Verhoeff J, Abels E et al (2020) Glioblastomas exploit truncated O - linked glycans for local and distant immune modulation via the macrophage galactose-type lectin. Proc Natl Acad Sci U S A 117:3693–3703

Elad-Sfadia G, Haklai R, Balan E et al (2004) Galectin-3 augments K-Ras activation and triggers a Ras signal that attenuates ERK but not phosphoinositide 3-kinase activity. J Biol Chem 279: 34922–34930

Freitas D, Campos D, Gomes J et al (2019) O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. EBioMedicine 40:349–362

Fritsch K, Mernberger M, Nist A et al (2016) Galectin-3 interacts with components of the nuclear ribonucleoprotein complex. BMC Cancer 16:502
Funes M, Miller JK, Lai C et al (2006) The mucin Muc4 potentiates neuregulin signaling by increasing the cell-surface populations of ErbB2 and ErbB3. J Biol Chem 281:19310–19319
Gabrilovich DI, Corak J, Ciernik IF et al (1997) Decreased antigen presentation by dendritic cells in patients with breast cancer. Clin Cancer Res 3:483–490
Gao X, Balan V, Tai G et al (2014) Galectin-3 induces cell migration via a calcium-sensitive MAPK/ERK1/2 pathway. Oncotarget 5:2077–2084
García-Vallejo JJ, Ilarregui JM, Kalay H et al (2014) CNS myelin induces regulatory functions of DC-SIGN-expressing, antigen-presenting cells via cognate interaction with MOG. J Exp Med 211:1465–1483
Geijtenbeek TB, Torensma R, van Vliet SJ et al (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100:575–585
Geijtenbeek TBH, van Vliet SJ, Engering A et al (2004) Self- and nonself-recognition by C-type lectins on dendritic cells. Annu Rev Immunol 22:33–54
Gendler SJ, Spicer AP (1995) Epithelial mucin genes. Annu Rev Physiol 57:607–634
Gendler SJ (2001) MUC1, the renaissance molecule. J Mammary Gland Biol Neoplasia 6:339–353
Gendler S, Taylor-Papadimitriou J, Duhig T et al (1988) A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J Biol Chem 263:12820–12823
Gill DJ, Chia J, Senewiratne J et al (2010) Regulation of O-glycosylation through Golgi-to-ER relocation of initiation enzymes. J Cell Biol 189:843–858
Gill DJ, Clausen H, Bard F (2011) Location, location, location: new insights into O-GalNAc protein glycosylation. Trends Cell Biol 21:149–158
Gringhuis SI, den Dunnen J, Litjens M et al (2007) C-type lectin DC-SIGN modulates toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. Immunity 26:605–616
Gringhuis SI, den Dunnen J, Litjens M et al (2009) Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to mycobacterium tuberculosis, HIV-1 and helicobacter pylori. Nat Immunol 10:1081–1088
Gringhuis SI, Kaptein TM, Wevers BA et al (2014a) Fucose-specific DC-SIGN signalling directs T helper cell type-2 responses via IKKε- and CYLD-dependent Bcl3 activation. Nat Commun 5: 3898
Gringhuis SI, Kaptein TM, Wevers BA et al (2014b) Fucose-based PAMPs prime dendritic cells for follicular T helper cell polarization via DC-SIGN-dependent IL-27 production. Nat Commun 5: 5074
Haas Q, Boligan KF, Jandus C et al (2019) Siglec-9 regulates an effector memory CD8 + T-cell subset that congregates in the melanoma tumor microenvironment. Cancer Immunol Res 7:707–718
Hanson RL, Hollingsworth MA (2016) Functional consequences of differential O-glycosylation of MUC1, MUC4, and MUC16 (downstream effects on signaling). Biomol Ther 6:34
Haridas D, Ponnusamy MP, Chugh S et al (2014) MUC16: molecular analysis and its functional implications in benign and malignant conditions. FASEB J 28:4183–4199
Hattrup CL, Gendler SJ (2008) Structure and function of the cell surface (tethered) mucins. Annu Rev Physiol 70:431–457
Hawiger D, Inaba K, Dorsett Y et al (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 194:769–779
Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132:344–362
Higashi N, Fujioka K, Denda-Nagai K et al (2002) The macrophage C-type lectin specific for galactose/N-acetylgalactosamine is an endocytic receptor expressed on monocyte-derived immature dendritic cells. J Biol Chem 277:20686–20693
Hirabayashi J, Hashidate T, Arata Y et al (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim Biophys Acta 1572:232–254

Hofmann BT, Schlüter L, Lange P et al (2015) COSMC knockdown mediated aberrant O-glycosylation promotes oncogenic properties in pancreatic cancer. Mol Cancer 14:109
Holbro T, Hynes NE (2004) ErbB receptors: directing key signaling networks throughout life. Annu Rev Pharmacol Toxicol 44:195–217
Hollingsworth MA, Swanson BJ (2004) Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer 4:45–60
Ho MK, Springer TA (1982) Mac-2, a novel 32,000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. Immunol 128:1221–1228
Hönig E, Ringer K, Dewes J et al (2018) Galectin-3 modulates the polarized surface delivery of β1-integrin in epithelial cells. J Cell Sci 131:jcs213199
Hönig E, Schneider K, Jacob R (2015) Recycling of galectin-3 in epithelial cells. Eur J Cell Biol 94: 309–315
Huang L, Chen D, Liu D et al (2005) MUC1 oncoprotein blocks glycogen synthase kinase 3beta-mediated phosphorylation and degradation of beta-catenin. Cancer Res 65:10413–10422
Irigoyen JP, Muñoz-Cánoves P, Montero L et al (1999) The plasminogen activator system: biology and regulation. Cell Mol Life Sci 56:104–132
Iurisci I, Tinari N, Natoli C et al (2000) Concentrations of galectin-3 in the sera of normal controls and cancer patients. Clin Cancer Res 6:1389–1393
Iwai T, Inaba N, Naundorf A (2002) Molecular cloning and characterization of a novel UDP-GlcNAc:GalNAc-peptide beta1,3-N-acetylglucosaminyltransferase (beta 3Gn-T6), an enzyme synthesizing the core 3 structure of O-glycans. J Biol Chem 277:12802–12809
Jandus C, Boligan KF, Chijioke O et al (2014) Interactions between Siglec-7/9 receptors and ligands influence NK cell-dependent tumor immunosurveillance. J Clin Invest 124:1810–1820
Engelman JA (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 9:550–562
Jégouzo SAF, Quintero-Martínez A, Ouyang X et al (2013) Organization of the extracellular portion of the macrophage galactose receptor: a trimeric cluster of simple binding sites for N-acetylgalactosamine. Glycobiology 23:853–864
Julien S, Videira PA, Delannoy P (2012) Sialyl-tn in cancer: (how) did we miss the target? Biomol Ther 2:435–466
Ju T, Brewer K, D'Souza A et al (2002) Cloning and expression of human core 1 beta1,3-galactosyltransferase. J Biol Chem 277:178–186
Ju T, Cummings RD (2002) A unique molecular chaperone Cosmc required for activity of the mammalian core 1 beta 3-galactosyltransferase. Proc Natl Acad Sci U S A 99:16613–16618
Ju T, Lanneau GS, Gautam T et al (2008) Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc. Cancer Res 68:1636–1646
Karanikas V, Hwang LA, Pearson J et al (1997) Antibody and T cell responses of patients with adenocarcinoma immunized with mannan-MUC1 fusion protein. J Clin Invest 100:2783–2792
Kharband A, Rajabi H, Jin C et al (2014) Targeting the oncogenic MUC1-C protein inhibits mutant EGFR-mediated signaling and survival in non-small cell lung cancer cells. Clin Cancer Res 20: 5423–5434
Komatsu M, Carraway CA, Fregien NL et al (1997) Reversible disruption of cell-matrix and cell-cell interactions by overexpression of sialomucin complex. J Biol Chem 272:33245–33254
Kominsky SL, Argani P, Korz D et al (2003) Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene 22:2021–2033
Kozloski GA, Carraway CAC, Carraway KL (2010) Mechanistic and signaling analysis of Muc4-ErbB2 signaling module: new insights into the mechanism of ligand-independent ErbB2 activity. J Cell Physiol 224:649–657
Kudelka MR, Ju T, Heimburg-Molinaro J et al (2015) Simple sugars to complex disease—mucin-type O-glycans in cancer. Adv Cancer Res 126:53–135
Kufe D, Inghirami G, Abe M et al (1984) Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. Hybridoma 3:223–332

Kufe DW (2009) Mucins in cancer: function, prognosis and therapy. Nat Rev Cancer 9:874–885
Kufe DW (2013) MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. Oncogene 32:1073–1081
Kwon DS, Gregorio G, Bitton N et al (2002) DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. Immunity 16:135–144
Lakshmanan I, Seshacharyulu P, Haridas D et al (2015) Novel HER3/MUC4 oncogenic signaling aggravates the tumorigenic phenotypes of pancreatic cancer cells. Oncotarget 6:21085–21099
Lakshmanan I, Ponnusamy MP, Das S et al (2012) MUC16 induced rapid G2/M transition via interactions with JAK2 for increased proliferation and anti-apoptosis in breast cancer cells. Oncogene 31:805–817
Läubli H, Pearce OMT, Schwarz F et al (2014) Engagement of myelomonocytic Siglecs by tumor-associated ligands modulates the innate immune response to cancer. Proc Natl Acad Sci U S A 111:14211–14216
Lei Z, Maeda T, Tamura A et al (2012) EpCAM contributes to formation of functional tight junction in the intestinal epithelium by recruiting claudin proteins. Dev Biol 371:136–145
Leng Y, Cao C, Ren J (2007) Nuclear import of the MUC1-C oncoprotein is mediated by nucleoporin Nup62. J Biol Chem 282:19321–19330
Levitin F, Stern O, Weiss M et al (2005) The MUC1 SEA module is a self-cleaving domain. J Biol Chem 280:33374–33386
Li D, Romain G, Flamar AL (2012) Targeting self- and foreign antigens to dendritic cells via DC-ASGPR generates IL-10-producing suppressive CD4+ T cells. J Exp Med 209:109–121
Lillehoj EP, Kim H, Chun EY et al (2004) Pseudomonas aeruginosa stimulates phosphorylation of the airway epithelial membrane glycoprotein Muc1 and activates MAP kinase. Am J Physiol Lung Cell Mol Physiol 287:L809–L815
Lin JC, Wu YY, Wu JY et al (2012) TROP2 is epigenetically inactivated and modulates IGF-1R signalling in lung adenocarcinoma. EMBO Mol Med 4:472–485
Lipinski M, Parks DR, Rouse RV et al (1981) Human trophoblast cell-surface antigens defined by monoclonal antibodies. Proc Natl Acad Sci U S A 78:5147–5150
Liu FT, Patterson RJ, Wang JL (2002) Intracellular functions of galectins. Biochim Biophys Acta 1572:263–273
Liu FT, Rabinovich GA (2005) Galectins as modulators of tumour progression. Nat Rev Cancer 5: 29–41
Li Y, Liu D, Chen D et al (2003) Human DF3/MUC1 carcinoma-associated protein functions as an oncogene. Oncogene 22:6107–6110
Li Y, Ren J, Yu W et al (2001) The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin. J Biol Chem 276:35239–35242
Lu J, Gu J (2015) Significance of β-galactoside α2,6 sialyltranferase 1 in cancers. Molecules 20: 7509–7527
Lu Z, Ding L, Hong H et al (2011) Claudin-7 inhibits human lung cancer cell migration and invasion through ERK/MAPK signaling pathway. Exp Cell Res 317:1935–1946
Macao B, Johansson DGA, Hansson GC et al (2006) Autoproteolysis coupled to protein folding in the SEA domain of the membrane-bound MUC1 mucin. Nat Struct Mol Biol 13:71–76
Macauley MS, Crocker PR, Paulson JC (2014) Siglec-mediated regulation of immune cell function in disease. Nat Rev Immunol 14:653–666
Maeda T, Inoue M, Koshiba S et al (2004) Solution structure of the SEA domain from the murine homologue of ovarian cancer antigen CA125 (MUC16). Int J Biol Marker 13:179–187
Mantovani A, Marchesi F, Malesci A et al (2017) Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol 14:399–416
Masri AA, Gendler SJ (2005) Muc1 affects c-Src signaling in PyV MT-induced mammary tumorigenesis. Oncogene 24:5799–5808

Mitchell DA, Fadden AJ, Drickamer K (2001) A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands. J Biol Chem 276:28939–28945

Moniaux N, Escande F, Porchet N et al (2001) Structural organization and classification of the human mucin genes. Front Biosci 6:D1192–D1206

Monti P, Leone BE, Zerbi A et al (2004) Tumor-derived MUC1 mucins interact with differentiating monocytes and induce IL-10highIL-12low regulatory dendritic cell. J Immunol 172:7341–7349

Mori Y, Akita K, Yashiro M et al (2015) Binding of galectin-3, a β-galactoside-binding lectin, to MUC1 protein enhances phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt, promoting tumor cell malignancy. J Biol Chem 290:26125–26140

Mori Y, Akita K, Tanida S et al (2014) MUC1 protein induces urokinase-type plasminogen activator (uPA) by forming a complex with NF-κB p65 transcription factor and binding to the uPA promoter, leading to enhanced invasiveness of cancer cells. J Biol Chem 289:35193–35204

Mori Y, Akita K, Ojima K et al (2019) Trophoblast cell surface antigen 2 (Trop-2) phosphorylation by protein kinase C α/δ (PKCα/δ) enhances cell motility. J Biol Chem 294:11513–11524

Morris S, Ahmad N, André S et al (2004) Quaternary solution structures of galectins-1, -3, and -7. Glycobiology 14:293–300

Moustafa AEA, Alaoui-Jamali MA, Batist G et al (2002) Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. Oncogene 21:2634–2640

Mukherjee P, Tinder TL, Basu GD et al (2005) MUC1 (CD227) interacts with lck tyrosine kinase in Jurkat lymphoma cells and normal T cells. J Leukoc Biol 77:90–99

Munkley J (2016) The role of Sialyl-Tn in cancer. Int J Mol Sci 17:275

Nagy P, Friedländer E, Tanner M et al (2005) Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. Cancer Res 65:473–482

Napoletano C, Zizzari IG, Rughetti A et al (2012) Targeting of macrophage galactose-type C-type lectin (MGL) induces DC signaling and activation. Eur J Immunol 42:936–945

Nakatsukasa M, Kawasaki S, Yamasaki K et al (2010) Tumor-associated calcium signal transducer 2 is required for the proper subcellular localization of claudin 1 and 7: implications in the pathogenesis of gelatinous drop-like corneal dystrophy. Am J Pathol 177:1344–1355

Nath D, Hartnell A, Happerfield L et al (1999) Macrophage-tumour cell interactions: identification of MUC1 on breast cancer cells as a potential counter-receptor for the macrophage-restricted receptor, sialoadhesin. Immunology 98:213–219

Nath S, Mukherjee P (2014) MUC1: a multifaceted oncoprotein with a key role in cancer progression. Trends Mol Med 20:332–342

Nieminen J, Kuno A, Hirabayashi J et al (2007) Visualization of galectin-3 oligomerization on the surface of neutrophils and endothelial cells using fluorescence resonance energy transfer. J Biol Chem 282:1374–1383

O'Brien TJ, Beard JB, Underwood LJ et al (2001) The CA 125 gene: an extracellular superstructure dominated by repeat sequences. Tumour Biol 22:348–366

Ohta M, Ishida A, Toda M et al (2010) Immunomodulation of monocyte-derived dendritic cells through ligation of tumor-produced mucins to Siglec-9. Biochem Biophys Res Commun 402: 663–669

Orford K, Crockett C, Jensen JP et al (1997) Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem 272:24735–24738

Ozaki K, Lee RT, Lee YC et al (1995) The differences in structural specificity for recognition and binding between asialoglycoprotein receptors of liver and macrophages. Glycoconj J 12:268–274

Padler-Karavani V, Hurtado-Ziola N, Yung-Chi C et al (2014) Rapid evolution of binding specificities and expression patterns of inhibitory CD33-related Siglecs in primates. FASEB J 28: 1280–12893

Pandey P, Kharbanda S, Kufe D (1995) Association of the DF3/MUC1 breast cancer antigen with Grb2 and the Sos/Ras exchange protein. Cancer Res 55:4000–4003

Perdicchio M, Ilarregui JM, Verstege MI et al (2016a) Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and de novo induction of regulatory T cells. Proc Natl Acad Sci U S A 113:3329–3334

Perdicchio M, Cornelissen LAM, Streng-Ouwehand I et al (2016b) Tumor sialylation impedes T cell mediated anti-tumor responses while promoting tumor associated-regulatory T cells. Oncotarget 7:8771–8782

Piyush T, Chacko AR, Sindrewicz P et al (2017) Interaction of galectin-3 with MUC1 on cell surface promotes EGFR dimerization and activation in human epithelial cancer cells. Cell Death Differ 24:1937–1947

Ponnusamy MP, Singh AP, Jain M et al (2008) MUC4 activates HER2 signalling and enhances the motility of human ovarian cancer cells. Br J Cancer 99:520–526

Pröpster JM, Yang F, Rabbani S et al (2016) Structural basis for sulfation-dependent self-glycan recognition by the human immune-inhibitory receptor Siglec-8. Proc Natl Acad Sci U S A 113: E4170–E4179

Qu CF, Li Y, Song YJ et al (2004) MUC1 expression in primary and metastatic pancreatic cancer cells for in vitro treatment by (213)Bi-C595 radioimmunoconjugate. Br J Cancer 91:2086–2093

Rahn JJ, Shen Q, Mah BK et al (2004) MUC1 initiates a calcium signal after ligation by intercellular adhesion molecule-1. J Biol Chem 279:29386–29390

Raina D, Ahmad R, Rajabi H et al (2012) Targeting cysteine-mediated dimerization of the MUC1-C oncoprotein in human cancer cells. Int J Oncol 40:1643–1649

Ramasamy S, Duraisamy S, Barbashov S et al (2007) The MUC1 and galectin-3 oncoproteins function in a microRNA-dependent regulatory loop. Mol Cell 27:992–1004

Rao M, Atay SM, Shukla V et al (2016) Mithramycin depletes specificity protein 1 and activates p53 to mediate senescence and apoptosis of malignant pleural mesothelioma cells. Clin Cancer Res 22:1197–1210

Rao TD, Tian H, Ma X et al (2015) Expression of the carboxy-terminal portion of MUC16/CA125 induces transformation and tumor invasion. PLoS One 10:e0126633

Ren J, Raina D, Wen C et al (2006) MUC1 oncoprotein functions in activation of fibroblast growth factor receptor signaling. Mol Cancer Res 4:873–883

RodrÍguez E, Schetters STT, van Kooyk Y (2018) The tumour glyco-code as a novel immune checkpoint for immunotherapy. Nat Rev Immunol 18:204–211

Roy LD, Sahraei M, Subramani DB et al (2011) MUC1 enhances invasiveness of pancreatic cancer cells by inducing epithelial to mesenchymal transition. Oncogene 30:1449–1459

Ruvolo PP (2016) Galectin 3 as a guardian of the tumor microenvironment. Biochim Biophys Acta 1863:427–437

Saeland E, van Vliet SJ, Bäckström M et al (2007) The C-type lectin MGL expressed by dendritic cells detects glycan changes on MUC1 in colon carcinoma. Cancer Immunol Immunother 56: 1225–1236

Sancho D, e Sousa CR (2012) Signaling by myeloid C-type lectin receptors in immunity and homeostasis. Annu Rev Immunol 30:491–529

Sato S, Hughes RC (1994) Regulation of secretion and surface expression of Mac-2, a galactoside-binding protein of macrophages. J Biol Chem 269:4424–4430

Saussez S, Lorfevre F, Lequeux T et al (2008) The determination of the levels of circulating galectin-1 and -3 in HNSCC patients could be used to monitor tumor progression and/or responses to therapy. Oral Oncol 44:86–93

Schroeder JA, Masri AA, Adriance MC et al (2004) MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation. Oncogene 23:5739–5747

Schroeder JA, Thompson MC, Gardner MM et al (2001) Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland. J Biol Chem 276:13057–13064

Sciacchitano S, Lavra L, Morgante A et al (2018) Galectin-3: one molecule for an alphabet of diseases from A to Z. Int J Mol Sci 19:379

Shimura T, Takenaka Y, Tsutsumi S (2004) Galectin-3, a novel binding partner of beta-catenin. Cancer Res 64:6363–6367

Shukla SK, Purohit V, Mehla K et al (2017) MUC1 and HIF-1alpha signaling crosstalk induces anabolic glucose metabolism to impart gemcitabine resistance to pancreatic cancer. Cancer Cell 32:71–87

Siddiqui J, Abe M, Hayes D et al (1988) Isolation and sequencing of a cDNA coding for the human DF3 breast carcinoma-associated antigen. Proc Natl Acad Sci U S A 85:2320–2323

Sidenius N, Blasi F (2003) The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy. Cancer Metastasis Rev 22:205–222

Sindrewicz P, Lian LY, Yu LG (2016) Interaction of the oncofetal Thomsen-Friedenreich antigen with galectins in cancer progression and metastasis. Front Oncol 6:79

Singh PK, Wen Y, Swanson BJ et al (2007) Platelet-derived growth factor receptor beta-mediated phosphorylation of MUC1 enhances invasiveness in pancreatic adenocarcinoma cells. Cancer Res 67:5201–5210

Singh PK, Hollingsworth MA (2006) Cell surface-associated mucins in signal transduction. Trends Cell Biol 16:467–476

Singh PK, Behrens ME, Eggers JP et al (2008) Phosphorylation of MUC1 by Met modulates interaction with p53 and MMP1 expression. J Biol Chem 283:26985–26995

Singh SK, Streng-Ouwehand I, Litjens M et al (2009) Characterization of murine MGL1 and MGL2 C-type lectins: distinct glycan specificities and tumor binding properties. Mol Immunol 46: 1240–1249

Stanczak MA, Siddiqui SS, Trefny MP et al (2018) Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells. J Clin Invest 128:4912–4923

Streetly MJ, Maharaj L, Joel S et al (2010) GCS-100, a novel galectin-3 antagonist, modulates MCL-1, NOXA, and cell cycle to induce myeloma cell death. Blood 115:3939–3948

Strous GJ, Dekker J (1992) Mucin-type glycoproteins. Crit Rev Biochem Mol Biol 27:57–92

Sun Q, Ju T, Cummings RD (2011) The transmembrane domain of the molecular chaperone Cosmc directs its localization to the endoplasmic reticulum. J Biol Chem 286:11529–11542

Swanson BJ, McDermott KM, Singh PK et al (2007) MUC1 is a counter-receptor for myelin-associated glycoprotein (Siglec-4a) and their interaction contributes to adhesion in pancreatic cancer perineural invasion. Cancer Res 67:10222–10229

Swindall AF, Londoño-Joshi AI, Schultz MJ et al (2013) ST6Gal-I protein expression is upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines. Cancer Res 73:2368–2378

Takaoka M, Nakamura T, Ban Y et al (2007) Phenotypic investigation of cell junction-related proteins in gelatinous drop-like corneal dystrophy. Invest Ophthalmol Vis Sci 48:1095–1101

Tanida S, Mori Y, Ishida A et al (2014) Galectin-3 binds to MUC1-N-terminal domain and triggers recruitment of β-catenin in MUC1-expressing mouse 3T3 cells. Biochim Biophys Acta 1840: 1790–1797

Tanida S, Akita K, Ishida A et al (2013) Binding of the sialic acid-binding lectin, Siglec-9, to the membrane mucin, MUC1, induces recruitment of β-catenin and subsequent cell growth. J Biol Chem 288:31842–31852

Tanos B, Rodriguez-Boulan E (2008) The epithelial polarity program: machineries involved and their hijacking by cancer. Oncogene 27:6939–6957

Thériault C, Pinard M, Comamala M et al (2011) MUC16 (CA125) regulates epithelial ovarian cancer cell growth, tumorigenesis and metastasis. Gynecol Oncol 121:434–443

Thomas DB, Winzler RJ (1969) Structural studies on human erythrocyte glycoproteins. Alkali-labile oligosaccharides. J Biol Chem 244:5943–5946

Toda M, Hisano R, Yurugi H et al (2009) Ligation of tumour-produced mucins to CD22 dramatically impairs splenic marginal zone B-cells. Biochem J 417:673–683

Trerotola M, Cantanelli P, Guerra E et al (2013a) Upregulation of Trop-2 quantitatively stimulates human cancer growth. Oncogene 32:222–233
Trerotola M, Jernigan DL, Qin L et al (2013b) Trop-2 promotes prostate cancer metastasis by modulating β(1) integrin functions. Cancer Res 73:3155–3167
Unger WWJ, van Beelen AJ, Bruijns SC et al (2012) Glycan-modified liposomes boost CD4+ and CD8+ T-cell responses by targeting DC-SIGN on dendritic cells. J Control Release 160:88–95
Usami Y, Chiba H, Nakayama F et al (2006) Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. Hum Pathol 37:569–577
van de Wall S, Santegoets KCM, van Houtum EJH et al (2020) Sialoglycans and Siglecs can shape the tumor immune microenvironment. Trends Immunol 41:274–285
van Gisbergen KP, Aarnoudse CA, Meijer GA et al (2005) Dendritic cells recognize tumor-specific glycosylation of carcinoembryonic antigen on colorectal cancer cells through dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin. Cancer Res 65:5935–5944
van Kooyk Y, Ilarregui JM, van Vliet SJ et al (2015) Novel insights into the immunomodulatory role of the dendritic cell and macrophage-expressed C-type lectin MGL. Immunobiology 220: 185–192
van Vliet SJ, Bay S, Vuist IM et al (2013) MGL signaling augments TLR2-mediated responses for enhanced IL-10 and TNF-α secretion. J Leukoc Biol 94:315–323
van Vliet SJ, van Liempt E, Saeland E et al (2005) Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells. Int Immunol 17:661–669
van Vliet SJ, Saeland E, van Kooyk Y (2008) Sweet preferences of MGL: carbohydrate specificity and function. Trends Immunol 29:83–90
van Vliet SJ, Gringhuis SI, Geijtenbeek TBH et al (2006) Regulation of effector T cells by antigen-presenting cells via interaction of the C-type lectin MGL with CD45. Nat Immunol 7:1200–1208
Varki A, Angata T (2006) Siglecs—the major subfamily of I-type lectins. Glycobiology 16:1R–27R
Vermeer PD, Einwalter LA, Moninger TO et al (2003) Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. Nature 422:322–326
Wang J, Ryan D, Yiyu D et al (2008) Identification of Trop-2 as an oncogene and an attractive therapeutic target in colon cancers. Mol Cancer 7:280–285
Wei X, Hai X, Kufe D (2006) MUC1 oncoprotein stabilizes and activates estrogen receptor alpha. Mol Cell 21:295–305
Wesseling J, van der Valk SW, Hilkens J (1996) A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucin episialin/MUC1. Mol Biol Cell 7:565–577
Workman HC, Sweeney C, Carraway KL 3rd (2009) The membrane mucin Muc4 inhibits apoptosis induced by multiple insults via ErbB2-dependent and ErbB2-independent mechanisms. Cancer Res 69:2845–2852
Xiao H, Woods EC, Vukojicic P et al (2016) Precision glycocalyx editing as a strategy for cancer immunotherapy. Proc Natl Acad Sci U S A 85:2320–2323
Xie J, Mølck C, Paquet-Fifield S et al (2016) High expression of TROP2 characterizes different cell subpopulations in androgen-sensitive and androgen-independent prostate cancer cells. Oncotarget 7:44492–44504
Yamamoto M, Bharti A, Li Y et al (1997) Interaction of the DF3/MUC1 breast carcinoma-associated antigen and beta-catenin in cell adhesion. J Biol Chem 272:12492–12494
Yamashita T, Mori Y, Alzaaqi SM et al (2019) Induction of Trop-2 expression through the binding of galectin-3 to MUC1. Biochem Biophys Res Commun 516:44–49
Yang RY, Hill PN, Hsu DK et al (1998) Role of the carboxyl-terminal lectin domain in self-association of galectin-3. Biochemistry 37:4086–4092
Yin BW, Lloyd KO (2001) Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. J Biol Chem 276:27371–27375

Yu LG, Andrews N, Zhao Q et al (2007) Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion. J Biol Chem 282:773–781

Zeng P, Chen MB, Zhou LN et al (2016) Impact of TROP2 expression on prognosis in solid tumors: a systematic review and meta-analysis. Sci Rep 6:33658

Zhao Q, Guo X, Nash GB et al (2009) Circulating galectin-3 promotes metastasis by modifying MUC1 localization on cancer cell surface. Cancer Res 69:6799–6806

Zhao Q, Barclay M, Hilkens J et al (2010) Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis. Mol Cancer 9:154

Zizzari IG, Martufi P, Battisti F et al (2015) The macrophage galactose-type C-type lectin (MGL) modulates regulatory T cell functions. PLoS One 10:e0132617

Part V
Clinical Trials Targeting Glycosignals

Immunotherapy of Neuroblastoma Targeting GD2 and Beyond

Jung-Tung Hung and Alice L. Yu

Abstract Neuroblastoma is the most common extracranial solid tumor in children. Although low-risk neuroblastoma is mostly curable, the outcome of high-risk neuroblastoma, which comprises ~50% of neuroblastoma, is dismal. Abundant expression of disialoganglioside GD2 antigens is a feature of neuroblastoma. The success of immunotherapy targeting the GD2 antigen with dinutuximab has fueled the interest in developing improved immunotherapeutics including humanized anti-GD2 antibodies, GD2 mimotopes, cytokine-fused GD2-specific antibodies, GD2-specific chimeric antigen receptor T/NKT cells, GD2 vaccine, anti-GD2 idiotype monoclonal antibody, and anti-*O*-acetyl GD2 antibody. Advance in these active or passive cancer immunotherapies hold promises to further improve the outcome of neuroblastoma.

1 Introduction

Neuroblastoma is the most common malignancy in children aged under 1 year. It is a neuroendocrine tumor that originates from embryonal neural crest tissue. The common lesions of primary tumors are the adrenal medulla, neck, chest, and pelvis. About 8–10% of all childhood cancer cases are neuroblastoma. The incidence of neuroblastoma is 10.54 cases per million young children (<15 years old) per year. According to the International Neuroblastoma Risk Group classification system based on clinical and molecular features, the high-risk group, which represents about 50% of neuroblastoma, has a poor prognosis (Cohn et al. 2009). Genetic

J.-T. Hung
Institute of Stem Cell and Translational Cancer Research, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan

A. L. Yu (✉)
Institute of Stem Cell and Translational Cancer Research, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan

Department of Pediatric, University of California San Diego, La Jolla, CA, USA
e-mail: a1yu@health.ucsd.edu

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_10

alterations are frequent in neuroblastoma. *MYCN* amplification, ranging from 100 kb to >1 Mb (Amler and Schwab 1989), is associated with the rapid progression of neuroblastoma (Seeger et al. 1985). *ALK* with gain-of-function mutations (Mosse et al. 2008) and paired mesoderm homeobox protein 2B (*PHOX2B*) with loss-of-function mutations (Trochet et al. 2004) are two predisposing factors for familial neuroblastoma. Lin-28 homologue B (LIN28B), which can suppress let-7 to increase MYCN expression, is an independent risk factor for high-risk neuroblastoma (Molenaar et al. 2012). On the other hand, altered glycosylation is a hallmark during tumorigenesis (Reis et al. 2010). GD2 is a tumor-associated carbohydrate antigen abundantly expressed on the surface of neuroblastoma cells with ~10^7 GD2 molecules per neuroblastoma cell (Wu et al. 1986), but limited expression in normal tissues, making it an attractive target for the development of immunotherapies against neuroblastoma.

Disialoganglioside GD2 is a b-series ganglioside consisting of GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1ceramide. Several enzymes are involved in the synthesis of GD2, including galactosyltransferase I, GM3 synthase, GD3 synthase, and GM2/GD2 synthase (Furukawa et al. 2002; Kolter et al. 2002). The biological functions of GD2 have not been fully explored. GD2 ceramide inhibited proliferation and induced apoptosis of T cells (Ladisch et al. 1992; Biswas et al. 2009). Recently, Siglect-7 has been identified to be a ligand for GD2 (Theruvath et al. 2022). Upon binding to GD2, Siglect-7 might suppress immune cell activity through its cytoplasmic immunoreceptor-tyrosine-based inhibitory motif domain. In normal tissues, GD2 expression is weak and restricted to the central nervous system, peripheral pain fibers, and skin melanocytes (Svennerholm et al. 1994; Yuki et al. 1997). In contrast, GD2 is overexpressed in a variety of cancers that include neuroblastoma, small cell lung cancer, melanoma, glioma, osteosarcoma, rhabdomyosarcoma, and Ewing sarcoma (Schulz et al. 1984; Heiner et al. 1987; Tsuchida et al. 1987; Kailayangiri et al. 2012). In addition, expression of GD2 has been reported on cancer stem cells of breast cancer (Battula et al. 2012; Liang et al. 2013) and malignant phyllodes tumors of the breast (Lin et al. 2014). Thus, GD2 is an ideal target for cancer immunotherapy.

Prior to 2015, the standard therapy for high-risk neuroblastoma is chemotherapy, surgical resection, myeloablative therapy, autologous hematopoietic stem cell transplantation, and isotretinoin. Addition of immunotherapy with anti-GD2 (dinutuximab), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-2 (IL-2) to maintenance phase of treatment significantly improved the 2-year event-free survival from 46% to 66% (Yu et al. 2010), leading to the regulatory approval of dinutuximab in 2015. Thus, immunotherapy with dinutuximab has since been considered as the standard of care for high-risk neuroblastoma. In this chapter, we will focus on the immunotherapeutic strategies including immune checkpoint blockade, adoptive cellular therapies, antibody therapies, and cancer vaccines for further improvement of the outcome of neuroblastoma.

2 Antibody Therapies

Several GD2-specific antibodies have been investigated for the treatment of neuroblastoma, such as murine monoclonal antibody, monoclonal antibody fused with cytokine, chimeric monoclonal antibody, humanized monoclonal antibody, and bispecific antibody.

2.1 Murine Anti-GD2 Antibodies

Monoclonal antibody (mAb) 14G2a (Fig. 1a) is an IgG isotype switch variant of a GD2-specific murine IgG3 14.18 (Mujoo et al. 1989). The mAb 14G2a could change morphology and increase apoptosis of GD2-positive mouse lymphoma EL4 cells (Doronin et al. 2014). Similarly, mAb 14G2a activated caspase 3 to induce apoptosis of human neuroblastoma IMR-32 cells (Kowalczyk et al. 2009). Moreover, mAb 14G2a decreased metalloproteinase-2 to inhibit invasion of SJSA-1, MG-62, and Saos-2 cells (Liu et al. 2014). Besides, many studies showed that mAb 14G2a could inhibit p70S6, Akt, 4E-BP1, mTOR, MYCN, Aurora kinases, an inhibitor of DNA binding 1, and T cell leukemia homeobox 2, and increase p53, PHLDA1, JUN, Ras association RalGDS/AF-6 domain family member 6, and supervillain (Wierzbicki et al. 2008; Horwacik et al. 2013, 2015; Durbas et al. 2015).

Another murine anti-GD2 IgG3 mAb 3F8 (Fig. 1a) has been extensively investigated by Cheng's group. It altered morphology of SH-SY5Y-TrkB cells by Src-dependent activation of *N*-methyl-D-aspartate receptor signaling (Tong et al. 2015). The mAb 3F8 also activated caspases 3, 7, and 8 and inhibited survivin and AKT, leading to apoptosis of human melanoma HTB63 cells (Tsao et al. 2015).

The mAb 14G2a not only induced apoptosis of cancer cells but also killed cancer cells by complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (Mujoo et al. 1989). Moreover, mAb 14G2a suppressed the growth of M21 xenografts in athymic nude mice (Mueller et al. 1990). Phase I clinical trials of mAb 14G2a was conducted in patients with neuroblastoma or osteosarcoma. The dosage of mAb 14G2a was escalated up to 500 mg/m^2/course. Infusion of mAb 14G2a caused pain, hypotension, fever, urticarial, hypertension, tachycardia, and hyponatremia (Saleh et al. 1992; Murray et al. 1994; Uttenreuther-Fischer et al. 1995a). The pain was attributable to the binding of mAb 14G2a to peripheral nerve fibers, which express GD2 (Svennerholm et al. 1994). Some patients with partial or mixed responses were observed in these early phase trials (Saleh et al. 1992; Murray et al. 1994).

In a phase I study, mAb 3F8 had similar side effects as mAb 14G2a (Cheung et al. 1987). A phase II clinical trial of mAb 3F8 showed long-term survival (79–130+ months) of three patients (3/16) with stage 4 neuroblastoma (Cheung et al. 1998). Other phase II studies showed 62% 5-year EFS for stage 4 patients in the first remission who received mAb 3F8 + subcutaneous GM-CSF + cis-retinoic acid

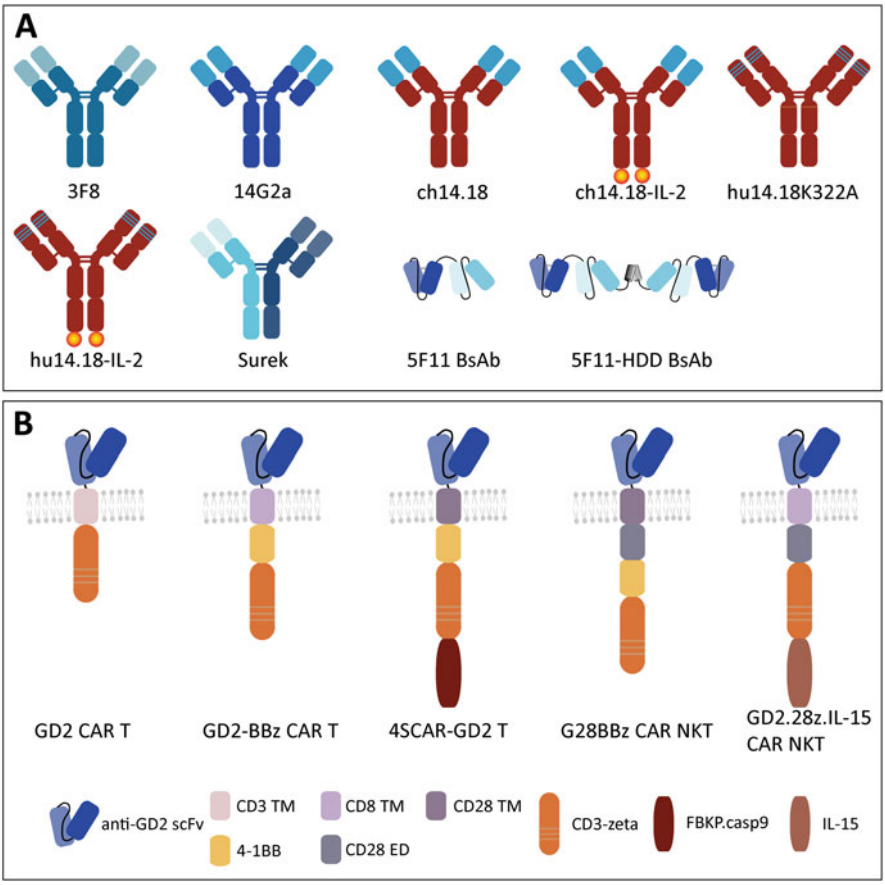


Fig. 1 Various GD2-specific antibodies and CAR constructs. (**a**) Various GD2-specific antibodies are shown: 3F8, 14G2a, chimeric 14.18 (ch14.18), ch14.18-IL-2, hu14.18K322A, hu14.18-IL-2, Surek (anti-GD2 x anti-mouse-CD3 trifunctional bispecific antibody), 5F11 BsAb (anti-GD2 x anti-CD3 bispecific antibody), and 5F11-HDD BsAb. (HDD: dimerization domain of human hepatocyte nuclear factor 1α). (**b**) Various GD2-specific CAR constructs are shown: GD2 CAR, GD2-BBz CAR, and 4SCAR-GD2 for T cells and G28BBz CAR and GD2.28z.IL-15 CAR for NKT cells. (BB: 4-1BB, z: CD3 zeta signaling domain, 28: CD28, TM: transmembrane domain, ED: endodomain)

(Cheung et al. 2012a; Cheung et al. 2014). In these studies, patients with the FCGR2A (R/R) genotype had a better outcome (Cheung et al. 2006). ^{131}I-labeled mAb 3F8 showed anticancer efficacy in human neuroblastoma xenografts in mice (Cheung et al. 1986). A phase I trial of ^{131}I-labeled mAb 3F8 given by intrathecal injection was conducted in 3 patients with recurrent metastatic central nervous system neuroblastoma. Extended survival (43, 41, and 33 months) of these patients was observed (Kramer et al. 2010). However, ^{131}I-labeled mAb 3F8 at dose levels of 4 mCi/kg/day × 5 days resulted in primary hypothyroidism (Bhandari et al. 2010).

Thus, to avoid potential adverse effects, protection of the thyroid gland will be needed for therapy of human neuroblastoma with ^{131}I-labeled mAb 3F8.

2.2 *Chimeric Anti-GD2 Monoclonal Antibody*

To reduce the induction of human antimouse antibodies, a chimeric mAb ch14.18 (Fig. 1a) was generated by fusing the variable regions of mAb 14G2a with constant regions of human IgG1-k (Gillies et al. 1989). The mAb ch14.18 triggered CDC and ADCC reactions to kill neuroblastoma cells in vitro and suppressed experimental liver metastasis in an NXS2 neuroblastoma animal model in vivo (Barker et al. 1991; Zeng et al. 2005). Moreover, the mAb ch14.18 displays 50–100-fold greater ADCC activity than mAb 14G2a (Mueller et al. 1990). In phase I studies, when compared to mAb 14G2a, mAb ch14.18 has a longer half-life (66.6 ± 27.4 h vs. 18.3 ± 11.8 h) (Handgretinger et al. 1995; Uttenreuther-Fischer et al. 1995a, b) but similar toxicity profiles (Handgretinger et al. 1995; Yu et al. 1998). The German Collaborative Neuroblastoma Study NB97 showed mydriasis and accommodation deficiency in 10/85 patients with high-risk neuroblastoma who received mAb ch14.18 treatment, but these ocular symptoms were reversible with time (Kremens et al. 2002). Notably, some patients with refractory/relapsed neuroblastoma in the phase I studies of mAb ch14.18 showed complete response (CR) and partial response (PR) (Handgretinger et al. 1995; Yu et al. 1998). To further enhance the anticancer efficacy, mAb ch14.18 was combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2, both of which enhance ADCC (Hank et al. 1990; Tarr 1996). Of 17 refractory/recurrent neuroblastoma, 5 CRs and 3 stable diseases (SDs) were observed in a pilot study of mAb ch14.18 + GM-CSF (Yu et al. 1995). Subsequently, a phase II study of mAb ch14.18 + GM-CSF showed 2 CRs, 2 PRs, and 1 mixed response (Yu et al. 1997). Subsequently, the MTD of mAb ch14.18 in combination with GM-CSF and IL-2 was 25 mg/m^2/d for 4 days when administered following consolidation therapy and stem cell transplant (Gilman et al. 2009). The toxicity of the regimen of mAb ch14.18 + GM-CSF + IL-2 was manageable and reversible (Hank et al. 1990). Consequently, a randomized phase III trial (COG study ANBL0032) of mAb ch14.18 with GM-CSF, interleukin-2, and isotretinoin was conducted in children with high-risk neuroblastoma after completing stem-cell transplantation and radiotherapy. Two-year event-free survival and overall survival rates were superior in patients treated with immunotherapy, *that is,* ch14.18 (dinutuximab) + GM-CSF + IL-2 + isotretinoin, than those treated with isotretinoin only (66 ± 5% vs. 46 ± 5%, $P = 0.01$ and 86 ± 4% vs. 75 ± 5%, $P = 0.02$, respectively) (Yu et al. 2010). Based on this data, US FDA and European medicines agency approved dinutuximab for the treatment of high-risk neuroblastoma in 2015. Since then, treatment with dinutuximab has become a standard of care for high-risk neuroblastoma. This is the first successful immunotherapy targeting a tumor-associated glycolipid antigen. Recently, a long-term follow-up (median 9.97 years) of the COG ANBL0032 study showed that mAb ch14.18 + GM-CSF + IL-

2 + isotretinoin significantly improved the 5-year EFS (57% vs. 46%) and OS (73% vs. 57%) of patients with high-risk neuroblastoma (Yu et al. 2021, 2022), although the benefits have decreased due to late relapses. Notably, immune correlative analyses revealed that patients with high affinity FCGR3A genotype, and KIR2DL2/C1 or KIR3DL1/HLA-B-Bw4-T80 genotypes had better outcome with anti-GD2 immunotherapy (Erbe et al. 2017, 2018; Desai et al. 2022).

To improve the efficacy of dinutuximab by combining with chemotherapy, COG conducted a randomized phase II selection design trial in patients with relapsed/refractory neuroblastoma. Eligible patients were randomly assigned to irinotecan and temozolomide plus either temsirolimus or dinutuximab + GM-CSF. Irinotecan–temozolomide–dinutuximab-GMCSF (I/T/DIN/GM-CSF) showed superior antitumor activity with 9/17 (53%) objective responses, versus 1/18 (6%) patients treated with I/T/temsirolimus (Mody et al. 2017). The trial was expanded to recruit additional 36 patients nonrandomly assigned to I/T/DIN/GM-CSF. In total, 22/53 patients showed objective responses (41·5%) (Mody et al. 2020). These findings provided the rationales for the ongoing development of a COG phase III study of combining dinutuximab with induction chemotherapy in high-risk neuroblastoma.

In view of the limited access of mAb ch14.18 produced by murine myeloma SP2/0 cells in Europe, the International Society of Paediatric Oncology European Neuroblastoma Group (SIOPEN) uses mAb ch14.18 generated by Chinese hamster ovary cells, designated as mAb ch14.18/CHO (dinutuximab beta) for clinical investigations. As compared to mAb ch14.18/SP2/0, similar toxicology and comparable pharmacokinetics were observed in phase I bridging study of mAb ch14.18/CHO in patients with neuroblastoma (Ladenstein et al. 2013). Subsequently, a randomized SIOPEN study of mAb ch14.18/CHO with or without IL2 showed similar efficacy for high-risk neuroblastoma (Ladenstein et al. 2018), but the combination of IL-2 with mAb ch14.18/CHO resulted in more toxicities than mAb ch14.18/CHO alone. In 2017, mAb ch14.18/CHO received regulatory approval by the European Medicines Agency, and designated as Dinutuximab-beta.

2.3 Humanized Anti-GD2 Antibody

Humanization of murine antibodies can reduce immunogenicity, enhance effector function, and increase half-life in the serum. A humanized mAb 14.18 (hu14.18) antibody was generated by CDR grafting of mAb ch14.18 V regions onto variable regions of human IgG1 heavy and kappa light chains (Metelitsa et al. 2002). In addition, a point mutation K322A at the Fc region was introduced to reduce complement activation, which causes neuropathic pain induced by fixation of C5a complement on anti-GD2-binding neuron (Sorkin et al. 2010). Furthermore, YB2/0 rat myeloma cells, which have low fucosylation activity leading to increased ADCC (Niwa et al. 2004), were used to produce the mAb hu14.18K322A (Fig. 1a). Indeed, the mAb hu14.18K322A displayed ADCC activity but induced negligible CDC and less allodynia than mAb ch14.18 in a rat model (Sorkin et al. 2010). A phase I

clinical trial of mAb hu14.18K322A showed that the maximum tolerated dose was 60 mg/m2/d for 4 days (Navid et al. 2014). Reversible ocular toxicities were seen in patients treated with mAb hu14.18K322A (Tse et al. 2015). A phase II study of mAb hu14.18K322A in combination with 6 courses of chemotherapy was conducted in patients with newly diagnosed high-risk neuroblastoma. After the first two chemo-immunotherapy cycles, an impressive 67% PR or better responses were noted in 42/63 patients (Furman et al. 2019). At the end-of-induction 60 of 62 patients (97%) had a partial response or better. Updated results of the induction chemotherapy with hu14.18K322A have demonstrated that 3-year EFS and OS were 73.7% and 86%, respectively (Furman et al. 2022). In addition, treatment of children with recurrent/refractory neuroblastoma with hu14.18K322A in combination with chemotherapeutic agents and haploidentical NK cell therapy resulted in 3 CRs, 1 very good partial response, 4 PRs, and 5 stable diseases (Federico et al. 2017).

In 2012, a humanized mAb 3F8 (hu3F8) was generated, and its binding activity to GD2 was similar to murine mAb 3F8. When compared to murine mAb 3F8, mAb hu3F8 had more potent ADCC but lower CDC activity (Cheung et al. 2012a, b). Based on in silico assisted design, a D32H (located at the L-CDR1) mutation was introduced in mAb hu3F8 to enhance the binding affinity to GD2. Although the binding affinity and ADCC of mAb hu3F8D32H were increased in vitro, its antitumor efficacy in IMR-32-bearing mice was not further improved (Zhao et al. 2015). A phase I trial of mAb hu3F8 in combination with GM-CSF for patients with resistant neuroblastoma demonstrated reversible neuropathic pain, low immunogenicity, and prolonged progression-free survival (Cheung et al. 2017; Kushner et al. 2018). Two single-arm, open-label trials (NCT03363373 and NCT01757626) were conducted to evaluate the antitumor efficacy of mAb hu3F8 in patients with relapsed/refractory neuroblastoma in the bone or bone marrow. The overall response rate for NCT03363373 and NCT01757626 was 45% and 34%, respectively (FDA 2020). These findings led to the FDA approval for mAb hu3F8 (naxitamab) for the treatment of relapsed/refractory high-risk neuroblastoma in 2020.

2.4 Cytokine-Conjugated GD2-Specific Antibodies

Another strategy to enhance anticancer efficacy is to link an antibody with cytokine to generate immunocytokine fusion protein, thereby inducing high concentration of cytokine in the tumor microenvironment to stimulate cellular immune responses against cancer cells. A fusion protein of hu14.18 and IL-2, hu14.18-IL2 (Fig. 1a), prolonged survival of NXS2-bearing mice with the involvement of NK cells and T cells (Neal et al. 2004a, b). Furthermore, upon binding to tumor cells, hu14.18-IL2 remained on the surface of tumor cells for a prolonged time, leading to the recruitment of NK cells to kill tumor cells (Buhtoiarov et al. 2011). In a phase I trial of patients with recurrent/refractory neuroblastoma, MTD of hu14.18-IL2 (EMD 273063) was 12 mg/m^2/day with expected toxicities similar to the combination of anti-GD2 and IL-2, and antitumor activity was observed in three patients (Osenga

et al. 2006). In a phase II study, hu14.18-IL2 resulted in 5 CR (5/23) for patients with disease evaluable by ^{123}I-metaiodobenzylguanidine (MIBG) imaging and/or bone marrow histology, while patients with measurable disease had no responses (Shusterman et al. 2010). Moreover, the mismatch between KIR and KIR-ligand was associated with better clinical response to hu14.18-IL2 therapy (Delgado et al. 2010).

2.5 Bispecific Antibody

It is an attractive therapeutic approach to combine fragments of two monoclonal antibodies to make a bispecific antibody that binds to two different antigens. Many bispecific antibody formats against GD2 have been generated and evaluated in vitro and in vivo. Generated by electrofusion of hybridoma cells, a trifunctional bispecific antibody TRBs07 was shown to redirect T cells to kill GD2-expressing melanoma cell lines (Ruf et al. 2004). Surek (anti-GD2 17A2 x anti-mouse CD3 Me361 with an intact Fc) (Fig. 1a), a trifunctional bispecific antibody generated by the TRION antibody platform technology, could not only prolong the survival of mice but also induce a long-term memory response to tumor cells (Eissler et al. 2012; Ruf et al. 2012). Interestingly, subcutaneous and intravenous injection of the Surek showed similar antitumor efficacy in mice; however, only subcutaneously injected Surek reduced CTLA-4 expression on $CD4^+$ and $CD8^+$ T cells (Deppisch et al. 2015). Subsequently, the anti-mouse CD3 in Surek was replaced with an antihuman CD3 to generate an Ektomun (anti-GD2 x anti-human CD3). Upon coculture of human neuroblastoma cells with PBMCs, Ektomun could activate effector cells to release proinflammatory cytokines and exert cytotoxic effects (Zirngibl et al. 2021). Another bispecific antibody anti-CD3 x anti-GD2 (3F8BiAb) generated by chemical heteroconjugation could redirect T cells to target neuroblastoma cells expressing GD2 (Yankelevich et al. 2012). A humanized hu3F8-BsAb showed GD2-specific cytotoxicity to neuroblastoma cells in vitro and anticancer efficacy in vivo (Xu et al. 2015). An MDX-260 BsAb prepared by crosslinking F(ab') fragments of mAb 7A4 (anti-GD2) and mAb 22 (anti-FcγRI) using bis-maleimide could trigger neutrophils to kill tumor in vitro (Michon et al. 1995).

A 5F11-BsAb (Fig. 1a) has been generated by genetic engineering with a gene construct comprised of VH-VL of anti-GD2 mAb 5F11, VHS44C and VLA100C mutations to stabilize disulfide bond, $(GGGGS)_3$ linker to link anti-huCD3 scFv, and an affinity maturation mutation VHP104Y. In IMR-32-bearing BALB/cA-*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice where human PBMCs were used as effector cells, the 5F11-BsAb effectively suppressed tumor growth (Cheng et al. 2015). However, renal clearance of 5F11-BsAb was rapid due to its small size. Thus, to prevent the rapid renal clearance and enhance the potency of 5F11-BsAb, a dimerization domain (residues 1-32) of human hepatocyte nuclear factor 1α (HNF1α) was added to the C-terminus of the 5F11-BsAb to generate 5F11-HDD-BsAb (Fig. 1a). The HNF1α dimerization domain could increase molecular size and the avidity of 5F11-BsAb, without

introducing immunogenicity. Surface plasmon resonance assay showed that 5F11-HDD-BsAb had a better affinity to GD2 than 5F11-BsAb, while both had a similar binding affinity to CD3. Moreover, the 5F11-HDD-BsAb significantly reduced tumor growth and prolonged survival in the IMR-32-bearing animal model. Notably, serum pharmacokinetic analysis revealed that 5F11-HDD-BsAb displayed a significantly longer half-life than 5F11-BsAb (Ahmed et al. 2015).

3 Adoptive Cellular Therapies

Adoptive cellular therapy (ACT) is a rapidly emerging immunotherapy approach, fueled by the FDA approval of two CAR T-cell therapies in 2017, one for the treatment of children with acute lymphoblastic leukemia (ALL) and the other for adults with advanced lymphomas (Mullard 2021). CAR is a synthetic receptor that combines a tumor-antigen-specific single-chain variable fragment with a transmembrane domain and an intracellular signaling domain (Rafiq et al. 2020). Upon binding to cancer cells by single-chain variable fragment, the intracellular signaling domain of CAR can transduce a signal to activate CAR T cells. Thus, CAR T cells can eliminate cancer cells in a major histocompatibility complex class I–independent fashion. Several GD2-directed CAR-T therapeutics are under evaluation in clinical trials. Epstein-Barr virus-specific T lymphocytes expressing a CAR specific for the GD2 (Fig. 1b, GD2 CAR T) have been generated and used for the treatment of patients with high-risk neuroblastoma. Tumor necrosis or complete remission was observed in 2 and 1 patients, respectively, at 6 weeks after treatment (Pule et al. 2008). Moreover, a long-term follow-up showed that the patients with persistence of GD2-CAR-T cells (>6 weeks) had a prolonged time to progression (Louis et al. 2011). Furthermore, tumor cells infected with oncolytic viruses to express RANTES and IL-15 ectopically could enhance the antitumor function of the GD2-CAR-T cells (Nishio and Dotti 2015). Another anti-GD2-BB-ζ CAR T cells (Fig. 1b), which comprised of scFv of mAb 126 (anti-GD2 IgM), the transmembrane domain of human CD8a hinge, human 4-1BB, and signaling domain of human CD3-ζ, killed neuroblastoma cells in vitro and suppressed tumor growth SH-SY5Y-bearing NOD. scid mice (Prapa et al. 2015). In a phase I clinical trial, six patients with refractory/recurrent neuroblastoma showed stable disease 6 months after treatment with 4SCAR-GD2 T cells (Fig. 1b), which contained intracellular signaling domain of CD28/4-1BB/CD3ζ-iCasp9, with an inducible caspase 9 gene in the anti-GD2-CAR construct (Yu et al. 2021, 2022). Adverse events associated with the 4SCARGD2 T-cell therapy, which included cytokine release syndrome and neuropathic pain, are manageable (Yu et al. 2021, 2022). In addition, the frequency of MDSC in human peripheral blood is a negative predictor of response to GD2-CAR-T (14G2a scFv-CD28-4-1BB-CD3ζ) cell therapy (Tumino et al. 2021).

In addition to CAR-T cells, different forms of ACT are being actively pursued. Natural killer T (NKT) cells, which are a sublineage of T cells with Vα24 invariant T cell receptors, play a role in neuroblastoma (Metelitsa et al. 2004; Song et al. 2007;

Liao et al. 2021). A G28BBz-CAR construct, which consisted of mAb 14G2a scFv, CD28 transmembrane domain, CD28 endodomain, 4-1BB endodomain, and ζ chain endodomain (Fig. 1b), was used to transfect primary human NKT cells to generate CAR.GD2 NKT cells. The CAR.GD2 NKT cells could infiltrate into the tumor site to exhibit potent cytotoxic activity (Heczey et al. 2014). Another CAR.GD2 NKT is generated using CAR construct (GD2.28z.15), which consisted of mAb 14G2a scFv, CD8 hinge and transmembrane regions, CD28 endodomain, CD3ζ signaling domain, 2A sequence of the equine rhinitis A virus, and IL-15 (Fig. 1b). It showed superior antitumor activity in CHLA-255 neuroblastoma-bearing mice (Xu et al. 2019). Thus, a first-in-human phase I trial of GD2.28z.15 CAR NKTs for children with relapsed/refractory neuroblastoma was conducted. An interim analysis of the first 3 patients showed that the GD2.28z.15 CAR NKTs could expand in vivo and migrate to tumor sites, and one patient had a partial response (Heczey et al. 2020). Thus, GD2-CAR T/NKT cell therapy will likely bring hope for the treatment of neuroblastoma in the near future. Nevertheless, it should be cautioned that it is still early days for CAR T cells and other forms of ACT. Whether they will ever be as effective against solid tumors as hematological malignancies remains to be demonstrated.

4 Cancer Vaccine

4.1 Racotumomab Vaccine

The majority (85%) of neuroblastoma expressed *N*-glycolyl GM3 (NeuGcGM3), regardless of NMYC amplification (Scursoni et al. 2011). NeuGcGM3 was undetectable in healthy human tissues (Irie et al. 1998), making it a potential target for neuroblastoma therapy. An anti-idiotype murine mAb racotumomab (also known as 1E10) (Vazquez et al. 1998) against anti-NeuGcGM3 mAb P3 (Vazquez et al. 1995) has been generated. In B16 melanoma- and F3II mammary tumor-bearing animal models, vaccination with racotumomab decreased tumor growth (Vazquez et al. 2000). Treatment of neuroblastoma patients with racotumomab showed grade 1–2 toxicity at the injection site and induction of IgG and IgM against NeuGcGM3 in the phase I trial (Cacciavillano et al. 2015). A phase II of racotumomab for children with high-risk neuroblastoma is ongoing (NCT02998983). Importantly, progression-free and overall survival were significantly prolonged in patients with non-small cell lung cancer who were vaccinated with racotumomab in a randomized phase II/III trial (Alfonso et al. 2014). Racotumomab was approved for the treatment of patients with recurrent/advanced non-small cell lung cancer in Cuba and Argentina in 2013.

4.2 GD2 Vaccines

In general, carbohydrate or glycolipid molecular including GD2 are poor antigens for inducing immune responses. Several approaches have been applied to enhance the immunogenicity of GD2. For example, conjugation of GD2 to keyhole limpet hemocyanin (KLH) protein derived from the *Megathura crenulato*, which is a T-cell-dependent antigen, can stimulate the immune system to produce antibodies (Swaminathan et al. 2014). The use of a potent immunostimulatory adjuvant, such as QS21 isolated from the *Quillaja saponaria*, can activate dendritic cells and $CD169^+$ macrophages to elicit strong immune responses. Another adjuvant, β-glycans, which are yeast cell wall carbohydrates, can activate immune cells and enhance ADCC (Cheung and Modak 2002; Dambuza and Brown 2015). GD2-KLH and GD3-KLH vaccines were produced by chemically crosslinking KLH to either GD2 or GD3. In a phase I trial, children with high-risk neuroblastoma in second or later remission were treated with GD2-KLH, GD3-KLH, a QS-21 equivalent OPT-821, and b-glucans. This regimen did not cause major toxicity and induced antibody responses against GD2/GD3 in 12 of 15 patients (Kushner et al. 2014). Subsequently, a phase II of the GD2-KLH/GD3-KLH/OPT-821/b-glucan in patients with high-risk neuroblastoma in remission was conducted. Multivariable analyses showed anti-GD2 IgG1 titer $>=$ 150 ng/mL by week 8 could contribute to favorable PFS and OS (Cheung et al. 2021).

4.3 GD2 Mimotope

GD2 mimotopes are carbohydrate-mimetic peptides that stimulate immune response to induce GD2-specific T cells and antibodies. Using a decamer phage display library, Forster-Waldl et al. obtained GD2 peptide mimotopes that could compete for binding to mAb ch14.18 (Forster-Waldl et al. 2005). To enhance immunogenicity, the GD2 peptide mimotopes were linked to KLH. The GD2 mimotope-KLH vaccine could elicit IgG antibodies to GD2 (Riemer et al. 2006). Another GD2 mimotope 47-LDA obtained from X(15) phage display library had increased mimicry to GD2 by molecular modeling. The 47-LDA-encoded plasmid DNA vaccine could induce GD2 cross-reactive IgG antibody response in vivo (Bolesta et al. 2005). Moreover, the 47-LDA DNA vaccine could induce cytotoxic T lymphocyte response to prolong survival of syngeneic neuroblastoma NXS2-bearing mice (Wierzbicki et al. 2008). In addition, computer modeling of mAb ch14.18 was used to design a GD2 mimicry MD (C-DGGWLSKGSW-C). A pSA-MA vector encoding for T-cell helper epitope and GD2-mimicking MD peptide was generated and transfected into *S. typhimurium* SL7207. Induction of tumor protective immunity was observed in NXS2-bearing mice, which were immunized by oral gavage of SL7207 carrying the pSA-MA vectors. Notably, the vaccine could increase NK cell activity, IFN-γ secretion, $CD4^+$ T cells, and $CD8^+$ T cells (Fest et al. 2006). In summary, GD2

mimotopes can induce not only humoral response but also cellular response, providing strong evidence for further development.

4.4 Anti-GD2 Idiotype Monoclonal Antibody

Danish immunologist Niels Kaj Jerne conceived the idiotypic network theory that antibodies (termed "Ab2" antibody) react to antigen-specific antibodies (termed "Ab1" antibody) (Jerne 1974). Anti-idiotypic (anti-Id) antibodies, which belong to Ab2, can bind to the idiotope of another antibody. An idiotope is a unique region within the Fv region of an antibody. Thus, the antigen-binding domain of anti-Id antibodies can structurally resemble the antigen epitope. The mAb 14G2a was used to generate an anti-idiotype antibody mAb 1A7 (also known as TriGem), which mimics the GD2 antigen (Saleh et al. 1993). Besides, DNA vaccines encoding scFv of mAb 1A7 could induce immune sera against GD2 in vivo (Zeytin et al. 2000).

A phase I clinical trial required 47 patients with advanced melanoma to study the toxicity and immune response of TriGem. Minimal toxicity was observed, including mild fever, chills, and local reaction at the injection site. Induction of anti-GD2 IgG antibody (Ab3) was found in 40 patients (85.1%, 40/47). Notably, one complete response and 12 stable diseases occurred in 47 patients with TriGem therapy (Foon et al. 1998, 2000).

In addition, 31 patients with high-risk neuroblastoma in first or subsequent complete remission or very good partial remission after frontline therapy were enrolled in a clinical trial of mAb 1A7 vaccine mixed with QS21 (Yu et al. 2001). Adverse reactions were transient fever, chills, serum sickness, and local reactions. Anti-mAb 1A7 was detected in all patients. Immune sera from some patients lysed human neuroblastoma cells in the ADCC and CDC reactions. At a median follow-up of 6.8 years, 16/21 patients (76.1%) remained in first remission with no evidence of disease (Yu et al. 2001).

Other idiotypic antibodies mimicking GD2 were generated. The mAb 4B5 was generated by fusing murine myeloma cell line Ag8 with peripheral blood lymphocytes of a patient who received mAb 14G2a treatment (Saleh et al. 1993). A clinical trial of human mAb 4B5 + GM-CSF was conducted in patients with stage III/IV melanoma (NCT00004184), but no results have been published. In addition, the mAb 3F8 was used to generate rat anti-3F8-idiotypic antibodies C2D8, Idio-2, AIG4, C2H7, C4E4, and A2A6. These anti-Id antibodies could inhibit the binding of mAb 3F8 to GD2 and induce GD2-specific antibodies in mice (Cheung et al. 1993). So far, these anti-Id antibodies have not been tested in a clinical trial.

In summary, immunotherapy with anti-Id GD2 vaccines can induce humoral responses to GD2-expressing tumor cells. Its therapeutic efficacy awaits verification in randomized clinical trials.

5 *O*-Acetyl GD2-Specific Antibody

A 9(7)-*O*-acetyl transferase can add an *O*-acetyl ester to the external sialic acid of GD2 to form an *O*-acetyl GD2 (Sjoberg and Varki 1993). An *O*-acetyl GD2-specific mAb 8B6 was generated by screening antibodies that bind to alkali-labile ganglioside *O*-acetyl GD2 after immunizing A/J mice with LAN-1 cells (Cerato et al. 1997). The mAb 8B6 could bind to many human tumor tissues, including neuroblastoma, melanoma, small cell line cancer, and renal tumors, but not pain nerve fibers (Alvarez-Rueda et al. 2011). Moreover, a human-mouse chimeric mAb c.8B6 not only exhibited ADCC and CDC activity in vitro but also reduced neuroblastoma liver metastasis in vivo. Importantly, the mAb ch14.18-inducing allodynia in a rat model was not observed in animals treated with the mAb c.8B6 (Terme et al. 2014). In addition, the mAb 8B6 could induce apoptosis and cell cycle arrest to inhibit the growth of GD2-expressing cancer cells (Cochonneau et al. 2013), as well as targeting *O*-acetyl GD2+ breast cancer stem cells to suppress tumor growth in vivo (Cheng et al. 2021). These promising preclinical findings provide a strong rationale for developing the *O*-acetyl GD2-targeting immunotherapeutics.

6 Immune Checkpoint Inhibitors

Blockade of immune checkpoint molecules cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) has shown remarkable therapeutic efficacy in some cancer patients. A few clinical trials of immune checkpoint inhibitors in neuroblastoma are now underway. Expression of programed death-ligand 1 (PD-L1) in clinical specimens of neuroblastoma has been reported to range from 18.9% to 35% in several studies (Zuo et al. 2020) (Saletta et al. 2017). Another study showed 25% (5/20) and 65% (13/20) of neuroblastoma at diagnosis and after 5-cycles of chemotherapy, respectively, expressed PD-L1 (Shirinbak et al. 2021). The clinical relevance of PD-L1 expression to the efficacy of anti-PD-1/PD-L1 in neuroblastoma remains to be determined. In preclinical studies, mAb PD-1 alone and mAb PD-L1 alone had no effect on tumor progression in syngeneic mice bearing with PD-L1-expressing Neuro2a and NXS2 neuroblastoma cells (Rigo et al. 2017). In a phase 1-2 trial of nivolumab (3 mg/kg) in children with measurable neuroblastoma, five of ten patients had stable disease (Davis et al. 2020). In another phase I clinical trial of ipilimumab, one neuroblastoma patient had stable disease for 2 months after single dose of ipilimumab at 10 mg/kg (Merchant et al. 2016). To improve the efficacy of ICI inhibitor in neuroblastoma, anti-GD2 in combination with PD-1 blockade was shown to significantly reduce tumor volume in A/J mice bearing neuroblastoma NXS2-HGW cells (Siebert et al. 2017). Based on this preclinical finding, two patients with refractory neuroblastoma were treated with the combination of Nivolumab and dinutuximab beta. One patient attained complete remission for 6 months, and the other showed complete disappearance of soft tissue

lesions and regression of skeletal lesions (Ehlert et al. 2020). Another checkpoint molecule CD47, which is a potent "don't eat me" signal to prevent phagocytosis by macrophages, is a potential therapeutic target for cancer immunotherapy. Combination of CD47 blockade and anti-GD2 showed significant antitumor efficacy in TH-MYCN syngeneic neuroblastoma model (Theruvath et al. 2022). In another study, inhibition of CSF-1R$^+$ myeloid cells with a tyrosine kinase inhibitor BLZ945 was shown to improve the anticancer efficacy of blocking antibodies against both PD-1 and PD-L1 in spontaneous neuroblastoma TH-*MYCN* mice (Mao et al. 2016). These findings suggest that neuroblastoma patients may benefit from ICI in combination with immunotherapy or other immune-modulating agents.

7 Conclusions

Improved outcomes in high-risk neuroblastoma patient after postconsolidation immunotherapy with dinutuximab have fueled further interest in refining immunotherapy against neuroblastoma. Strategies that aim for ameliorating the toxicities of the dinutuximab, improving efficacy by combination with chemotherapy or ICI, or developing new immunotherapeutics targeting GD2, *O*-acetyl GD2, GD3, NeuGcGM3 are flourishing. These endeavors will likely further improve the outcome of neuroblastoma.

References

Ahmed M, Cheng M, Cheung IY, Cheung NK (2015) Human derived dimerization tag enhances tumor killing potency of a T-cell engaging bispecific antibody. Onco Targets Ther 4:e989776. https://doi.org/10.4161/2162402X.2014.989776

Alfonso S, Valdes-Zayas A, Santiesteban ER, Flores YI, Areces F, Hernandez M, Viada CE, Mendoza IC, Guerra PP, Garcia E, Ortiz RA, de la Torre AV, Cepeda M, Perez K, Chong E, Hernandez AM, Toledo D, Gonzalez Z, Mazorra Z, Crombet T, Perez R, Vazquez AM, Macias AE (2014) A randomized, multicenter, placebo-controlled clinical trial of racotumomab-alum vaccine as switch maintenance therapy in advanced non-small cell lung cancer patients. Clin Cancer Res 20:3660–3671. https://doi.org/10.1158/1078-0432.CCR-13-1674

Alvarez-Rueda N, Desselle A, Cochonneau D, Chaumette T, Clemenceau B, Leprieur S, Bougras G, Supiot S, Mussini JM, Barbet J, Saba J, Paris F, Aubry J, Birkle S (2011) A monoclonal antibody to O-acetyl-GD2 ganglioside and not to GD2 shows potent anti-tumor activity without peripheral nervous system cross-reactivity. PLoS One 6:e25220. https://doi.org/10.1371/journal.pone.0025220

Amler LC, Schwab M (1989) Amplified N-myc in human neuroblastoma cells is often arranged as clustered tandem repeats of differently recombined DNA. Mol Cell Biol 9:4903–4913. https://doi.org/10.1128/mcb.9.11.4903-4913.1989

Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA (1991) Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. Cancer Res 51:144–149

Battula VL, Shi Y, Evans KW, Wang RY, Spaeth EL, Jacamo RO, Guerra R, Sahin AA, Marini FC, Hortobagyi G, Mani SA, Andreeff M (2012) Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. J Clin Invest 122:2066–2078. https://doi.org/10.1172/JCI59735

Bhandari S, Cheung NK, Kushner BH, Kramer K, Modak S, Larson SM, Yeh S, Heller G, Sklar CA (2010) Hypothyroidism after 131I-monoclonal antibody treatment of neuroblastoma. Pediatr Blood Cancer 55:76–80. https://doi.org/10.1002/pbc.22452

Biswas S, Biswas K, Richmond A, Ko J, Ghosh S, Simmons M, Rayman P, Rini B, Gill I, Tannenbaum CS, Finke JH (2009) Elevated levels of select gangliosides in T cells from renal cell carcinoma patients is associated with T cell dysfunction. J Immunol 183:5050–5058. https://doi.org/10.4049/jimmunol.0900259

Bolesta E, Kowalczyk A, Wierzbicki A, Rotkiewicz P, Bambach B, Tsao CY, Horwacik I, Kolinski A, Rokita H, Brecher M, Wang X, Ferrone S, Kozbor D (2005) DNA vaccine expressing the mimotope of GD2 ganglioside induces protective GD2 cross-reactive antibody responses. Cancer Res 65:3410–3418. https://doi.org/10.1158/0008-5472.CAN-04-2164

Buhtoiarov IN, Neal ZC, Gan J, Buhtoiarova TN, Patankar MS, Gubbels JA, Hank JA, Yamane B, Rakhmilevich AL, Reisfeld RA, Gillies SD, Sondel PM (2011) Differential internalization of hu14.18-IL2 immunocytokine by NK and tumor cell: impact on conjugation, cytotoxicity, and targeting. J Leukoc Biol 89:625–638. https://doi.org/10.1189/jlb.0710422

Cacciavillano W, Sampor C, Venier C, Gabri MR, de Davila MT, Galluzzo ML, Guthmann MD, Fainboim L, Alonso DF, Chantada GL (2015) A phase I study of the anti-idiotype vaccine racotumomab in neuroblastoma and other pediatric refractory malignancies. Pediatr Blood Cancer 62:2120–2124. https://doi.org/10.1002/pbc.25631

Cerato E, Birkle S, Portoukalian J, Mezazigh A, Chatal JF, Aubry J (1997) Variable region gene segments of nine monoclonal antibodies specific to disialogangliosides (GD2, GD3) and their O-acetylated derivatives. Hybridoma 16:307–316

Cheng M, Ahmed M, Xu H, Cheung NK (2015) Structural design of disialoganglioside GD2 and CD3-bispecific antibodies to redirect T cells for tumor therapy. Int J Cancer 136:476–486. https://doi.org/10.1002/ijc.29007

Cheng JY, Hung JT, Lin J, Lo FY, Huang JR, Chiou SP, Wang YH, Lin RJ, Wu JC, Yu J, Yu AL (2021) O-acetyl-GD2 as a therapeutic target for breast cancer stem cells. Front Immunol 12:791551. https://doi.org/10.3389/fimmu.2021.791551

Cheung NK, Modak S (2002) Oral (1-->3),(1-->4)-beta-D-glucan synergizes with antiganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma. Clin Cancer Res 8:1217–1223

Cheung NK, Landmeier B, Neely J, Nelson AD, Abramowsky C, Ellery S, Adams RB, Miraldi F (1986) Complete tumor ablation with iodine 131-radiolabeled disialoganglioside GD2-specific monoclonal antibody against human neuroblastoma xenografted in nude mice. J Natl Cancer Inst 77:739–745

Cheung NK, Lazarus H, Miraldi FD, Abramowsky CR, Kallick S, Saarinen UM, Spitzer T, Strandjord SE, Coccia PF, Berger NA (1987) Ganglioside GD2 specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. J Clin Oncol 5:1430–1440

Cheung NK, Canete A, Cheung IY, Ye JN, Liu C (1993) Disialoganglioside GD2 anti-idiotypic monoclonal antibodies. Int J Cancer 54:499–505

Cheung NK, Kushner BH, Yeh SD, Larson SM (1998) 3F8 monoclonal antibody treatment of patients with stage 4 neuroblastoma: a phase II study. Int J Oncol 12:1299–1306. https://doi.org/10.3892/ijo.12.6.1299

Cheung NK, Sowers R, Vickers AJ, Cheung IY, Kushner BH, Gorlick R (2006) FCGR2A polymorphism is correlated with clinical outcome after immunotherapy of neuroblastoma with anti-GD2 antibody and granulocyte macrophage colony-stimulating factor. J Clin Oncol 24:2885–2890. https://doi.org/10.1200/JCO.2005.04.6011

Cheung NK, Cheung IY, Kushner BH, Ostrovnaya I, Chamberlain E, Kramer K, Modak S (2012a) Murine anti-GD2 monoclonal antibody 3F8 combined with granulocyte-macrophage colony-

stimulating factor and 13-cis-retinoic acid in high-risk patients with stage 4 neuroblastoma in first remission. J Clin Oncol 30:3264–3270. https://doi.org/10.1200/JCO.2011.41.3807

Cheung NK, Guo H, Hu J, Tassev DV, Cheung IY (2012b) Humanizing murine IgG3 anti-GD2 antibody m3F8 substantially improves antibody-dependent cell-mediated cytotoxicity while retaining targeting in vivo. Onco Targets Ther 1:477–486

Cheung NK, Cheung IY, Kramer K, Modak S, Kuk D, Pandit-Taskar N, Chamberlain E, Ostrovnaya I, Kushner BH (2014) Key role for myeloid cells: phase II results of anti-G(D2) antibody 3F8 plus granulocyte-macrophage colony-stimulating factor for chemoresistant osteomedullary neuroblastoma. Int J Cancer 135:2199–2205. https://doi.org/10.1002/ijc.28851

Cheung IY, Kushner BH, Modak S, Basu EM, Roberts SS, Cheung NV (2017) Phase I trial of anti-GD2 monoclonal antibody hu3F8 plus GM-CSF: impact of body weight, immunogenicity and anti-GD2 response on pharmacokinetics and survival. Onco Targets Ther 6:e1358331. https://doi.org/10.1080/2162402X.2017.1358331

Cheung IY, Cheung NV, Modak S, Mauguen A, Feng Y, Basu E, Roberts SS, Ragupathi G, Kushner BH (2021) Survival impact of anti-GD2 antibody response in a phase II ganglioside vaccine trial among patients with high-risk neuroblastoma with prior disease progression. J Clin Oncol 39:215–226. https://doi.org/10.1200/JCO.20.01892

Cochonneau D, Terme M, Michaud A, Dorvillius M, Gautier N, Frikeche J, Alvarez-Rueda N, Bougras G, Aubry J, Paris F, Birkle S (2013) Cell cycle arrest and apoptosis induced by O-acetyl-GD2-specific monoclonal antibody 8B6 inhibits tumor growth in vitro and in vivo. Cancer Lett 333:194–204. https://doi.org/10.1016/j.canlet.2013.01.032

Cohn SL, Pearson AD, London WB, Monclair T, Ambros PF, Brodeur GM, Faldum A, Hero B, Iehara T, Machin D, Mosseri V, Simon T, Garaventa A, Castel V, Matthay KK, Force IT (2009) The international neuroblastoma risk group (INRG) classification system: an INRG task force report. J Clin Oncol 27:289–297. https://doi.org/10.1200/JCO.2008.16.6785

Dambuza IM, Brown GD (2015) C-type lectins in immunity: recent developments. Curr Opin Immunol 32:21–27. https://doi.org/10.1016/j.coi.2014.12.002

Davis KL, Fox E, Merchant MS, Reid JM, Kudgus RA, Liu X, Minard CG, Voss S, Berg SL, Weigel BJ, Mackall CL (2020) Nivolumab in children and young adults with relapsed or refractory solid tumours or lymphoma (ADVL1412): a multicentre, open-label, single-arm, phase 1-2 trial. Lancet Oncol 21:541–550. https://doi.org/10.1016/S1470-2045(20)30023-1

Delgado DC, Hank JA, Kolesar J, Lorentzen D, Gan J, Seo S, Kim K, Shusterman S, Gillies SD, Reisfeld RA, Yang R, Gadbaw B, DeSantes KB, London WB, Seeger RC, Maris JM, Sondel PM (2010) Genotypes of NK cell KIR receptors, their ligands, and Fc-gamma receptors in the response of neuroblastoma patients to Hu14.18-IL2 immunotherapy. Cancer Res 70:9554–9561. https://doi.org/10.1158/0008-5472.CAN-10-2211

Deppisch N, Ruf P, Eissler N, Neff F, Buhmann R, Lindhofer H, Mocikat R (2015) Efficacy and tolerability of a GD2-directed trifunctional bispecific antibody in a preclinical model: subcutaneous administration is superior to intravenous delivery. Mol Cancer Ther 14:1877–1883. https://doi.org/10.1158/1535-7163.MCT-15-0156

Desai AV, Gilman AL, Ozkaynak MF, Naranjo A, London WB, Tenney SC, Diccianni M, Hank JA, Parisi MT, Shulkin BL, Smith M, Moscow JA, Shimada H, Matthay KK, Cohn SL, Maris JM, Bagatell R, Sondel PM, Park JR, Yu AL (2022) Outcomes following anti-GD2 antibody-based post-consolidation therapy after cessation of randomization on ANBL0032: a report from the children's oncology group. J Clin Oncol

Doronin II, Vishnyakova PA, Kholodenko IV, Ponomarev ED, Ryazantsev DY, Molotkovskaya IM, Kholodenko RV (2014) Ganglioside GD2 in reception and transduction of cell death signal in tumor cells. BMC Cancer 14:295. https://doi.org/10.1186/1471-2407-14-295

Durbas M, Horwacik I, Boratyn E, Kamycka E, Rokita H (2015) GD2 ganglioside specific antibody treatment downregulates PI3K/Akt/mTOR signaling network in human neuroblastoma cell lines. Int J Oncol 47:1143–1159. https://doi.org/10.3892/ijo.2015.3070

Ehlert K, Hansjuergens I, Zinke A, Otto S, Siebert N, Henze G, Lode H (2020) Nivolumab and dinutuximab beta in two patients with refractory neuroblastoma. J Immunother Cancer 8(1): e000540. https://doi.org/10.1136/jitc-2020-000540

Eissler N, Ruf P, Mysliwietz J, Lindhofer H, Mocikat R (2012) Trifunctional bispecific antibodies induce tumor-specific T cells and elicit a vaccination effect. Cancer Res 72:3958–3966. https://doi.org/10.1158/0008-5472.CAN-12-0146

Erbe AK, Wang W, Reville PK, Carmichael L, Kim K, Mendonca EA, Song Y, Hank JA, London WB, Naranjo A, Hong F, Hogarty MD, Maris JM, Park JR, Ozkaynak MF, Miller JS, Gilman AL, Kahl B, Yu AL, Sondel PM (2017) HLA-Bw4-I-80 isoform differentially influences clinical outcome as compared to HLA-Bw4-T-80 and HLA-A-Bw4 isoforms in rituximab or dinutuximab-based cancer immunotherapy. Front Immunol 8:675. https://doi.org/10.3389/fimmu.2017.00675

Erbe AK, Wang W, Carmichael L, Kim K, Mendonca EA, Song Y, Hess D, Reville PK, London WB, Naranjo A, Hank JA, Diccianni MB, Reisfeld RA, Gillies SD, Matthay KK, Cohn SL, Hogarty MD, Maris JM, Park JR, Ozkaynak MF, Gilman AL, Yu AL, Sondel PM (2018) Neuroblastoma patients' KIR and KIR-ligand genotypes influence clinical outcome for dinutuximab-based immunotherapy: a report from the children's oncology group. Clin Cancer Res 24:189–196. https://doi.org/10.1158/1078-0432.CCR-17-1767

FDA (2020) FDA grants accelerated approval to naxitamab for high-risk neuroblastoma in bone or bone marrow Journal 2022:U.S. Food & Drug

Federico SM, McCarville MB, Shulkin BL, Sondel PM, Hank JA, Hutson P, Meagher M, Shafer A, Ng CY, Leung W, Janssen WE, Wu J, Mao S, Brennan RC, Santana VM, Pappo AS, Furman WL (2017) A pilot trial of humanized anti-GD2 monoclonal antibody (hu14.18K322A) with chemotherapy and natural killer cells in children with recurrent/refractory neuroblastoma. Clin Cancer Res 23:6441–6449. https://doi.org/10.1158/1078-0432.CCR-17-0379

Fest S, Huebener N, Weixler S, Bleeke M, Zeng Y, Strandsby A, Volkmer-Engert R, Landgraf C, Gaedicke G, Riemer AB, Michalsky E, Jaeger IS, Preissner R, Forster-Wald E, Jensen-Jarolim-E, Lode HN (2006) Characterization of GD2 peptide mimotope DNA vaccines effective against spontaneous neuroblastoma metastases. Cancer Res 66:10567–10575. https://doi.org/10.1158/0008-5472.CAN-06-1158

Foon KA, Sen G, Hutchins L, Kashala OL, Baral R, Banerjee M, Chakraborty M, Garrison J, Reisfeld RA, Bhattacharya-Chatterjee M (1998) Antibody responses in melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. Clin Cancer Res 4:1117–1124

Foon KA, Lutzky J, Baral RN, Yannelli JR, Hutchins L, Teitelbaum A, Kashala OL, Das R, Garrison J, Reisfeld RA, Bhattacharya-Chatterjee M (2000) Clinical and immune responses in advanced melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. J Clin Oncol 18:376–384

Forster-Waldl E, Riemer AB, Dehof AK, Neumann D, Bramswig K, Boltz-Nitulescu G, Pehamberger H, Zielinski CC, Scheiner O, Pollak A, Lode H, Jensen-Jarolim E (2005) Isolation and structural analysis of peptide mimotopes for the disialoganglioside GD2, a neuroblastoma tumor antigen. Mol Immunol 42:319–325. https://doi.org/10.1016/j.molimm.2004.07.011

Furman WL, Federico SM, McCarville MB, Shulkin BL, Davidoff AM, Krasin MJ, Sahr N, Sykes A, Wu J, Brennan RC, Bishop MW, Helmig S, Stewart E, Navid F, Triplett B, Santana VM, Bahrami A, Anthony G, Yu AL, Hank J, Gillies SD, Sondel PM, Leung WH, Pappo AS (2019) A phase II trial of Hu14.18K322A in combination with induction chemotherapy in children with newly diagnosed high-risk neuroblastoma. Clin Cancer Res 25:6320–6328. https://doi.org/10.1158/1078-0432.CCR-19-1452

Furman WL, McCarville B, Shulkin BL, Davidoff A, Krasin M, Hsu CW, Pan H, Wu J, Brennan R, Bishop MW, Helmig S, Stewart E, Navid F, Triplett B, Santana V, Santiago T, Hank JA, Gillies SD, Yu A, Sondel PM, Leung WH, Pappo A, Federico SM (2022) Improved outcome in children with newly diagnosed high-risk neuroblastoma treated with chemoimmunotherapy:

updated results of a phase II study using hu14.18K322A. J Clin Oncol 40:335–344. https://doi.org/10.1200/JCO.21.01375

Furukawa K, Takamiya K, Furukawa K (2002) Beta1,4-N-acetylgalactosaminyltransferase--GM2/GD2 synthase: a key enzyme to control the synthesis of brain-enriched complex gangliosides. Biochim Biophys Acta 1573:356–362

Gillies SD, Lo KM, Wesolowski J (1989) High-level expression of chimeric antibodies using adapted cDNA variable region cassettes. J Immunol Methods 125:191–202

Gilman AL, Ozkaynak MF, Matthay KK, Krailo M, Yu AL, Gan J, Sternberg A, Hank JA, Seeger R, Reaman GH, Sondel PM (2009) Phase I study of ch14.18 with granulocyte-macrophage colony-stimulating factor and interleukin-2 in children with neuroblastoma after autologous bone marrow transplantation or stem-cell rescue: a report from the children's oncology group. J Clin Oncol 27:85–91. https://doi.org/10.1200/JCO.2006.10.3564

Handgretinger R, Anderson K, Lang P, Dopfer R, Klingebiel T, Schrappe M, Reuland P, Gillies SD, Reisfeld RA, Neithammer D (1995) A phase I study of human/mouse chimeric antiganglioside GD2 antibody ch14.18 in patients with neuroblastoma. Eur J Cancer 31A:261–267

Hank JA, Robinson RR, Surfus J, Mueller BM, Reisfeld RA, Cheung NK, Sondel PM (1990) Augmentation of antibody dependent cell mediated cytotoxicity following in vivo therapy with recombinant interleukin 2. Cancer Res 50:5234–5239

Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, Gao X, Guo L, Yvon E, Hicks J, Liu H, Dotti G, Metelitsa LS (2014) Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. Blood 124:2824–2833. https://doi.org/10.1182/blood-2013-11-541235

Heczey A, Courtney AN, Montalbano A, Robinson S, Liu K, Li M, Ghatwai N, Dakhova O, Liu B, Raveh-Sadka T, Chauvin-Fleurence CN, Xu X, Ngai H, Di Pierro EJ, Savoldo B, Dotti G, Metelitsa LS (2020) Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. Nat Med 26:1686–1690. https://doi.org/10.1038/s41591-020-1074-2

Heiner JP, Miraldi F, Kallick S, Makley J, Neely J, Smith-Mensah WH, Cheung NK (1987) Localization of GD2-specific monoclonal antibody 3F8 in human osteosarcoma. Cancer Res 47:5377–5381

Horwacik I, Durbas M, Boratyn E, Wegrzyn P, Rokita H (2013) Targeting GD2 ganglioside and aurora A kinase as a dual strategy leading to cell death in cultures of human neuroblastoma cells. Cancer Lett 341:248–264. https://doi.org/10.1016/j.canlet.2013.08.018

Horwacik I, Durbas M, Boratyn E, Sawicka A, Wegrzyn P, Krzanik S, Gorka A, Drozniak J, Augustyniak E, Kowalczyk A, Rokita H (2015) Analysis of genes involved in response to doxorubicin and a GD2 ganglioside-specific 14G2a monoclonal antibody in IMR-32 human neuroblastoma cells. Acta Biochim Pol 62:423–433. https://doi.org/10.18388/abp.2015_1035

Irie A, Koyama S, Kozutsumi Y, Kawasaki T, Suzuki A (1998) The molecular basis for the absence of N-glycolylneuraminic acid in humans. J Biol Chem 273:15866–15871. https://doi.org/10.1074/jbc.273.25.15866

Jerne NK (1974) Towards a network theory of the immune system. Ann Immunol (Paris) 125C:373–389

Kailayangiri S, Altvater B, Meltzer J, Pscherer S, Luecke A, Dierkes C, Titze U, Leuchte K, Landmeier S, Hotfilder M, Dirksen U, Hardes J, Gosheger G, Juergens H, Rossig C (2012) The ganglioside antigen G(D2) is surface-expressed in Ewing sarcoma and allows for MHC-independent immune targeting. Br J Cancer 106:1123–1133. https://doi.org/10.1038/bjc.2012.57

Kolter T, Proia RL, Sandhoff K (2002) Combinatorial ganglioside biosynthesis. J Biol Chem 277:25859–25862. https://doi.org/10.1074/jbc.R200001200

Kowalczyk A, Gil M, Horwacik I, Odrowaz Z, Kozbor D, Rokita H (2009) The GD2-specific 14G2a monoclonal antibody induces apoptosis and enhances cytotoxicity of chemotherapeutic drugs in IMR-32 human neuroblastoma cells. Cancer Lett 281:171–182. https://doi.org/10.1016/j.canlet.2009.02.040

Kramer K, Kushner BH, Modak S, Pandit-Taskar N, Smith-Jones P, Zanzonico P, Humm JL, Xu H, Wolden SL, Souweidane MM, Larson SM, Cheung NK (2010) Compartmental intrathecal radioimmunotherapy: results for treatment for metastatic CNS neuroblastoma. J Neuro-Oncol 97:409–418. https://doi.org/10.1007/s11060-009-0038-7

Kremens B, Hero B, Esser J, Weinel P, Filger-Brillinger J, Fleischhack G, Graf N, Gruttner HP, Niemeyer C, Schulz A, Wickmann L, Berthold F (2002) Ocular symptoms in children treated with human-mouse chimeric anti-GD2 mAb ch14.18 for neuroblastoma. Cancer Immunol Immunother 51:107–110. https://doi.org/10.1007/s00262-001-0259-x

Kushner BH, Cheung IY, Modak S, Kramer K, Ragupathi G, Cheung NK (2014) Phase I trial of a bivalent gangliosides vaccine in combination with beta-glucan for high-risk neuroblastoma in second or later remission. Clin Cancer Res 20:1375–1382. https://doi.org/10.1158/1078-0432.CCR-13-1012

Kushner BH, Cheung IY, Modak S, Basu EM, Roberts SS, Cheung NK (2018) Humanized 3F8 anti-GD2 monoclonal antibody dosing with granulocyte-macrophage Colony-stimulating factor in patients with resistant neuroblastoma: A phase 1 clinical trial. JAMA Oncol 4:1729–1735. https://doi.org/10.1001/jamaoncol.2018.4005

Ladenstein R, Weixler S, Baykan B, Bleeke M, Kunert R, Katinger D, Pribill I, Glander P, Bauer S, Pistoia V, Michon J, Garaventa A, Lode HN (2013) Ch14.18 antibody produced in CHO cells in relapsed or refractory stage 4 neuroblastoma patients: a SIOPEN phase 1 study. MAbs 5:801–809. https://doi.org/10.4161/mabs.25215

Ladenstein R, Potschger U, Valteau-Couanet D, Luksch R, Castel V, Yaniv I, Laureys G, Brock P, Michon JM, Owens C, Trahair T, Chan GCF, Ruud E, Schroeder H, Beck Popovic M, Schreier G, Loibner H, Ambros P, Holmes K, Castellani MR, Gaze MN, Garaventa A, Pearson ADJ, Lode HN (2018) Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. Lancet Oncol 19:1617–1629. https://doi.org/10.1016/S1470-2045(18)30578-3

Ladisch S, Becker H, Ulsh L (1992) Immunosuppression by human gangliosides: I. Relationship of carbohydrate structure to the inhibition of T cell responses. Biochim Biophys Acta 1125:180–188

Liang YJ, Ding Y, Levery SB, Lobaton M, Handa K, Hakomori SI (2013) Differential expression profiles of glycosphingolipids in human breast cancer stem cells vs. cancer non-stem cells. Proc Natl Acad Sci U S A 110:4968–4973. https://doi.org/10.1073/pnas.1302825110

Liao YM, Hung TH, Tung JK, Yu J, Hsu YL, Hung JT, Yu AL (2021) Low expression of IL-15 and NKT in tumor microenvironment predicts poor outcome of MYCN-non-amplified neuroblastoma. J Pers Med 11(2):122. https://doi.org/10.3390/jpm11020122

Lin JJ, Huang CS, Yu J, Liao GS, Lien HC, Hung JT, Lin RJ, Chou FP, Yeh KT, Yu AL (2014) Malignant phyllodes tumors display mesenchymal stem cell features and aldehyde dehydrogenase/disialoganglioside identify their tumor stem cells. Breast Cancer Res 16:R29. https://doi.org/10.1186/bcr3631

Liu B, Wu Y, Zhou Y, Peng D (2014) Endothelin A receptor antagonism enhances inhibitory effects of anti-ganglioside GD2 monoclonal antibody on invasiveness and viability of human osteosarcoma cells. PLoS One 9:e93576. https://doi.org/10.1371/journal.pone.0093576

Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, Rossig C, Russell HV, Diouf O, Liu E, Liu H, Wu MF, Gee AP, Mei Z, Rooney CM, Heslop HE, Brenner MK (2011) Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood 118:6050–6056. https://doi.org/10.1182/blood-2011-05-354449

Mao Y, Eissler N, Blanc KL, Johnsen JI, Kogner P, Kiessling R (2016) Targeting suppressive myeloid cells potentiates checkpoint inhibitors to control spontaneous neuroblastoma. Clin Cancer Res 22:3849–3859. https://doi.org/10.1158/1078-0432.CCR-15-1912

Merchant MS, Wright M, Baird K, Wexler LH, Rodriguez-Galindo C, Bernstein D, Delbrook C, Lodish M, Bishop R, Wolchok JD, Streicher H, Mackall CL (2016) Phase I clinical trial of ipilimumab in pediatric patients with advanced solid tumors. Clin Cancer Res 22:1364–1370. https://doi.org/10.1158/1078-0432.CCR-15-0491

Metelitsa LS, Gillies SD, Super M, Shimada H, Reynolds CP, Seeger RC (2002) Antidisialoganglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis. Blood 99:4166–4173

Metelitsa LS, Wu HW, Wang H, Yang Y, Warsi Z, Asgharzadeh S, Groshen S, Wilson SB, Seeger RC (2004) Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2. J Exp Med 199:1213–1221. https://doi.org/10.1084/jem.20031462

Michon J, Moutel S, Barbet J, Romet-Lemonne JL, Deo YM, Fridman WH, Teillaud JL (1995) In vitro killing of neuroblastoma cells by neutrophils derived from granulocyte colony-stimulating factor-treated cancer patients using an anti-disialoganglioside/anti-Fc gamma RI bispecific antibody. Blood 86:1124–1130

Mody R, Naranjo A, Van Ryn C, Yu AL, London WB, Shulkin BL, Parisi MT, Servaes SE, Diccianni MB, Sondel PM, Bender JG, Maris JM, Park JR, Bagatell R (2017) Irinotecan-temozolomide with temsirolimus or dinutuximab in children with refractory or relapsed neuroblastoma (COG ANBL1221): an open-label, randomised, phase 2 trial. Lancet Oncol 18:946–957. https://doi.org/10.1016/S1470-2045(17)30355-8

Mody R, Yu AL, Naranjo A, Zhang FF, London WB, Shulkin BL, Parisi MT, Servaes SE, Diccianni MB, Hank JA, Felder M, Birstler J, Sondel PM, Asgharzadeh S, Glade-Bender J, Katzenstein H, Maris JM, Park JR, Bagatell R (2020) Irinotecan, temozolomide, and dinutuximab with GM-CSF in children with refractory or relapsed neuroblastoma: A report from the children's oncology group. J Clin Oncol 38:2160–2169. https://doi.org/10.1200/JCO.20.00203

Molenaar JJ, Domingo-Fernandez R, Ebus ME, Lindner S, Koster J, Drabek K, Mestdagh P, van Sluis P, Valentijn LJ, van Nes J, Broekmans M, Haneveld F, Volckmann R, Bray I, Heukamp L, Sprussel A, Thor T, Kieckbusch K, Klein-Hitpass L, Fischer M, Vandesompele J, Schramm A, van Noesel MM, Varesio L, Speleman F, Eggert A, Stallings RL, Caron HN, Versteeg R, Schulte JH (2012) LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. Nat Genet 44:1199–1206. https://doi.org/10.1038/ng.2436

Mosse YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, Laureys G, Speleman F, Kim C, Hou C, Hakonarson H, Torkamani A, Schork NJ, Brodeur GM, Tonini GP, Rappaport E, Devoto M, Maris JM (2008) Identification of ALK as a major familial neuroblastoma predisposition gene. Nature 455:930–935. https://doi.org/10.1038/nature07261

Mueller BM, Romerdahl CA, Gillies SD, Reisfeld RA (1990) Enhancement of antibody-dependent cytotoxicity with a chimeric anti-GD2 antibody. J Immunol 144:1382–1386

Mujoo K, Kipps TJ, Yang HM, Cheresh DA, Wargalla U, Sander DJ, Reisfeld RA (1989) Functional properties and effect on growth suppression of human neuroblastoma tumors by isotype switch variants of monoclonal antiganglioside GD2 antibody 14.18. Cancer Res 49:2857–2861

Mullard A (2021) FDA approves fourth CAR-T cell therapy. Nat Rev Drug Discov 20:166. https://doi.org/10.1038/d41573-021-00031-9

Murray JL, Cunningham JE, Brewer H, Mujoo K, Zukiwski AA, Podoloff DA, Kasi LP, Bhadkamkar V, Fritsche HA, Benjamin RS et al (1994) Phase I trial of murine monoclonal antibody 14G2a administered by prolonged intravenous infusion in patients with neuroectodermal tumors. J Clin Oncol 12:184–193

Navid F, Sondel PM, Barfield R, Shulkin BL, Kaufman RA, Allay JA, Gan J, Hutson P, Seo S, Kim K, Goldberg J, Hank JA, Billups CA, Wu J, Furman WL, McGregor LM, Otto M, Gillies SD, Handgretinger R, Santana VM (2014) Phase I trial of a novel anti-GD2 monoclonal antibody, Hu14.18K322A, designed to decrease toxicity in children with refractory or recurrent neuroblastoma. J Clin Oncol 32:1445–1452. https://doi.org/10.1200/JCO.2013.50.4423

Neal ZC, Imboden M, Rakhmilevich AL, Kim KM, Hank JA, Surfus J, Dixon JR, Lode HN, Reisfeld RA, Gillies SD, Sondel PM (2004a) NXS2 murine neuroblastomas express increased

levels of MHC class I antigens upon recurrence following NK-dependent immunotherapy. Cancer Immunol Immunother 53:41–52. https://doi.org/10.1007/s00262-003-0435-2

Neal ZC, Yang JC, Rakhmilevich AL, Buhtoiarov IN, Lum HE, Imboden M, Hank JA, Lode HN, Reisfeld RA, Gillies SD, Sondel PM (2004b) Enhanced activity of hu14.18-IL2 immunocytokine against murine NXS2 neuroblastoma when combined with interleukin 2 therapy. Clin Cancer Res 10:4839–4847. https://doi.org/10.1158/1078-0432.CCR-03-0799

Nishio N, Dotti G (2015) Oncolytic virus expressing RANTES and IL-15 enhances function of CAR-modified T cells in solid tumors. Onco Targets Ther 4:e988098. https://doi.org/10.4161/21505594.2014.988098

Niwa R, Shoji-Hosaka E, Sakurada M, Shinkawa T, Uchida K, Nakamura K, Matsushima K, Ueda R, Hanai N, Shitara K (2004) Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. Cancer Res 64:2127–2133. https://doi.org/10.1158/0008-5472.can-03-2068

Osenga KL, Hank JA, Albertini MR, Gan J, Sternberg AG, Eickhoff J, Seeger RC, Matthay KK, Reynolds CP, Twist C, Krailo M, Adamson PC, Reisfeld RA, Gillies SD, Sondel PM, Children's Oncology Group (2006) A phase I clinical trial of the hu14.18-IL2 (EMD 273063) as a treatment for children with refractory or recurrent neuroblastoma and melanoma: a study of the children's oncology group. Clin Cancer Res 12:1750–1759. https://doi.org/10.1158/1078-0432.CCR-05-2000

Prapa M, Caldrer S, Spano C, Bestagno M, Golinelli G, Grisendi G, Petrachi T, Conte P, Horwitz EM, Campana D, Paolucci P, Dominici M (2015) A novel anti-GD2/4-1BB chimeric antigen receptor triggers neuroblastoma cell killing. Oncotarget 6:24884–24894. https://doi.org/10.18632/oncotarget.4670

Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, Huls MH, Liu E, Gee AP, Mei Z, Yvon E, Weiss HL, Liu H, Rooney CM, Heslop HE, Brenner MK (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 14:1264–1270. https://doi.org/10.1038/nm.1882

Rafiq S, Hackett CS, Brentjens RJ (2020) Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nat Rev Clin Oncol 17:147–167. https://doi.org/10.1038/s41571-019-0297-y

Reis CA, Osorio H, Silva L, Gomes C, David L (2010) Alterations in glycosylation as biomarkers for cancer detection. J Clin Pathol 63:322–329. https://doi.org/10.1136/jcp.2009.071035

Riemer AB, Forster-Waldl E, Bramswig KH, Pollak A, Zielinski CC, Pehamberger H, Lode HN, Scheiner O, Jensen-Jarolim E (2006) Induction of IgG antibodies against the GD2 carbohydrate tumor antigen by vaccination with peptide mimotopes. Eur J Immunol 36:1267–1274. https://doi.org/10.1002/eji.200535279

Rigo V, Emionite L, Daga A, Astigiano S, Corrias MV, Quintarelli C, Locatelli F, Ferrini S, Croce M (2017) Combined immunotherapy with anti-PDL-1/PD-1 and anti-CD4 antibodies cures syngeneic disseminated neuroblastoma. Sci Rep 7:14049. https://doi.org/10.1038/s41598-017-14417-6

Ruf P, Jager M, Ellwart J, Wosch S, Kusterer E, Lindhofer H (2004) Two new trifunctional antibodies for the therapy of human malignant melanoma. Int J Cancer 108:725–732. https://doi.org/10.1002/ijc.11630

Ruf P, Schafer B, Eissler N, Mocikat R, Hess J, Ploscher M, Wosch S, Suckstorff I, Zehetmeier C, Lindhofer H (2012) Ganglioside GD2-specific trifunctional surrogate antibody Surek demonstrates therapeutic activity in a mouse melanoma model. J Transl Med 10:219. https://doi.org/10.1186/1479-5876-10-219

Saleh MN, Khazaeli MB, Wheeler RH, Dropcho E, Liu T, Urist M, Miller DM, Lawson S, Dixon P, Russell CH et al (1992) Phase I trial of the murine monoclonal anti-GD2 antibody 14G2a in metastatic melanoma. Cancer Res 52:4342–4347

Saleh MN, Stapleton JD, Khazaeli MB, LoBuglio AF (1993) Generation of a human anti-idiotypic antibody that mimics the GD2 antigen. J Immunol 151:3390–3398

Saletta F, Vilain RE, Gupta AK, Nagabushan S, Yuksel A, Catchpoole D, Scolyer RA, Byrne JA, McCowage G (2017) Programmed death-ligand 1 expression in a large cohort of pediatric patients with solid tumor and association with clinicopathologic features in neuroblastoma. JCO Precis Oncol 1:1–12. https://doi.org/10.1200/PO.16.00049

Schulz G, Cheresh DA, Varki NM, Yu A, Staffileno LK, Reisfeld RA (1984) Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. Cancer Res 44:5914–5920

Scursoni AM, Galluzzo L, Camarero S, Lopez J, Lubieniecki F, Sampor C, Segatori VI, Gabri MR, Alonso DF, Chantada G, de Davila MT (2011) Detection of N-glycolyl GM3 ganglioside in neuroectodermal tumors by immunohistochemistry: an attractive vaccine target for aggressive pediatric cancer. Clin Dev Immunol 2011:245181. https://doi.org/10.1155/2011/245181

Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D (1985) Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 313:1111–1116. https://doi.org/10.1056/NEJM198510313131802

Shirinbak S, Chan RY, Shahani S, Muthugounder S, Kennedy R, Hung LT, Fernandez GE, Hadjidaniel MD, Moghimi B, Sheard MA, Epstein AL, Fabbri M, Shimada H, Asgharzadeh S (2021) Combined immune checkpoint blockade increases CD8+CD28+PD-1+ effector T cells and provides a therapeutic strategy for patients with neuroblastoma. Onco Targets Ther 10: 1838140. https://doi.org/10.1080/2162402X.2020.1838140

Shusterman S, London WB, Gillies SD, Hank JA, Voss SD, Seeger RC, Reynolds CP, Kimball J, Albertini MR, Wagner B, Gan J, Eickhoff J, DeSantes KB, Cohn SL, Hecht T, Gadbaw B, Reisfeld RA, Maris JM, Sondel PM (2010) Antitumor activity of hu14.18-IL2 in patients with relapsed/refractory neuroblastoma: a children's oncology group (COG) phase II study. J Clin Oncol 28:4969–4975. https://doi.org/10.1200/JCO.2009.27.8861

Siebert N, Zumpe M, Juttner M, Troschke-Meurer S, Lode HN (2017) PD-1 blockade augments anti-neuroblastoma immune response induced by anti-GD2 antibody ch14.18/CHO. Onco Targets Ther 6:e1343775. https://doi.org/10.1080/2162402X.2017.1343775

Sjoberg ER, Varki A (1993) Kinetic and spatial interrelationships between ganglioside glycosyltransferases and O-acetyltransferase(s) in human melanoma cells. J Biol Chem 268: 10185–10196

Song L, Ara T, Wu HW, Woo CW, Reynolds CP, Seeger RC, DeClerck YA, Thiele CJ, Sposto R, Metelitsa LS (2007) Oncogene MYCN regulates localization of NKT cells to the site of disease in neuroblastoma. J Clin Invest 117:2702–2712. https://doi.org/10.1172/JCI30751

Sorkin LS, Otto M, Baldwin WM 3rd, Vail E, Gillies SD, Handgretinger R, Barfield RC, Ming Yu H, Yu AL (2010) Anti-GD(2) with an FC point mutation reduces complement fixation and decreases antibody-induced allodynia. Pain 149:135–142. https://doi.org/10.1016/j.pain.2010.01.024

Svennerholm L, Bostrom K, Fredman P, Jungbjer B, Lekman A, Mansson JE, Rynmark BM (1994) Gangliosides and allied glycosphingolipids in human peripheral nerve and spinal cord. Biochim Biophys Acta 1214:115–123

Swaminathan A, Lucas RM, Dear K, McMichael AJ (2014) Keyhole limpet haemocyanin - a model antigen for human immunotoxicological studies. Br J Clin Pharmacol 78:1135–1142. https://doi.org/10.1111/bcp.12422

Tarr PE (1996) Granulocyte-macrophage colony-stimulating factor and the immune system. Med Oncol 13:133–140. https://doi.org/10.1007/BF02990841

Terme M, Dorvillius M, Cochonneau D, Chaumette T, Xiao W, Diccianni MB, Barbet J, Yu AL, Paris F, Sorkin LS, Birkle S (2014) Chimeric antibody c.8B6 to O-acetyl-GD2 mediates the same efficient anti-neuroblastoma effects as therapeutic ch14.18 antibody to GD2 without antibody induced allodynia. PLoS One 9:e87210. https://doi.org/10.1371/journal.pone.0087210

Theruvath J, Menard M, Smith BAH, Linde MH, Coles GL, Dalton GN, Wu W, Kiru L, Delaidelli A, Sotillo E, Silberstein JL, Geraghty AC, Banuelos A, Radosevich MT, Dhingra S, Heitzeneder S, Tousley A, Lattin J, Xu P, Huang J, Nasholm N, He A, Kuo TC, Sangalang ERB, Pons J, Barkal A, Brewer RE, Marjon KD, Vilches-Moure JG, Marshall PL, Fernandes R, Monje M, Cochran JR, Sorensen PH, Daldrup-Link HE, Weissman IL, Sage J,

Majeti R, Bertozzi CR, Weiss WA, Mackall CL, Majzner RG (2022) Anti-GD2 synergizes with CD47 blockade to mediate tumor eradication. Nat Med 28:333–344. https://doi.org/10.1038/s41591-021-01625-x

Tong W, Maira M, Gagnon M, Saragovi HU (2015) Ligands binding to cell surface ganglioside GD2 cause Src-dependent activation of N-methyl-D-aspartate receptor signaling and changes in cellular morphology. PLoS One 10:e0134255. https://doi.org/10.1371/journal.pone.0134255

Trochet D, Bourdeaut F, Janoueix-Lerosey I, Deville A, de Pontual L, Schleiermacher G, Coze C, Philip N, Frebourg T, Munnich A, Lyonnet S, Delattre O, Amiel J (2004) Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. Am J Hum Genet 74:761–764. https://doi.org/10.1086/383253

Tsao CY, Sabbatino F, Cheung NV, Hsu JC, Villani V, Wang X, Ferrone S (2015) Anti-proliferative and pro-apoptotic activity of GD2 ganglioside-specific monoclonal antibody 3F8 in human melanoma cells. Onco Targets Ther 4:e1023975. https://doi.org/10.1080/2162402X.2015.1023975

Tse BC, Navid F, Billups CA, O'Donnell T, Hoehn ME (2015) Ocular abnormalities in patients treated with a novel anti-GD2 monoclonal antibody, hu14.18K322A. J AAPOS 19:112–115. https://doi.org/10.1016/j.jaapos.2014.11.005

Tsuchida T, Saxton RE, Morton DL, Irie RF (1987) Gangliosides of human melanoma. J Natl Cancer Inst 78:45–54

Tumino N, Weber G, Besi F, Del Bufalo F, Bertaina V, Paci P, Quatrini L, Antonucci L, Sinibaldi M, Quintarelli C, Maggi E, De Angelis B, Locatelli F, Moretta L, Vacca P, Caruana I (2021) Polymorphonuclear myeloid-derived suppressor cells impair the anti-tumor efficacy of GD2.CAR T-cells in patients with neuroblastoma. J Hematol Oncol 14:191. https://doi.org/10.1186/s13045-021-01193-0

Uttenreuther-Fischer MM, Huang CS, Reisfeld RA, Yu AL (1995a) Pharmacokinetics of anti-ganglioside GD2 mAb 14G2a in a phase I trial in pediatric cancer patients. Cancer Immunol Immunother 41:29–36

Uttenreuther-Fischer MM, Huang CS, Yu AL (1995b) Pharmacokinetics of human-mouse chimeric anti-GD2 mAb ch14.18 in a phase I trial in neuroblastoma patients. Cancer Immunol Immunother 41:331–338

Vazquez AM, Alfonso M, Lanne B, Karlsson KA, Carr A, Barroso O, Fernandez LE, Rengifo E, Lanio ME, Alvarez C et al (1995) Generation of a murine monoclonal antibody specific for N-glycolylneuraminic acid-containing gangliosides that also recognizes sulfated glycolipids. Hybridoma 14:551–556. https://doi.org/10.1089/hyb.1995.14.551

Vazquez AM, Perez A, Hernandez AM, Macias A, Alfonso M, Bombino G, Perez R (1998) Syngeneic anti-idiotypic monoclonal antibodies to an anti-NeuGc-containing ganglioside monoclonal antibody. Hybridoma 17:527–534. https://doi.org/10.1089/hyb.1998.17.527

Vazquez AM, Gabri MR, Hernandez AM, Alonso DF, Beausoleil I, Gomez DE, Perez R (2000) Antitumor properties of an anti-idiotypic monoclonal antibody in relation to N-glycolyl-containing gangliosides. Oncol Rep 7:751–756. https://doi.org/10.3892/or.7.4.751

Wierzbicki A, Gil M, Ciesielski M, Fenstermaker RA, Kaneko Y, Rokita H, Lau JT, Kozbor D (2008) Immunization with a mimotope of GD2 ganglioside induces CD8+ T cells that recognize cell adhesion molecules on tumor cells. J Immunol 181:6644–6653

Wu ZL, Schwartz E, Seeger R, Ladisch S (1986) Expression of GD2 ganglioside by untreated primary human neuroblastomas. Cancer Res 46:440–443

Xu H, Cheng M, Guo H, Chen Y, Huse M, Cheung NK (2015) Retargeting T cells to GD2 pentasaccharide on human tumors using bispecific humanized antibody. Cancer Immunol Res 3:266–277. https://doi.org/10.1158/2326-6066.CIR-14-0230-T

Xu X, Huang W, Heczey A, Liu D, Guo L, Wood M, Jin J, Courtney AN, Liu B, Di Pierro EJ, Hicks J, Barragan GA, Ngai H, Chen Y, Savoldo B, Dotti G, Metelitsa LS (2019) NKT cells co-expressing a GD2-specific chimeric antigen receptor and IL15 show enhanced in vivo persistence and antitumor activity against neuroblastoma. Clin Cancer Res 25:7126–7138. https://doi.org/10.1158/1078-0432.CCR-19-0421

Yankelevich M, Kondadasula SV, Thakur A, Buck S, Cheung NK, Lum LG (2012) Anti-CD3 x anti-GD2 bispecific antibody redirects T-cell cytolytic activity to neuroblastoma targets. Pediatr Blood Cancer 59:1198–1205. https://doi.org/10.1002/pbc.24237

Yu AL, Uttenreuther-Fischer MM, Kamps A (1995) Combined use of a human mouse chimeric anti-GD2 (ch14.18) and GM-CSF in the treatment of refractory neuroblastoma. Antibody Immunocon Radiopharm 8:12

Yu AL, Batova A, Alvarado C, Rao VJ, Castleberry RP (1997) Usefulness of a chimeric anti-GD2 (ch14.18) and GM-CSF for refractory neuroblastoma: a POG phase II study. PROC ASCO 16: 1846

Yu AL, Uttenreuther-Fischer MM, Huang CS, Tsui CC, Gillies SD, Reisfeld RA, Kung FH (1998) Phase I trial of a human-mouse chimeric anti-disialoganglioside monoclonal antibody ch14.18 in patients with refractory neuroblastoma and osteosarcoma. J Clin Oncol 16:2169–2180

Yu AL, Eskenazi A, Strother D (2001) A piot study of anti-idiotype monoclonal antibody as tumor vaccine in patients with high risk neuroblastoma. Proc Am Soc Clin Oncol 20(abstr 1470):18s

Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, Smith M, Anderson B, Villablanca JG, Matthay KK, Shimada H, Grupp SA, Seeger R, Reynolds CP, Buxton A, Reisfeld RA, Gillies SD, Cohn SL, Maris JM, Sondel PM (2010) Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med 363:1324–1334. https://doi.org/10.1056/NEJMoa0911123

Yu AL, Gilman AL, Ozkaynak MF, Naranjo A, Diccianni MB, Gan J, Hank JA, Batova A, London WB, Tenney SC, Smith M, Shulkin BL, Parisi M, Matthay KK, Cohn SL, Maris JM, Bagatell R, Park JR, Sondel PM (2021) Long-term follow-up of a phase III study of ch14.18 (dinutuximab) + cytokine immunotherapy in children with high-risk neuroblastoma: COG study ANBL0032. Clin Cancer Res 27:2179–2189. https://doi.org/10.1158/1078-0432.CCR-20-3909

Yu L, Huang L, Lin D, Lai X, Wu L, Liao X, Liu J, Zeng Y, Liang L, Zhang G, Wang B, Wu Z, Tao S, Liu Y, Jiao C, Chang LJ, Yang L (2022) GD2-specific chimeric antigen receptor-modified T cells for the treatment of refractory and/or recurrent neuroblastoma in pediatric patients. J Cancer Res Clin Oncol 148(10):2643–2652. https://doi.org/10.1007/s00432-021-03839-5

Yuki N, Yamada M, Tagawa Y, Takahashi H, Handa S (1997) Pathogenesis of the neurotoxicity caused by anti-GD2 antibody therapy. J Neurol Sci 149:127–130

Zeng Y, Fest S, Kunert R, Katinger H, Pistoia V, Michon J, Lewis G, Ladenstein R, Lode HN (2005) Anti-neuroblastoma effect of ch14.18 antibody produced in CHO cells is mediated by NK-cells in mice. Mol Immunol 42:1311–1319. https://doi.org/10.1016/j.molimm.2004.12.018

Zeytin HE, Tripathi PK, Bhattacharya-Chatterjee M, Foon KA, Chatterjee SK (2000) Construction and characterization of DNA vaccines encoding the single-chain variable fragment of the anti-idiotype antibody 1A7 mimicking the tumor-associated antigen disialoganglioside GD2. Cancer Gene Ther 7:1426–1436. https://doi.org/10.1038/sj.cgt.7700240

Zhao Q, Ahmed M, Guo HF, Cheung IY, Cheung NK (2015) Alteration of electrostatic surface potential enhances affinity and tumor killing properties of anti-ganglioside GD2 monoclonal antibody hu3F8. J Biol Chem 290:13017–13027. https://doi.org/10.1074/jbc.M115.650903

Zirngibl F, Ivasko SM, Grunewald L, Klaus A, Schwiebert S, Ruf P, Lindhofer H, Astrahantseff K, Andersch L, Schulte JH, Lode HN, Eggert A, Anders K, Hundsdoerfer P, Kunkele A (2021) GD2-directed bispecific trifunctional antibody outperforms dinutuximab beta in a murine model for aggressive metastasized neuroblastoma. J Immunother Cancer 9(7):e002923. https://doi.org/10.1136/jitc-2021-002923

Zuo S, Sho M, Sawai T, Kanehiro H, Maeda K, Yoshida M, Tsukada R, Nomura M, Okuyama H (2020) Potential role of the PD-L1 expression and tumor-infiltrating lymphocytes on neuroblastoma. Pediatr Surg Int 36:137–143. https://doi.org/10.1007/s00383-019-04616-9