Koichi Furukawa · Minoru Fukuda
Editors

Glycosignals in Cancer: Mechanisms of Malignant Phenotypes

Springer

Glycosignals in Cancer: Mechanisms of Malignant Phenotypes

Koichi Furukawa • Minoru Fukuda
Editors

Glycosignals in Cancer: Mechanisms of Malignant Phenotypes

Springer

Editors
Koichi Furukawa
Department of Biochemistry II
Nagoya University Graduate School of Medicine
Nagoya, Aichi
Japan

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

ISBN 978-4-431-55937-5 ISBN 978-4-431-55939-9 (eBook)
DOI 10.1007/978-4-431-55939-9

Library of Congress Control Number: 2016930299

Springer Tokyo Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer Japan KK is part of Springer Science+Business Media (www.springer.com)

Preface

Along with recent progress in genomics and proteomics, new findings in the significance of complex carbohydrates in the regulation of cell signals have been elucidated in a wide variety of biological events. We may call it the real glycomics. This volume provides a comprehensive summary of glycosignals and their involvement in cancer, covering numerous topics such as glycosylation machineries, regulation of phenotypes, cell signaling, immune regulation, complex carbohydrate organization, and clinical applications. The contents have been organized to promote ready understanding, covering basic to clinical research and studies on micromolecules, to animal/human cells and tissues. This book is an essential read for students and young researchers interested in cancers and carbohydrates. Specialists in glycobiology will also benefit from the new results and approaches detailed here, which provide insights into future directions of functional studies of sugar chains in both basic and applied research.

The incidence of cancers has been increasing and will keep doing so in the future. The most serious issue in our life and health, i.e., cancer, is and will be the most important theme in biomedical research, and the roles of carbohydrates in the altered phenotypes of malignant cells will attract interest as key mechanisms and key molecules in the construction of therapeutic strategies of cancers. I believe that the contents of this volume should encourage young researchers to step into the world of glycobiology and to promote integrative and novel studies fusing glycobiology and their own specialized fields.

I deeply thank Ms. Yoshiko Shikano of Springer for her great assistance.

Nagoya, Japan
October, 2015

Koichi Furukawa
Nagoya University

Contents

Part I Changes of Glycosylation Machineries in Cancer Cells

1 Roles of Fucosyltransferases in Cancer Phenotypes 3
Eiji Miyoshi, Naofumi Uozumi, Tomoaki Sobajima, Shinji Takamatsu, and Yoshihiro Kamada

2 Glycosyltransferases and Gastric Cancer 17
Celso A. Reis and Ana Magalhães

Part II Regulation of Cancer Phenotypes by Carbohydrates

3 Differential Roles of Mammalian Sialidases in Cancers 35
Taeko Miyagi

Part III Altered Cell Signaling in Cancer Cells

4 Regulatory Mechanisms for Malignant Properties of Cancer Cells with Disialyl and Monosialyl Gangliosides 57
Koichi Furukawa, Yuki Ohkawa, Yasuyuki Matsumoto, Yuhsuke Ohmi, Noboru Hashimoto, and Keiko Furukawa

5 Regulation of Growth Factor Receptors by Glycosphingolipids . . . 77
Sen-itiroh Hakomori and Kazuko Handa

6 Nutrient Regulation of Cancer Cells by O-GlcNAcylation 95
Xin Liu and Gerald W. Hart

Part IV Regulation of Immune Systems and Cancers by Carbohydrates

7 Function of Unique *O*-Glycan Structures in Protecting Gastric Mucosa Against *Helicobacter pylori* Infection and Gastric Cancer Development 111
Jun Nakayama

8 Sialylation and Immune Surveillance of Cancer by Siglecs 125
Bindu Mohan and Paul R. Crocker

Part V Changes in the Organization of Complex Carbohydrates in Cancers

9 Expression and Function of Poly-*N*-Acetyllactosamine Type Glycans in Cancer 141
Motohiro Nonaka and Minoru Fukuda

10 Role of Glycans in Cancer Cell Death: A Deadly Relationship 163
Inka Brockhausen

Part VI Implication of Sugars in Cancer Diagnostics and Therapeutics

11 Targeting Glycans for Immunotherapy of Human Cancers 197
Jung-Tung Hung and Alice L. Yu

Part I
Changes of Glycosylation Machineries in Cancer Cells

Chapter 1
Roles of Fucosyltransferases in Cancer Phenotypes

Eiji Miyoshi, Naofumi Uozumi, Tomoaki Sobajima, Shinji Takamatsu, and Yoshihiro Kamada

Abstract Fucosylation is one of the most important types of glycosylation in carcinogenesis. Fucosylation is linked to certain processes in cell-cell interaction and dynamic regulation of growth factor receptor signaling on cell surface, and changes in fucosylation result in differences of biological phenotype in cancer cells. Eleven fucosyltransferases are involved in the synthesis of fucosylated glycans and belong to some family of fucosyltransferases. To regulate cellular fucosylation, GDP-fucose, a donor substrate of fucosyltransferases, and GDP-fucose transporter are also important. Terminal fucosylation (Lewis-type fucosylation) is associated with the synthesis of sialyl Lewis antigens, leading to cancer metastasis. In contrast, core fucosylation is linked to the regulation of membrane-anchored glycoproteins, such as growth factor receptors and adhesion molecules. Target glycoproteins for each fucosyltransferase might be different in various kinds of cancer. In this chapter, we describe the roles of fucosyltransferase in several kinds of cancer, particularly gastroenterological cancers.

Keywords Fucosylation • Fucosyltransferases • Pancreatic cancer • Colon cancer • HCC • Lewis antigen • Cancer biomarker • CA19-9

1.1 Introduction

Fucosylation is one of the most important types of glycosylation involved in cancer and inflammation (Miyoshi et al. 2008). Cancer fucosylation is mainly divided into three types: α1-2 fucosylation, α1-3/1-4 fucosylation, and α1-6 fucosylation, as shown in Fig. 1.1. All fucosylations are regulated by orchestration of many fucosyltransferases (FUTs), guanosine 5′-diphosphate (GDP)-fucose synthetic enzymes, and GDP-fucose transporter(s). FUT1 and FUT2 have been shown to be responsible for α1-2 fucosylation (Larsen et al. 1990; Kelly et al. 1995). A family of

E. Miyoshi (✉) • N. Uozumi • T. Sobajima • S. Takamatsu • Y. Kamada
Department of Molecular Biochemistry and Clinical Investigation, Osaka University Graduate School of Medicine, 1-7, Yamada-oka, Suita, Osaka 565-0871, Japan
e-mail: emiyoshi@sahs.med.osaka-u.ac.jp; bpcqd109@tcct.zaq.ne.jp; stomoaki@sahs.med.osaka-u.ac.jp; shinjit@sahs.med.osaka-u.ac.jp; ykamada@gh.med.osaka-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_1

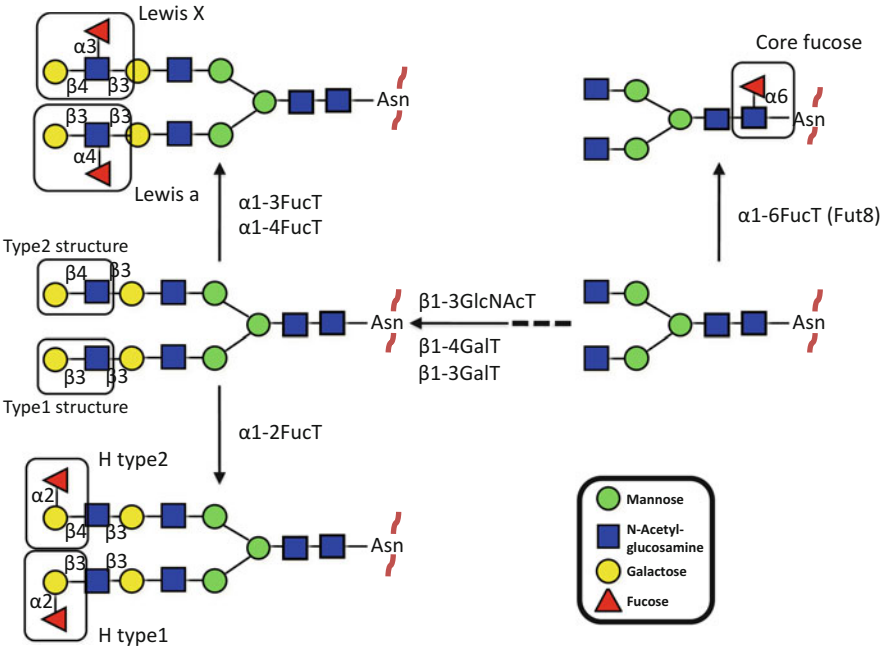

Fig. 1.1 Cancer fucosylation is mainly divided into 3 groups: α1-2 fucosylation, α1-3/1-4 fucosylation, and α1-6 fucosylation. Representative biosynthetic pathways and structures of the H, Lewis A, and Lewis X antigens. Type 1 and type 2 structures differ in the linkage of the outermost galactose (β1-3 and β1-4, respectively) and in the linkage of the fucose moiety to the internal GlcNAc (α1-4 and α1-3, respectively). The core fucose structure is synthesized by the glycosyltransferase FUT8, which catalyzes the transfer of a fucose residue from the donor substrate, guanosine 5′-diphosphate (GDP)-β-L-fucose, to the reducing terminal GlcNAc of the core structure of asparagine-linked oligosaccharide via an α1-6 linkage

α1-3 fucosyltransferases, including FUT3 (Kukowska-Latallo et al. 1990), FUT4 (Goelz et al. 1990), FUT5 (Weston et al. 1992), FUT6 (Koszdin and Bowen 1992), FUT7 (Natsuka et al. 1994), and FUT9 (Kudo et al. 1998), is involved in the synthesis of Lewis blood group antigens. FUTs 3–7 can synthesize the sialyl Lewis X (sLe^x) structure, NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ-R, and FUTs 3–6 and FUT9 can synthesize the Le^x structure, Galβ1-4(Fucα1-3)GlcNAcβ-R. Only FUT3 exhibits α1-4 fucosyltransferase activity, resulting in the synthesis of type 1 Lewis antigens such as Le^a, Le^b, and sialyl Le^a (sLe^a) (Galβ1-3(Fucα1-4)GlcNAcβ-R, (Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ-R, and NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ-R, respectively). Therefore, individuals with FUT3 mutations, comprising approximately 10 % of the Japanese population, have a problem regarding diagnosis of pancreatic cancer using the CA19-9 antigen, sLe^a (Narimatsu et al. 1998). In contrast, FUT8 is the only α1-6 fucosyltransferase involved in core fucosylation at the innermost *N*-acetylglucosamine on *N*-glycans (Uozumi et al. 1996). Among the many fucosyltransferases, Fut8-deficient mice show severe phenotypes, including mortality rates of 70–80 % after birth, and the

survivors show severe growth disturbances and lung emphysema (Wang et al. 2005). The phenotype of Fut8 knockout mice is due to insufficient signaling via membrane-anchored receptors. Mice with a deficiency of FX (GDP-4-keto-6-deoxy-mannose-3, 5-epimerase-4-reductase), a rate-limiting enzyme for GDP-fucose synthesis, show severe phenotypes, which are mostly caused by the loss of Lewis-type fucosylation (Smith et al. 2002). There are two pathways of GDP-fucose synthesis: de novo and alternative (Miyoshi et al. 2008). Because FX is involved in the de novo pathway, the salvage pathway compensates the synthesis of GDP-fucose in FX knockout mice (Smith et al. 2002). These reports suggest that core fucosylation is associated with growth factor receptor-mediated cell signaling and Lewis-type fucosylation is linked to lymphocyte/white blood cell adhesion through selectin and $sLe^{x/a}$ interaction. In cases of many cancers, fucosylation levels are increased. The biological significance of increased fucosylation in each cancer has some common and some different aspects. In this review article, we discuss the roles of fucosyltransferase in several kinds of cancer, with a particular focus on gastroenterological cancers.

1.2 Fucosylation in Colorectal Cancer

α1-2 Fucosylation, as seen in H, Lewis B, and Lewis Y antigens, is regulated by FUT1 and FUT2 and plays pivotal roles in colorectal cancer. Increased α1-2 fucosylation is observed in the progression of colorectal cancer (Misonou et al. 2009). Based on mass spectrometry analysis of neutral and acidic glycosphingolipids, structures of normal colorectal epithelial cells are characterized by the dominant expression of neutral type-1 chain oligosaccharides. Three specific alterations were observed in malignant transformation: increased ratios of type-2 oligosaccharides, increased α2-3 and/or α2-6 sialylation, and increased α1-2 fucosylation. Pendu et al. have published several papers regarding the biological function of α1-2 fucosylation in colorectal cancer cells (Goupille et al. 2000; Cordel et al. 2000). They used gene manipulation techniques, but did not identify target glycoproteins/glycolipids for α1-2 fucosylation, which regulates biological functions. When the FUT1 expression vector was transfected into colorectal cancer cell lines, the cells showed resistance to serum-starved apoptosis and anticancer drug treatment. In contrast, transfection with antisense cDNA against FUT1 induces apoptosis under these conditions. An increase in tumorigenicity was observed in rat colon carcinoma cells after transfection with rat α1, 2-fucosyltransferase FTA (human FUT1) antisense cDNA (Hallouin et al. 1999). Antisense transfection of a cDNA fragment of the FTB enzyme (human FUT2) decreased the cell-surface levels of H-antigen and concomitantly decreased tumorigenicity. Interestingly, these phenomena were observed only in synergic animals but not in immunodeficient mice. These results suggest that FTA and FTB fucosylate distinct glycan chains in the same cell, leading to opposite effects, under the control of the immune system. The immune system regulated by α1-2 fucosylation is not mediated by NK

cell cytotoxicity, but lymphokine-activated killer cytotoxicity (Marionneau et al. 2000). In the case of α1-3 fucosylation, increased expression of sLe^x in colorectal cancer is associated with liver metastasis (Nakamori et al. 1993). This is due to enhanced selectin-mediated cell adhesion. Selectins are intrinsic ligands for sLe^x, and the interaction between selectin and oligosaccharides plays a pivotal role in the rolling of white blood cells and the initial adhesion of cancer cells to metastatic organs. Overexpression of FUT7 induces the expression of sLe^x antigen, resulting in the promotion of cell migration and invasion in a colon cancer cell line, LoVo (Li et al. 2010). The authors identified CD24 as a carrier molecule for the sLe^x antigen, which is a well-known cell-surface marker for cancer stem cells. Administration of disaccharides blocked colorectal cancer cells from forming selectin ligands and inhibited adhesion to immobilized selectins, suggesting that glycosides might prove useful for interfering with tumor cell adhesion and metastasis (Brown et al. 2003). This approach opens up the possibility of clinical glycotherapy with no gene manipulations. Apart from fucosyltransferases, the sialidase NEU4 inhibits the synthesis of Le^x antigens on *O*-glycans (Shiozaki et al. 2011). Cell adhesion to and motility and growth on E-selectin are significantly reduced by NEU4. Under hypoxia conditions, whereby sLe^x antigens are increased concomitantly with several sialyl- and fucosyltransferases, NEU4 expression is markedly decreased. These results suggest that NEU4 plays an important role in the control of sLe^x expression and its impairment is involved in colon cancer progression. The epithelial-mesenchymal transition (EMT) is involved in cancer metastasis, and fibroblast growth factor (FGF)/basic FGF (bFGF)-mediated EMT induced increases cancer invasion and metastasis. During the EMT, transcript levels of the glycosyltransferase genes ST3GAL1/3/4 and FUT3 were significantly elevated, and that of FUT2 was markedly suppressed (Sakuma et al. 2012). GDP-mannose-4,6-dehydratase (GMDS) is a rate-limiting enzyme in GDP-fucose synthesis, in addition to FX (Miyoshi et al. 2008). Mutation of the GMDS gene was found in the colon cancer cell line HCT116 (Moriwaki et al. 2009). HCT116 cells showed complete loss of all types of fucosylation, and the fucosylation level was recovered upon transfection of a wild-type GMDS expression vector. Unexpectedly, HCT116 cells showed higher metastatic potential through escape from NK cell-mediated immune surveillance. The molecular mechanisms underlying the inhibition of cell death by NK cell killing are suppressed during death signaling through the Fas or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors. Interestingly DR5, a TRAIL receptor, has neither *N*-glycans nor *O*-glycans, although DR5-mediated cell death is inhibited in HCT116 cells, but not in fucosylation-rescued HCT116 cells (Moriwaki et al. 2011). There is an unknown pathway in fucosylation and receptor-mediated cell signaling. Mutation of the GMDS gene was found in approximately 10 % of original colorectal cancer tissues and 15 % of metastatic colorectal cancer tissues, but not in the normal colon (Nakayama et al. 2013). This report suggests a novel type of metastatic pathway due to the loss of fucosylation in colon carcinogenesis.

1.3 Fucosylation in Pancreatic Cancer

Pancreatic cancer is one of the worst diseases in terms of prognosis. The reason for the poor prognosis of pancreatic cancer is the difficulty in early diagnosis and high metastatic potential. Sialyl Lewis A, referred to as CA19-9, is a representative cancer biomarker for pancreatic cancer and is a fucosylated glycan (Kannagi et al. 2004). Measurement of serum CA19-9 levels in patients with pancreatic cancer is dependent on the levels of several kinds of mucins that carry many sialyl Lewis A molecules. Recently, we identified another type of CA19-9 carrier molecule, microlipid membranes, in both bile and sera of patients with pancreatic cancer (Uozumi et al. 2010). Details of the synthetic pathway of CA19-9, as well as its carrier molecules, remain unknown, but this appears to be one of the most interesting topics in glycobiology research. Fucosylated haptoglobin has been found in the sera of patients with pancreatic cancer (Okuyama et al. 2006), and we have developed a lectin ELISA system for measuring fucosylated haptoglobin (Kamada et al. 2013b). Various screening analyses using this ELISA assay have revealed that fucosylated haptoglobin is increased in several cancers and liver diseases (Kamada et al. 2013a; Takeda et al. 2012). Fucosylated haptoglobin is probably produced in the liver upon metastasis of colon/pancreatic cancers, and this is the reason why the positive rate of fucosylated haptoglobin is much higher in pancreatic cancer, which easily metastasizes at the early clinical stages (Okuyama et al. 2006). Detailed oligosaccharide analysis of fucosylated haptoglobin showed that most of the fucosylation is the Lewis type, and there are small amounts of core fucosylation. Interestingly, site 3 of the *N*-glycan on haptoglobin has a unique oligosaccharide structure with unique fucosylation, compared to the other 3 sites (Nakano et al. 2008). High expression of sLe^x antigen is involved in the metastasis of pancreatic cancer as well as colorectal cancer (Mas et al. 1998). The restoration of α1-2 fucosyltransferase (FUT1) activity decreases the adhesive and metastatic properties of human pancreatic cancer cells, although the molecular mechanisms are not clarified (Aubert et al. 2000; Mathieu et al. 2004).

1.4 Fucosylation in Hepatocellular Carcinoma

Fucosylation in the liver is different from that in other organs because the expression of α1-6 fucosyltransferase (FUT8) is quite low in normal hepatocytes (Noda et al. 1998a). Inflammation induces the expression of fucosylation regulatory genes, resulting in increases in Lewis-type fucosylation on hepatic glycoproteins. FUT6 is important in the synthesis of Lewis-type fucosylation in the liver but is a pseudogene in the mouse. Therefore, FUT8 is the main fucosyltransferase involved in cellular fucosylation in the mouse liver. FUT8-deficient mice show dramatic inhibition of hepatic glycoproteins in bile (Nakagawa et al. 2006), suggesting that fucosylation might be a sorting signal for the secretion of liver glycoproteins into bile. This hypothesis could explain the molecular mechanisms underlying the production of

fucosylated cancer biomarkers in hepatocellular carcinoma (HCC). α-Fetoprotein (AFP) is clinically used as cancer biomarker but has the drawback that serum AFP levels are increased in certain cases of chronic liver disease such as chronic hepatitis and liver cirrhosis (Taketa 1990). In contrast, fucosylated AFP, referred to as AFP-L3, is specifically increased in the sera of patients with HCC. AFP-L3-positive HCC showed worse prognosis than did AFP-L3-negative HCC (Yamashita et al. 1996). Although it has been reported that AFP-L3 could be a cancer biomarker for early HCC (Sato et al. 1993), many clinicians think that AFP-L3 is a marker for HCC with poor prognosis and that AFP-L3-positive HCC should be treated with hepatic resection, but not percutaneous radiofrequency ablation. However, a highly sensitive AFP-L3 assay might provide another possibility as a biomarker for the early diagnosis of HCC (Kumada et al. 2013). While FUT8 is involved in the synthesis of AFP-L3, the expression of FUT8 is increased in chronic liver disease (Noda et al. 1998b). In contrast, the level of GDP-fucose, a donor substrate of FUT8, is higher in HCC tissue than in the surrounding tissue (Noda et al. 2003). Increased levels of GDP-fucose are caused by high FX expression in HCC tissue. However, the most important factor involved in the production of AFP-L3 seems to be an abnormal sorting system for fucosylated proteins in HCC (Nakagawa et al. 2012). Fucosylated proteins produced by normal hepatocytes are secreted into bile, but this system is disrupted in HCC. This disruption might be due to the loss of the intrahepatic bile duct or the loss of cargo receptors for core fucose in HCC. In the case of hepatitis B virus-related HCC, it is reported that FUT8 is directly involved in the progression of HCC (Ji et al. 2013). In this report, downregulation of FUT8 in cancer cells was found to cause a decrease in cell growth in HCC cell lines as in other cancer cell lines. Although liver diseases have different etiologies and each disease has a characteristic biomarker, common changes in protein glycosylation in liver diseases are hyperfucosylation and an increase in the branching structure of *N*-glycans (Blomme et al. 2009). Moreover, mass spectrometry analysis of *N*-glycans on hepatic glycoproteins in HCC tissue revealed that the characteristic changes in glycan structure involve increases in tetra-antennary *N*-linked glycan but not in core fucosylation (Mehta et al. 2012). These papers further support the presence of an abnormal sorting system of fucosylated proteins in liver diseases. A previous report suggests that fucosyltransferase activity in serum, plasma, and tissue is different in patients with liver cirrhosis and HCC (Hutchinson et al. 1991), although its biological significance remains unknown.

1.5 Fucosylation in Other Cancers

Gastric cancer is the most popular carcinoma and the second cause of cancer-related death in Japan. In addition to hepatocarcinogenesis, inflammation induced by *Helicobacter pylori* infection plays a key role in the carcinogenesis of gastric cancer (Wang et al. 2013). Interestingly, polymorphisms of fucosyltransferases are involved in infection by *Helicobacter pylori* (Ikehara et al. 2001). Both IL-1 and IL-6, which are associated with inflammation-related cytokines, regulate the expression of

fucosyltransferases in gastric cancer cell lines (Padro et al. 2011). This finding is similar to the observed induction of fucosylation regulatory genes in HCC cell lines with IL-6 treatment (Narisada et al. 2008). Interestingly, FUT3 gene expression, involved in the synthesis of the sialyl Lewis A antigen, CA19-9, is regulated by DNA methylation (Serpa et al. 2006). Hypo-methylation of the FUT3 gene promoter in gastric cancer leads to the production of CA19-9. The expression pattern of fucosyltransferases alters oligosaccharides of mucins produced in normal and cancer tissue (Lopez-Ferrer et al. 2000). In the case of prostate cancer, α1-3 fucosyltransferases are very important in the regulation of cell behaviors such as adhesion, trafficking, and cell growth (Barthel et al. 2009; Inaba et al. 2003). Furthermore, FUT6 is involved in the bone metastasis of prostate cancer (Li et al. 2013). It has recently been reported that the high expression of GnT-IX and FUT8 is associated with the malignant phenotype of prostate cancer (Lange et al. 2012). Serum levels of fucosylated haptoglobin are also increased in patients with prostate cancer (Fujimura et al. 2008). Several papers on mammary cancer and fucosylation have been published, and their contents are similar to observations in other cancers (Yang et al. 2013, 2014; Julien et al. 2011). A very old article reported in *Science* indicates that a decrease in human serum fucosyltransferase is an indicator of successful tumor therapy in breast cancer (Bauer et al. 1978). A recent interesting paper on cholangiocarcinoma suggests that FUT2 and FUT3 genotypes determine the cutoff value for CA19-9 in differential diagnosis of cancer in patients with sclerosing cholangitis (Wannhoff et al. 2013). The involvement of FUT8 in EMT and EGF signaling has been reported in lung cancer (Liu et al. 2011; Chen et al. 2013).

1.6 Closing

Both FUT10 and FUT11 are novel types of α1-3 fucosyltransferases (Mollicone et al. 2009; Kumar et al. 2013; Both et al. 2011). These FUTs might play specific roles in carcinogenesis or may have other pathological functions that have not been mentioned in this review. A summary of FUT genes, including their chromosomal localization, is provided in Table 1.1. Interestingly, many fucosyltransferase genes are localized on chromosome 19. The amino acid homology of α1-3/1-4 fucosyltransferases is shown in Fig. 1.2. Here, we should note the structural characteristics of α1-3 fucosyltransferases. FUT3 is a unique glycosyltransferase that has the enzymatic activities of both α1-3 and 1-4 fucosyltransferase. FUT3 plays an important role in the synthesis of Lewis antigen in blood type, and loss of Fut3 activity leads to deficiency of the Lewis antigen in blood type, as well as of the Lewis A antigen (CA19-9). However, the amino acid sequence of FUT3 is very similar to those of other α1-3 fucosyltransferases. PCR primers for each fucosyltransferase should be designed carefully, due to their high gene/amino acid homologies. While this number is not so much, compared to other research fields, the number is relatively high in glycobiology research. Recently, O-fucosylation and notch signaling are two of the most important issues in glycobiology (Stanley 2007). Since notch signaling is involved in EGF

Table 1.1 Papers on the chromosomal localization and cloning of fucosyltransferases (FUTs)

FUTs	Official full name	Location (Human)	Gene ID (Human)	References (cDNA cloning)
FUT1	Fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)	19q13.3	2523	Larsen RD, et al. *Proc Natl Acad Sci U S A* 87, 6674–6678 (1990)
FUT2	Fucosyltransferase 2 (secretor status included)	19q13.3	2524	Kelly RJ, et al. *J Biol Chem* 270, 4640–4649 (1995)
FUT3	Fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group)	19p13.3	2525	Kukowska-Latallo JF, et al. *Genes Dev* 4, 1288–1303 (1990)
FUT4	Fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid specific)	11q21	2526	Goelz SE, et al. *Cell* 63, 1349–1356 (1990)
FUT5	Fucosyltransferase 5 (alpha (1,3) fucosyltransferase)	19p13.3	2527	Weston BW, et al. *J Biol Chem* 267, 4152–4160 (1992)
FUT6	Fucosyltransferase 6 (alpha (1,3) fucosyltransferase)	19p13.3	2528	Koszdin KL, et al. *Biochem Biophys Res Commun* 187, 152–157 (1992)
FUT7	Fucosyltransferase 7 (alpha (1,3) fucosyltransferase)	9q34.3	2529	Natsuka S, et al. *J Biol Chem* 269, 16789–16794 (1994),
FUT8	Fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	14q24.3	2530	Uozumi N, et al. *J Biol Chem* 271, 27810–27817 (1996)
FUT9	Fucosyltransferase 9 (alpha (1,3) fucosyltransferase)	6q16	10690	Kudo T, et al. *J Biol Chem* 273, 26729–26738 (1998)
FUT10	Fucosyltransferase 10 (alpha (1,3) fucosyltransferase)	8p12	84750	Mollicone R, et al. *J Biol Chem* 284, 4723–4738 (2009)
FUT11	Fucosyltransferase 11 (alpha (1,3) fucosyltransferase)	10q22.2	170384	Mollicone R, et al. *J Biol Chem* 284, 4723–4738 (2009)

receptor-mediated cell signaling, O-fucosylation might be important in cancer biology. Globally, if we search for all fucosyltransferases and cancer, the number is 588. If we search for all sialyltransferases and cancer, the number is 780. We believe that fucosylation, sialylation, and branching are three major glycosylations involved in cancer. Taken together, each fucosylation shows commonalities in each gastroenterological cancer and also shows differences in different cancers, suggesting that the target glycoproteins for fucosylation might be different in each cancer phenotype.

Fig. 1.2 Amino acid homology of α1-3 fucosyltransferases (Fut3–Fut11). (**A**) High-homology region of α1-3 fucosyltransferases (Fut3–Fut11). (**B**) Total amino acid structure of α1-3 fucosyltransferases (Fut3–Fut11)

References

Aubert M, Panicot L, Crotte C, Gibier P, Lombardo D, Sadoulet MO, Mas E (2000) Restoration of alpha(1,2) fucosyltransferase activity decreases adhesive and metastatic properties of human pancreatic cancer cells. Cancer Res 60(5):1449–1456

Barthel SR, Wiese GK, Cho J, Opperman MJ, Hays DL, Siddiqui J, Pienta KJ, Furie B, Dimitroff CJ (2009) Alpha 1,3 fucosyltransferases are master regulators of prostate cancer cell trafficking. Proc Natl Acad Sci USA 106(46):19491–19496. doi:10.1073/pnas.0906074106, 0906074106 [pii]

Bauer CH, Reutter WG, Erhart KP, Kottgen E, Gerok W (1978) Decrease of human serum fucosyltransferase as an indicator of successful tumor therapy. Science 201(4362):1232–1233

Blomme B, Van Steenkiste C, Callewaert N, Van Vlierberghe H (2009) Alteration of protein glycosylation in liver diseases. J Hepatol 50(3):592–603. doi:10.1016/j.jhep.2008.12.010, S0168-8278(08)00802-7 [pii]

Both P, Sobczak L, Breton C, Hann S, Nobauer K, Paschinger K, Kozmon S, Mucha J, Wilson IB (2011) Distantly related plant and nematode core alpha1,3-fucosyltransferases display similar

trends in structure-function relationships. Glycobiology 21(11):1401–1415. doi:10.1093/glycob/cwr056, cwr056 [pii]

Brown JR, Fuster MM, Whisenant T, Esko JD (2003) Expression patterns of alpha 2,3-sialyltransferases and alpha 1,3-fucosyltransferases determine the mode of sialyl Lewis X inhibition by disaccharide decoys. J Biol Chem 278(26):23352–23359. doi:10.1074/jbc.M303093200, M303093200 [pii]

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 USA 110(2):630–635. doi:10.1073/pnas.1220425110, 1220425110 [pii]

Cordel S, Goupille C, Hallouin F, Meflah K, Le Pendu J (2000) Role for alpha1,2-fucosyltransferase and histo-blood group antigen H type 2 in resistance of rat colon carcinoma cells to 5-fluorouracil. Int J Cancer 85(1):142–148. doi:10.1002/(SICI)1097-0215(20000101)85:1<142::AID-IJC24>3.0.CO;2-K [pii]

Fujimura T, Shinohara Y, Tissot B, Pang PC, Kurogochi M, Saito S, Arai Y, Sadilek M, Murayama K, Dell A, Nishimura S, Hakomori SI (2008) Glycosylation status of haptoglobin in sera of patients with prostate cancer vs. benign prostate disease or normal subjects. Int J Cancer 122(1):39–49. doi:10.1002/ijc.22958

Goelz SE, Hession C, Goff D, Griffiths B, Tizard R, Newman B, Chi-Rosso G, Lobb R (1990) ELFT: a gene that directs the expression of an ELAM-1 ligand. Cell 63(6):1349–1356, doi:0092-8674(90)90430-M [pii]

Goupille C, Marionneau S, Bureau V, Hallouin F, Meichenin M, Rocher J, Le Pendu J (2000) Alpha1,2Fucosyltransferase increases resistance to apoptosis of rat colon carcinoma cells. Glycobiology 10(4):375–382, doi:cwd035 [pii]

Hallouin F, Goupille C, Bureau V, Meflah K, Le Pendu J (1999) Increased tumorigenicity of rat colon carcinoma cells after alpha1,2-fucosyltransferase FTA anti-sense cDNA transfection. Int J Cancer 80(4):606–611. doi:10.1002/(SICI)1097-0215(19990209)80:4<606::AID-IJC20>3.0.CO;2-M [pii]

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, doi:S0270913991000812 [pii]

Ikehara Y, Nishihara S, Yasutomi H, Kitamura T, Matsuo K, Shimizu N, Inada K, Kodera Y, Yamamura Y, Narimatsu H, Hamajima N, Tatematsu M (2001) Polymorphisms of two fucosyltransferase genes (Lewis and Secretor genes) involving type I Lewis antigens are associated with the presence of anti-Helicobacter pylori IgG antibody. Cancer Epidemiol Biomarkers Prev 10(9):971–977

Inaba Y, Ohyama C, Kato T, Satoh M, Saito H, Hagisawa S, Takahashi T, Endoh M, Fukuda MN, Arai Y, Fukuda M (2003) Gene transfer of alpha1,3-fucosyltransferase increases tumor growth of the PC-3 human prostate cancer cell line through enhanced adhesion to prostatic stromal cells. Int J Cancer 107(6):949–957. doi:10.1002/ijc.11513

Ji J, Gu X, Fang M, Zhao Y, Yi C, Wang A, Gao C (2013) Expression of alpha 1,6-fucosyltransferase 8 in hepatitis B virus-related hepatocellular carcinoma influences tumour progression. Dig Liver Dis 45(5):414–421. doi:10.1016/j.dld.2012.12.007, S1590-8658(12)00467-7 [pii]

Julien S, Ivetic A, Grigoriadis A, QiZe D, Burford B, Sproviero D, Picco G, Gillett C, Papp SL, Schaffer L, Tutt A, Taylor-Papadimitriou J, Pinder SE, Burchell JM (2011) Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers. Cancer Res 71(24):7683–7693. doi:10.1158/0008-5472.CAN-11-1139, 0008–5472.CAN-11-1139 [pii]

Kamada Y, Akita M, Takeda Y, Yamada S, Fujii H, Sawai Y, Doi Y, Asazawa H, Nakayama K, Mizutani K, 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(6):e66328. doi:10.1371/journal.pone.0066328, PONE-D-13-02561 [pii]

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. doi:10.1016/j.cca.2012.12.014, S0009-8981(12)00585-2 [pii]

Kannagi R, Izawa M, Koike T, Miyazaki K, Kimura N (2004) Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. Cancer Sci 95(5):377–384

Kelly RJ, Rouquier S, Giorgi D, Lennon GG, Lowe JB (1995) Sequence and expression of a candidate for the human Secretor blood group alpha(1,2)fucosyltransferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. J Biol Chem 270(9):4640–4649

Koszdin KL, Bowen BR (1992) The cloning and expression of a human alpha-1,3 fucosyltransferase capable of forming the E-selectin ligand. Biochem Biophys Res Commun 187(1):152–157, doi:S0006-291X(05)81472-X [pii]

Kudo T, Ikehara Y, Togayachi A, Kaneko M, Hiraga T, Sasaki K, Narimatsu H (1998) Expression cloning and characterization of a novel murine alpha1, 3-fucosyltransferase, mFuc-TIX, that synthesizes the Lewis x (CD15) epitope in brain and kidney. J Biol Chem 273 (41):26729–26738

Kukowska-Latallo JF, Larsen RD, Nair RP, Lowe JB (1990) A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group alpha(1,3/1,4)fucosyltransferase. Genes Dev 4(8):1288–1303

Kumada T, Toyoda H, Tada T, Kiriyama S, Tanikawa M, Hisanaga Y, Kanamori A, Tanaka J, Kagebayashi C, Satomura S (2013) High-sensitivity lens culinaris agglutinin-reactive alpha-fetoprotein assay predicts early detection of hepatocellular carcinoma. J Gastroenterol. doi:10.1007/s00535-013-0883-1

Kumar A, Torii T, Ishino Y, Muraoka D, Yoshimura T, Togayachi A, Narimatsu H, Ikenaka K, Hitoshi S (2013) The Lewis X-related alpha1,3-fucosyltransferase, Fut10, is required for the maintenance of stem cell populations. J Biol Chem 288(40):28859–28868. doi:10.1074/jbc.M113.469403, M113.469403 [pii]

Lange T, Ullrich S, Muller I, Nentwich MF, Stubke K, Feldhaus S, Knies C, Hellwinkel OJ, Vessella RL, Abramjuk C, Anders M, Schroder-Schwarz J, Schlomm T, Huland H, Sauter G, Schumacher U (2012) Human prostate cancer in a clinically relevant xenograft mouse model: identification of beta(1,6)-branched oligosaccharides as a marker of tumor progression. Clin Cancer Res 18(5):1364–1373. doi:10.1158/1078-0432.CCR-11-2900, 1078–0432.CCR-11-2900 [pii]

Larsen RD, Ernst LK, Nair RP, Lowe JB (1990) Molecular cloning, sequence, and expression of a human GDP-L-fucose:beta-D-galactoside 2-alpha-L-fucosyltransferase cDNA that can form the H blood group antigen. Proc Natl Acad Sci USA 87(17):6674–6678

Li W, Zhang W, Luo J, Cao A, Zhang Y, Huang D, Sheng W, Cai S, Li J (2010) Alpha1,3 fucosyltransferase VII plays a role in colorectal carcinoma metastases by promoting the carbohydration of glycoprotein CD24. Oncol Rep 23(6):1609–1617

Li J, Guillebon AD, Hsu JW, Barthel SR, Dimitroff CJ, Lee YF, King MR (2013) Human fucosyltransferase 6 enables prostate cancer metastasis to bone. Br J Cancer. doi:10.1038/bjc.2013.690 bjc2013690 [pii]

Liu YC, Yen HY, Chen CY, Chen CH, Cheng PF, Juan YH, 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 USA 108 (28):11332–11337. doi:10.1073/pnas.1107385108, 1107385108 [pii]

Lopez-Ferrer A, de Bolos C, Barranco C, Garrido M, Isern J, Carlstedt I, Reis CA, Torrado J, Real FX (2000) Role of fucosyltransferases in the association between apomucin and Lewis antigen expression in normal and malignant gastric epithelium. Gut 47(3):349–356

Marionneau S, Bureau V, Goupille C, Hallouin F, Rocher J, Vaydie B, Le Pendu J (2000) Susceptibility of rat colon carcinoma cells to lymphokine activated killer-mediated

cytotoxicity is decreased by alpha1,2-fucosylation. Int J Cancer 86(5):713–717. doi:10.1002/(SICI)1097-0215(20000601)86:5<713::AID-IJC17>3.0.CO;2-V [pii]

Mas E, Pasqualini E, Caillol N, El Battari A, Crotte C, Lombardo D, Sadoulet MO (1998) Fucosyltransferase activities in human pancreatic tissue: comparative study between cancer tissues and established tumoral cell lines. Glycobiology 8(6):605–613, doi:cwb066 [pii]

Mathieu S, Prorok M, Benoliel AM, Uch R, Langlet C, Bongrand P, Gerolami R, El-Battari A (2004) Transgene expression of alpha(1,2)-fucosyltransferase-I (FUT1) in tumor cells selectively inhibits sialyl-Lewis x expression and binding to E-selectin without affecting synthesis of sialyl-Lewis a or binding to P-selectin. Am J Pathol 164(2):371–383, doi:S0002-9440(10)63127-6 [pii]

Mehta A, Norton P, Liang H, Comunale MA, Wang M, Rodemich-Betesh L, Koszycki A, Noda K, Miyoshi E, Block T (2012) Increased levels of tetra-antennary N-linked glycan but not core fucosylation are associated with hepatocellular carcinoma tissue. Cancer Epidemiol Biomarkers Prev 21(6):925–933. doi:10.1158/1055-9965.EPI-11-1183, 1055–9965.EPI-11-1183 [pii]

Misonou Y, Shida K, Korekane H, Seki Y, Noura S, Ohue M, Miyamoto Y (2009) Comprehensive clinico-glycomic study of 16 colorectal cancer specimens: elucidation of aberrant glycosylation and its mechanistic causes in colorectal cancer cells. J Proteome Res 8(6):2990–3005. doi:10.1021/pr900092r

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

Mollicone R, Moore SE, Bovin N, Garcia-Rosasco M, Candelier JJ, Martinez-Duncker I, Oriol R (2009) Activity, splice variants, conserved peptide motifs, and phylogeny of two new alpha1,3-fucosyltransferase families (FUT10 and FUT11). J Biol Chem 284(7):4723–4738. doi:10.1074/jbc.M809312200, M809312200 [pii]

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(1):188–198. doi:10.1053/j.gastro.2009.04.002, e181–e182

Moriwaki K, Shinzaki S, Miyoshi E (2011) GDP-mannose-4,6-dehydratase (GMDS) deficiency renders colon cancer cells resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor- and CD95-mediated apoptosis by inhibiting complex II formation. J Biol Chem 286(50):43123–43133. doi:10.1074/jbc.M111.262741, M111.262741 [pii]

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. doi:10.1074/jbc.M605697200, M605697200 [pii]

Nakagawa T, Moriwaki K, Terao N, Miyamoto Y, Kamada Y, Miyoshi E (2012) Analysis of polarized secretion of fucosylated alpha-fetoprotein in HepG2 cells. J Proteome Res 11(5):2798–2806. doi:10.1021/pr201154k

Nakamori S, Kameyama M, Imaoka S, Furukawa H, Ishikawa O, Sasaki Y, Kabuto T, Iwanaga T, Matsushita Y, Irimura T (1993) Increased expression of sialyl Lewisx antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study. Cancer Res 53(15):3632–3637

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(10):2301–2309. doi:10.1002/ijc.23364

Nakayama K, Moriwaki K, Imai T, Shinzaki S, Kamada Y, Murata K, Miyoshi E (2013) Mutation of GDP-mannose-4,6-dehydratase in colorectal cancer metastasis. PLoS ONE 8(7):e70298. doi:10.1371/journal.pone.0070298, PONE-D-13-03666 [pii]

Narimatsu H, Iwasaki H, Nakayama F, Ikehara Y, Kudo T, Nishihara S, Sugano K, Okura H, Fujita S, Hirohashi S (1998) Lewis and secretor gene dosages affect CA19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. Cancer Res 58(3):512–518

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(3):792–796. doi:10.1016/j.bbrc.2008.10.061, S0006-291X(08)02016-0 [pii]

Natsuka S, Gersten KM, Zenita K, Kannagi R, Lowe JB (1994) Molecular cloning of a cDNA encoding a novel human leukocyte alpha-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. J Biol Chem 269(32):20806

Noda K, Miyoshi E, Uozumi N, Gao CX, Suzuki K, Hayashi N, Hori M, Taniguchi N (1998a) High expression of alpha-1-6 fucosyltransferase during rat hepatocarcinogenesis. Int J Cancer 75 (3):444–450. doi:10.1002/(SICI)1097-0215(19980130)75:3<444::AID-IJC19>3.0.CO;2-8 [pii]

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 (1998b) Gene expression of alpha1-6 fucosyltransferase in human hepatoma tissues: a possible implication for increased fucosylation of alpha-fetoprotein. Hepatology 28(4):944–952. doi:10.1002/hep.510280408, S0270913998004005 [pii]

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(19):6282–6289

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(11):2803–2808. doi:10.1002/ijc.21728

Padro M, Mejias-Luque R, Cobler L, Garrido M, Perez-Garay M, Puig S, Peracaula R, de Bolos C (2011) Regulation of glycosyltransferases and Lewis antigens expression by IL-1beta and IL-6 in human gastric cancer cells. Glycoconj J 28(2):99–110. doi:10.1007/s10719-011-9327-4

Sakuma K, Aoki M, Kannagi R (2012) Transcription factors c-Myc and CDX2 mediate E-selectin ligand expression in colon cancer cells undergoing EGF/bFGF-induced epithelial-mesenchymal transition. Proc Natl Acad Sci USA 109(20):7776–7781. doi:10.1073/pnas.1111135109, 1111135109 [pii]

Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, Taketa K, Endo Y, Nagataki S (1993) Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med 328(25):1802–1806. doi:10.1056/NEJM199306243282502

Serpa J, Mesquita P, Mendes N, Oliveira C, Almeida R, Santos-Silva F, Reis CA, LePendu J, David L (2006) Expression of Lea in gastric cancer cell lines depends on FUT3 expression regulated by promoter methylation. Cancer Lett 242(2):191–197. doi:10.1016/j.canlet.2005.11.009, S0304-3835(05)00988-2 [pii]

Shiozaki K, Yamaguchi K, Takahashi K, Moriya S, Miyagi T (2011) Regulation of sialyl Lewis antigen expression in colon cancer cells by sialidase NEU4. J Biol Chem 286 (24):21052–21061. doi:10.1074/jbc.M111.231191, M111.231191 [pii]

Smith PL, Myers JT, Rogers CE, Zhou L, Petryniak B, Becker DJ, Homeister JW, Lowe JB (2002) Conditional control of selectin ligand expression and global fucosylation events in mice with a targeted mutation at the FX locus. J Cell Biol 158(4):801–815. doi:10.1083/jcb.200203125, 200203125 [pii]

Stanley P (2007) Regulation of Notch signaling by glycosylation. Curr Opin Struct Biol 17 (5):530–535. doi:10.1016/j.sbi.2007.09.007, S0959-440X(07)00149-2 [pii]

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(12):3036–3043. doi:10.1002/cncr.26490

Taketa K (1990) Alpha-fetoprotein: reevaluation in hepatology. Hepatology 12(6):1420–1432

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

Uozumi N, Gao C, Yoshioka T, Nakano M, Moriwaki K, Nakagawa T, Masuda T, Tanabe M, Miyoshi E (2010) Identification of a novel type of CA19-9 carrier in human bile and sera of cancer patients: an implication of the involvement in nonsecretory exocytosis. J Proteome Res 9(12):6345–6353. doi:10.1021/pr100600u

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 USA 102(44):15791–15796. doi:10.1073/pnas.0507375102, 0507375102 [pii]

Wang F, Meng W, Wang B, Qiao L (2013) Helicobacter pylori-induced gastric inflammation and gastric cancer. Cancer Lett. doi:10.1016/j.canlet.2013.08.016, S0304-3835(13)00591-0 [pii]

Wannhoff A, Hov JR, Folseraas T, Rupp C, Friedrich K, Anmarkrud JA, Weiss KH, Sauer P, Schirmacher P, Boberg KM, Stremmel W, Karlsen TH, Gotthardt DN (2013) FUT2 and FUT3 genotype determines CA19-9 cut-off values for detection of cholangiocarcinoma in patients with primary sclerosing cholangitis. J Hepatol 59(6):1278–1284. doi:10.1016/j.jhep.2013.08.005, S0168-8278(13)00558-8 [pii]

Weston BW, Nair RP, Larsen RD, Lowe JB (1992) Isolation of a novel human alpha (1,3) fucosyltransferase gene and molecular comparison to the human Lewis blood group alpha (1,3/1,4)fucosyltransferase gene. Syntenic, homologous, nonallelic genes encoding enzymes with distinct acceptor substrate specificities. J Biol Chem 267(6):4152–4160

Yamashita F, Tanaka M, Satomura S, Tanikawa K (1996) Prognostic significance of Lens culinaris agglutinin A-reactive alpha-fetoprotein in small hepatocellular carcinomas. Gastroenterology 111(4):996–1001, doi:S0016-5085(96)70067-7 [pii]

Yang X, Liu S, Yan Q (2013) Role of fucosyltransferase IV in epithelial-mesenchymal transition in breast cancer cells. Cell Death Dis 4:e735. doi:10.1038/cddis.2013.241, cddis2013241 [pii]

Yang X, Wang J, Liu S, Yan Q (2014) HSF1 and Sp1 regulate FUT4 gene expression and cell proliferation in breast cancer cells. J Cell Biochem 115(1):168–178. doi:10.1002/jcb.24645

Chapter 2
Glycosyltransferases and Gastric Cancer

Celso A. Reis and Ana Magalhães

Abstract The present chapter describes the pattern of glycosylation and the functional role of glycans in gastric tissues. The mechanisms of biosynthesis of gastric cell oligosaccharide chains and the glycosyltransferases involved in these processes are discussed in normal and pathological settings.

Particular focus is given to the glycosyltransferases controlling the biosynthesis of the fucosylated and sialylated histo-blood group antigens that act as receptors for the outer membrane adhesins of the gastric pathogenic bacteria *Helicobacter pylori*.

This chapter also addresses the glycosylation alterations and the underlying molecular mechanisms occurring during the gastric carcinogenic pathways, including the expression of simple mucin-type carbohydrate antigens, such as sialyl-Tn.

Moreover, the frequent glycosylation modifications observed in gastric carcinoma, the glycosyltransferases regulating important cancer cell adhesion molecules and signaling pathways, and the glycan-mediated modulation of cell biological functions are also discussed.

The decoding of the functional role of glycans in gastric cells may constitute the basis for defining biomarkers with potential to improve diagnosis and prognosis, contributing for stratification of patients and the development of better therapies.

Keywords Gastric cancer • Gastric cell glycosylation • *Helicobacter pylori* • Glycan receptors • Histo-blood group antigens • Sialylated antigens • E-cadherin N-glycans • Biomarkers in cancer

C.A. Reis (✉)
Institute for Research and Innovation in Health (i3S) & Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal

Medical Faculty of the University of Porto (FMUP), Porto, Portugal

Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, Porto, Portugal
e-mail: celsor@ipatimup.pt

A. Magalhães
Institute for Research and Innovation in Health (i3S) & Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_2

2.1 Glycosylation in Normal Gastric Mucosa

2.1.1 The Human Gastric Mucosa Glycosylation Profile

The glycosylation profile of the human gastric mucosa is partially defined by the expression of the heavily glycosylated mucins. In healthy conditions, mucins show a well-defined distribution pattern and the gastric superficial epithelial cells express the membrane-associated MUC1 and secrete MUC5AC, whereas the deep glands of the gastric mucosa produce the secreted MUC6 mucin (Reis et al. 1997, 1998, 2000; Teixeira et al. 2002).

The mucin-type O-glycosylation is a stepwise process regulated by different glycosyltransferases. The first step is the transfer of GalNAc from a sugar donor UDP-GalNAc to serine and threonine residues and is controlled by UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts) (Clausen and Bennett 1996; Hassan et al. 2000; Ten Hagen et al. 2003). To date, 20 distinct members of the mammalian ppGalNAc-T family have been identified (Bennett et al. 2012). They control the first level of complexity of O-glycosylation: the sites and density of O-glycan occupancy of the mucin tandem repeat. This is because ppGalNAc-Ts, although catalyzing the same enzymatic step, have different kinetic properties and acceptor substrate specificities (Bennett et al. 1998; Ten Hagen et al. 2003; Wandall et al. 1997).

In addition, ppGalNAc-Ts are unique among glycosyltransferases due to their ricin-like lectin domain (Bennett et al. 2012; Ten Hagen et al. 2003). This domain binds O-GalNAc and therefore promotes secondary GalNAc glycosylation on neighboring positions in the polypeptide sequence (Pedersen et al. 2011; Wandall et al. 2007). The ppGalNAc-T enzymatic specificity leads to different functions depending on the cell type and organ in which it is expressed.

The second level of complexity in O-glycosylation is the processing of carbohydrate chains by other glycosyltransferases. After the first glycan (GalNAc) is added forming the Tn antigen, a Gal-transferase (C1GalT-1) adds Gal to GalNAc, forming the core 1 structure. Alternatively, Tn and T antigens can be sialylated by sialyltransferases forming the sialyl-Tn, sialyl-T, and disialyl-T antigens (Brockhausen 1999) (Fig. 2.1).

In normal cells the O-glycans can be branched with a GlcNAc attached to core 1 and is termed core 2. This synthesis is performed by C2GnT family of enzymes (Bierhuizen et al. 1994; Schwientek et al. 1999). This O-glycan structure can be further elongated by galactosyltransferases and N-acetylglucosaminyltransferases and terminated by fucosyltransferases and sialyltransferases (Fig. 2.1).

Structural analysis of the glycan composition of gastric mucins has revealed a major expression of terminal neutral and highly fucosylated glycan structures (Rossez et al. 2012). The regulated cell-specific mucin distribution pattern in combination with fine-tuned glycosyltransferases expression results in co-expression of MUC5AC and type 1 Lewis antigens (Lewis a and b) in superficial epithelium and MUC6 and type 2 Lewis antigens (Lewis x and y) in the stomach

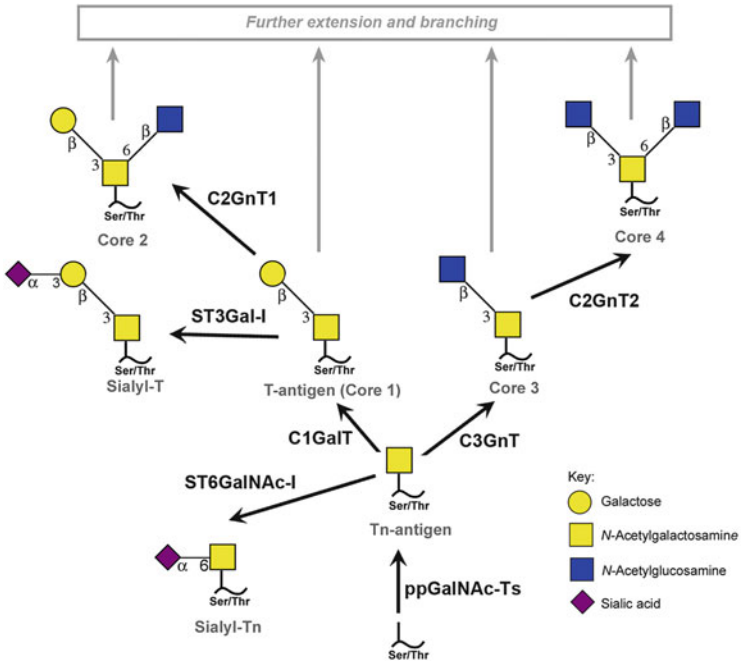

Fig. 2.1 Schematic representation of O-GalNAc glycan biosynthesis initiation pathways. For image simplicity, only representative enzymes were included

deeper glandular region (Lopez-Ferrer et al. 2000; Nordman et al. 2002). MUC6 is also frequently modified by terminal 1,4-linked N-acetylglucosamine (α1,4-GlcNAc) (Ferreira et al. 2006; Kawakubo et al. 2004).

Although, in healthy conditions, the gastric glycosylation profile is similar among individuals within the same blood group and sharing the same Lewis and secretor status, individual genetic polymorphisms in glycosyltransferase genes are associated with distinct gastric glycophenotypes.

2.1.2 Glycosyltransferases Involved in the Biosynthesis of H-Type and Lewis Antigens

The biosynthesis of H-type and Lewis antigens depends on the expression of specific glycosyltransferases, which is determined by the individual genotype. The capacity to synthesize the terminal alpha1,2-fucosylated structure H-type 1 in body secretions depends on the activity of the FUT2 enzyme (Kelly et al. 1995). Individuals are classified as secretors, when they have this capacity, or non-secretors, if they are unable to synthesize this structure (Mollicone et al. 1985). Several genetic polymorphisms on the FUT2 gene have been described. The G428A

mutation is the most common mutation found in European and African populations (Kelly et al. 1995; Liu et al. 1998), while the A385T mutation is found at high frequencies in nonsecretors from East Asian populations (Soejima et al. 2008). Secretor individuals constitute approximately 80 % of Caucasian population.

The Lewis histo-blood group antigens correspond to a group of structurally related alpha1-3(4)-fucosylated glycans that are built up from type 1 (Galbeta1-3GlcNAc) and type 2 (Galbeta1-4GlcNAc) precursor chains. The substitution of the terminal Gal with an alpha1,2-fucose residue on type 1 or type 2 chains results in the H-type 1 or H-type 2 antigens, respectively. These structures can be further fucosylated by alpha1,4/3fucosyltransferases that add a fucose in an alpha1,4-linkage to the type 1 GlcNAc producing the difucosylated Lewis b antigen or in an alpha1,3-linkage to type 2 structures producing an isomer structure, the Lewis y antigen. Alternatively, the type 1 and type 2 chain backbones may be first modified by alpha1,4/3fucosyltransferases leading to the biosynthesis of the mono-fucosylated Lewis a and Lewis x structures, respectively. Otherwise, the terminal Gal residue of type 1 and type 2 chains can be first modified by sialyltransferases by addition of an alpha2,3 sialic acid residue, followed then by fucosylation of the GlcNAc in alpha1,4 or alpha1,3 linkage originating the sialyl-Lewis a or sialyl-Lewis x antigens, respectively (Fig. 2.2).

The transfer of sialic acid to terminal Gal residues on type 1 and type 2 chains is mediated by the alpha2,3 sialyltransferases ST3Gal III, ST3Gal IV, and ST3Gal VI (Carvalho et al. 2010; Harduin-Lepers et al. 2012; Sasaki et al. 1993). The ST3Gal III uses preferentially type 1 chains leading to sialyl-Lewis a biosynthesis, whereas ST3Gal IV and ST3Gal VI modify preferentially type 2 sequences originating the formation of sialyl-Lewis x structure (Harduin-Lepers et al. 2012).

Regarding the fucosyltransferase activity, both FUT3 and FUT5 can transfer alpha1,4-linked fucose to type 1 chains, resulting in Lewis a, Lewis b, and sialyl-Lewis a synthesis (Holgersson and Löfling 2006). The modification with alpha1,3-linked fucose to type 2 chains can be mediated by FUT3, FUT4-7, and FUT9 fucosyltransferases (Miyoshi et al. 2008) (Fig. 2.2).

2.1.3 Helicobacter pylori *Adhesion Mediated by Glycan Receptors*

The terminal histo-blood group antigens present on gastric mucosal surface are recognized as attachment factors for bacteria, namely, for the gastric pathogen *Helicobacter pylori* (*H. pylori*).

H. pylori is a carcinogenic bacteria that colonizes half of the world population (Salama et al. 2013). Infection with *H. pylori* causes chronic gastric mucosa inflammation, and in a subset of infected individuals, long-term colonization is associated with the development of gastric and duodenal ulcers, mucosa-associated

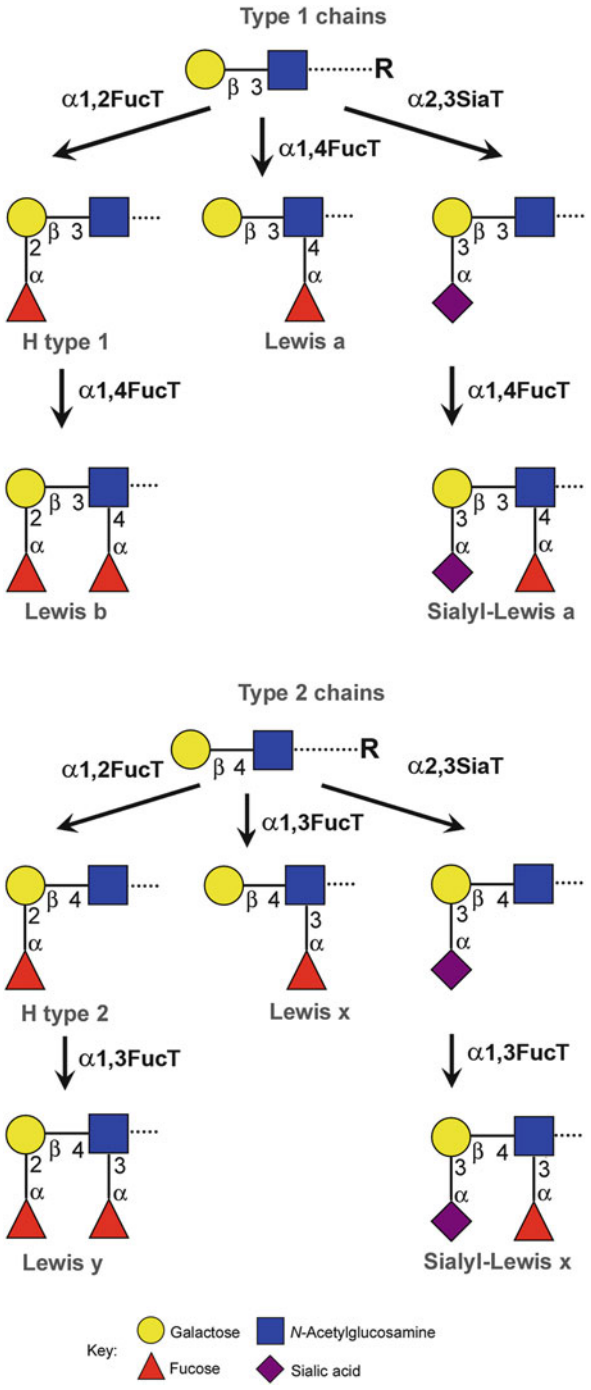

Fig. 2.2 Schematic representation of terminal type 1 and type 2 Lewis antigen biosynthesis pathways

lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma (Wroblewski et al. 2010).

H. pylori attachment to the gastric epithelium constitutes an important step for stomach colonization and establishment of a successful infection, providing bacterial protection from clearance mechanisms such as liquid flow, peristaltic movements, or renewal of the mucous layer. Bacterial binding to gastric mucosa is mediated by bacterial outer membrane proteins, with lectin-like domains that have affinity for glycan receptors expressed on the host gastric tissue.

The *H. pylori* glycan receptors include neutral fucosylated histo-blood group antigens (Boren et al. 1993; Ilver et al. 1998) and di-N-acetyllactosamine (lacdiNAc) motifs (Rossez et al. 2014), and glycans with charged groups, such as sialic acid (Mahdavi et al. 2002) or sulfate (Namavar et al. 1998; Huesca et al. 1996).

Bacterial binding to the fucosylated histo-blood group antigens H-type 1 and Lewis b is mediated by the blood group antigen-binding adhesin (BabA) (Boren et al. 1993; Ilver et al. 1998). Since H-type 1 and Lewis b structures are extensively present on the MUC5AC mucin, BabA-expressing strains have been described to adhere to the MUC5AC-rich gastric mucus layer (Linden et al. 2002; Van de Bovenkamp et al. 2003; Van den Brink et al. 2000). Additionally to H-type 1 and Lewis b structures, some *H. pylori* strains also bind to further modified fucosylated structures A-Lewis b (GalNAc-Lewis b) and B-Lewis b (Gal-Lewis b). The strains displaying these broader affinity properties are named generalists. On the contrary, strains that are only able to bind to naked Lewis b or exclusively to A-Lewis b are termed specialists (Aspholm-Hurtig et al. 2004).

The individual secretor status has been associated with *H. pylori* infection susceptibility (Azevedo et al. 2008; Ikehara et al. 2001; Lee et al. 2006). Using a nonsecretor animal model, the homozygous FUT2-null mice, it was demonstrated that the loss of glycans carrying the H-epitope resulted in impaired *H. pylori* BabA-mediated adhesion (Magalhaes et al. 2009a) (Fig. 2.3).

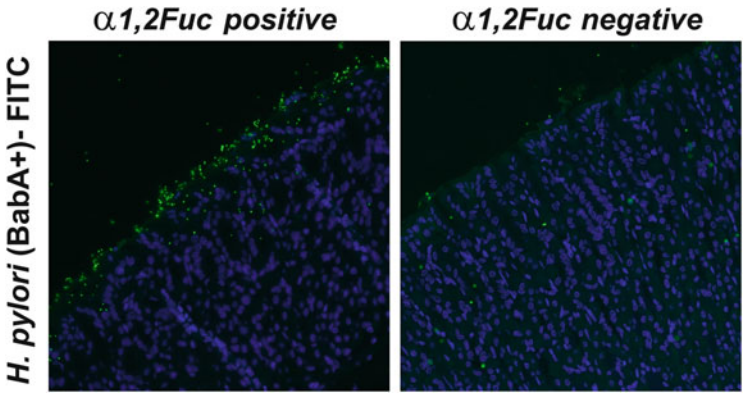

Fig. 2.3 Adhesion of fluorescein (FITC)-labeled *H. pylori* strain 17875/Leb (*green*) to gastric mucosa tissue sections of wild-type (alpha1,2-fucose positive) and Fut2-null (alpha1,2-fucose negative) mice. Magnification ×200

Persistent *H. pylori* infection results in a remodeling of the gastric mucosa glycophenotype, with de novo expression of negatively charged sialylated glycan structures (Magalhães et al. 2015; Mahdavi et al. 2002; Ota et al. 1998). These include sialyl-Lewis a/x, the receptors of the *H. pylori* sialic acid-binding adhesin (SabA) (Mahdavi et al. 2002). The three-dimensional structure of the extracellular adhesion domain of the SabA adhesin showed a cavity lined by conserved amino acids at the SabA N-terminal domain that is likely to function as a highly selective glycan-binding site (Pang et al. 2013).

Besides the very well-characterized BabA and SabA *H. pylori* adhesins, other protein–glycan interactions have been described as relevant for *H. pylori* colonization. These include the neutrophil-activating protein (NAP), which binds sulfated structures such as sulfo-Lewis a, sulfo-Gal, and sulfo-GalNAc expressed on salivary mucins, and the heat shock protein Hsp70 that mediates binding to sulfoglycosphingolipids (Huesca et al. 1996; Namavar et al. 1998). Additionally, *H. pylori* proteins with affinity for heparan sulfate proteoglycans (heparan sulfate-binding proteins, HSBPs) have been identified and demonstrated to participate in bacterial binding to gastric cells (Guzman-Murillo et al. 2001; Ruiz-Bustos et al. 2001). In accordance, *H. pylori*-infected individuals show increased expression of the heparan sulfate-rich proteoglycan syndecan-4 (Magalhaes et al. 2009b).

2.2 Alteration of Glycosylation During Gastric Carcinogenesis

2.2.1 Expression of Terminal Sialylated Glycan Structures in Gastritis

H. pylori chronic infection and the concomitant host inflammatory response are associated with increased expression of terminal sialylated glycan structures, including sialyl-Lewis a and sialyl-Lewis x, in human gastric mucosa (Mahdavi et al. 2002; Ota et al. 1998). Similarly, it has been observed that experimentally infected rhesus monkeys and Mongolian gerbils showed increased expression of sialylated antigens in inflamed gastric tissue (Lindén et al. 2008; Ohno et al. 2011). *H. pylori* infection and host inflammatory cytokines, namely, tumor necrosis factor alpha (TNF-α), induce the expression of the beta3GnT5 glycosyltransferase, which results in increased expression of sialyl-Lewis x in human gastric cells and favors SabA-mediated binding (Magalhães et al. 2015; Marcos et al. 2008).

H. pylori's ability to modulate outer membrane protein expression allows the bacteria to adapt its adhesin expression profile to the changes in the host gastric glycosylation profile and to escape from the inflammatory response, favoring long-term colonization and chronic gastric mucosa inflammation.

2.2.2 *Expression of Simple Mucin-Type Carbohydrate Antigens in Pathological Conditions*

The expression of simple mucin-type Tn and sialyl-Tn antigens is frequently found in several pathological conditions. The expression of this altered glycosylation can be due to various molecular mechanisms that can be modified in disease. Differential expression of ppGalNAc-Ts and variation in the Golgi localization of these enzymes may contribute for the aberrant expression of Tn. Furthermore, the overexpression of ST6GalNAc-I, the α2-6 sialyltransferase responsible for the biosynthesis of sialyl-Tn, may induce the expression of this antigen in pathological tissues. Another mechanism leading to the expression of truncated O-glycans sialyl-Tn and Tn antigens in cancer is the deficiency in Cosmc, a chaperon protein known to stabilize T synthase, the enzyme responsible for Core 1 synthesis. Deficiencies in this chaperone lead to the synthesis of Tn and sialyl-Tn antigen (Ju et al. 2008; Wang et al. 2010).

2.2.2.1 Sialyltransferases and the Biosynthesis of Sialyl-Tn Antigen

The increased expression of terminal sialylated glycans in cancer results from an altered expression of glycosyltransferases, including the increased expression of sialyltransferases (Nakamori et al. 1999). Twenty different sialyltransferases have been described to catalyze the transfer of sialic acid residues from a donor substrate CMP-sialic acid to the oligosaccharide side chain of the glycoconjugates (Harduin-Lepers et al. 2012). Sialyltransferases show cell- and tissue-specific expression pattern and differ in substrate specificities and types of linkage formed (Harduin-Lepers et al. 2005). Depending on these characteristics, STs are classified in four families – ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia (Harduin-Lepers et al. 2012).

Six different human GalNAc α2,6-sialyltransferases catalyze the transfer of sialic acid residues in α2,6-linkage to the proximal GalNAc residue of O-glycosylproteins (ST6GalNAc I, ST6GalNAc II, ST6GalNAc IV) or onto GalNAc residues of glycolipids like GM1b (ST6GalNAc III, ST6GalNAc V, ST6GalNAc VI) (Harduin-Lepers 2010). Among the α2,6-sialyltransferases, both ST6GalNAc I and ST6GalNAc II have shown similar enzymatic activity in vitro, but only ST6GalNAc I has been shown to lead to the biosynthesis of sialyl-Tn in gastrointestinal tissues (Marcos et al. 2004, 2011) (Fig. 2.1).

2.2.2.2 Expression of Simple O-Linked Glycans in Intestinal Metaplasia

In the human stomach, intestinal metaplasia is a premalignant lesion characterized by a transdifferentiation of gastric epithelium into intestinal epithelium and is associated with an increased risk for gastric carcinoma development. Intestinal metaplasia expresses several intestinal markers, such as mucin MUC2 and sialyl-Tn, which

co-localize at the mucinous vacuoles of goblet cells (Ferreira et al. 2006; Pinto et al. 2012; Reis et al. 1999). The expression of ST6GalNAc-I is observed in all intestinal metaplasia cases (Marcos et al. 2011) and co-localizes at the tissue and cell level with the sialyl-Tn antigen expression (Marcos et al. 2004, 2011). At the subcellular level, the expression of ST6GalNAc I is observed at the Golgi of the epithelial cells, and a high expression is observed in Goblet cells of metaplastic glands (Marcos et al. 2011).

2.3 Glycosylation Alterations in Gastric Cancer

2.3.1 The Alterations of Glycosylation in Gastric Carcinoma

Different alterations of glycosylation have been described in gastric cancer cells. These include alterations in O-glycans, N-glycans, and terminal glycan structures present in different types of glycoconjugates (Pinho and Reis 2015).

In the case of O-glycosylation, gastric carcinoma is characterized by the expression of immature simple mucin-type carbohydrate antigens. As discussed above, the aberrant expression of Tn and sialyl-Tn antigens may be due to the differential expression of glycosyltransferases, variation in the Golgi localization of these enzymes, and mutations in protein chaperones.

2.3.1.1 ppGalNAc-Transferase Expression in Gastric Cancer

Different ppGalNAc transferases have been described as being expressed in gastric epithelial cells and gastric cell lines (Gomes et al. 2009; Marcos et al. 2003). In addition, various studies have reported altered expression of ppGalNAc transferases in gastric cancer, being one of the mechanisms underlying the changes in O-glycosylation during malignant transformation. This is the case of the expression of ppGalNAc-T10 that has been shown to be associated with the histological type of the carcinoma (Gao et al. 2013) and ppGalNAc-T6, which has been extensively evaluated in human gastric tissues, including in normal gastric mucosa, intestinal metaplasia, and in gastric carcinomas. ppGalNAc-T6 is expressed in normal gastric mucosa and in intestinal metaplasia of the stomach (Gomes et al. 2009). Furthermore, ppGalNAc-T6 has been shown to be heterogeneous expressed in gastric carcinomas. ppGalNAc-T6 was expressed in 79 % of gastric carcinoma cases, and its expression level was associated with the presence of venous invasion. This association with clinical features of the cases may stem from the contribution of this enzyme for the O-glycosylation of specific sites on specific proteins that are relevant in the biology of the gastric tumor progression. Another member of the ppGalNAc family, the ppGalNAc-T2, has been described as inducing reduced cell proliferation, adhesion, and invasion in a gastric cancer cell line (Hua et al. 2012), reflecting the multitude effects that variations of O-glycosylation may play in the biology of cancer cells depending on different ppGalNAc transferases.

2.3.2 *Glycans in Key Molecules Controlling Gastric Cancer Cell Biology*

2.3.2.1 N-Glycans Modulating Gastric Cancer Cell Adhesion and Invasion

The alterations of glycosylation in gastric cancer also include the modification of N-glycosylation of key molecules involved in cancer development. This is the case of E-cadherin, a protein codified by the CDH1 gene. This protein is involved in epithelial cell–cell adhesion and has been shown to be a key factor in cancer development and cancer cell invasion. In the diffuse type of gastric carcinoma, E-cadherin has been shown to display molecular abnormalities leading to defective intercellular adhesion (Corso et al. 2013; Paredes et al. 2012). In some cases, this is due to germline alterations (mostly mutations) of the CDH1 gene. This is the genetic cause of hereditary diffuse gastric cancer (HDGC) (Guilford et al. 1998). In the sporadic setting, various mechanisms of E-cadherin deregulation have been described, including genetic and epigenetic silencing of the CDH1 gene. Recently, several evidences have shown that during malignant transformation, E-cadherin displays substantial alterations of its glycans. The correct processing and glycosylation, particularly the bisecting GlcNAc N-glycans, catalyzed by GnT-III, has been shown to contribute for the function of this molecule and increased adherens junction stability of epithelial cells. Conversely, when E-cadherin is glycosylated with branched N-glycans, catalyzed by GnT-V, there are major effects on its function leading to the dysregulation in gastric carcinoma (Pinho et al. 2013).

Other key molecules involved in the biology of cancer cells are integrins, which are glycoproteins that play a key role in the cell interaction with the extracellular matrix. The alpha5beta1 integrin has been shown to be modulated with N-glycans that are required for the heterodimer formation and integrin–matrix interactions (Bellis 2004; Gu and Taniguchi 2004).

2.3.2.2 Terminal Sialylation in Gastric Cancer: Biosynthesis and Functional Role

Another common modification of glycosylation observed in gastric cancer is the increased expression of terminal sialylated structures, including the sialyl-Lewis X. The expression of sialyl-Lewis X has been reported in the majority of gastric carcinomas. In addition, this expression has been associated with features of aggressiveness of the cases (Amado et al. 1998).

The expression of this structure depends on the expression of the various glycosyltransferases involved in its biosynthesis (Carvalho et al. 2010). In gastric carcinoma this expression has been shown to affect the activation of receptor tyrosine kinases (RTK), such as the c-Met (Gomes et al. 2013b). This activation is capable of inducing cell signaling pathways that control cell invasion and

motility. These mechanisms are crucial for cancer cell biology. In general, solid tumors display alterations of metabolism that, together with altered transcription of several genes, including glycosyltransferases, may lead to increased sialylated glycosylation, leading to the modification of key signaling pathways controlling epithelial cell behavior, mediating cell motility, invasion, and metastasis.

2.4 Glycans as Biomarkers in Gastric Cancer

In gastric cancer, one of the major concerns of cancer clinical management is to improve the early diagnosis and the successful rate of the therapeutic strategies. Nowadays, most of the serological assays for detection of cancer are based on detection of glycoconjugates (glycoproteins and glycolipids) shed from the tumor cells into the bloodstream. Examples of these are the carcinoembryonic antigen (CEA) and the sialyl-Lewis a (CA19-9) for gastric carcinoma, the MUC16 (CA125) for ovarian cancer, and the MUC1 (CA15-3) for breast cancer. However, the reduced specificity and sensitivity of some of these serological assays for early detection of cancer set the ground for the search for novel biomarkers.

Recently, glycoproteomic strategies have revealed novel putative biomarkers based on altered glycosylation in specific proteins present in circulation of gastric cancer patients. The simple glycan antigen sialyl-Tn has been shown in circulating proteins of gastric carcinoma patients and in individuals with precursor lesions, such as intestinal metaplasia (Gomes et al. 2013a). In addition, glycomic investigations have identified increases in the levels of sialyl-Lewis x present on triantennary glycans accompanied by increased levels of core fucosylation of N-glycans present on IgG from gastric cancer patients (Bones et al. 2011). Recently, CD44 and GalNAc-T5 circulating with the STn glycoform were identified in gastric cancer patient's serum and further validated in gastric tumor tissues, supporting its biomarker potential in gastric cancer (Campos et al. 2015).

These glycosylation alterations detected in the serum of individuals may have valuable applications as biomarkers in order to improve the early diagnosis and prognosis of gastric cancer.

References

Amado M, Carneiro F, Seixas M, Clausen H, Sobrinho-Simoes M (1998) Dimeric sialyl-Le(x) expression in gastric carcinoma correlates with venous invasion and poor outcome. Gastroenterology 114(3):462–470

Aspholm-Hurtig M, Dailide G, Lahmann M, Kalia A, Ilver D, Roche N, Vikstrom S, Sjostrom R, Linden S, Backstrom A, Lundberg C, Arnqvist A, Mahdavi J, Nilsson UJ, Velapatino B, Gilman RH, Gerhard M, Alarcon T, Lopez-Brea M, Nakazawa T, Fox JG, Correa P, Dominguez-Bello MG, Perez-Perez GI, Blaser MJ, Normark S, Carlstedt I, Oscarson S, Teneberg S, Berg DE, Boren T (2004) Functional adaptation of BabA, the *H. pylori* ABO

blood group antigen binding adhesin. Science 305(5683):519–522. doi:10.1126/science.1098801

Azevedo M, Eriksson S, Mendes N, Serpa J, Figueiredo C, Resende LP, Ruvoën-Clouet N, Haas R, Borén T, Pendu JL, David L (2008) Infection by *Helicobacter pylori* expressing the BabA adhesin is influenced by the secretor phenotype. J Pathol 215(3):308–316

Bellis SL (2004) Variant glycosylation: an underappreciated regulatory mechanism for β1 integrins. Biochim Biophys Acta Biomembr 1663(1–2):52–60. doi:10.1016/j.bbamem.2004.03.012

Bennett EP, Hassan H, Mandel U, Mirgorodskaya E, Roepstorff P, Burchell J, Taylor-Papadimitriou J, Hollingsworth MA, Merkx G, van Kessel AG, Eiberg H, Steffensen R, Clausen H (1998) Cloning of a human UDP-N-acetyl-alpha-D-Galactosamine:polypeptide N-acetylgalactosaminyltransferase that complements other GalNAc-transferases in complete O-glycosylation of the MUC1 tandem repeat. J Biol Chem 273(46):30472–30481

Bennett EP, Mandel U, Clausen H, Gerken TA, Fritz TA, Tabak LA (2012) Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. Glycobiology 22(6):736–756. doi:10.1093/glycob/cwr182

Bierhuizen MF, Maemura K, Fukuda M (1994) Expression of a differentiation antigen and poly-N-acetyllactosaminyl O-glycans directed by a cloned core 2 beta-1,6-N-acetylglucosaminyl-transferase. J Biol Chem 269(6):4473–4479

Bones J, Byrne JC, O'Donoghue N, McManus C, Scaife C, Boissin H, Nastase A, Rudd PM (2011) Glycomic and glycoproteomic analysis of serum from patients with stomach cancer reveals potential markers arising from host defense response mechanisms. J Proteome Res 10(3):1246–1265. doi:10.1021/pr101036b

Boren T, Falk P, Roth KA, Larson G, Normark S (1993) Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science 262(5141):1892–1895. doi:10.1126/science.8018146

Brockhausen I (1999) Pathways of O-glycan biosynthesis in cancer cells. Biochim Biophys Acta Gen Subj 1473(1):67–95. doi:10.1016/S0304-4165(99)00170-1

Campos D, Freitas D, Gomes J, Magalhães A, Steentoft C, Gomes C, Vester-Christensen MB, Ferreira JA, Afonso LP, Santos LL, Pinto de Sousa J, Mandel U, Clausen H, Vakhrushev SY, Reis CA (2015) Probing the O-glycoproteome of gastric cancer cell lines for biomarker discovery. Mol Cell Proteomics 14(6):1616–1629. doi:10.1074/mcp.M114.046862

Carvalho AS, Harduin-Lepers A, Magalhães A, Machado E, Mendes N, Costa LT, Matthiesen R, Almeida R, Costa J, Reis CA (2010) Differential expression of α-2,3-sialyltransferases and α-1,3/4-fucosyltransferases regulates the levels of sialyl Lewis a and sialyl Lewis x in gastrointestinal carcinoma cells. Int J Biochem Cell Biol 42(1):80–89. doi:10.1016/j.biocel.2009.09.010

Clausen H, Bennett EP (1996) A family of UDP-GalNAc: polypeptide N-acetylgalactosaminyl-transferases control the initiation of mucin-type O-linked glycosylation. Glycobiology 6(6):635–646

Corso G, Carvalho J, Marrelli D, Vindigni C, Carvalho B, Seruca R, Roviello F, Oliveira C (2013) Somatic mutations and deletions of the E-Cadherin gene predict poor survival of patients with gastric cancer. J Clin Oncol 31(7):868–875. doi:10.1200/jco.2012.44.4612

Ferreira B, Marcos NT, David L, Nakayama J, Reis CA (2006) Terminal alpha1,4-linked N-acetylglucosamine in *Helicobacter pylori*-associated intestinal metaplasia of the human stomach and gastric carcinoma cell lines. J Histochem Cytochem 54(5):585–591. doi:10.1369/jhc.5A6836.2006

Gao Y, Liu Z, Feng J, Sun Q, Zhang B, Zheng W, Ma W (2013) Expression pattern of polypeptide N-acetylgalactosaminyltransferase-10 in gastric carcinoma. Oncol lett 5(1):113–116. doi:10.3892/ol.2012.980

Gomes J, Marcos NT, Berois N, Osinaga E, Magalhaes A, Pinto-de-Sousa J, Almeida R, Gartner F, Reis CA (2009) Expression of UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase-6 in gastric mucosa, intestinal metaplasia, and gastric carcinoma. J Histochem Cytochem 57(1):79–86. doi:10.1369/jhc.2008.952283

Gomes C, Almeida A, Ferreira JA, Silva L, Santos-Sousa H, Pinto-de-Sousa J, Santos LL, Amado F, Schwientek T, Levery SB, Mandel U, Clausen H, David L, Reis CA, Osório H (2013a) Glycoproteomic analysis of serum from patients with gastric precancerous lesions. J Proteome Res 12(3):1454–1466. doi:10.1021/pr301112x

Gomes C, Osorio H, Pinto MT, Campos D, Oliveira MJ, Reis CA (2013b) Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. PLoS ONE 8(6):e66737. doi:10.1371/journal.pone.0066737

Gu J, Taniguchi N (2004) Regulation of integrin functions by N-glycans. Glycoconj J 21(1–2): 9–15. doi:10.1023/b:glyc.0000043741.47559.30

Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE (1998) E-cadherin germ line mutations in familial gastric cancer. Nature 392(6674):402–405

Guzman-Murillo MA, Ruiz-Bustos E, Ho BOW, Ascencio F (2001) Involvement of the heparan sulphate-binding proteins of *Helicobacter pylori* in its adherence to HeLa S3 and Kato III cell lines. J Med Microbiol 50(4):320–329

Harduin-Lepers A (2010) Comprehensive analysis of sialyltransferases in vertebrate genomes. Glycobiol Insights 2010(2):29–61. doi:10.4137/gbi.s3123, 1870-GBI-Comprehensive-Analysis-of-Sialyltransferases-in-Vertebrate-Genomes.pdf

Harduin-Lepers A, Mollicone R, Delannoy P, Oriol R (2005) The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology 15(8):805–817. doi:10.1093/glycob/cwi063

Harduin-Lepers A, Krzewinski-Recchi MA, Colomb F, Foulquier F, Groux-Degroote S, Delannoy P (2012) Sialyltransferases functions in cancers. Front Biosci (Elite Ed) 4:499–515

Hassan H, Reis CA, Bennett EP, Mirgorodskaya E, Roepstorff P, Hollingsworth MA, Burchell J, Taylor-Papadimitriou J, Clausen H (2000) The lectin domain of UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase-T4 directs its glycopeptide specificities. J Biol Chem 275(49):38197–38205. doi:10.1074/jbc.M005783200

Holgersson J, Löfling J (2006) Glycosyltransferases involved in type 1 chain and Lewis antigen biosynthesis exhibit glycan and core chain specificity. Glycobiology 16(7):584–593. doi:10.1093/glycob/cwj090

Hua D, Shen L, Xu L, Jiang Z, Zhou Y, Yue A, Zou S, Cheng Z, Wu S (2012) Polypeptide N-acetylgalactosaminyltransferase 2 regulates cellular metastasis-associated behavior in gastric cancer. Int J Mol Med 30(6):1267–1274. doi:10.3892/ijmm.2012.1130

Huesca M, Borgia S, Hoffman P, Lingwood CA (1996) Acidic pH changes receptor binding specificity of Helicobacter pylori: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. Infect Immun 64(7):2643–2648

Ikehara Y, Nishihara S, Yasutomi H, Kitamura T, Matsuo K, Shimizu N, Inada K-i, Kodera Y, Yamamura Y, Narimatsu H, Hamajima N, Tatematsu M (2001) Polymorphisms of two fucosyltransferase genes (lewis and secretor genes) involving type I lewis antigens are associated with the presence of anti-*Helicobacter pylori* IgG antibody. Cancer Epidemiol Biomarkers Prev 10(9):971–977

Ilver D, Arnqvist A, Ogren J, Frick I-M, Kersulyte D, Incecik ET, Berg DE, Covacci A, Engstrand L, Boren T (1998) *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 279(5349):373–377. doi:10.1126/science.279.5349.373

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(6):1636–1646. doi:10.1158/0008-5472.can-07-2345

Kawakubo M, Ito Y, Okimura Y, Kobayashi M, Sakura K, Kasama S, Fukuda MN, Fukuda M, Katsuyama T, Nakayama J (2004) Natural antibiotic function of a human gastric mucin against *Helicobacter pylori* infection. Science 305(5686):1003–1006. doi:10.1126/science.1099250

Kelly RJ, Rouquier S, Giorgi D, Lennon GG, Lowe JB (1995) Sequence and expression of a candidate for the human secretor blood group α(1,2)Fucosyltransferase Gene (FUT2). J Biol Chem 270(9):4640–4649. doi:10.1074/jbc.270.9.4640

Lee HS, Choe G, Kim WH, Kim HH, Song J, Park KU (2006) Expression of Lewis antigens and their precursors in gastric mucosa: relationship with *Helicobacter pylori* infection and gastric carcinogenesis. J Pathol 209(1):88–94. doi:10.1002/path.1949

Linden S, Nordman H, Hedenbro J, Hurtig M, Thoma B, Carlstedt I (2002) Strain-and blood group dependent binding of *Helicobacter pylori* to human gastric MUC5AC glycoforms. Gastroenterology 123(6):1923–1930

Lindén S, Mahdavi J, Semino-Mora C, Olsen C, Carlstedt I, Borén T, Dubois A (2008) Role of ABO secretor status in mucosal innate immunity and *H. pylori* infection. PLoS Pathog 4(1):e2

Liu Y, Koda Y, Soejima M, Pang H, Schlaphoff T, du Toit ED, Kimura H (1998) Extensive polymorphism of the FUT2 gene in an African (Xhosa) population of South Africa. Hum Genet 103(2):204–210. doi:10.1007/s004390050808

Lopez-Ferrer A, de Bolos C, Barranco C, Garrido M, Isern J, Carlstedt I, Reis CA, Torrado J, Real FX (2000) Role of fucosyltransferases in the association between apomucin and Lewis antigen expression in normal and malignant gastric epithelium. Gut 47(3):349–356

Magalhaes A, Gomes J, Ismail MN, Haslam SM, Mendes N, Osorio H, David L, Le Pendu J, Haas R, Dell A, Boren T, Reis CA (2009a) Fut2-null mice display an altered glycosylation profile and impaired BabA-mediated *Helicobacter pylori* adhesion to gastric mucosa. Glycobiology 19(12):1525–1536. doi:10.1093/glycob/cwp131

Magalhaes A, Marcos NT, Carvalho AS, David L, Figueiredo C, Bastos J, David G, Reis CA (2009b) *Helicobacter pylori* cag pathogenicity island-positive strains induce syndecan-4 expression in gastric epithelial cells. FEMS Immunol Med Microbiol 56(3):223–232

Magalhães A, Marcos-Pinto R, Nairn AV, Dela Rosa M, Ferreira RM, Junqueira-Neto S, Freitas D, Gomes J, Oliveira P, Santos MR, Marcos NT, Xiaogang W, Figueiredo C, Oliveira C, Dinis-Ribeiro M, Carneiro F, Moremen KW, David L, Reis CA (2015) Helicobacter pylori chronic infection and mucosal inflammation switches the human gastric glycosylation pathways. Biochim Biophys Acta 1852(9):1928–1939. doi:10.1016/j.bbadis.2015.07.001

Mahdavi J, Sonden B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson K-A, Altraja S, Wadstrom T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson K-E, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarstrom L, Boren T (2002) *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science 297(5581):573–578. doi:10.1126/science.1069076

Marcos NT, Cruz A, Silva F, Almeida R, David L, Mandel U, Clausen H, Von Mensdorff-Pouilly S, Reis CA (2003) Polypeptide GalNAc-transferases, ST6GalNAc-transferase I, and ST3Gal-transferase I expression in gastric carcinoma cell lines. J Histochem Cytochem 51(6):761–771

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(19):7050–7057. doi:10.1158/0008-5472.can-04-1921

Marcos NT, Magalhães A, Ferreira B, Oliveira M, Carvalho A, Mendes N, Gilmartin T, Head S, Figueiredo C, David L, Santos-Silva F, Reis C (2008) *Helicobacter pylori* induces beta3GnT5 in human gastric cell lines, modulating expression of the SabA ligand sialyl-Lewis x. J Clin Invest 118(6):12

Marcos NT, Bennett EP, Gomes J, Magalhaes A, Gomes C, David L, Dar I, Jeanneau C, DeFrees S, Krustrup D, Vogel LK, Kure EH, Burchell J, Taylor-Papadimitriou J, Clausen H, Mandel U, Reis CA (2011) ST6GalNAc-I controls expression of sialyl-Tn antigen in gastrointestinal tissues. Front Biosci (Elite Ed) 3:1443–1455

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

Mollicone R, Bara J, Le Pendu J, Oriol R (1985) Immunohistologic pattern of type 1 (Lea, Leb) and type 2 (X, Y, H) blood group-related antigens in the human pyloric and duodenal mucosae. Lab Investig 53(2):9

Nakamori S, Nishihara S, Ikehara Y, Nagano H, Dono K, Sakon M, Narimatsu H, Monden M (1999) Molecular mechanism involved in increased expression of sialyl Lewis antigens in ductal carcinoma of the pancreas. J Exp Clin Cancer Res 18(3):425–432

Namavar F, Sparrius M, Veerman ECI, Appelmelk BJ, Vandenbroucke-Grauls CMJE (1998) Neutrophil-activating protein mediates adhesion of *Helicobacter pylori* to sulfated carbohydrates on high-molecular-weight salivary mucin. Infect Immun 66(2):444–447

Nordman H, Davies JR, Lindell G, de Bolós C, Real F, Carlstedt I (2002) Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution. Biochem J 364(1):191–200

Ohno T, Vallström A, Rugge M, Ota H, Graham DY, Arnqvist A, Yamaoka Y (2011) Effects of blood group antigen–binding adhesin expression during *Helicobacter pylori* infection of mongolian gerbils. J Infect Dis 203(5):726–735. doi:10.1093/infdis/jiq090

Ota H, Nakayama J, Momose M, Hayama M, Akamatsu T, Katsuyama T, Graham DY, Genta RM (1998) *Helicobacter pylori* infection produces reversible glycosylation changes to gastric mucins. Virchows Arch 433(5):419–426

Pang SS, Nguyen ST, Perry AJ, Day CJ, Panjikar S, Tiralongo J, Whisstock JC, Kwok T (2013) The three-dimensional structure of the extracellular adhesion domain of the sialic acid-binding adhesin SabA from Helicobacter pylori. J Biol Chem. doi:10.1074/jbc.M113.513135

Paredes J, Figueiredo J, Albergaria A, Oliveira P, Carvalho J, Ribeiro AS, Caldeira J, Costa AM, Simões-Correia J, Oliveira MJ, Pinheiro H, Pinho SS, Mateus R, Reis CA, Leite M, Fernandes MS, Schmitt F, Carneiro F, Figueiredo C, Oliveira C, Seruca R (2012) Epithelial E-and P-cadherins: role and clinical significance in cancer. Biochim Biophys Acta Rev on Cancer 1826(2):297–311. doi:10.1016/j.bbcan.2012.05.002

Pedersen JW, Bennett EP, Schjoldager KT, Meldal M, Holmer AP, Blixt O, Clo E, Levery SB, Clausen H, Wandall HH (2011) Lectin domains of polypeptide GalNAc transferases exhibit glycopeptide binding specificity. J Biol Chem 286(37):32684–32696. doi:10.1074/jbc.M111.273722

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

Pinho SS, Figueiredo J, Cabral J, Carvalho S, Dourado J, Magalhães A, Gärtner F, Mendonça AM, Isaji T, Gu J, Carneiro F, Seruca R, Taniguchi N, Reis CA (2013) E-cadherin and adherens-junctions stability in gastric carcinoma: functional implications of glycosyltransferases involving N-glycan branching biosynthesis, N-acetylglucosaminyltransferases III and V. Biochim Biophys Acta Gen Subj 1830(3):2690–2700. doi:10.1016/j.bbagen.2012.10.021

Pinto R, Carvalho AS, Conze T, Magalhaes A, Picco G, Burchell JM, Taylor-Papadimitriou J, Reis CA, Almeida R, Mandel U, Clausen H, Soderberg O, David L (2012) Identification of new cancer biomarkers based on aberrant mucin glycoforms by in situ proximity ligation. J Cell Mol Med 16(7):1474–1484. doi:10.1111/j.1582-4934.2011.01436.x

Reis CA, David L, Nielsen PA, Clausen H, Mirgorodskaya K, Roepstorff P, Sobrinho-Simões M (1997) Immunohistochemical study of MUC5AC expression in human gastric carcinomas using a novel monoclonal antibody. Int J Cancer 74(1):112–121

Reis CA, David L, Seixas M, Burchell J, Sobrinho-Simoes M (1998) Expression of fully and under-glycosylated forms of MUC1 mucin in gastric carcinoma. Int J Cancer 79(4):402–410

Reis CA, David L, Correa P, Carneiro F, Cd B, Garcia E, Mandel U, Clausen H, Sobrinho-Simoes M (1999) Intestinal metaplasia of human stomach displays distinct patterns of mucin (MUC1, MUC2, MUC5AC, and MUC6) expression. Cancer Res 59(5):1003–1007

Reis CA, David L, Carvalho F, Mandel U, de Bolos C, Mirgorodskaya E, Clausen H, Sobrinho-Simoes M (2000) Immunohistochemical study of the expression of MUC6 mucin and co-expression of other secreted mucins (MUC5AC and MUC2) in human gastric carcinomas. J Histochem Cytochem 48(3):377–388

Rossez Y, Maes E, Lefebvre Darroman T, Gosset P, Ecobichon C, Joncquel Chevalier Curt M, Boneca IG, Michalski JC, Robbe-Masselot C (2012) Almost all human gastric mucin O-glycans harbor blood group A, B or H antigens and are potential binding sites for Helicobacter pylori. Glycobiology 22(9):1193–1206. doi:10.1093/glycob/cws072

Rossez Y, Gosset P, Boneca IG, Magalhães A, Ecobichon C, Reis CA, Cieniewski-Bernard C, Joncquel Chevalier Curt M, Léonard R, Maes E, Sperandio B, Slomianny C, Sansonetti PJ, Michalski JC, Robbe-Masselot C (2014) The lacdiNAc-specific adhesin LabA mediates adhesion of Helicobacter pylori to human gastric mucosa. J Infect Dis 210(8):1286–1295. doi:10.1093/infdis/jiu239

Ruiz-Bustos E, Ochoa JL, Wadstrom T, Ascencio F (2001) Isolation and characterisation of putative adhesins from *Helicobacter pylori* with affinity for heparan sulphate proteoglycan. J Med Microbiol 50(3):215–222

Salama NR, Hartung ML, Muller A (2013) Life in the human stomach: persistence strategies of the bacterial pathogen *Helicobacter pylori*. Nat Rev Microbiol 11(6):385–399. doi:10.1038/nrmicro3016

Sasaki K, Watanabe E, Kawashima K, Sekine S, Dohi T, Oshima M, Hanai N, Nishi T, Hasegawa M (1993) Expression cloning of a novel Gal beta (1-3/1-4) GlcNAc alpha 2,3-sialyltransferase using lectin resistance selection. J Biol Chem 268(30):22782–22787

Schwientek T, Nomoto M, Levery SB, Merkx G, van Kessel AG, Bennett EP, Hollingsworth MA, Clausen H (1999) Control of O-Glycan branch formation: molecular cloning of human cDNA encoding a novel β1,6-N-acetylglucosaminyltransferase forming core 2 and core 4. J Biol Chem 274(8):4504–4512. doi:10.1074/jbc.274.8.4504

Soejima M, Nakajima T, Fujihara J, Takeshita H, Koda Y (2008) Genetic variation of FUT2 in Ovambos, Turks, and Mongolians. Transfusion 48(7):1423–1431. doi:10.1111/j.1537-2995.2008.01710.x

Teixeira A, David L, Reis CA, Costa J, Sobrinho-Simões M (2002) Expression of mucins (MUC1, MUC2, MUC5AC, and MUC6) and type 1 Lewis antigens in cases with and without *Helicobacter pylori* colonization in metaplastic glands of the human stomach. J Pathol 197(1):37–43

Ten Hagen KG, Fritz TA, Tabak LA (2003) All in the family: the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. Glycobiology 13(1):1R–16R. doi:10.1093/glycob/cwg007

Van de Bovenkamp JH, Mahdavi J, Korteland-Van Male AM, Buller HA, Einerhand AW, Boren T, Dekker J (2003) The MUC5AC glycoprotein is the primary receptor for *Helicobacter pylori* in the human stomach. Helicobacter 8(5):521–532

Van den Brink GR, Tytgat KMAJ, Van der Hulst RWM, Van der Loos CM, Einerhand AWC, Buller HA, Dekker J (2000) *H. pylori* colocalises with MUC5AC in the human stomach. Gut 46(5):601–607. doi:10.1136/gut.46.5.601

Wandall HH, Hassan H, Mirgorodskaya E, Kristensen AK, Roepstorff P, Bennett EP, Nielsen PA, Hollingsworth MA, Burchell J, Taylor-Papadimitriou J, Clausen H (1997) Substrate specificities of three members of the human UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase family, GalNAc-T1, −T2, and -T3. J Biol Chem 272(38):23503–23514

Wandall HH, Irazoqui F, Tarp MA, Bennett EP, Mandel U, Takeuchi H, Kato K, Irimura T, Suryanarayanan G, Hollingsworth MA, Clausen H (2007) The lectin domains of polypeptide GalNAc-transferases exhibit carbohydrate-binding specificity for GalNAc: lectin binding to GalNAc-glycopeptide substrates is required for high density GalNAc-O-glycosylation. Glycobiology 17(4):374–387. doi:10.1093/glycob/cwl082

Wang Y, Ju T, Ding X, Xia B, Wang W, Xia L, He M, Cummings RD (2010) Cosmc is an essential chaperone for correct protein O-glycosylation. Proc Natl Acad Sci USA 107(20):9228–9233. doi:10.1073/pnas.0914004107

Wroblewski LE, Peek RM, Wilson KT (2010) Helicobacter pylori and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev 23(4):713–739. doi:10.1128/cmr.00011-10

Part II
Regulation of Cancer Phenotypes by Carbohydrates

Chapter 3
Differential Roles of Mammalian Sialidases in Cancers

Taeko Miyagi

Abstract Aberrant sialylation has been implicated in malignant properties including invasiveness and metastasis. To cast light on the causes and the consequences of such alteration, our studies have focused on mammalian sialidases, which regulate the cellular sialic acid content by catalyzing the removal of sialic acid residues from glycoproteins and glycolipids. There are four types of mammalian sialidases, designated as NEU1, NEU2, NEU3, and NEU4, encoded by different genes differing in their major subcellular localization and enzymatic properties. Each has been found to play a unique role depending on its particular properties and to behave in a particular manner in cancers. Sialidases are involved in regulation of various signaling pathways critical to normal homeostasis and control of proliferation, differentiation, cell adhesion, and motility, so that dysregulation can contribute to tumor initiation, promotion, and progression. Human sialidases, thus, may provide potential targets for cancer diagnosis and therapy. The present review briefly summarizes our recent results on aberrant expression and roles of mammalian sialidases in cancers.

Keywords Sialidase • Sialic acid • Cancer • Progression • Invasion • Metastasis • Ganglioside • Glycoprotein • Transmembrane signaling

3.1 Introduction

Sialic acids are considered to play important roles in various biological processes, mainly through changing the conformation of glycoproteins, and by recognizing and masking of biological sites of functional molecules. Sialic acids show differences in quantity as well as structure during cell differentiation, proliferation, and carcinogenesis and may contribute as virulence factors in bacterial and viral infection. The removal of sialic acids catalyzed by sialidase (EC 3.2.1.18, also called neuraminidases), therefore, exerts great influence on many pathophysiological phenomena. Sialidases exist in common in metazoan animals, from echinoderms to mammals, and are also found in various microorganisms.

T. Miyagi (✉)
Cancer Glycosylation Research, Tohoku Pharmaceutical University, 981-8558 Sendai, Japan
e-mail: tmiyagi@tohoku-pharm.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_3

The subject of cell surface sialic acids on cancer cells received much attention in the 1960–1970s, and a large number of studies suggested increase in negative surface charge determined by electrophoretic mobility to be correlated with reduced adhesiveness of tumor cells. Incubation with bacterial sialidase can result in decreased surface charge followed by suppression of malignancy, indicating an importance of cell surface sialic acids to the cancer phenotype. Although bacterial sialidases are quite different from cellular endogenous enzymes in mammals, subsequent structural studies revealed that a general increase in branching of asparagine-linked glycans, increase in the number and/or length of polylactosaminoglycan chains, and increase in sialylation are often found in cell surface glycoproteins of malignant cells (Lau and Dennis 2008) and that altered sialylation of glycolipids is also observed as a ubiquitous phenotype, associated with the appearance of tumor-associated antigens, aberrant adhesion, and blocking of transmembrane signaling (Hakomori 2010). However, no definite conclusions could be drawn on physiological links between sialic acid contents and malignant properties, because of anomalous experimental results in many cases. To understand the causes and consequences of aberrant sialylation, we have focused on mammalian sialidases, which control the cellular sialic acid content in cooperation with sialyltransferases. Sialidase expression levels indeed change in response to various cellular phenomena and during carcinogenesis, and alteration of individual forms can impact on tumor initiation, promotion, and progression (Miyagi et al. 2012). Recent investigation of mammalian sialidases in cancer has uncovered important information concerning the molecular basis of aberrant sialylation related to malignancy.

3.2 Enzymatic Properties and Possible Functions of Mammalian Sialidases

3.2.1 General Properties of Mammalian Sialidases

Observations on the sialidase activity of cancer cells provided the first clues that the enzymes might be related to transformation and tumor invasiveness. However, it remained uncertain whether the activities were due to the same or different types of sialidase. Recent progress in molecular cloning has validated multiple forms of mammalian sialidases and facilitated elucidation of their functional roles (Miyagi and Yamaguchi 2012). Fundamental information on the four mammalian sialidases is briefly covered here. They are known to be localized predominantly in lysosomes (NEU1), cytosol (NEU2), and plasma membranes (NEU3), while a fourth sialidase (NEU4) is found in lysosomes and/or in the mitochondria and endoplasmic reticulum. However, more recent observations have revealed that they can vary their subcellular localizations with particular cell conditions and stimuli, thereby participating different cellular functions depending on their loci. In fact, NEU1 and

NEU4 may mobilize to the cell surface under certain conditions, as well as NEU3, and all contain several Asp boxes (-Ser-X-Asp-X-Gly-X- Thr-Trp-) and the Arg-Ileu-Pro sequence, which are conserved in microbial sialidases, despite no overriding structural similarities to the mammalian enzymes. Distinctive behavioral differences in response to inhibitors between mammalian and microbial sialidases are suggestive of molecular variation. For example, regarding anti-influenza drugs, Tamiflu (oseltamivir) was found to hardly affect any of the human sialidases in vitro, even at 1 mM, and Relenza (zanamivir) significantly inhibited NEU3 and NEU2 only in the micromolar range, contrasting with the low nanomolar concentrations in which activity of viral sialidases is blocked (Hata et al. 2008). The sialic acid analog 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA), Neu5Ac2en, is known to be a common inhibitor of almost all sialidases, but viral and bacterial sialidases are ten times more sensitive than their mammalian counterparts. Among human sialidases, the overall amino acid identity of NEU1 to the other sialidases is relatively low (19–24 %), while NEU2, NEU3, and NEU4 show 34–40 % homology to each other. Regarding comparative expression levels, NEU1 generally shows the strongest expression, 10–20 times greater than those of NEU3 and NEU4, while NEU2 expression is extremely low and at most only four- to ten-thousandth of the NEU1 value in a range of tissues (Hata et al. 2008), as assessed by quantitative real-time RT-PCR using a standard curve for each cDNA, although profiles differ among the human, rat, and mouse.

3.2.2 Sialidase NEU1

The lysosomal sialidase, NEU1, is involved in glycoconjugate catabolism in lysosomes, existing as a complex with a protective protein (carboxypeptidase A) and β-galactosidase whose dissociation leads to sialidase inactivation (D'Azzo et al. 1982; Galjart et al. 1988). The human NEU1 gene was identified as a major histocompatibility complex (MHC)-related sialidase gene responsible for sialidase deficiency (Bonten et al. 1996; Pshezhetsky et al. 1997; Milner et al. 1997). Taking advantage of the SM/J inbred strain of mice carrying a defective sialidase allele, the mouse *Neu1* gene was mapped near the H-2D end of the major histocompatibility complex (MHC) on chromosome 17 by linkage analysis, a region which is syntonic to human MHC on chromosome 6. NEU1 is linked to two neurodegenerative lysosomal storage disorders, sialidosis and galactosialidosis (d'Azzo and Bonten 2010). The former is caused by defects in genomic DNA, including frameshift insertions and missense mutations, and the latter features a combined deficiency of the sialidase and β-galactosidase due to the absence of a functional protective protein. NEU1 possesses narrow substrate specificity, with a synthetic substrate, 4-methylumbelliferyl-neuraminic acid (4MU-NeuAc), oligosaccharides, and glycopeptides serving as good substrates in in vitro assays. Sialyloligosaccharides become increased in sialidosis patients' urine and fibroblasts. However, recent observations revealed various subcellular distribution in plasma membrane as

well as within lysosomes under conditions of cell stimulation. In line with these findings, NEU1 was found to negatively regulate lysosomal exocytosis, a cellular process for the recruitment of lysosomes to the plasma membrane, resulting in an increase in extracellular proteolytic activity (Yogalingam et al. 2008). NEU1 is also involved in cellular signaling for immune responses and elastic fiber assembly through transportation to plasma membranes and contributes to the regulation of phagocytosis in macrophages and dendritic cells through the desialylation of surface receptors (Pshezhetsky and Hinek 2011). Furthermore, NEU1 plays an essential role for regulation of cell migration and invasion by desialylation of cell surface glycoproteins including β4-integrin, leading to suppression of cancer metastasis (Uemura et al. 2009).

3.2.3 Sialidase NEU2

NEU2 was the first mammalian sialidase for which cDNA cloning was achieved (Miyagi et al. 1993). The human ortholog was cloned from a genomic library of human skeletal muscle (Monti et al. 1999) and the three-dimensional structure was determined by X-ray crystallography (Chavas et al. 2005). In general, the occurrence of glycoconjugates in the cytosol may be considered as an unusual event, probably because of its lack of involvement in major glycosylation pathways. However, there are many examples of delivery of misfolded glycoproteins from the endoplasmic reticulum (ER) into the cytosol via the ER-associated degradation (ERAD) pathway, of free oligosaccharides deglycosylated from ERAD substrate glycoproteins by the cytosolic peptide:N-glycanase, and of gangliosides binding to vimentin or other intermediate filaments, suggesting that NEU2 plays physiologically significant roles in the cytosol. NEU2 is able to hydrolyze a wide range of glycoproteins, oligosaccharides, gangliosides, as well as 4MU-NeuAc at near-neutral pH of about 6.0–6.5. It participates in muscle cell and neuronal differentiation in murine cells, and the rat *Neu2* gene contains two E-box pairs in the 5′-flanking enhancer/promoter region (Sato and Miyagi 1995), known to be consensus binding sites for muscle-specific transcription factors, and exhibits transcriptional activity in murine myogenic cells. In PC12 cells the sialidase has been suggested to participate in neuronal differentiation on the basis of nerve-growth factor-induced transcriptional activation of the gene (Fanzani et al. 2004).

3.2.4 Sialidase NEU3

The plasma membrane-associated sialidase, NEU3, was first cloned from a bovine brain library, based on the peptide sequence information from the purified enzyme protein (Hata et al. 1998), as well as from a human brain cDNA library (Miyagi et al 1999). The bovine NEU3 and its mouse ortholog were found to be

predominantly located in plasma membranes by Percoll density gradient centrifugation of cell homogenates and by immunofluorescence staining of transfected cells. Unlike these sialidases, the human ortholog NEU3 is not always detected on cell surface and can be moved to and concentrated at leading edges in response to growth stimuli. With administration of the radio-labeled ganglioside GD1a to murine *Neu3*-transfected cells, the enzyme was shown to hydrolyze ganglioside substrates in intact living cells at a neutral pH mainly through cell-to-cell interactions (Papini et al. 2004), further supporting a plasma membrane topology for NEU3. The bovine and human enzymes almost specifically hydrolyze gangliosides, whereas the murine enzyme acts on oligosaccharides, 4MU-NeuAc, and glycoproteins to a certain extent. Gangliosides GD3, GM3, GD1a, and even GD1b, but not GM1 and GM2, are good substrates in the presence of Triton X-100. Unlike the bovine and murine enzymes, with only one activity peak at a pH near 4.6, the human enzyme shows two peaks at pH 4.5–4.8 and pH 6.0–6.5. Interestingly, the activity at neutral pH is elevated by phosphatidic acid (Shiozaki et al. 2015) NEU3 was proven to positively regulate neurite formation in mice and in human neuroblastoma cells and to enhance regeneration in rat hippocampus neurons. The sialidase was found to be located in rafts of neuroblastoma cells and in caveolae of HeLa cells, closely associated with caveolin-1 (Wang et al. 2002), probably through the caveolin-binding region, and was further shown to exert a great influence on signaling pathway including EGFR, integrin, and IL-6 signaling through ganglioside modulation (Miyagi et al. 2008).

3.2.5 Sialidase NEU4

The fourth sialidase, NEU4, was identified based on cDNA sequences in public databases (Comelli et al. 2003; Monti et al. 2004; Seyrantepe et al. 2004; Yamaguchi et al. 2005). With regard to the subcellular localization of the human ortholog, two different descriptions have been reported on the basis of gene transfection studies: one demonstrated targeting to the lysosomal lumen and the other to mitochondria and endoplasmic reticulum. The human enzyme seems to consist of isoforms differing in the presence or absence of 12 N-terminal amino acid residues which act in mitochondrial targeting, so that the long form may be in mitochondria and the short form in endoplasmic reticulum (Yamaguchi et al. 2005). The isoforms are also differentially expressed in a tissue-specific manner, the brain, muscle, and kidney containing both and the liver and colon possessing predominantly the short form, as assessed by RT-PCR. The mouse enzyme also presents as two isoforms (NEU4a and 4b) derived from alternating splicing (Shiozaki et al. 2009a), and NEU4a possessing an additional 23 amino acid stretch at the N-terminus exhibits lower enzymatic activity than NEU4b but interestingly more efficient hydrolysis of polysialic acids on the neural cell adhesion molecule (polySia-NCAM) (Takahashi et al. 2012). The mouse NEU4 gene is expressed dominantly in the brain and is only found at very low levels in other tissues. NEU4

possesses broad substrate specificity and, unlike other human sialidases, is capable of acting on mucins and polySia-NCAM. It may be involved in neuronal cell apoptosis, based on the observation that the NEU4 long form probably regulates levels of the GD3, known to be an apoptosis-related ganglioside, in mitochondria of neuroblastoma cells. In contrast to NEU3, NEU4 appears to negatively regulate neurite formation in Neuro2a cells and hippocampal neuron.

3.3 Alteration of Sialidase Expression in Cancer

Previous observations on the sialidase activity of cancer cells suggested a close link of mammalian sialidases with the malignant phenotype. For example, alterations of the sialidase activity against gangliosides were described as associated with malignancy in 3T3-transformed cells (Yogeeswaran and Hakomori 1975) and also in BHK-transformed cells (Schengrund et al. 1973). In mouse epidermal JB6 cells exposed to TPA and in their anchorage-independent transformants, decreased sialidase activity in the lysosomal fractions and contrasting increased activity in plasma membrane fractions were found as compared with the corresponding activities in the untreated JB6 cells (Miyagi et al. 1990). After validation of four types of mammalian sialidase by the development of gene cloning, our focus has been on identification of which sialidases are involved in alteration in cancer. Endogenous activity levels can be estimated using a differential assay procedure for each sialidase mainly based on its substrate specificity and major subcellular localization, in combination with measurement of mRNA levels by RT-PCR. Sometimes it is difficult to distinguish among forms because of their redundancy in their biochemical properties, but in most cases, the activity level has been found to be parallel the mRNA level.

When the sialidase expression level was evaluated in human cancers by activity assays and quantitative RT-PCR, NEU3 exhibited remarkable upregulation in colon, prostate, ovary, and renal cancers (Miyagi et al. 2012) but downregulation in acute lymphoblastic leukemia (Mandal et al. 2010). In contrast, NEU1and NEU4 showed a tendency for reduced expression in colon cancer, and NEU2 has proved to be hardly detectable in human normal and even cancer tissues. The mRNA level of NEU3 in surgical specimens of human colon cancer was found to be increased in all tissue specimens ($n = 50$) by 3- to 100-fold as compared with that in the adjacent noncancerous mucosa (Kakugawa et al. 2002). In-situ hybridization analysis showed NEU3 expression in the epithelial elements of adenocarcinomas. On the other hand, NEU4 showed a marked decrease in mRNA level in tumors as compared with nontumor mucosa, the average reduction being 2.8-fold, although a wide variety of values were obtained (Yamanami et al. 2007). Significant elevation of sialidase activity against gangliosides has also been observed in tumor tissues (8.9 ± 0.2 to 5.7 ± 0.1 units/mg protein, relative to mucosa). Although nontumor mucosa contained the higher gangliosides activity than expected, it is probably due to relatively high NEU4 expression in the mucosa. This implies that most of the

ganglioside sialidase activity observed in the cancerous tissues might actually be relevant to NEU3. A recent study (Yamaguchi et al. 2010) on NEU3 gene expression provided evidence of diverse regulation by Sp1/Sp3 transcription factors binding to alternative promoters. One of the mechanisms of NEU3 upregulation in cancers, therefore, may be attributed to transcriptional regulation by Sp1/Sp3 factors, which have been documented to play critical roles in regulating the transcription of genes involved in cell growth control and tumorigenesis (Wierstra 2008). In fact, NEU3 expression exhibits good correlations with those of Sp1 or Sp3 in cancer, implying a promoting role in the gene transcription. The mechanism of altered expression of NEU1 and NEU4 remains largely unknown.

3.4 Sialidase in Cancer Initiation and Promotion

3.4.1 Observations in Genetically Engineered Mouse Models

A possible role of NEU3 in promoting tumorigenesis in vivo has been demonstrated in human NEU3 transgenic mice treated with a carcinogen, azoxymethane for induction of precancerous colonic aberrant crypt foci (ACF) as shown in Fig. 3.1 (Shiozaki et al. 2009b). ACF were induced in the mice significantly more frequently than in their control wild-type counterparts. Enhanced phosphorylation of EGF receptor, Akt, and ERK and upregulation of Bcl-xL protein were observed in the transgenic colon mucosa, but no changes were found in cell proliferation, suggesting that the increased ACF formation was due to suppression of apoptosis. Thus, NEU3 upregulation may be important to the promotion stage of colorectal carcinogenesis in vivo. When *Neu3*-deficient mice were exposed to dimethylhydrazine, there were no differences in the incidence or growth of tumors from wild-type mice. On the other hand, the *Neu3*-deficient mice were less susceptible to colitis-associated colon carcinogenesis induced by azoxymethane and dextran sodium sulfate, indicating an involvement of NEU3 in inflammation-dependent tumor development (Fig. 3.2) (Yamaguchi et al. 2012). These results suggested a role of NEU3 in tumor promotion, and recent investigations indeed have demonstrated the evidence for its critical involvement in all the processes in tumorigenesis including tumor initiation, promotion, and progression (Fig. 3.3), as described below.

3.4.2 Anchorage-Independent Growth

NEU1 and NEU3 appear to have opposing actions in initiation of anchorage-independent growth of cancer cells. NEU1 transfection into murine melanoma B16 cells resulted in diminished overall and anchorage- independent growth, and

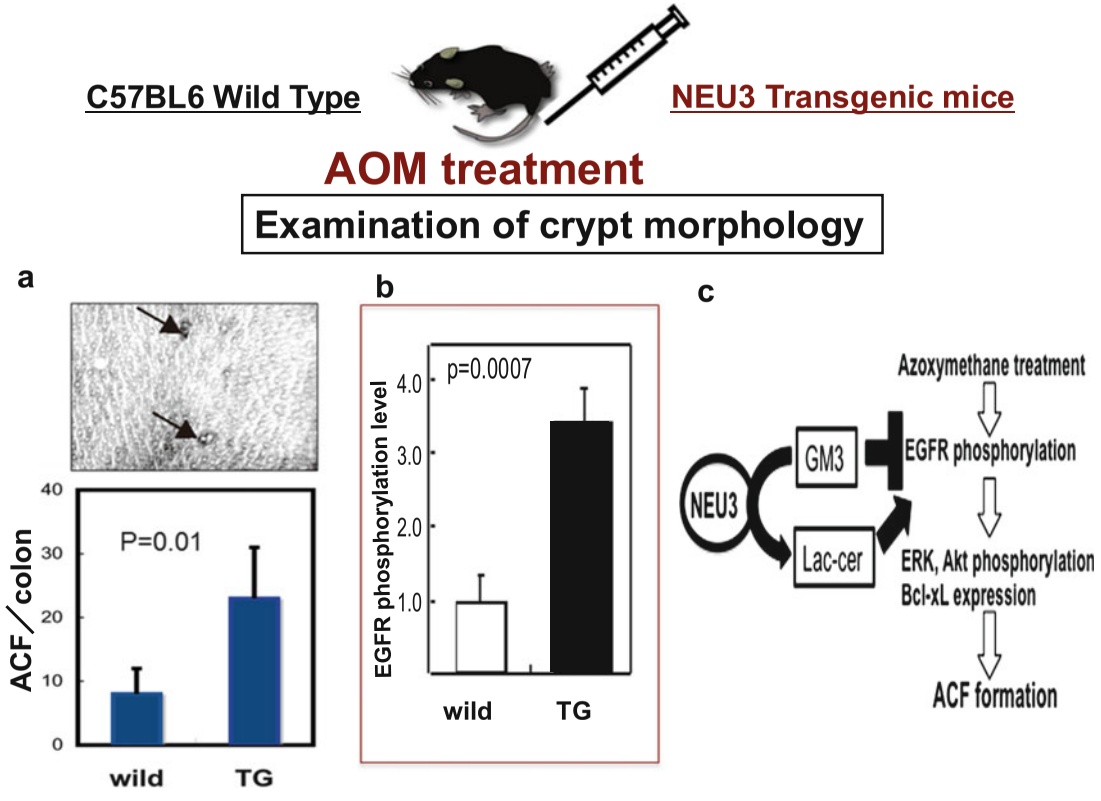

Fig. 3.1 Promotion of aberrant crypt foci (ACF) formation in colonic mucosa of azoxymethane (AOM)-injected human *NEU3* transgenic (TG) mice (Shiozaki et al. 2009b). (**a**) *Methylene blue-stained ACFs* are indicated by *arrows* in the colonic mucosa of NEU3 transgenic mice (*upper*), and the number of ACF was counted in *methylene blue-stained* colon (*lower*). (**b**) The phosphorylation levels of EGFR as measured by Western blot analysis. (**c**) Possible mechanism of increased ACF formation in TG mice

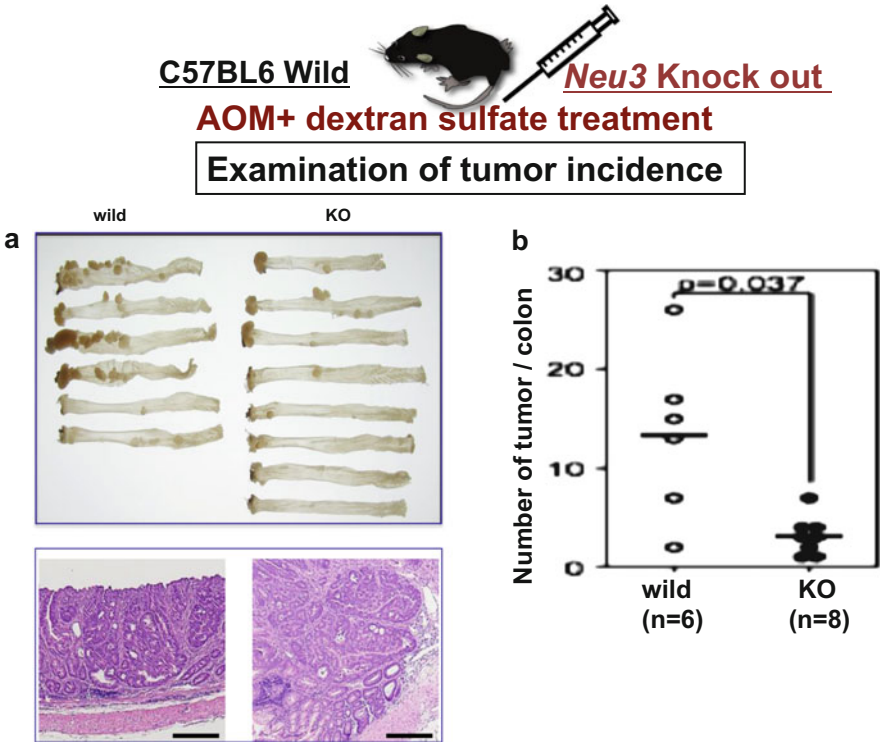

Fig. 3.2 Reduced susceptibility to colitis-associated colon carcinogenesis in *Neu3* knockout mice (Yamaguchi et al. 2012). (**a**) Macroscopic photograph of dissected colons of dextran sulfate and AOM-treated wild-type and *Neu3* knockout mice. Tumors that developed at the distal ends of colons are indicated by *arrowheads*. (**b**) Micrographs of the colon adenocarcinoma. Sections from paraffin-embedded tumors were stained with hematoxylin-eosin. Bars, 0.1 mm

increased sensitivity to apoptosis induced by suspension culture or serum depletion, compared with parental and control cells (Kato et al. 2001). Transfectants expressing the highest sialidase activity exhibited the greatest reduction in anchorage-independent growth.

In contrast to NEU1, downregulation of NEU3 in colon cancer cells was found to cause decreased colony formation, chemosensitivity, anchorage-independent growth, and in vivo tumor growth in nude mice. This complements the findings for association of NEU3 with augmented malignant properties including increased cell migration, invasiveness, and cell survival, through activation of Ras/ERK signaling described below. Our recent data for stem-like characteristics and tumorigenic potential also provided evidence of a close link between NEU3 expression and Wnt/β-catenin signaling in colon cancer cells (Takahashi et al. 2015). The available data strongly suggests NEU3 participation in tumor initiation and promotion, since constitutive activation of Wnt/β-catenin signaling is implicated in the maintenance of cancer stem cells and initiation of the process of colon carcinogenesis (Clevers 2006).

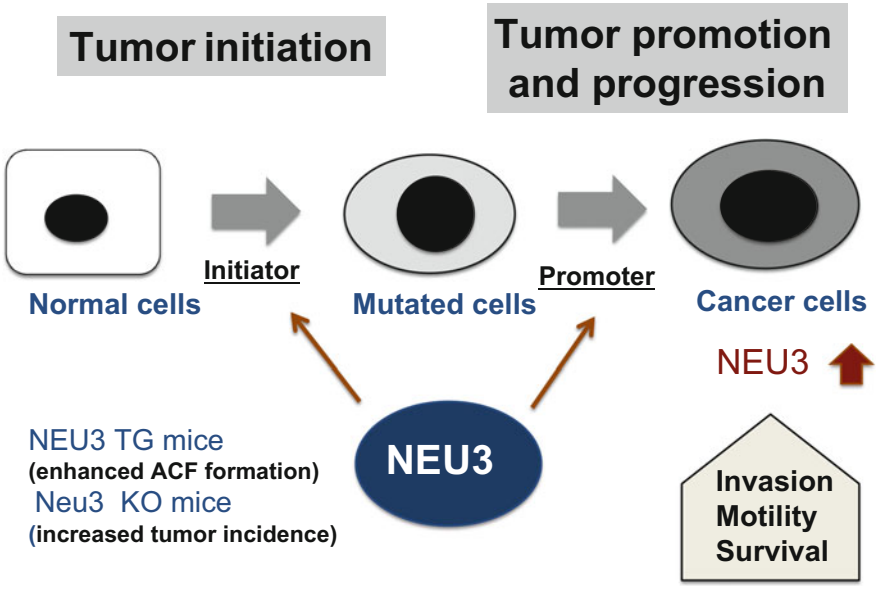

Fig. 3.3 Possible involvement of NEU3 in cancer initiation, promotion, and progression

3.4.3 Cell Apoptosis

A close link between NEU3 and cell survival has been revealed by silencing or overexpression of the encoding gene in various human cancer cells (Wada et al. 2007). Silencing caused apoptosis without specific stimuli, accompanied by decreased Bcl-xL and increased caspase, mda7 (melanoma differentiation associated gene-7) and GM3 synthase mRNA levels in HeLa cells, whereas overexpression resulted in the opposite effects. Human colon and breast carcinoma cell lines, HT-29 and MCF-7 cells, were similarly affected by treatment with NEU3 siRNA, but interestingly noncancerous human WI-38 and NHDF fibroblasts and NHEK keratinocytes showed no significant changes. NEU3 siRNA was found to inhibit and NEU3 overexpression to stimulate Ras activation with consequent influence on ERK and Akt. Ras activation by NEU3 was largely abrogated by PP2 (an src inhibitor) or AG1478 (an EGFR inhibitor), in line with the finding that siRNA introduction reduced the phosphorylation of EGFR whereas overexpression promoted its phosphorylation in response to EGF. The fact that NEU3 co-immunoprecipitates with EGFR and EGF stimulation yields a higher amount of immunoprecipitable NEU3 further indicates that it suppresses apoptosis of cancer cells by promoting EGFR phosphorylation, probably through its association with EGFR and consequent activation of Ras cascades, especially via the Ras/ERK pathway. Colon cancer tissue specimens and cancer cells exhibit marked accumulation of lactosylceramide, a possible NEU3 product, and addition of the glycolipid

to the culture reduced the number of apoptotic cells during sodium butyrate treatment (Kakugawa et al. 2002). These results indicate that high expression of NEU3 in cancer cells leads to protection against programmed cell death, whereas decrease in NEU3 induces apoptosis, implying a critical role in the survival of cancer cells through ganglioside modulation. Thus, siRNA targeting of the enzymatic protein could have utility for gene-based therapy of human cancers.

The fact that NEU4 mRNA levels are markedly decreased in human colon cancer may also have important implications for cell apoptosis (Yamanami et al. 2007). In cultured human colon cancer cells, the enzyme was found to be upregulated in early stages of apoptosis induced by either the death ligand TRAIL or serum depletion. Transfection of NEU4 into DLD-1 and HT-15 colon adenocarcinoma cells resulted in acceleration of apoptosis and decreased invasiveness and cellular motility. siRNA-mediated NEU4 targeting, on the other hand, caused a significant inhibition of apoptosis.

NEU2 may also be involved in cell apoptosis, *NEU2* gene introduction into leukemic K562 cells being associated with increased sensitivity to apoptotic stimuli by impairing Bcr-ABl/Src kinase signaling (Tringali et al. 2007). However, it is uncertain how NEU2 actually functions in human tissues and cells, because of its extremely low expression, even in cancer cells. The biological and phylogenic significance of markedly lower expression of NEU2 in man as opposed to other species remains to be elucidated.

3.5 Sialidase in Cancer Progression and Metastasis

Investigation of NEU3 upregulation in cancers also suggested involvement in progression. Immunohistochemical analysis of surgical specimens using an anti-NEU3 monoclonal antibody confirmed upregulation in several human cancers. In prostate cancer, the intensity of the histochemical staining showed a positive relationship with the Gleason score, related to the pathological progression stage (Kawamura et al. 2012). Furthermore, in head and neck cancer, increase in the mRNA and activity levels significantly correlated with the histological differentiation grade, lymphatic invasion, and lymph node metastasis (Shiga et al. 2015). In contrast to NEU3, NEU1 and NEU4 downregulation is likely to relate to increased progression and metastasis, for example, to venous invasion involved in hematogenous metastasis.

3.5.1 Cell Migration and Invasion

An increased NEU3 mRNA level has been reported in renal cell carcinomas (RCC) (Ueno et al. 2006), as compared with those in adjacent noncancerous tissues, significantly correlated with elevation in the expression of interleukin (IL)-6, a

pleiotropic cytokine which has been implicated in the immune responses to and pathogenesis of several cancers. Activity of the NEU3 sialidase in RCC in connection with IL-6 function was investigated using tumor tissues obtained from RCC patients and renal cancer ACHN cells. Upregulation of NEU3 in the tumor tissues was strongly linked to the IL-6 expression level; NEU3 in the renal cancer ACHN cells was activated by IL-6 in a positive feedback manner on the cytokine function, mainly through the PI3K/Akt pathway, resulting in suppression of apoptosis and promotion of migration. In human RCC ACHN cells, IL-6 treatment significantly enhanced endogenous sialidase activity and also increased the NEU3 promoter luciferase activity 2.5-fold. NEU3 transfection or IL-6 treatment both resulted in the suppression of apoptosis or promotion of cell motility, and their combination exerted synergistic effects. Glycolipid analysis revealed a decrease in the expression of the ganglioside GM3 and increase in that of Lac-cer after NEU3 transfection. Addition of these lipids to the culture also apparently affected the cellular apoptosis and motility. The results indicate that NEU3 activated by IL-6 promotes signaling largely via the PI3K/Akt cascade in a positive feedback manner and contributes to the expression of the malignant phenotype in renal cancer.

As noted above, upregulation of NEU3 was also detected in prostate cancer, showing a significant correlation with malignancy (Kawamura et al. 2012). In androgen-sensitive LNCaP cells, forced overexpression of *NEU3* significantly increased levels of a progression-related transcription factor EGR-1, androgen receptor (AR), and PSA both with and without androgen, the cells becoming sensitive to androgen. The NEU3-mediated induction was abrogated by inhibitors of PI-3 kinase and MAPK, increased phosphorylation of AKT and ERK1/2 in NEU3-overexpressing cells being confirmed. To understand further how NEU3 causes activation of AKT and ERK followed by elevation of EGR-1, AR, and PSA expression, we observed the upstream signaling including EGFR family expression, which has been proposed to escape androgen regulation and switch to androgen-independent cell growth (Traish and Morgentaler 2009). Consistent with other types of cancer, NEU3 was found to activate the PI3K and MAPK pathways, associated with increase in mRNA and protein levels of EGFR and ERBB2 under androgen-deficient conditions. For development of hormone-refractory progression, enhanced AR mRNA and protein expression is known as a critical factor in conversion to a hormone-refractory state in the majority of patients who do not have AR mutations or amplification. The mechanism of increased AR expression includes cross-talk between ARs and growth factor receptors such as EGFR and ERBB2. In this context, NEU3 upregulation may trigger off activation of AR pathway probably via EGFR family, leading to conversion to a hormone-refractory state. In fact, NEU3 siRNA introduction caused reduction of growth of androgen-independent PC-3 cells in culture and of transplanted tumors in nude mice (Kawamura et al. 2012). These data suggest that NEU3 regulates progression of prostate cancer through androgen receptor signaling. Interestingly, in the sera of prostate cancer patients, ganglioside sialidase activity has been detected which is generally low or lacking in the healthy subjects. Preliminary results have indicated no activity with 4MU-NeuAc, a good substrate for sialidases other than human

NEU3, and furthermore the protein responsible for the activity could be co-immunoprecipitated with anti-NEU3 antibodies, suggesting its activity is originated from NEU3 (Hata et al. 2015). Another report by Li et al. (2011) on NEU3 effect in prostate cancer PC-3 cells also demonstrated knockdown to significantly decrease invasion and migration accompanied with reduced expressions of the matrix metalloproteinases MMP-2 and MMP-9, and mice injected with PC-3 cell tumors developed fewer bone metastases on treatment with the NEU3 siRNA than controls.

While NEU4 mRNA levels showing marked decrease in human colon cancer were not significantly correlated with the histological differentiation or the pathological stage, the T/NT (tumor-to-nontumor expression ratio) value remained significant at $P = 0.025$ with venous invasion between v0 ($n = 28$) and v1–3 ($n = 13$) (Yamanami et al. 2007). To elucidate the significance of NEU4 downregulation in colon cancer, sialyl-Lewis antigens, sialyl-Le^a and sialyl-Le^x, as endogenous substrates for the sialidase were investigated (Shiozaki et al. 2011), because they are utilized as tumor markers, and their increase in cancer is associated with tumor progression. NEU4 was found to hydrolyze the antigens in vitro and decrease cell surface levels much more effectively than other sialidases. Western blot, thin-layer chromatography, and metabolic inhibition studies of desialylation products revealed NEU4 to preferentially catalyze sialyl-Lewis antigens expressed on O-glycans. Cell adhesion to and motility and growth on E-selectin were significantly reduced by NEU4. Furthermore, E-selectin stimulation of colon cancer cells enhanced cell motility through activation of the p38/Hsp27/actin reorganization pathway, whereas NEU4 attenuated the signaling. It is interesting to note in this context that the sialidase did not change the level of a normal glycan, disialyl-Le^a, generally expressed in nonmalignant epithelial cells. Although it has been proposed that glycosyltransferases are responsible for synthesis of these antigens, expression levels of the encoding genes have not always been found to correlate with sialyl-Lewis antigen contents, with even contradictory expression noted. It is feasible that desialylation by NEU4 may occur specifically with cancer-related sialyl-Lewis antigens, and thus maintenance of the normal glycan level can be achieved in colon mucosa highly expressing NEU4. PolySia-NCAM has been reported to be expressed in malignant tumors, including gliomas and lung and colon cancers, as well as nervous tissue, the presence being correlated with tumor development, invasion, and poor prognosis (Tanaka et al. 2001; Suzuki et al. 2005). Considering regulation of polySia by NEU4 (Takahashi et al. 2012), it is likely that downregulation of the enzyme might be involved in its presence in cancers, at least in the colon.

3.5.2 Cancer Metastasis

Various cancers show general tendencies for decreased NEU1expression. Interestingly, there is a good inverse relationship with metastatic ability, as evidenced with the results for several cell lines of different malignant potential. After src transformation, 3Y1 fibroblasts showed decrease in the sialidase activity, and furthermore,

v-*fos* transfer to the transformed cells caused even more severe effects with acquisition of higher lung metastatic potential (Miyagi et al. 1994). In this regard, it is interesting that various lysosomal enzymes other than sialidase were not appreciably affected by the transformation, suggesting that the alteration was specific for the sialidase. In mouse adenocarcinoma colon 26 cells, compared to low metastatic NL4 and NL44 cell lines, highly metastatic NL17 and NL 22 cells exhibited lower expression of NEU1 sialidase, accompanied by higher levels of sialyl Le^x and GM3 (Sawada et al. 2002). Introduction of the murine *Neu1* gene into Bl6 melanoma cells resulted in suppression of experimental pulmonary metastasis and tumor progression, with reduction of anchorage-independent growth and increased sensitivity to apoptosis (Kato et al. 2001). Since metastatic potential did not parallel the sialic acid contents in these cells, it is likely that altered sialidase expression was directly involved.

Overexpression of the human ortholog gene *NEU1* with protective protein brought about similar alterations of human cancer cells to those observed in the murine cells, with suppressed cell migration and invasion in colon adenocarcinoma HT-29 cells, whereas its knockdown resulted in the opposite. When *NEU1*-overexpressing cells were injected transsplenically into mice, in vivo liver metastatic potential was significantly reduced. One of the target molecules for NEU1 was found to be integrinβ4, undergoing desialylation and decreased phosphorylation associated with attenuation of FAK and ERK1/2 signaling and downregulation of matrix metalloproteinase-7 (Uemura et al. 2009). Biotinylation and immunofluorescence staining exhibited some NEU1 molecules to be at the cell surface accessible to the integrin. A microRNA, miR-125b, has been found to promote growth of prostate cancers in both intact and castrated male nude mice by downregulating proapoptotic and tumor suppressor genes (Shi et al. 2011). Interestingly, miR-125b targets eight transcripts including NEU1 as suppressor of metastasis, consistent with the NEU1 effects described above.

When the rat *Neu2* gene was transfected into highly metastatic mouse colon 26 adenocarcinoma cells, intravenous injection into syngeneic mice was associated with marked reduction in lung metastasis, invasion, and cell motility, with a concomitant decrease in sialyl Le^x and GM3 levels (Sawada et al. 2002). Treatment of the cells with antibodies against sialyl Le^x- and GM3-affected cell adhesion and/or cell motility, providing direct evidence that desialylation of these molecules, as natural substrates of the sialidase, is involved in the suppression of metastasis. As described earlier, sublines of cells featuring low spontaneous metastasis possessed a relatively high level of endogenous NEU1 sialidase, compared with highly metastatic cells, suggesting that even at endogenous levels, rat and murine *Neu2* gene expression may exert a negative influence on cell invasion, motility, and metastasis. Independent of sialidase expression level, on the other hand, highly metastatic cells exhibited rather decreased sialic acid contents, both total and cell surface, as compared to low metastasis counterparts.

These results all together indicate that reduced levels of sialidases such as NEU1 and NEU2 may be a determining factor for metastatic ability, independent of the cell type and the sialic acid content. In addition, NEU4 also may play a role in regulation of metastasis through efficient hydrolysis of sialyl-Lewis antigens,

because decreased NEU4 observed in human colon cancer was significantly correlated with venous invasion and therefore to the development of hematogenous metastasis.

3.6 Conclusions and Perspectives

Recent investigations of mammalian sialidases in cancer have greatly clarified the molecular basis and significance of aberrant sialylation. Altered expression of the sialidases indeed exerts marked influence on the malignancy, through desialylation of endogenous glycoconjugates, probably not simply dependent on the sialic acid content. In general, the three sialidases NEU1, NEU2, and NEU4 appear to be downregulated in carcinomas, and this facilitates metastasis by changing mainly adhesion mechanisms, whereas NEU3 upregulation observed in various carcinomas augments probably all the processes contributing to tumorigenesis, including initiation, promotion, and progression (Fig. 3.3). Alteration of sialidases, therefore, opens up potential applications in cancer diagnosis and cure as illustrated in Fig. 3.4. In particular, taking advantage of its limited effects on normal cells, downregulation of NEU3, as a key regulator for cancer cell survival, by treatment

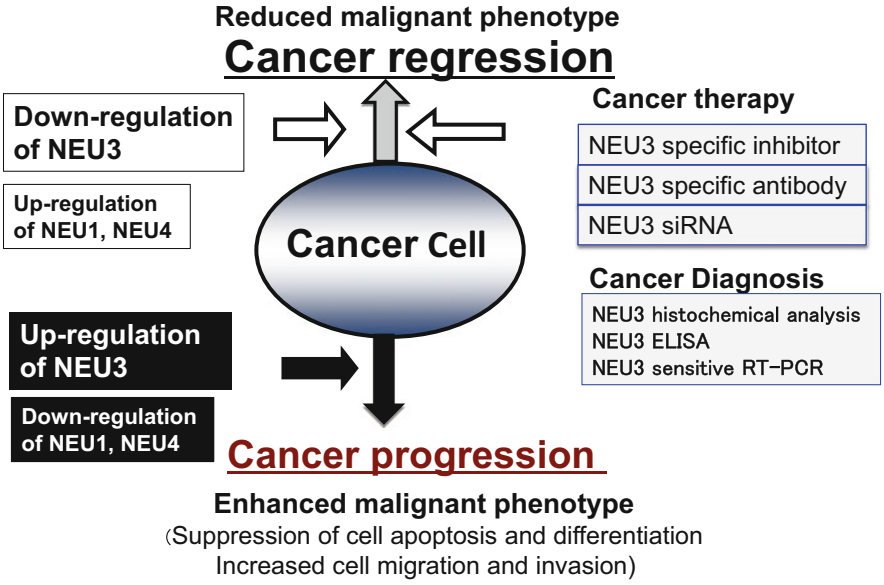

Fig. 3.4 Relationship of three human sialidases in human cancer and a possible role of NEU3 as a potential target for cancer diagnosis and therapy. In colon cancer, downregulation of NEU1 and NEU4 and upregulation of NEU3 result in tumor progression through enhancing malignant properties, whereas downregulation of NEU3 by a specific siRNA, antibody, or inhibitor could lead to cancer regression

with specific siRNAs, antibodies or inhibitors, might cause cancer cell apoptosis and lead to prevention of cancer progression.

References

Bonten E, van der Spoel A, Fornerod M, Grosveld G, d'Azzo A (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. Genes Dev 10:3156–3169

Chavas LM, Tringali C, Fusi P, Venerando B, Tettamanti G, Kato R, Monti E, Wakatsuki S (2005) Crystal structure of the human cytosolic sialidase Neu2. Evidence for the dynamic nature of substrate recognition. J Biol Chem 280:469–475

Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127:469–480

Comelli EM, Amado M, Lustig SR, Paulson JC (2003) Identification and expression of Neu4, a novel murine sialidase. Gene 321:155–161

D'Azzo A, Bonten E (2010) Molecular mechanisms of pathogenesis in a glycosphingolipid and a glycoprotein storage disease. Biochem Soc Trans 38:1453–1457

D'Azzo A, Hoogeveen A, Reuser AJ, Robinson D, Galjaard H (1982) Molecular defect in combined beta-galactosidase and neuraminidase deficiency in man. Proc Natl Acad Sci USA 79:4535–4539

Fanzani A, Colombo F, Giuliani R, Preti A, Marchesini S (2004) Cytosolic sialidase Neu2 upregulation during PC12 cells differentiation. FEBS Lett 566:178–182

Galjart NJ, Gillemans N, Harris A, van der Horst GT, Verheijen FW, Galjaard H, d'Azzo A (1988) Expression of cDNA encoding the human "protective protein" associated with lysosomal **β**-galactosidase and neuraminidase: homology to yeast proteases. Cell 54:755–764

Hakomori SI (2010) Glycosynaptic, microdomains controlling tumor cell phenotype through alteration of cell growth, adhesion, and motility. FEBS Lett 584:1901–1906

Hata K, Wada T, Hasegawa A, Kiso M, Miyagi T (1998) Purification and characterization of a membrane-associated ganglioside sialidase from bovine brain. J Biochem (Tokyo) 123:899–905

Hata K, Koseki K, Yamaguchi K, Moriya S, Suzuki Y, Yingsakmongkon S, Hirai G, Sodeoka M, von Itzstein M, Miyagi T (2008) Limited inhibitory effects of oseltamivir and zanamivir on human sialidases. Antimicrob Agents Chemother 52:3484–3491

Hata K, Tochigi T, Sato I, Kawamura S, Shiozaki K, Wada T, Takahashi K, Moriya S, Yamaguchi K, Hosono M, Miyagi T (2015) Increased sialidase activity in serum of cancer patients: identification of sialidase and inhibitor activities in human serum. Cancer Sci 106:383–389

Kakugawa Y, Wada T, Yamaguchi K, Yamanami H, Ouchi K, Sato I, Miyagi T (2002) Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression. Proc Natl Acad Sci USA 99:10718–10723

Kato T, Wang Y, Yamaguchi K, Milner CM, Shineha R, Satomi S, Miyagi T (2001) Overexpression of lysosomal-type sialidase leads to suppression of metastasis associated with reversion of malignant phenotype in murine B16 melanoma cells. Int J Cancer 92:797–804

Kawamura S, Sato I, Wada T, Yamaguchi K, Li Y, Li D, Zhao X, Ueno S, Aoki H, Tochigi T, Kuwahara M, Kitamura T, Takahashi K, Moriya S, Miyagi T (2012) Plasma membrane-associated sialidase (NEU3) regulates progression of prostate cancer to androgen- independent growth through modulation of androgen receptor signaling. Cell Death Differ 19:170–179

Lau KS, Dennis JW (2008) N-Glycans in cancer progression. Glycobiology 18:750–760

Li X, Zhang L, Shao Y, Liang Z, Shao C, Wang B, Guo B, Li N, Zhao X, Li Y, Xu D (2011) Effects of a human plasma membrane-associated sialidase siRNA on prostate cancer invasion. Biochem Biophys Res Commun 416:270–276

Mandal C, Tringali C, Mondal S, Anastasia L, Chandra S, Venerando B (2010) Down regulation of membrane-bound Neu3 constitutes a new potential marker for childhood acute lymphoblastic leukemia and induces apoptosis suppression of neoplastic cells. Int J Cancer 126:337–349

Milner CM, Smith SV, Carrillo MB, Taylor GL, Hollinshead M, Campbell RD (1997) Identification of a sialidase encoded in the human major histocompatibility complex. J Biol Chem 272:4549–4558

Miyagi T, Sagawa J, Kuroki T, Matsuya Y, Tsuiki S (1990) Tumor-promoting phorbol ester induces alterations of sialidase and sialyltransferase activities of JB6 cells. Jpn J Cancer Res 81:1286–1292

Miyagi T, Konno K, Emori Y, Kawasaki H, Suzuki K, Yasui A, Tsuik S (1993) Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase. J Biol Chem 268:26435–26440

Miyagi T, Sato K, Hata K, Taniguchi S (1994) Metastatic potential of transformed rat 3Y1 cell lines is inversely correlated with lysosomal-type sialidase activity. FEBS Lett 349:255–259

Miyagi T, Wada T, Iwamatsu A, Hata K, Yoshikawa Y, Tokuyama S, Sawada M (1999) Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. J Biol Chem 274:5004–5011

Miyagi T, Wada T, Yamaguchi K, Hata K, Shiozaki K (2008) Plasma membrane-associated sialidase as a crucial regulator of transmembrane signalling. J Biochem 144:279–285

Miyagi T, Takahashi K, Hata K, Shiozaki K, Yamaguchi K (2012) Sialidase significance for cancer progression. Glycoconj J 29:567–577

Miyagi T, Yamaguchi K (2012) Mammalian sialidase: physiological and pathological roles in cellular functions. Glycobiology 22:880–896

Monti E, Preti A, Rossi E, Ballabio A, Borsani G (1999) Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. Genomics 57:137–143

Monti E, Bassi MT, Bresciani R, Civini S, Croci GL, Papini N, Riboni M, Zanchetti G, Ballabio A, Preti A, Tettamanti G, Venerando B, Borsani G (2004) Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family. Genomics 83:445–453

Papini N, Anastasia L, Tringali C, Croci G, Bresciani R, Yamaguchi K, Miyagi T, Preti A, Prinetti A, Prioni S et al (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

Pshezhetsky AV, Hinek A (2011) Where catabolism meets signalling: neuraminidase 1 as a modulator of cell receptors. Glycoconj J 28:441–452

Pshezhetsky AV, Richard C, Michaud L, Igdoura S, Wang S, Elsliger MA, Qu J, Leclerc D, Gravel R, Dallaire L et al (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. Nat Genet 15:316–320

Sato K, Miyagi T (1995) Genomic organization and the 5′-upstream sequence of the rat cytosolic sialidase gene. Glycobiology 5:511–516

Sawada M, Moriya S, Saito S, Shineha R, Satomi S, Yamori T, Tsuruo T, Kannagi R, Miyagi T (2002) Reduced sialidase expression in highly metastatic variants of mouse colon adenocarcinoma 26 and retardation of their metastatic ability by sialidase overexpression. Int J Cancer 97:180–185

Schengrund CL, Lausch RN, Rosenberg A (1973) Sialidase activity in transformed cells. J Biol Chem 248:4424–4428

Seyrantepe V, Landry K, Trudel S, Hassan JA, Morales CR, Pshezhetsky AV (2004) Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells. J Biol Chem 279:37021–37029

Shi XB, Xue L, Ma AH, Tepper CG, Kung HJ, White RW (2011) miR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. Prostate 71:538–549

Shiga K, Takahashi K, Sato I, Kato K, Saijo S, Moriya S, Hosono M, Miyagi T (2015) Upregulation of sialidase NEU3 in head and neck squamous cell carcinoma associated with lymph node metastasis. Cancer Sci. in press

Shiozaki K, Koseki K, Yamaguchi K, Shiozaki M, Narimatsu H, Miyagi T (2009a) Developmental change of sialidase neu4 expression in murine brain and its involvement in the regulation of neuronal cell differentiation. J Biol Chem 284:21157–21164

Shiozaki K, Yamaguchi K, Sato I, Miyagi T (2009b) Plasma membrane-associated sialidase (NEU3) promotes formation of colonic aberrant crypt foci in azoxymethane-treated transgenic mice. Cancer Sci 100:588–594

Shiozaki K, Yamaguchi K, Takahashi K, Moriya S, Miyagi T (2011) Regulation of sialyl Lewis antigen expression in colon cancer cells by sialidase NEU4. J Biol Chem 286:21052–21061

Shiozaki K, Takahashi K, Hosono M, Yamaguchi K, Hata K, Shiozaki M, Bassi R, Prinetti A, Sonnino S, Nitta K, Miyagi T (2015) Phosphatidic acid-mediated activation and translocation to the cell surface of sialidase NEU3, promoting signaling for cell migration. FASEB J 29(5):2099–2111

Suzuki M, Nakayama J, Suzuki A, Angata K, Chen S, Sakai K, Hagihara K, Yamaguchi Y, Fukuda M (2005) Polysialic acid facilitates tumor invasion by glioma cells. Glycobiology 15:887–894

Takahashi K, Mitoma J, Shiozaki K, Hosono M, Sato C, Yamaguchi K, Kitajima K, Higashi H, Nitta K, Shima H, Miyagi T (2012) Sialidase neu4 hydrolyzes polysialic acids of neural cell adhesion molecules and negatively regulates neurite formation by hippocampal neurons. J Biol Chem 287:14816–14826

Takahashi K, Hosono M, Sato I, Hata K, Wada T, Yamaguchi K, Nitta K, Shima H, Miyagi T (2015) Sialidase NEU3 contributes neoplastic potential on colon cancer cells as a key modulator of gangliosides by regulating Wnt signaling. Int J Cancer 137:1560–1573

Tanaka F, Otake Y, Nakagawa T, Kawano Y, Miyahara R, Li M, Yanagihara K, Inui K, Oyanagi H, Yamada T, Nakayama J, Fujimoto I, Ikenaka K, Wada H (2001) Prognostic significance of polysialic acid expression in resected non-small cell lung cancer. Cancer Res 61:1666–1670

Traish AM, Morgentaler A (2009) Epidermal growth factor receptor expression escapes androgen regulation in prostate cancer: a potential molecular switch for tumour growth. Br J Cancer 101:1949–1956

Tringali C, Lupo B, Anastasia L, Papini N, Monti E, Bresciani R, Tettamanti G, Venerando B (2007) Expression of sialidase Neu2 in leukemic K562 cells induces apoptosis by impairing Bcr-Abl/Src kinases signaling. J Biol Chem 282:14364–14372

Uemura T, Shiozaki K, Yamaguchi K, Miyazaki S, Satomi S, Kato K, Sakuraba H, Miyagi T (2009) Contribution of sialidase NEU1 to suppression of metastasis of human colon cancer cells through desialylation of integrin beta4. Oncogene 28:1218–1229

Ueno S, Saito S, Wada T, Yamaguchi K, Satoh M, Arai Y, Miyagi T (2006) Plasma membrane-associated sialidase is up-regulated in renal cell carcinoma and promotes interleukin-6-induced apoptosis suppression and cell motility. J Biol Chem 281:7756–7764

Wada T, Hata K, Yamaguchi K, Shiozaki K, Koseki K, Moriya S, Miyagi T (2007) A crucial role of plasma membrane- associated sialidase in the survival of human cancer cells. Oncogene 26:2483–2490

Wang Y, Yamaguchi K, Wada T, Hata K, Zhao X, Fujimoto T, Miyagi T (2002) A close association of the ganglioside-specific sialidase Neu3 with caveolin in membrane microdomains. J Biol Chem 277:26252–26259

Wierstra I (2008) Sp1: emerging roles—beyond constitutive activation of TATA-less housekeeping genes. Biochem Biophys Res Commun 372:1–13

Yamaguchi K, Hata K, Koseki K, Shiozaki K, Akita H, Wada T, Moriya S, Miyagi T (2005) Evidence for mitochondrial localization of a novel human sialidase (NEU4). Biochem J 390:85–93

Yamaguchi K, Koseki K, Shiozaki M, Shimada Y, Wada T, Miyagi T (2010) Regulation of plasma-membrane-associated sialidase NEU3 gene by Sp1/ Sp3 transcription factors. Biochem J 430:107–117

Yamaguchi K, Shiozaki K, Moriya S, Koseki K, Wada T, Tateno H, Sato I, Asano M, Iwakura Y, Miyagi T (2012) Reduced susceptibility to colitis-associated colon carcinogenesis in mice lacking plasma membrane-associated sialidase. PLoS One 7:41132

Yamanami H, Shiozaki K, Wada T, Yamaguchi K, Uemura T, Kakugawa Y, Hujiya T, Miyagi T (2007) Down-regulation of sialidase NEU4 may contribute to invasive properties of human colon cancers. Cancer Sci 98:299–307

Yogalingam G, Bonten EJ, van de Vlekkert D, Hu H, Moshiach S, Connell SA, d'Azzo A (2008) Neuraminidase 1 is a negative regulator of lysosomal exocytosis. Dev Cell 15:74–86

Yogeeswaran G, Hakomori S (1975) Cell contact-dependent ganglioside changes in mouse 3T3 fibroblasts and a suppressed sialidase activity on cell contact. Biochemistry 14:2151–2156

Part III
Altered Cell Signaling in Cancer Cells

Chapter 4
Regulatory Mechanisms for Malignant Properties of Cancer Cells with Disialyl and Monosialyl Gangliosides

Koichi Furukawa, Yuki Ohkawa, Yasuyuki Matsumoto, Yuhsuke Ohmi, Noboru Hashimoto, and Keiko Furukawa

Abstract Using mouse and human monoclonal antibodies, a number of cancer-associated antigens were defined, and many of their epitopes have turned out to be carbohydrates. Besides as a tumor marker, some of them really play roles as regulatory molecules on the cell membrane. Generally, disialyl glycosphingolipids (disialyl gangliosides) such as GD3 and GD2 transduce activating signals to enhance malignant properties of cancer cells, while monosialyl gangliosides such as GM1 transduce suppressive signals on cancer properties. In particular, roles of GD3 in melanomas and GD2 in small cell lung cancers, osteosarcomas, and breast cancers have been well investigated. Recently, roles of GM1 in the suppression of cancer metastasis have been demonstrated using a murine lung cancer system. These regulatory functions of cancer-associated glycolipids are performed at membrane microdomains named glycolipid-enriched microdomain (GEM)/rafts. As mechanisms for these regulatory functions, molecular interactions between glycolipids and various membrane proteins existing in the vicinity of them at GEM/rafts are of importance and now under vigorous investigation.

Keywords Ganglioside • Cancer-associated antigen • Glycosyltransferase • Metastasis • Melanoma • Lipid rafts • Integrin

K. Furukawa (✉) • Y. Ohkawa • K. Furukawa
Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan

Department of Biomedical Sciences, Chubu University College of Life and Health Sciences, 1200 Matsumoto-cho, Kasugai 487-8501, Japan
e-mail: koichi@med.nagoya-u.ac.jp

Y. Matsumoto • Y. Ohmi • N. Hashimoto
Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_4

4.1 Introduction

Unique carbohydrates on proteins and lipids expressed in cancer cells have been considered to be cancer-associated antigens and some of them have been used as tumor markers in the clinical fields (Brockhausen 1999; Hakomori 2002a; Aksoy and Akinci 2004). Mechanisms for evolution of cancer-associated glycosylation have been also investigated in a number of researchers (Dall'Olio et al. 2012; Kannagi et al. 2008). Some of those antigens have been used as targets of tumor immunotherapy (Durrant et al. 2012). Main gangliosides and their synthetic pathway were shown in Fig. 4.1.

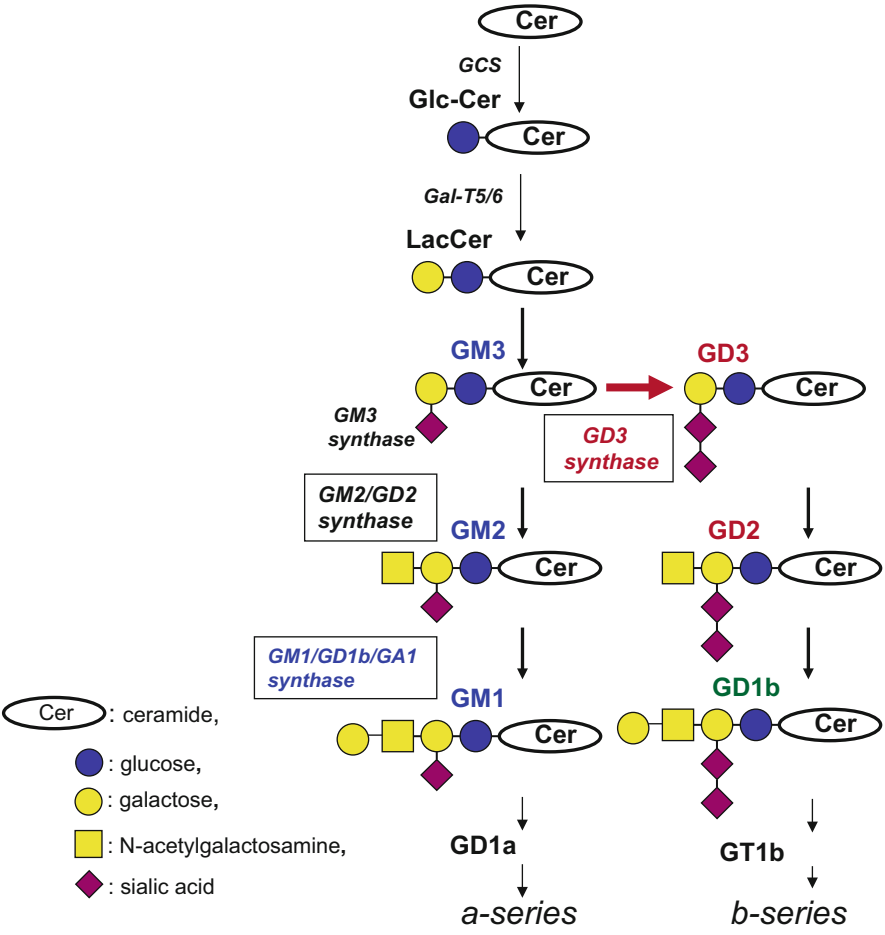

Fig. 4.1 Synthetic pathway of major gangliosides described in this review. GD3 synthase (ST8SIA1) is a key enzyme for the synthesis of b-series gangliosides, such as GD3 and GD2

Gangliosides, sialic acid-containing glycosphingolipids, have been considered to be useful markers for many neuroectoderm-derived cancers and some kind of leukemias (Old 1981; Siddiqui et al. 1984). For instance, ganglioside GD3 was defined as a melanoma-associated antigen (Portoukalian et al. 1979), and GD2 was identified as a neuroblastoma-associated ganglioside (Saito et al. 1985). GD2 was also reported to be a characteristic antigen for small cell lung cancers (Cheresh et al. 1986), osteosarcomas (Heiner et al. 1987), breast cancers (Cazet et al. 2010), and metastatic stage of melanomas (Thurin et al. 1986). Many of these disialyl gangliosides have been shown to play roles in the enhancement of malignant properties in cancer cells with some exception (Malisan and Testi 2002). On the other hand, monosialyl gangliosides, such as GM3 and GM1, have been reported to play roles for the suppression of malignant properties of cancer cells (Furukawa et al. 2012b). Thus, gangliosides appear to modulate cell signaling with positive and negative directions (Fishman and Brady 1976; Nagai 1995; Kaucic et al. 2006). Changes in cell signaling due to the expression of various gangliosides were summarized in Fig. 4.2. Although details in the mechanisms for the distinct effects between disialyl and monosialyl gangliosides on cancer properties are largely unknown, modes of action in membrane microdomains, i.e., lipid rafts or glycolipid-enriched microdomain (GEM)/rafts, might be crucially different depending on the quantity of sialic acid(s) on glycosphingolipids (Hakomori et al. 1998).

Implication of GEM/rafts in cancer cells has been increasingly recognized (Simons and Ikonen 1997; Patra 2008; Simons and Gerl 2010). Among proposed functions of lipid rafts (Simons and Ikonen 1997), roles in the regulation of signal transduction have been intensively investigated. Pioneering studies on GEM/rafts (Hakomori et al. 1998; Hakomori 2002b) have given insights into glycobiological researches. In particular, molecular interaction between proteins and proteins or proteins and lipids in GEM/rafts might be the most intriguing and important issue to be solved in cancer biology (Simons and Gerl 2010).

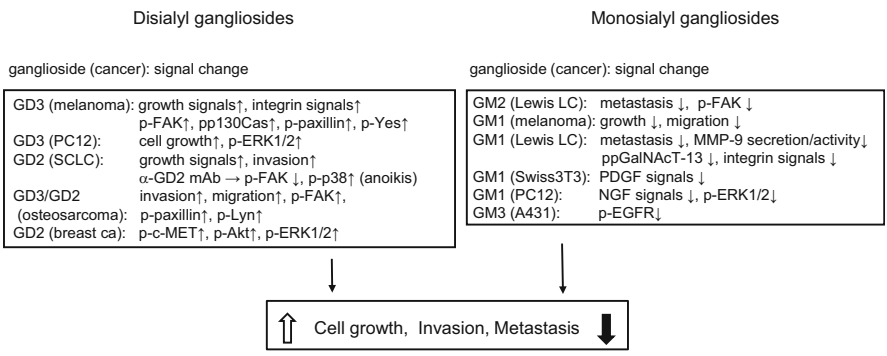

Fig. 4.2 Enhanced signaling under GD3 expression in melanoma cells. GD3 expression-induced convergence of two major signals, i.e., adhesion signals mediated by integrins and growth signals triggered by growth factor/receptors, leading to synergistic effects on cell adhesion, invasion, growth, and migration and resistance to apoptosis

Cancer metastasis is a major cause of death in almost all human cancers. If metastasis can be suppressed, prognosis of cancer patients should be much better (Yilmaz et al. 2013). However, mechanisms for the development of cancer metastasis have not been well understood. Metastasis is a complex event and consists of multiple steps such as expansion and invasion into surrounding tissues, release from primary tumor tissues, intravasation, reach distant organs via blood/lymph stream, adhesion to vascular walls, extravasation, and formation of new foci (Meng et al. 2012). Although a number of studies on ganglioside expression and their implications in cancer properties have been reported, clear evidence to indicate that gangliosides are involved in cancer metastasis can be scarcely found in the past articles.

In this review, involvement of sugar chains in malignant properties of cancer cells and molecular mechanisms for their actions were summarized with focus on our recent results and related reports from other laboratories.

4.2 Contrastive Effects of GM1 Synthase and GD3 Synthase in PC12 Cells

In a rat pheochromocytoma line, PC12, remodeling experiments of glycolipid expression pattern were performed. PC12 responds well to nerve growth factor (NGF) by neurite extension and activation of Ras/Raf/MEK/MAPK signaling pathway (Vaudry et al. 2002) and has been frequently used for the studies of the signaling of neuronal cell differentiation (Greene and Tischler 1976). While PC12 died within several hours when serum was deprived from culture medium, addition of ganglioside GM1 could rescue the cells from apoptosis, suggesting its role as a neurotrophic factor (Ferrari et al. 1995). Furthermore, GM1 bound a NGF receptor, TrkA, and enhanced differentiation of PC12 (Mutoh et al. 1995). However, these results were obtained by observing effects of exogenously added gangliosides into the culture medium. Effects of endogenously generated gangliosides were examined by transfecting GM1/GD1b/GA1 synthase (B3galt4) cDNA (Miyazaki et al. 1997) and GD3 synthase (ST8SIA1) cDNA (Haraguchi et al. 1994), showing different results from those expected based on previous experiments. Overexpression of GM1/GD1b/GA1 synthase cDNA resulted in the unresponsiveness to NGF (Nishio et al. 2004). Moreover, neither phosphorylation of TrkA nor subsequent signaling molecules could be found after NGF treatment. Interestingly, a dramatic alteration in the intracellular localization of TrkA and other signaling molecules such as $p75^{NTR}$ and H-Ras was observed, i.e., a shift from inside to outside of GEM/rafts. These results were not in accordance with previous studies (Ferrari et al. 1995; Mutoh et al. 1995). Probably, relative GM1 levels on the cell surface might be crucial for the determination of cell signaling caused by GM1. On the other hand, overexpression of GD3 synthase cDNA resulted in the constitutive activation of TrkA and Erk1/2 and unresponsiveness to NGF (Fukumoto et al. 2000). These cells showed uncontrolled cell growth. GD3 synthase cDNA-

transfected cells may represent the features of "neuronal stem cells" in which GD3 is a dominant ganglioside (Yanagisawa et al. 2005). Neuronal stem cells are defined as neural progenitor cells that are enriched in fetal brain tissues and can be differentiated into both neurons and glial cells such as astrocytes and oligodendrocytes. These results demonstrated that gangliosides regulate proliferation and differentiation of neural cells by modulating cell signaling, and GM1/GD1b synthase and GD3 synthase exert their actions to opposite directions as summarized in Fig. 4.2.

4.3 Enhancing Function of Disialyl Gangliosides in Various Cancer Cells

4.3.1 Malignant Melanomas

Gangliosides GD3 and GD2 (or GM2) have been considered as melanoma-associated glycolipid antigens (Lloyd and Old 1989). Synthetic pathway of these gangliosides was shown in Fig. 4.1. Since early biochemical analysis was reported (Portoukalian et al. 1979; Carubia et al. 1984; Rodeck and Herlyn 1988; Hersey 1991), immunological studies using monoclonal antibodies have been rigorously performed (Pukel et al. 1982; Thurin et al. 1986; Ravindranath et al. 1991). Not only mouse mAbs but many human-origin mAbs reactive with melanoma gangliosides were generated (Yamaguchi et al. 1987; Irie and Morton 1986; Furukawa and Lloyd 1990). Furthermore, genetic approaches have been increasingly performed based on the expanded availability of cDNAs of glycosyltransferases to examine the implication of gangliosides in cancers (Daniotti et al. 2013). The first study on activated signals in GD3-expressing melanomas was reported in 2005 (Hamamura et al. 2005). Using a GD3-lacking mutant of SK-MEL-28, GD3 synthase cDNA-transfected cells were compared with control cells transfected with an expression vector alone, and highly tyrosine-phosphorylated molecules were searched in the transfected cells. Consequently, adaptor molecules, p130Cas and paxillin, were identified to undergo stronger activation in GD3+ cells than in GD3- cells (Hamamura et al. 2005). Subsequently, focal adhesion kinase (FAK) was also identified as a critical molecule to be activated more strongly in GD3+ cells (Hamamura et al. 2008). Furthermore, Src family kinase, Yes, was identified as a kinase to be constitutively activated under GD3 expression (Hamamura et al. 2011). All these molecules were shown to form a molecular complex in the vicinity of cell membrane (Furukawa et al. 2006), and these molecules seemed to be candidates for the molecular-targeted therapy of melanomas (Furukawa et al. 2008). In addition to the signaling via growth factor/receptor, another signaling pathway that is critical for cancer cell natures, adhesion-mediated signaling, is also intimately regulated by gangliosides. GD3 expression enhanced cell adhesion to various extracellular matrices (Ohkawa et al. 2010). Gene silencing of integrin

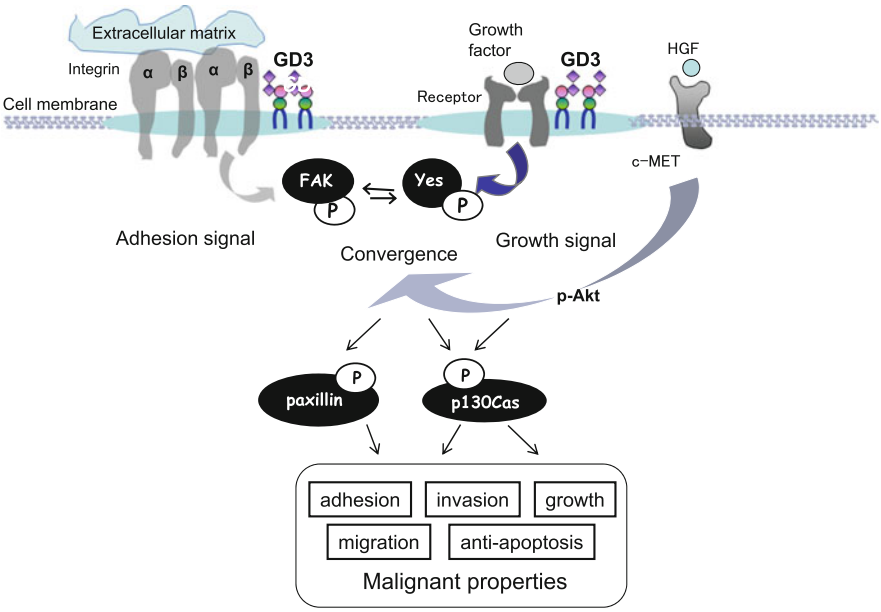

Fig. 4.3 Contrastive effects of monosialyl and disialyl gangliosides on the cell signals in various cancer cells. Generally, disialogangliosides are frequently considered as cancer-associated carbohydrate antigens and enhance malignant properties such as rapid cell growth, increased invasion activity, and metastatic potential. On the other hand, monosialyl gangliosides generally suppress these phenotypes

(β1) resulted in marked reduction of phosphorylation levels of p130Cas, FAK, and paxillin as well as of adhesion activity (Ohkawa et al. 2008). Interestingly, copresence of adhesion signals and FCS (growth) signals was needed to induce definite activation of p130Cas, FAK, and paxillin, suggesting their collaborative mode of action. Furthermore, it was demonstrated that integrins shifted to GEM/rafts only in GD3+ cells after serum stimulation, suggesting that interaction of GD3 with integrins and/or these signaling molecules in GEM/rafts might generate signals that drive malignant properties of melanoma cells (Furukawa et al. 2006). GEM/rafts (lipid rafts) are microdomains formed on the cell surface containing high levels of cholesterol, sphingolipids, GPI-anchored proteins, and some membrane molecules. They are usually prepared as detergent-resistant fractions by sucrose density gradient ultracentrifugation of cell/tissue extracts with Triton X-100. All these results were summarized in Fig. 4.3.

4.3.2 Ganglioside GD2 in Small Cell Lung Cancers (SCLCs)

Expression analysis of gangliosides on human lung cancer cell lines revealed that SCLC cells express GD2, while NSCLC cells mainly express GM2 (Yoshida et al. 2001). Although these findings were reported many years ago (Hanibuchi et al. 1996; Grant et al. 1996), the responsible glycosyltransferase gene for each group was demonstrated for the first time in 2001. Namely, GM2/GD2 synthase was expressed commonly in all cell types of lung cancers, while GD3 synthase was expressed exclusively in SCLC cell lines (Yoshida et al. 2001) as shown in Fig. 4.1. The most intriguing point was that anti-GD2 mAb induced apoptosis by reducing tyrosine phosphorylation levels of FAK, leading to anoikis. Anoikis means a mode of cell death due to loss of cell attachment in adherent cells. Binding of anti-GD2 mAb eventually induced phosphorylation of p38 and DNA ladder formation (Aixinjueluo et al. 2005). Stable expression of short hairpin (sh) RNA against GD3 synthase gene in lung cancer cell lines also resulted in the suppression of cell growth and migration and DNA ladder formation (Ko et al 2006). These results strongly encouraged the application of anti-GD2 mAbs for the treatment of SCLC patients, since mere binding of mAb could induce apoptosis of cancer cell. The combination therapy of SCLC with anti-GD2 mAb and chemotherapy reagents also seemed very promising (Yoshida et al. 2002). Antibody therapy of SCLC targeting GD2 (Zhan et al. 2013) or GD3 (Blackhall and Shepherd 2007) is now being considered.

4.3.3 GD2/GD3 in Osteosarcoma

As reported previously (Heiner et al. 1987), majority of osteosarcoma cell lines expressed high levels of GD2. Effects of expression of these disialyl gangliosides in osteosarcoma cells were analyzed (Shibuya et al. 2012). GD2/GD3 expression enhanced cell invasion and motility with increased activation of either FAK or Lyn, leading to the activation of a common target, paxillin. Simultaneous knockdown of FAK and Lyn completely suppressed phosphorylation of paxillin and increased cell invasion and motility based on GD2/GD3 expression. These results suggested cooperative effects of two parallel signaling pathways in osteosarcoma cells. In contrast with melanoma cells, cell growth was not affected by the expression of disialyl gangliosides in osteosarcoma cells (Hamamura et al. 2005).

In accordance with these differences in the effects of GD3/GD2 expression on the tumor phenotypes, intriguing differences in the cell adhesion were demonstrated as analyzed by RT-CES. When four glycotypes of a osteosarcoma line (GD3+, GD2+, GD3+/GD2+, GD2-/GD3-) were compared about their phenotypes and signaling, GD3+/GD2+ cells did not show any adhesion as presented by cell index in RT-CES, while these cells showed the strongest phosphorylation of paxillin during cell “adhesion.” Interestingly, the intensity in the phosphorylation

of paxillin and that in cell adhesion was completely opposite. This paradoxical relationship between the weakest adhesion and the strongest activation of paxillin in GD3+/GD2+ is hard to be explained at this moment (Furukawa et al. 2012a). These results were quite contrastive with those observed in melanomas, in which increased phosphorylation of p130Cas, paxillin, and FAK resulted in the enhanced cell growth, invasion, and adhesion (Ohkawa et al. 2010).

4.3.4 GD2 in Breast Cancers

GD2 expression in breast cancer cell lines resulted in the increased cell proliferation and invasion (Cazet et al. 2010). Recently, GD2 was reported to be a stem cell marker of breast cancers (Battula et al. 2012). As a mechanism for action of GD2, activation of HGF receptor cMET in GD2+ cells was shown (Cazet et al. 2010). In turn, expression of GD3 in melanoma cells did not increase cMET activation after HGF treatment (Furukawa et al. 2014). Therefore, effects of disialyl gangliosides were largely different depending on different cell lineages. In melanoma cells, it was shown that GD3 expression resulted in the convergence and synergy between HGF/cMET signals and adhesion signals in melanomas (Furukawa et al. 2014). Effects of GD2 overexpression in melanomas remain to be examined, while its role in the metastasis has been long expected (Thurin et al. 1986).

4.3.5 GD2/GD3 in Neuroblastomas, Gliomas, and Leukemia Cells

Gangliosides in neuroblastomas have been long examined (Schengrund and Shochat 1988). Indeed, neuroblastoma cells specifically express GD2, and therefore, anti-GD2 antibody therapy has been widely tried. Antibody therapies with human-mouse chimeric antibody or mouse mAb 3F8 have been performed. Another option with combination of mAb and other treatment such as cytokines (Fukuda et al. 1998) or irradiation (Matthay et al. 2012) has been tried. Consequently, antibody therapies performed during the disease remission can cause extension of 5-year survival and/or disease-free duration in severe cases (Raffaghello et al. 2003; Castel et al. 2010; Parsons et al. 2013). Some of gangliosides have been noted as cancer-associated antigens in gliomas (Fredman et al. 1986; Yates 1988; Wikstrand et al. 1994). However, no clear expression patterns of gangliosides depending on the histopathological tumor types or clinical stages of tumors have been demonstrated. Ganglioside expression on human leukemia cells was reported since a long time ago. GD3 was detected in T-cell acute lymphoblastic leukemia (T-ALL) cells (Siddiqui et al. 1984; Merritt et al. 1987) and also peripheral T lymphocytes activated by various reagents (Welte et al. 1987; Yamashiro et al. 1995). Adult

T-cell leukemia cell lines and human lymphotropic virus type I-infected T cells also expressed ganglioside GD2 (Furukawa et al. 1993), while leukemia cells from patients with adult T-cell leukemia expressed significant levels of GD2 only after culture in vitro (Okada et al. 1996).

4.4 Suppressive Effects of Monosialyl Gangliosides on Malignant Signals

4.4.1 Suppressive Effects of GM1 Synthase in Mouse Swiss 3T3

As for anticancer property of GM1 synthase, several examples using GM1 synthase-transfected cell lines were reported by us. Swiss3T3 cells overexpressing GM1 synthase showed lowered growth rates and reduced responses to PDGF stimulation (Mitsuda et al. 2002). A shift of a PDGF receptor from GEM/rafts to non-GEM/rafts with reduced phosphorylation levels was shown in GM1 synthase cDNA-transfected cells (Mitsuda et al. 2002). This was the first example to demonstrate that GM1 expression suppressed cancer properties in contrast to GD3/GD2 as shown in Fig. 4.3.

4.4.2 Suppressive Effects of GM1 Synthase in Human Melanomas

In human melanomas, overexpression of GM1 synthase cDNA in a melanoma cell line SK-MEL 37 also resulted in reduced cell growth and invasion activity (Dong et al. 2010). Along with neo-expression of GM1 and GD1b in the transfected cells of GM1 synthase cDNA, enrichment of GD3 in GEM/rafts was reduced, leading to the dispersion of GD3 to non-GEM/raft fractions. Accordingly, cell growth rate and invasion activity were decreased, suggesting the suppressive roles of GM1 synthase and/or its products. Interestingly, ratios of gangliosides with saturated fatty acids in GEM/rafts were reduced in GM1 synthase-transfected cells. A caveolae-specific protein, caveolin-1, has been reported to generally suppress cell signals at lipid rafts, although reverse effects were sometimes observed in some cancers (Williams and Lisanti 2005). Changes in the intracellular distribution of GD3 were also observed in a melanoma cell line, SK-MEL-28 transfected with caveolin-1 cDNA (Nakashima et al. 2007). In this study, increased ratios of GD3 with unsaturated fatty acids outside of GEM/rafts were observed. Consequently, GM1 synthase (or GM1) and caveolin-1 seem to have a common function in the suppression of signaling transduced via GEM/rafts and also affect intracellular distribution pattern

of GD3 with changes in the saturation/unsaturation patterns of fatty acids in ceramide portion of GD3.

Thus, suppressive function of GM1 appears to be similar with that of caveolin-1 (Quann et al. 2013; Razani et al. 2001). It seems to be interesting that both GM1 and caveolin-1 have been considered as markers of GEM/rafts, while no essential linkages between these two molecules have been demonstrated so far.

4.5 Suppression of Metastatic Potential of Murine Lewis Lung Cancer (LLC) by Monosialyl Gangliosides, GM2 and GM1

4.5.1 Overexpression of GM2/GD2 Synthase cDNA Resulted in the Suppression of Metastatic Potential of Murine LLC Cells

Lewis lung cancer has been used as a cancer metastasis model (Takenaga 1986). To investigate roles of gangliosides in cancer metastasis, a cDNA expression vector of GM2/GD2 synthase was transfected into a LCC subline (Chen et al. 2003). Since GM2 has been considered as a tumor-associated antigen in lung cancers (Hanibuchi et al. 1996) as well as in melanomas (Irie et al. 1989; Yamaguchi et al. 1990), increase of metastatic potential in the transfectants was expected. Consequently, they showed reduced number of metastasis foci. Phosphorylation levels of focal adhesion kinase (FAK) were also suppressed in the transfectants compared with controls, suggesting that newly expressed GM2 suppressed adhesion signals (Chen et al. 2003).

Suppression of EGF signaling and phosphorylation of EGF receptor by ganglioside GM3 in A431 cells was also reported (Yoon et al. 2005), suggesting that negative regulation of growth signaling by monosialyl gangliosides is a universal phenomena as summarized in Fig. 4.2.

4.5.2 Alteration of Surface Molecules in Established High Metastatic Sublines of LLC

To identify molecules and genes responsible for the metastatic potential, low metastatic LLC lines were repeatedly injected into C57BL/6 mice iv or sc (Zhang et al. 2006). Several high metastatic sublines for each of parent line were established. In order to clarify alteration in the expression of surface molecules, various surface antigens were analyzed, i.e., integrin families, CD44, gangliosides, etc. None of those antigens except ganglioside GM1 showed definite changes along with increased metastatic potential. Only GM1 showed marked reduction in

common among all high metastatic sublines compared with individual parent lines (Zhang et al. 2006), suggesting that GM1 suppresses metastatic phenotypes.

Indeed, silencing of GM1 synthase by transfection of RNAi expression vector resulted in the establishment of GM1 low LLC sublines with high metastatic potentials. Consequently, it was demonstrated that reduced GM1 expression induced a shift of MMP-9 and integrins to GEM/rafts and promoted secretion and activation of MMP-9, leading to the increased metastatic property (Zhang et al. 2006).

Together with results of analysis on the surface molecules in the high metastatic LLC sublines, all these data suggested that GM1 and/or GM1 synthase induced suppression of cancer properties, at least partly, due to the alteration in the location of cognate membrane receptors and modulation of their functions in GEM/rafts. However, no clear mode of action of GM1 has yet been demonstrated.

4.5.3 Trimeric Tn on Syndecan-1 Binds Integrin α5β1 and Enhances Cell Adhesion

In order to comprehensively understand the mechanisms for the increased metastatic potentials in high metastatic LCC sublines, gene expression profiling was performed using DNA microarray (Matsumoto et al. 2012). Unique point in our study among similar studies reported was to search metastasis-related genes by multiple combinations of "high" metastatic lines and "low" metastatic lines and to pick up genes that is commonly up- or downregulated along with increased metastatic property (Matsumoto et al. 2012). Thus, we could identify more universal and reliable genes responsible for cancer metastasis.

One of genes identified to be upregulated in high metastatic lines both in GM1 synthase gene-silenced cells and in high metastatic sublines to lymph nodes was ppGalNAc-T13 (Matsumoto et al. 2012). ppGalNAc-T13 is a family member of GalNAc transferase involved in the initiation reaction of O-glycan synthesis by transferring alpha-GalNAc to Ser/Thr residues mainly in mucin proteins (Zhang et al. 2003). There are now 20 family members, and 16 out of them are shown to have enzyme activities (Brooks et al. 2007). Among them, ppGalNAc-T13 is very unique in terms of its restricted expression in brain tissues (Zhang et al. 2003). One more unique feature of ppGalNAc-T13 is its activity to generate trimeric Tn structure.

Tn antigen is the most famous tumor antigen (Ju et al. 2011, 2013). Although biological functions of this cancer-associated carbohydrate antigen have been clarified based on the genetic remodeling of carbohydrate patterns on the cell surface membrane, concrete implication of Tn structure in tumor phenotypes has not yet been demonstrated to date. Consequently, its role in cancer metastasis has never been well recognized.

Trimeric Tn structures were reported first in 1989 by Nakada et al (Matsumoto-Takasaki et al. 2012) in colon cancer cell lines using monoclonal antibody LS186. This unique structure was distributed in colon cancers and was shown to be involved in their malignant properties (Morita et al. 2009), while mechanisms for its function in malignant properties of cancers have not been clarified to date.

As described above, real function of Tn antigen in cancers has been long unknown, while it has been considered as a representative tumor-associated antigen. To investigate the implication of trimeric Tn in cancer metastasis, carrier proteins of trimeric Tn were investigated by immunoprecipitation with an anti-trimeric Tn antibody. Consequently, syndecan-1 was identified as a representative carrier protein in high metastatic LCC sublines (Matsumoto et al. 2013).

Syndecan-1 has been known as a representative heparan sulfate proteoglycan involved in various aspects of cancer cells including cell adhesion, proliferation, and motility (Munesue et al. 2002). In many cases, roles of glycosaminoglycan on the molecules have been recognized as key moiety in the interaction with other molecules (Munesue et al. 2007). ppGalNAc-T13-transfected cells showed markedly enhanced cell adhesion to fibronectin in an integrin-dependent manner in RT-CES (Matsumoto et al. 2013), suggesting that syndecan and integrins were tightly collaborating. As expected, it was eventually demonstrated that binding of syndecan-1 to integrins via trimeric Tn in lipid rafts markedly enhanced functions of integrins and integrin-mediated signaling. These molecular interactions were shown in Fig. 4.4.

Furthermore, tertiary complex consisting of trimeric Tn-carrying syndecan-1, MMP-9 and integrin β1/α5 could be found in GEM/rafts only in high metastatic lines including ppGalNAc-T13-transfected cells. These results suggested that strong cell adhesion promoting cancer metastasis takes place in GEM/rafts.

A number of findings on integrin-mediated signaling have been reported (Margadant et al. 2011). Upon adhesion of cells to extracellular matrix, FAK and/or Src family kinases are activated, and subsequent activation of p130Cas, ILK, paxillin, etc. takes place (Margadant et al. 2011). In the case of LLC, strong activation of FAK and phosphorylation of paxillin based on the expression of ppGalNAc-T13 were observed as shown in Fig. 4.4.

The identification of ppGalNAc-T13 as one of the responsible genes for metastasis of LCC was achieved using GM1 synthase-silenced cell lines and the parent line (Zhang et al. 2006; Matsumoto et al. 2012). Indeed, knockdown of GM1 synthase resulted in the upregulation of ppGalNAc-T13, leading to increased cell growth and invasion activities. Therefore, GM1-mediated signals might regulate the gene expression of ppGalNAc-T13, while precise mechanisms are not clear now.

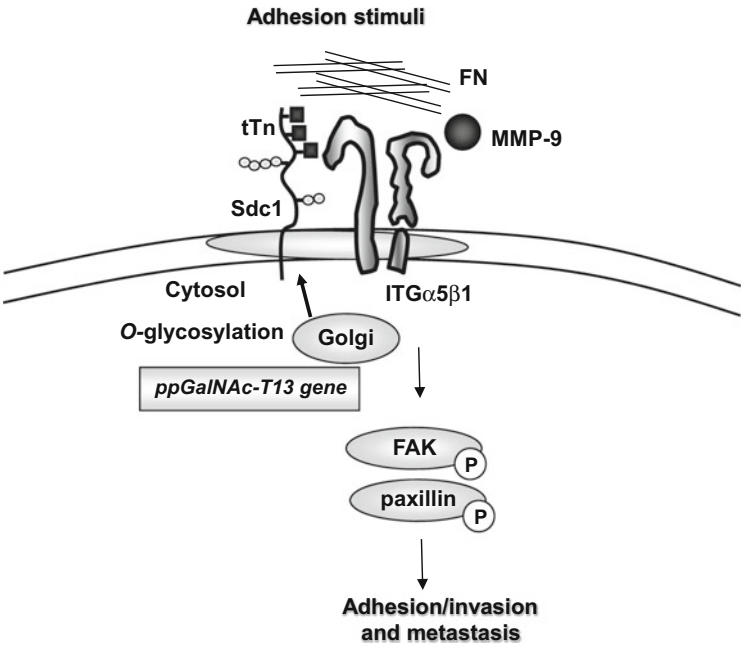

Fig. 4.4 ppGalNAc-T13 is induced by reduced expression of GM1, resulting in the expression of trimeric Tn on syndecan-1 and enhancement of integrin function. Unique product of ppGalNAc-T13, trimeric Tn on syndecan-1, is a key structure to bind integrins, resulting in the dramatic increase of cell adhesion to ECM and high metastatic potential. These interactions occur mainly in GEM/rafts and highly activate FAK and paxillin as well as MMP-9. ITG, integrin; Sdc1, syndecan-1; FN, fibronectin; tTn, trimeric Tn; MMP-9, metalloproteinase-9

4.6 Glycosphingolipids Regulate Cell Signals and Fates by Modulating GEM/Rafts

Since Simons reported lipid rafts (Simons and Ikonen 1997), a number of studies on the physicochemical aspects (Brown and London 1998) and biological functions (Simons and Gerl 2010) of rafts have been performed. Normal and disordered lipid rafts have been reported to be associated with various biological processes and pathological states. Among functions of lipid/rafts claimed by many researchers, regulation of cell signaling (Simons and Toomre 2000) is the most intriguing and universal issues particularly for the glycobiology field. This is partly because majority of glycosphingolipids reside in lipid rafts, and they contain common lipid moiety and polymorphic carbohydrate portions. The former determines intracellular localization of glycolipids and the latter exerts interacting probes with various extrinsic (*trans*) ligands and/or intrinsic (*cis*) ones. Moreover, alteration of the carbohydrate moieties seems to control natures of lipid rafts and localization of membrane molecules such as integrins and growth factor receptors. Thus, glycolipids characteristically expressed on malignant cancer cells might play

essential roles to enhance malignant properties of cancer cells via controlling behaviors of lipid rafts.

However, substantial mechanisms by which glycolipids control lipid rafts have not been well understood. Even the existence of lipid rafts has long been a controversial issue (Munro 2003). To clarify how glycosphingolipids play roles in GEM/rafts, new challenges have been tried. For example, single molecule imaging with ultrahigh spatiotemporal resolution should have become very efficient approaches to address substantial natures of GEM/rafts (Suzuki et al 2013). Molecular interactions in GEM/rafts can be dissected at millisecond resolution, leading to further understanding of their dynamic physical natures (Suzuki et al 2012). Substantial basis for cluster formation of glycolipids and for molecular complex is also being demonstrated beyond known concepts (Kusumi et al 2012). One more example is new methods to define glycolipid-associating molecules on the living cell membrane. Enzyme-mediated activation of radical sources (EMARS) is an efficient method to define membrane molecules in the vicinity of cancer-associated carbohydrates (Kotani et al 2008). In our experience, majority of molecules defined by EMARS reaction turned out to be physically associated with glycolipids used as a target antigen (Hashimoto et al 2012). This EMARS reaction is strongly expected to serve in the investigation of the essential roles of glycosphingolipids in vivo.

4.7 Ending Remarks

There have been a number of reports on cancer-associated carbohydrate antigens (Hakomori 1985; Zhang et al. 1997). In addition to their roles as tumor markers, functional implications in cancer phenotypes of those antigens have been demonstrated (Hollingsworth and Swanson 2004; Hamamura et al. 2005; Furukawa et al. 2012a, b). In order to well understand mechanisms for enhancement of cancer properties such as increased cell growth and invasion and high metastatic potential by cancer-related glyco-chains, identification of interacting molecules with individual sugar structures seems to be critical (Lopez and Schnaar 2009). Actually, many membrane proteins have been considered as targets of gangliosides (Yates et al. 1995). In particular, it is intriguing that involvement of integrins in the functions of sugar chains has been frequently observed in our studies (Zhang et al. 2006; Ohkawa et al. 2010; Matsumoto et al. 2013) and other studies (Hakomori and Handa 2002; Cabodi et al. 2010; Hakomori 2010). Indeed, cell adhesion, motility, invasion, angiogenesis, and anti-apoptosis have been considered to be regulated via the actions of cancer-associated glycosylations. Furthermore, cell-to-cell interaction in cancer niche via cytokines (Miyata et al. 2014) and other unknown vesicles also seems essential for the development of micro-foci of transformed cells (Peinado et al. 2012). Environmental factors around micro-foci of transforming cells could be subjects of studies on carcinogenesis with focus on the alteration in glycosylation machineries.

References

Aixinjueluo W, Furukawa K, Zhang Q, Hamamura K, Tokuda N, Yoshida S, Ueda R (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

Aksoy N, Akinci OF (2004) Mucin macromolecules in normal, adenomatous, and carcinomatous colon: evidence for the neotransformation. Macromol Biosci 4:483–496

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

Blackhall FH, Shepherd FA (2007) Small cell lung cancer and targeted therapies. Curr Opin Oncol 19:103–108

Brockhausen I (1999) Pathways of O-glycan biosynthesis in cancer cells. Biochim Biophys Acta 1473:67–95

Brooks SA, Carter TM, Bennett EP, Clausen H, Mandel U (2007) Immunolocalisation of members of the polypeptide N-acetylgalactosaminyl transferase (ppGalNAc-T) family is consistent with biologically relevant altered cell surface glycosylation in breast cancer. Acta Histochem 109:273–284

Brown DA, London E (1998) Structure and origin of ordered lipid domains in biological membranes. J Membr Biol 164:103–114

Cabodi S, del Pilar Camacho-Leal M, Di Stefano P, Defilippi P (2010) Integrin signalling adaptors: not only figurants in the cancer story. Nat Rev Cancer 10:858–870

Carubia JM, Yu RK, Macala LJ, Kirkwood JM, Varga JM (1984) Gangliosides of normal and neoplastic human melanocytes. Biochem Biophys Res Commun 120:500–504

Castel V, Segura V, Cañete A (2010) Treatment of high-risk neuroblastoma with anti-GD2 antibodies. Clin Transl Oncol 12:788–793

Cazet A, Lefebvre J, Adriaenssens E, Julien S, Bobowski M, Grigoriadis A, Tutt A, Tulasne D, Le Bourhis X, Delannoy P (2010) GD_3 synthase expression enhances proliferation and tumor growth of MDA-MB-231 breast cancer cells through c-Met activation. Mol Cancer Res 8:1526–1535

Chen HH, Fukumoto S, Furukawa K, Nakao A, Akiyama S, Urano T (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

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

Dall'Olio F, Malagolini N, Trinchera M, Chiricolo M (2012) Mechanisms of cancer-associated glycosylation changes. Front Biosci (Landmark Ed) 17:670–99

Daniotti JL, Vilcaes AA, Torres Demichelis V, Ruggiero FM, Rodriguez-Walker M (2013) Glycosylation of glycolipids in cancer: basis for development of novel therapeutic approaches. Front Oncol 3:306

Dong Y, Ikeda K, Hamamura K, Zhang Q, Kondo Y, Matsumoto Y, Ohmi Y, Yamauchi Y, Furukawa K, Taguchi R (2010) 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

Durrant LG, Noble P, Spendlove I (2012) Immunology in the clinic review series; focus on cancer: glycolipids as targets for tumour immunotherapy. Clin Exp Immunol 167:206–215

Ferrari G, Anderson BL, Stephens RM, Kaplan DR, Greene LA (1995) Prevention of apoptotic neuronal death by GM1 ganglioside. Involvement of Trk neurotrophin receptors. J Biol Chem 270:3074–3080

Fishman PH, Brady RO (1976) Biosynthesis and function of gangliosides. Science 194:906–915

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

Fukuda M, Horibe K, Furukawa K (1998) Enhancement of in vitro and in vivo anti-tumor activity of anti-GD2 monoclonal antibody 220–51 against human neuroblastoma by granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor. Int J Mol Med 2:471–475

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

Furukawa K, Hamamura K, Aixinjueluo W (2006) Biosignals modulated by tumor-associated carbohydrate antigens: novel targets for cancer therapy. Ann N Y Acad Sci 1086:185–198

Furukawa K, Hamamura K, Nakashima H (2008) Molecules in the signaling pathway activated by gangliosides can be targets of therapeutics for malignant melanomas. Proteomics 8:3312–3316

Furukawa K, Hamamura K, Ohkawa Y, Ohmi Y (2012a) Disialyl gangliosides enhance tumor phenotypes with differential modalities. Glycoconj J 29:579–584

Furukawa K, Ohkawa Y, Yamauchi Y, Hamamura K, Ohmi Y (2012b) Fine tuning of cell signals by glycosylation. J Biochem 151:573–578

Furukawa K, Kambe M, Miyata M, Ohkawa Y, Tajima O (2014) Ganglioside GD3 induces convergence and synergism of adhesion and hepatocyte growth factor/Met signals in melanomas. Cancer Sci 105:52–63

Furukawa K, Lloyd KO (1990) Gangliosides in melanoma. In: Ferrone S (ed) Human melanoma: from basic research to clinical application. Springer, Heidelberg, pp 15–30

Furukawa K, Akagi T, Nagata Y, Yamada Y, Shimotohno K, Cheung NK, Shiku H (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 USA 90:1972–1976

Grant SC, Kostakoglu L, Kris MG, Yeh SD, Larson SM, Finn RD, Oettgen HF, Cheung NV (1996) Targeting of small-cell lung cancer using the anti-GD2 ganglioside monoclonal antibody 3F8: a pilot trial. Eur J Nucl Med 23:145–149

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73:2424–2428

Hakomori S (1985) Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. Cancer Res 45:2405–2414

Hakomori S, Yamamura S, Handa AK (1998) Signal transduction through glyco- (sphingo)lipids. Introduction and recent studies on glyco(sphingo)lipid-enriched microdomains. Ann N Y Acad Sci 845:1–10

Hakomori S (2002a) Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci USA 99:10231–10233

Hakomori SI (2002b) The glycosynapse. Proc Natl Acad Sci USA 99:225–232

Hakomori S, Handa K (2002) Glycosphingolipid-dependent cross-talk between glycosynapses interfacing tumor cells with their host cells: essential basis to define tumor malignancy. FEBS Lett 531:88–92

Hakomori SI (2010) Glycosynaptic microdomains controlling tumor cell phenotype through alteration of cell growth, adhesion, and motility. FEBS Lett 584:1901–1906

Hamamura K, Tsuji M, Ohkawa Y, Nakashima H, Miyazaki S, Urano T, Yamamoto N, Ueda M, 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 1780:513–519

Hamamura K, Furukawa K, Hayashi T, Hattori T, Nakano J, Nakashima H, Okuda T, Mizutani H, Hattori H, Ueda M, Urano T, Lloyd KO (2005) Ganglioside GD3 promotes cell growth and

invasion through p130Cas and paxillin in malignant melanoma cells. Proc Natl Acad Sci USA 102:11041–11046

Hamamura K, Tsuji M, Hotta H, Ohkawa Y, Takahashi M, Shibuya H, Nakashima H, Yamauchi Y, Hashimoto N, Hattori H, Ueda M, 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

Hanibuchi M, Yano S, Nishioka Y, Yanagawa H, Sone S (1996) Anti-ganglioside GM2 monoclonal antibody-dependent killing of human lung cancer cells by lymphocytes and monocytes. Jpn J Cancer Res 87:497–504

Haraguchi M, Yamashiro S, Yamamoto A, Furukawa K, Takamiya K, Lloyd KO, Shiku H (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 USA 91:10455–10459

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 12:3154–3163

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

Hersey P (1991) Ganglioside antigens in tissue sections of skin, naevi, and melanoma-- implications for treatment of melanoma. Cancer Treat Res 54:137–151

Hollingsworth MA, Swanson BJ (2004) Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer 4:45–60

Irie RF, Morton DL (1986) Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside GD2. Proc Natl Acad Sci USA 83:8694–8698

Irie RF, Matsuki T, Morton DL (1989) Human monoclonal antibody to ganglioside GM2 for melanoma treatment. Lancet 1:786–787

Ju T, Otto VI, Cummings RD (2011) The Tn antigen-structural simplicity and biological complexity. Angew Chem Int Ed Engl 50:1770–1791

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

Kannagi R, Yin J, Miyazaki K, Izawa M (2008) Current relevance of incomplete synthesis and neo-synthesis for cancer associated alteration of carbohydrate determinants--Hakomori's concepts revisited. Biochim Biophys Acta 1780:525–531

Kaucic K, Liu Y, Ladisch S (2006) Modulation of growth factor signaling by gangliosides: positive or negative? Methods Enzymol 417:168–185

Ko K, Furukawa K, Takahashi T, Urano T, Sanai Y, Nagino Y, Nimura Y, Furukawa K (2006) Fundamental study of small interfering RNAs for ganglioside GD3 synthase as a therapeutic target of lung cancers. Oncogene 25:6924–6935

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 USA 105:7405–7409

Kusumi A, Fujiwara TK, Morone N, Yoshida KJ, Chadda R, Xie M, Kasai RS, Suzuki KG (2012) Membrane mechanisms for signal transduction: the coupling of the meso-scale raft domains to membrane-skeleton-induced compartments and dynamic protein complexes. Semin Cell Dev Biol 23:126–144

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

Lopez PH, Schnaar RL (2009) Gangliosides in cell recognition and membrane protein regulation. Curr Opin Struct Biol 19:549–557

Malisan F, Testi R (2002) GD3 ganglioside and apoptosis. Biochim Biophys Acta 1585:179–187

Margadant C, Monsuur HN, Norman JC, Sonnenberg A (2011) Mechanisms of integrin activation and trafficking. Curr Opin Cell Biol 23:607–614

Matsumoto Y, Zhang Q, Akita K, Nakada H, Hamamura K, Tsuchida A, Okajima T, Furukawa K, Urano T (2013) Trimeric Tn antigen on syndecan 1 produced by ppGalNAc-T13 enhances cancer metastasis via a complex formation with integrin α5β1 and matrix metalloproteinase 9. J Biol Chem 288:24264–24276

Matsumoto Y, Zhang Q, Akita K, Nakada H, Hamamura K, Tokuda N, Tsuchida A, Matsubara T, Hori T, Okajima T, Furukawa K, Urano T (2012) pp-GalNAc-T13 induces high metastatic potential of murine Lewis lung cancer by generating trimeric Tn antigen. Biochem Biophys Res Commun 419:7–13

Matsumoto-Takasaki A, Hanashima S, Aoki A, Yuasa N, Ogawa H, Sato R, Kawakami H, Mizuno M, Nakada H, Yamaguchi Y, Fujita-Yamaguchi Y (2012) Surface plasmon resonance and NMR analyses of anti Tn-antigen MLS128 monoclonal antibody binding to two or three consecutive Tn-antigen clusters. J Biochem 151:273–282

Matthay KK, George RE, Yu AL (2012) Promising therapeutic targets in neuroblastoma. Clin Cancer Res 18:2740–2753

Meng X, Zhong J, Liu S, Murray M, Gonzalez-Angulo AM (2012) A new hypothesis for the cancer mechanism. Cancer Metastasis Rev 31:247–268

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 (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

Miyata M, Ichihara M, Tajima O, Sobue S, Kambe M, Sugiura K, Furukawa K (2014) UVB-irradiated keratinocytes induce melanoma-associated ganglioside GD3 synthase gene in melanocytes via secretion of tumor necrosis factor α and interleukin 6. Biochem Biophys Res Commun 445:504–510

Miyazaki H, Fukumoto S, Okada M, Hasegawa T, Furukawa K (1997) Expression cloning of rat cDNA encoding UDP-galactose: GD2 beta 1,3-galactosyltransferase that determines the expression of GD1b/GM1/GA1. J Biol Chem 272:24794–24799

Morita N, Yajima Y, Asanuma H, Nakada H, Fujita-Yamaguchi Y (2009) Inhibition of cancer cell growth by anti-Tn monoclonal antibody MLS128. Biosci Trends 3:32–37

Munesue S, Kusano Y, Oguri K, Itano N, Yoshitomi Y, Nakanishi H, Yamashina I, Okayama M (2002) The role of syndecan-2 in regulation of actin-cytoskeletal organization of Lewis lung carcinoma-derived metastatic clones. Biochem J 363:201–209

Munesue S, Yoshitomi Y, Kusano Y, Koyama Y, Nishiyama A, Nakanishi H, Miyazaki K, Ishimaru T, Miyaura S, Okayama M, Oguri K (2007) A novel function of syndecan-2, suppression of matrix metalloproteinase-2 activation, which causes suppression of metastasis. J Biol Chem 282:28164–28174

Munro S (2003) Lipid rafts: elusive or illusive? Cell 115:377–388

Mutoh T, Tokuda A, Miyadai T, Hamaguchi M, Fujiki N (1995) Ganglioside GM1 binds to the Trk protein and regulates receptor function. Proc Natl Acad Sci USA 92:5087–5091

Nagai Y (1995) Functional roles of gangliosides in bio-signaling. Behav Brain Res 66:99–104

Nakashima H, Hamamura K, Houjou T, Taguchi R, Yamamoto N, Mitsudo K, Tohnai I, Ueda M, Urano T, 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

Nishio M, Fukumoto S, Furukawa K, Ichimura A, Miyazaki H, Kusunoki S, Urano T (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 279:33368–33378

Ohkawa Y, Miyazaki S, Miyata M, Hamamura 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

Ohkawa Y, Miyazaki S, Hamamura K, Kambe M, Miyata M, Tajima O, Ohmi Y, Yamauchi Y, 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

Okada M, Furukawa K, Yamashiro S, Yamada Y, Haraguchi M, Horibe K, Kato K, Tsuji Y (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. Clowes Memorial lecture. Cancer Res 41:361–375

Parsons K, Bernhardt B, Strickland B (2013) Targeted immunotherapy for high-risk neuroblastoma--the role of monoclonal antibodies. Ann Pharmacother 47:210–218

Patra SK (2008) Dissecting lipid raft facilitated cell signaling pathways in cancer. Biochim Biophys Acta 1785:182–206

Peinado H et al (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 18:883–891

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

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

Quann K, Gonzales DM, Mercier I, Wang C, Sotgia F, Pestell RG, Lisanti MP, Jasmin JF (2013) Caveolin-1 is a negative regulator of tumor growth in glioblastoma and modulates chemosensitivity to temozolomide. Cell Cycle 12:1510–1520

Raffaghello L, Marimpietri D, Pagnan G, Pastorino F, Cosimo E, Brignole C, Ponzoni M, Montaldo PG (2003) Anti-GD2 monoclonal antibody immunotherapy: a promising strategy in the prevention of neuroblastoma relapse. Cancer Lett 197:205–209

Ravindranath MH, Tsuchida T, Morton DL, Irie RF (1991) Ganglioside GM3: GD3 ratio as an index for the management of melanoma. Cancer 67:3029–3035

Razani B, Schlegel A, Liu J, Lisanti MP (2001) Caveolin-1, a putative tumour suppressor gene. Biochem Soc Trans 29:494–499

Rodeck U, Herlyn M (1988) Characteristics of cultured human melanocytes from different stages of tumor progression. Cancer Treat Res 43:3–16

Saito M, Yu RK, Cheung NK (1985) Ganglioside GD2 specificity of monoclonal antibodies to human neuroblastoma cell. Biochem Biophys Res Commun 127:1–7

Schengrund CL, Shochat SJ (1988) Gangliosides in neuroblastomas. Neurochem Pathol 8:189–202

Shibuya H, Hamamura K, Hotta H, Matsumoto Y, Nishida Y, Hattori H, Furukawa K, Ueda M (2012) Enhancement of malignant properties of human osteosarcoma cells with disialyl gangliosides GD2/GD3. Cancer Sci 103:1656–1664

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

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569–572

Simons K, Gerl MJ (2010) Revitalizing membrane rafts: new tools and insights. Nat Rev Mol Cell Biol 11:688–699

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31–39

Suzuki KG, Kasai RS, Hirosawa KM, Nemoto YL, Ishibashi M, Miwa Y, Fujiwara TK, Kusumi A (2012) Transient GPI-anchored protein homodimers are units for raft organization and function. Nat Chem Biol 8:774–783

Suzuki KG, Kasai RS, Fujiwara TK, Kusumi A (2013) Single-molecule imaging of receptor-receptor interactions. Methods Cell Biol 117:373–390

Takenaga K (1986) Modification of the metastatic potential of tumor cells by drugs. Cancer Metastasis Rev 5:67–75

Thurin J, Thurin M, Herlyn M, Elder DE, Steplewski Z, Clark WH, Koprowski H (1986) GD2 ganglioside biosynthesis is a distinct biochemical event in human melanoma tumor progression. FEBS Lett 208:17–22

Vaudry D, Stork PJ, Lazarovici P, Eiden LE (2002) Signaling pathways for PC12 cell differentiation: making the right connections. Science 296:1648–1649

Welte K, Miller G, Chapman PB, Yuasa H, Natoli E, Kunicka JE, Cordon-Cardo C, Buhrer C, Old LJ, Houghton AN (1987) Stimulation of T lymphocyte proliferation by monoclonal antibodies against GD3 ganglioside. J Immunol 139:1763–1771

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

Williams TM, Lisanti MP (2005) Caveolin-1 in oncogenic transformation, cancer, and metastasis. Am J Physiol Cell Physiol 288:C494–506

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 USA 84:2416–2420

Yamaguchi H, Furukawa K, Fortunato SR, Livingston PO, Lloyd KO, Oettgen HF, Old LJ (1990) Human monoclonal antibody with dual GM2/GD2 specificity derived from an immunized melanoma patient. Proc Natl Acad Sci USA 87:3333–3337

Yamashiro S, Okada M, Haraguchi M, Furukawa K, Lloyd KO, Shiku H (1995) Expression of alpha 2,8-sialyltransferase (GD3 synthase) gene in human cancer cell lines: high level expression in melanomas and up-regulation in activated T lymphocytes. Glycoconj J 12:894–900

Yanagisawa M, Taga T, Nakamura K, Ariga T, Yu RK (2005) Characterization of glycoconjugate antigens in mouse embryonic neural precursor cells. J Neurochem 95:1311–1320

Yates AJ (1988) Glycolipids and gliomas. Neurochem Pathol 8:157–180

Yates AJ, Saqr HE, Van Brocklyn J (1995) Ganglioside modulation of the PDGF receptor. A model for ganglioside functions. J Neurooncol 24:65–73

Yilmaz YE, Lawless JF, Andrulis IL, Bull SB (2013) Insights from mixture cure modeling of molecular markers for prognosis in breast cancer. J Clin Oncol 31:2047–2054

Yoon SJ, Nakayama K, Hikita T, Handa K, Hakomori SI (2005) Epidermal growth factor receptor tyrosine kinase is modulated by GM3 interaction with N-linked GlcNAc termini of the receptor. Proc Natl Acad Sci USA 103:18987–18991

Yoshida S, Kawaguchi H, Sato S, Ueda R, Furukawa K (2002) An anti-GD2 monoclonal antibody enhances apoptotic effects of anti-cancer drugs against small cell lung cancer cells via JNK (c-Jun terminal kinase) activation. Jpn J Cancer Res 93:816–824

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

Zhan J, Han Q, Wang K (2013) Development of antibody therapeutics for small cell lung cancer. Expert Opin Investig Drugs 22:235–244

Zhang Q, Furukawa K, Chen HH, Sakakibara T, Urano T (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

Zhang S, Cordon-Cardo C, Zhang HS, Reuter VE, Adluri S, Hamilton WB, Lloyd KO, Livingston PO (1997) Selection of tumor antigens as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. Int J Cancer 73:42–49

Zhang Y, Iwasaki H, Wang H, Kudo T, Kalka TB, Hennet T, Kubota T, Cheng L, Inaba N, Gotoh M, Togayachi A, Guo J, Hisatomi H, Nakajima K, Nishihara S, Nakamura M, Marth JD, Narimatsu H (2003) Cloning and characterization of a new human UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase, designated pp-GalNAc-T13, that is specifically expressed in neurons and synthesizes GalNAc alpha-serine/threonine antigen. J Biol Chem 278:573–584

Chapter 5
Regulation of Growth Factor Receptors by Glycosphingolipids

Sen-itiroh Hakomori and Kazuko Handa

Abstract Complex glycosphingolipids (GSLs) are ubiquitous components of animal cell plasma membranes, and many have been structurally characterized. GSLs, including gangliosides, are involved in crucial biological processes such as cell growth, differentiation, and motility. Certain GSLs have been identified as tumor-associated antigens in various types of cancer cells. Early studies of inhibitory effects of gangliosides on cell growth led to the discovery that GSLs modulate (inhibit or enhance) the activation of growth factor (GF) receptor-associated tyrosine kinase, which is triggered by the binding between a GF and its specific receptor. GSLs are localized as clusters at unique microdomains of the plasma membrane, termed glycolipid-enriched microdomains, lipid rafts, or glycosynapses. Various GF receptors (GFRs), including epidermal GFR and hepatocyte GFR, are localized in such membrane microdomains. There is increasing evidence that GSLs modulate the activation of GFR kinases in such microdomains, in which other signaling molecules and regulatory molecules such as integrins and tetraspanins are also localized.

Keywords Glycosphingolipids • Growth factor • Growth factor receptor • Membrane microdomain • Aberrant glycosylation • Cell phenotype • Cancer • Cell signaling • Tetraspanin

S.-i. Hakomori (✉)
Division of Biomembrane Research, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122, USA

Departments of Pathobiology and Global Health, University of Washington, Seattle, WA 98040, USA
e-mail: hakomori@u.washington.edu

K. Handa
Division of Biomembrane Research, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122, USA

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_5

5.1 Glycosphingolipids

5.1.1 Structures

Two major groups of glycosphingolipids (GSLs) have been described: (1) those derived from GalβCer (only four members of this group are known: cerebroside (3-O-sulfated cerebroside), sulfatide (Galα1- 4GalβCer), diGalCer (GM4-ganglioside), and Sialα3GalβCer) and (2) those derived from GlcβCer. This group includes ~140 members: ~80 "lacto-series GSLs" having either Galβ3GlcNAcβ3Galβ4GlcβCer (lacto-type 1) core or Galβ4GlcNAcβ3Galβ4GlcβCer (lacto-type 2) core; ~50 "ganglio-series GSLs" having either Sialα3Galβ4GlcβCer core (GM3) or Sialα3[GalNAcβ4]Galβ4GlcβCer core (GM2 and other higher gangliosides); and ~10 "globo-series" GSLs having Galα4Galβ4GlcβCer core (Hakomori 1983). The structural basis for these groups of GSLs is shown in Fig. 5.1.

The major contributions by our laboratory to knowledge of GSL structure are (1) the common core structure of all types of globo-series GSLs was identified as Galα4Galβ4GlcβCer (Hakomori et al. 1971); (2) human blood group A, B, H, and Le^b antigens and Le^x (lactofucopentaosyl ceramide) were isolated and characterized using a large quantity of human blood cells (Hakomori and Strycharz 1968) and human colorectal carcinoma tissue (Yang and Hakomori 1971), respectively; and (3) the type 1 lacto-series structure (Galβ3GlcNAcβ3Galβ4GlcβCer) can be repeated two times, but cannot be branched, with α4 fucosylation to the β3GlcNAc

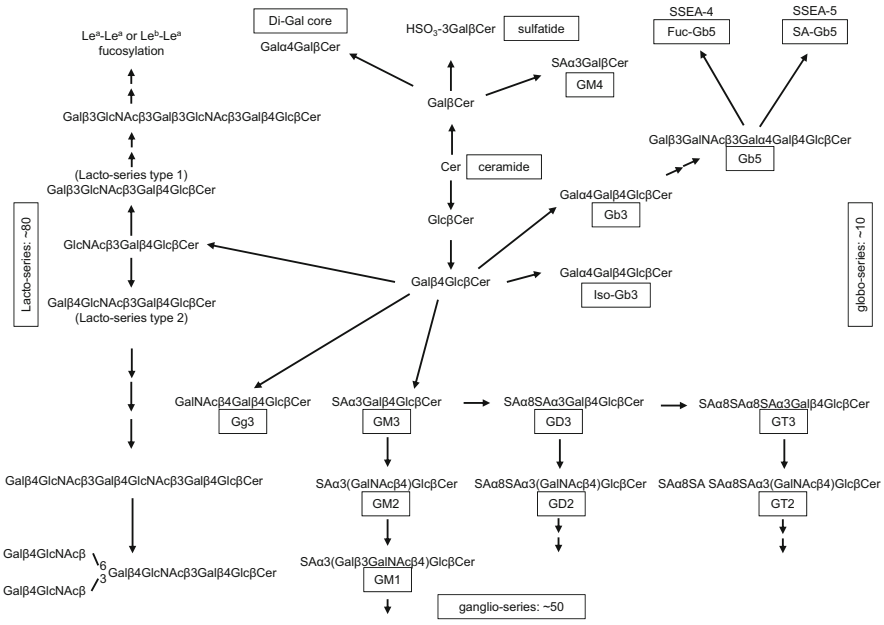

Fig. 5.1 General structures of GSLs

and/or α2 fucosylation to the terminal Gal. The whole antigens were well characterized as Le^a-Le^a (Le^a on Le^a) (Stroud et al. 1991) and Le^b-Le^a (Le^b on Le^a) (Stroud et al. 1992). A mouse monoclonal antibody (mAb) against Le^a-Le^a epitope was found to inhibit human colon cancer cell growth and metastasis in in vitro and in vivo xenograft assay through antibody-dependent cell-mediated cytotoxicity (Watanabe et al. 1991). A similar study using a mAb directed to Le^b-Le^a epitope revealed strong complement-dependent cell-mediated cytotoxicity against human colonic cancer Colo205 cells (Ito et al. 1992). In contrast to type 1 lacto-series, type 2 lacto-series antigens can have three or more times repetition of the Galβ4GlcNAcβ3Galβ4Glcβ structure and branching structures such as R-Galβ4GlcNAcβ6[R-Galβ4GlcNAcβ2]Galβ4GlcNAcβ3Gal (R could be Sialα3 or Fucα2 as terminal structure or include further extension).

Biosynthesis of GSLs occurs in the endoplasmic reticulum or Golgi apparatus in a stepwise fashion. Glucose (Glc) or galactose (Gal) is initially transferred to ceramide (Cer), and subsequent sugars are added from nucleotide sugar donors by specific glycosyltransferases. Various glycosyltransferases responsible for GSL synthesis have been purified, and their genes identified. The GSL synthesis pathways catalyzed by these glycosyltransferases have been studied extensively and described systematically in previous reviews (Taniguchi et al. 2002). Knockout mouse models deficient in specific glycosyltransferases have been used to extensively investigate the functional roles of GSLs, particularly gangliosides (Proia 2003; Furukawa et al. 2011; Yu et al. 2012).

5.1.2 Functions

The presence of GSLs as ubiquitous components of cell membranes and their structures were well established during the first half of the twentieth century by pioneers such as J.L.W. Thudichum, H. Thierfelder, E. Klenk, G. Blix, and T. Yamakawa. GSLs were known initially as allogeneic histo-blood groups and heterophile antigens, but their biological roles in defining cellular functions did not become clear until the mid-1960s. We examined the effect of oncogenic transformation of cells on GSL expression patterns and found that ganglioside GM3 expression in polyoma virus-transformed baby hamster kidney (BHK) cells decreased significantly along with enhanced expression of lactosyl-Cer. Such transformation caused "incomplete or defective synthesis" of certain GSLs (Hakomori and Murakami 1968). Around the same time, R.O. Brady and colleagues observed a clear decrease of mono- and disialogangliosides in mouse 3 T3 cells following oncogenic transformation by RNA viruses. Similar changes in ganglioside expression were observed in spontaneously transformed cells (Mora et al. 1969). Differing expression levels of various gangliosides were found in rat

hepatocytes and hepatomas (Brady et al. 1969). The transformation process was shown to be reversible under defined conditions (Miura et al. 2004).

Another approach to understanding functional roles of cell surface GSLs involved possible changes of GSL expression patterns in relation to cell population density. Cell growth and motility were known to change following cell-to-cell contact, and "contact inhibition" of growth and motility was displayed by many normal cells but not by transformed cells (Abercrombie and Heaysman 1954). No studies prior to 1970 examined the possible correlation between GSL expression and contact inhibition. We compared GSL expression patterns in NIL and BHK cells grown at low density (without contact inhibition) and at high density (with contact inhibition). The gangliosides Gb3, GM3, and GD3 showed clearly enhanced expression in contact-inhibited cells. Such enhanced expression was lost in transformed cells without contact inhibition (Hakomori 1970). Around the same time, other groups reported similar GSL expression changes associated with cell social functions and oncogenic transformation (Robbins and Macpherson 1971; Sakiyama et al. 1972).

Following introduction of mAb techniques (Kohler and Milstein 1975) in tumor and developmental biology, a surprisingly large number of tumor-associated antigens, or antigens expressed at specific stages of early embryonic development, were identified as carbohydrate epitopes, usually expressed as GSLs (Hakomori and Handa 2002; Hakomori 2008). These findings led to the concept that aberrant glycosylation is associated with changes in cell phenotype, particularly malignancy (Hakomori 2003). D. Solter and colleagues applied mAb approaches in studies of mouse early embryonic development and established several mAbs specific to antigens expressed at certain developmental stages, termed "stage-specific embryonic antigens" (SSEAs). One such antigen, SSEA-1, which is expressed only at the 8–32-cell stage and plays an essential role in cellular adhesion and autoaggregation at the morula stage leading to compaction (Abercrombie and Heaysman 1954), was found to carry type 2 lactofucopentaose II, i.e., Le^x structure. We showed that cell autoaggregation was inhibited by trivalent Le^x but not by its stereoisomer Le^a (Hakomori 2008), indicating a functional role of SSEA-1 during the compaction process. The structure of SSEA-1 expressed on mouse embryonic stem cells was identified as long non-branched type 2 chain with repeated Le^x (Sakiyama et al. 1972; Kannagi et al. 1982). SSEA-3 and SSEA-4, which are expressed at an earlier stage (0.5–1 days) following fertilization, were identified as extended globo-series structures with a Galα4Galβ4Glc core (Kannagi et al. 1983a; Kannagi et al. 1983b). The functional roles of SSEA-3 and SSEA-4 remain to be elucidated.

The above findings led to a concept that cell membrane GSLs, particularly gangliosides, are involved in regulation of cellular phenotypes. This concept was supported by studies that utilized the amphipathic property of GSLs to incorporate certain GSLs into cell membranes (Hakomori 1981). For example, incubation of human leukemia HL60 cells in GM3-containing culture medium led to GM3 incorporation and differentiation into monocytes/macrophages, which are characterized by high GM3 expression (Nojiri et al. 1986). When sialylparagloboside (SPG) was incorporated into HL60 cells, they underwent differentiation into

granulocytes, which are characterized by high SPG expression (Nojiri et al. 1988). In other studies, ganglioside GQ1b was shown to be involved in neurite formation (neuritogenesis), a terminal differentiation process of neuronal cells (Ito et al. 1989). To the contrary, GSL expression has been decreased by blocking GlcCer synthesis using the UDP-Glc:ceramide Glc-transferase inhibitors 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (Radin et al. 1993) and D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (Et-P4) (Lee et al. 1999).

5.2 Effects of Glycosphingolipids on Growth Factor Receptors

Beginning in the 1950s, many growth factors (GFs) that induce cell growth and alter cellular phenotypes have been described. These include epidermal GF (EGF) (Cohen 1965), nerve GF (NGF) (Levi-Montalcini and Cohen 1956), the fibroblast GF (FGF) family (Gospodarowicz 1974), and platelet-derived GF (PDGF) (Paul et al. 1971). Specific receptors for each type of GFs were identified subsequently (Yarden and Ullrich 1988; Ullrich and Schlessinger 1990). The GF receptors (GFRs) are expressed as transmembrane molecules at the cell surface. The extracellular domain of each receptor has a binding site specific to the GF, and the intracellular domain displays kinase activity. Following activation of the receptor through binding to its specific GF, receptor tyrosine kinases phosphorylate tyrosine residues on the receptor protein and/or specific signaling molecules. These findings led to a steadily increasing number of studies on regulatory roles of GSLs in GFR functions, either inhibitory or enhancing effects, depending on: GSL type, cell type, and experimental conditions (Kaucic et al. 2006).

5.2.1 PDGF Receptor/FGF Receptor

In view of the changes of GSL composition and structure associated with cell phenotype changes as described above, the possible effects of GSLs on cell growth and functions of related GFs were investigated. We showed that GSLs affect cell cycle (Lingwood and Hakomori 1977) and cell growth. GM3 and GM1 added exogenously to BHK fibroblast cells in chemically defined culture media were incorporated equally well by the cells. GM3, but not GM1, had a significant growth inhibitory effect. GM3-treated cells showed a reduced response to the cell growth stimulation by FGF, whereas the response of GM1-treated cells to FGF stimulation was similar to that of control cells. Radiolabeled FGF accumulated on the surface of GM3-treated cells, but not on GM1-treated cells. Neither GM3 nor GM1 showed direct interaction with FGF (Bremer and Hakomori 1982). In contrast, PDGF-

induced activation of PDGF receptors was inhibited strongly by GM1 but not by other GSLs tested (GM3, NeuAcnLc4, Gb4) (Bremer and Hakomori 1984; Bremer et al. 1984). These findings, together with those from EGF receptor studies as described below, suggested that GSLs regulate the activity of various GFs by modulating the activities of receptor-associated kinases.

5.2.2 EGF Receptor

Studies of human epidermoid carcinoma A431 cells demonstrated that GM3 inhibits EGF-induced autophosphorylation of EGF receptor. Analysis of phosphoamino acids of the EGF receptor prepared by immunoaffinity purification revealed that exogenous addition of GM3, but not GM1, greatly reduced the amount of phosphotyrosine, a major phosphorylated amino acid of the EGF receptor following EGF stimulation (Bremer et al. 1986). Similar effects of GM3 were observed under more physiological conditions using ldlD cells, a UDP-Gal-4-epimerase-deficient mutant of Chinese hamster ovary (CHO) cells that expresses GM3 only when cultured in Gal-containing medium (Weis and Davis 1990). Numerous studies supported the concept that gangliosides modulate cell functions through their effect on GF-associated tyrosine kinases (see for review (Yates and Rampersaud 1998).

We subsequently examined the effects of various modified forms of GM3 (Fig. 5.2) in comparison to regular GM3 on EGF receptor kinase activity. This activity, determined by in vitro assays, was greatly affected by detergent concentration and ATP concentration. GM3 affected the activity in a biphasic manner, under physiological ATP concentration, i.e., GM3 was inhibitory at low detergent concentration and stimulatory at high detergent concentration. In contrast, lyso-GM3 displayed a monophasic inhibitory effect under a wide range of detergent concentrations, while lyso-CDH had no detectable effect on the kinase activity. The

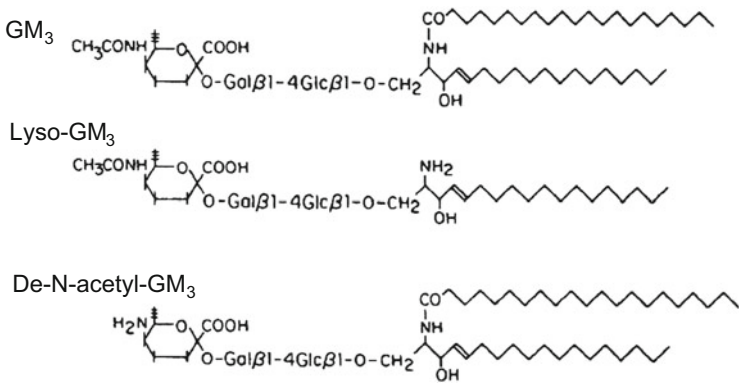

Fig. 5.2 Structures of GM_3 and some of its derivatives

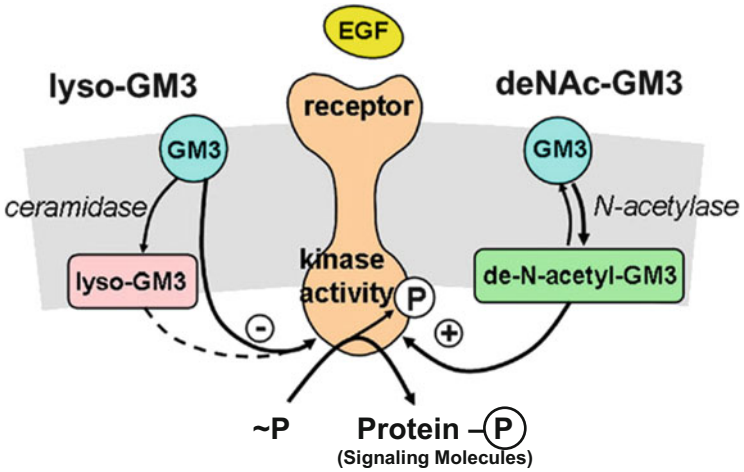

Fig. 5.3 Effects of GM_3, Lyso-GM_3, and de-N-acetyl-GM_3 on EGFR

present of lyso-GM3 in A431 cells suggested that de-N-fatty acylation of gangliosides, particularly GM3, might be an effective way to modulate EGF receptor function in membranes (Hanai et al. 1988b). To examine this possibility, we established mAb DH5 directed to a novel ganglioside, de-N-acetyl-GM3, which has a neuraminosyl-lactosyl group instead of the sialosyl-lactosyl group found in regular GM3 (Dohi et al. 1988). DH5 blotting revealed a small amount of de-N-acetyl-GM3 in A431 cells and in mouse melanoma B16 cells. De-N-acetyl-GM3 prepared from GM3 by chemical modification (Hanai et al. 1987; Nores et al. 1988) strongly enhanced both EGF receptor kinase activity and EGF-induced growth of A431, Swiss 3 T3, and B16 cells. Exogenously added de-N-acetyl-GM3 had no effect on the affinity of EGF binding to its receptor, in contrast to the notable inhibitory effect of GM3 and lyso-GM3 (Hanai et al. 1988a). We found that GM3 inhibits tyrosine phosphorylation, whereas de-N-acetyl-GM3 enhances serine phosphorylation of EGF receptor (Zhou et al. 1994) (Fig. 5.3).

For further systematic studies, we synthesized de-N-acetyl-lyso-GM3, lyso-GM3, de-N-acetyl-GM3 with N-acetyl-sphingosine, and GM3 with N-acetyl-sphingosine (Nores et al. 1989). De-N-acetyl-GM3 and lyso-GM3 had been considered as the major modulators of EGF-induced EGF receptor activity in A431 cells, but we found that de-N-acetyl-lyso-GM3, N-acetyl-sphingosine, and N-fatty acyl-sphingosine also function as minor modulators (Nores et al. 1988; Hanai et al. 1988a). De-N-acetyl-GM3 was later reported to be expressed highly in metastatic/invasive human melanoma cells and related to their high migration (Liu et al. 2009). The findings of the 1980 studies suggested that the N-acetyl group of sialosyl residue and the N-fatty acyl group of ceramide moiety of gangliosides may cause modification of essential functional organization of GSLs in cell membrane microdomains. This concept was more clearly formulated in the 1990s

and subsequently extended to the context of gangliosides, GFRs, and other signaling molecules as described below.

To investigate the role of the sialosyl group of GM3 in its inhibitory activity, a gene encoding a soluble Mr 42,000 sialidase was transfected into A431 cells. In comparison with control cells, the transfectant cells showed reduced surface expression of lipid-bound sialic acids, faster growth, and enhanced EGF receptor autophosphorylation. The degree of sialidase expression had no effect on binding of EGF to its receptor (Meuillet et al. 1999). Another group cloned mouse plasma membrane-associated sialidase (NEU3), which degrades cell membrane GM3 and GD1a (Hasegawa et al. 2000; Papini et al. 2004). The *NEU3* gene was shown to be upregulated in human cancers, and enhanced expression of NEU3 resulted in suppression of apoptosis. Knockdown of *NEU3* expression by small interfering RNA (siRNA) inhibited Ras activation, such inhibition was blocked by EGF receptor kinase inhibitor AG1478, and NEU3 overexpression enhanced EGF-induced EGF receptor autophosphorylation (Wada et al. 2007). Enhanced EGF receptor phosphorylation was also detected in NEU3 transgenic mice, suggesting that upregulation of NEU3 is involved in carcinogenesis (Shiozaki et al. 2009). NEU3 was found to be highly expressed in human melanoma cells (Miyata et al. 2011).

Based on evidence that GM3 interacts directly with EGF receptor, E.G. Bremer's group measured the interaction of various gangliosides with extracellular domain of recombinant human EGF receptor prepared in insect cells. The order of binding intensity was GM3 $\gg$ GM2, GD3, GM4 $>$ GM1, GD1a, GD1b, GT1b, GD2, GQ1b $>$ lactosyl-Cer, consistent with the inhibitory effect of these gangliosides on EGF receptor activation (Miljan et al. 2002).

5.2.3 NGF Receptor

Glycolipids, particularly gangliosides, are accumulated in vertebrate brains, and their expression profiles change during development (Wiegandt 1985). Various gangliosides have been shown to play important roles in nerve system function. GM1 has been well studied in this regard because of its abundance in brain. Brain ganglioside structures and expression levels are generally conserved among mammals (Tettamanti et al. 1973). GM1, GD1a, GD1b, and GT1b comprise >90 % of total gangliosides in human brain.

NGF was the first identified member of a family of neurotrophic factors that promote survival and differentiation of neuronal cells (Levi-Montalcini 1987). NGF was found to induce differentiation of rat pheochromocytoma PC12 cells into cells resembling sympathetic neurons (Greene and Tischler 1976), and the PC12 model has been widely used for studies of NGF function. Gangliosides, particularly GM1, were shown to have activities similar to those of trophic factors, and the B subunit of cholera toxin, which binds specifically to GM1, was found to promote NGF function (Mutoh et al. 1993). These findings suggested a functional

connection between GM1 and Trk receptor, also known as high-affinity NGF receptor or neurotrophic tyrosine kinase receptor, which is responsible for NGF-induced axon outgrowth. Follow-up studies demonstrated that GM1 is associated specifically with Trk on the PC12 cell surface and enhances NGF function. Other tested polysialogangliosides did not bind to Trk, and GM1 did not bind to EGF receptor (Mutoh et al. 1995).

Our systematic studies of basic (cationic) lipids in human brain using cation exchange, Florisil, and iatrobead chromatographic procedures resulted in separation and identification of five compounds. The two major cationic lipids, found in brain white matter but not gray matter, were identified as cyclic plasmalogens, i.e., aliphatic aldehyde acetals linked at different hydroxyl groups of the galactosyl residue of psychosine (P-D-galactopyranosyl-1-sphingosine). Compound A had a 3, 4-cyclic acetal linkage, and the slower migrating compound B had a 4, 6-cyclic acetal linkage (Fig. 5.4). Compounds A and B both had a weak inhibitory effect on protein kinase C (PKC) and were not cytotoxic. In contrast, psychosine displayed strong PKC inhibitory effect and cytotoxicity (Nudelman et al. 1992).

Subsequent studies using chemically synthesized compounds demonstrated the neurotrophic activity of compound B, plasmalopsychosine (PLPS). PLPS, like NGF, enhanced TrkA phosphorylation and mitogen-activated protein kinase (MAPK) activity and consequently induced PC12 neurite outgrowth. Tyrosine kinase inhibitors known to inhibit the neurotrophic effect of NGF also inhibited that of PLPS, suggesting a common signaling cascade for the two molecules, although PLPS did not compete with the high-affinity binding of NGF to TrkA (Sakakura et al. 1996).

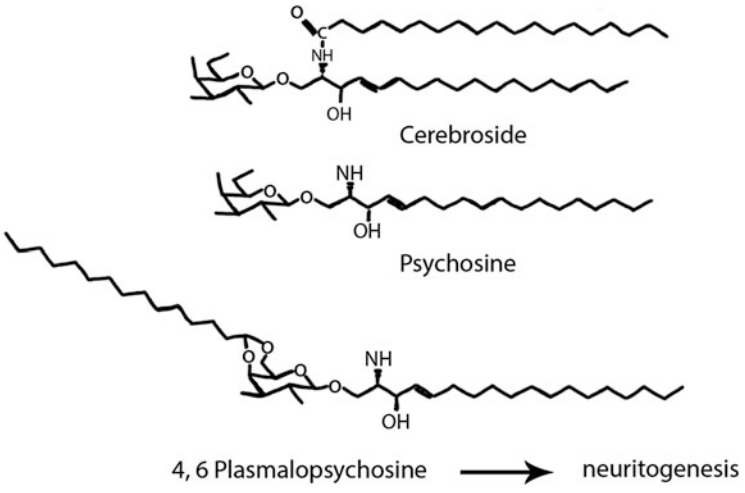

Fig. 5.4 Structures of cerebroside (β-galactosylceramide), psychosine (β-galactosyl-Sph), and 4, 6 plasmalopsychosine

5.2.4 *Met Receptor*

The receptor tyrosine kinase Met was discovered in the mid-1980s (Park et al. 1987) and shown to bind exclusively to hepatocyte GF (HGF) (Nakamura et al. 1989; Bottaro et al. 1991), also known as scatter factor (SF) (Gherardi et al. 1989). Later studies demonstrated the role of HGF/SF-Met signaling in uncontrolled cell growth and enhanced cell motility (Rong et al. 1994; Bladt et al. 1995).

Our studies on the effect of GSLs on Met receptor using human bladder cell lines that express different levels of GM2 and/or GM3 revealed that GM2 depletion by Et-P4 treatment significantly enhanced HGF-induced Met tyrosine kinase activity and motility in one of the cell lines (Todeschini et al. 2007). A follow-up study showed that GM2 and GM3 form a ganglioside heterodimer complex (GM2/GM3) in the presence of Ca^{2+}, and that this complex had a stronger inhibitory effect than GM2 alone on Met tyrosine kinase activity and HGF-induced cell motility (Todeschini et al. 2008). The tetraspanin CD82 was shown to be involved in such inhibitory activity, as described below.

5.3 The Mechanisms of GSL Effects on GFRs

Membrane microdomains variously termed as detergent-insoluble membrane microdomains (Brown and London 1997, 2000), GSL-enriched microdomains (GEM), caveolae (Stan et al. 1997), and lipid rafts (Simons and Ikonen 1997) have been proposed to function as platforms for GF-induced signal transduction. These microdomains are enriched in GSLs, cholesterol, glycosylphosphatidylinositol-anchored proteins, and sphingomyelin. GSLs in cell surface membrane microdomains are associated or complexed with signal transducers (Src family kinases and small G proteins), tetraspanins (e.g., CD9, CD81, CD82), GFRs, and integrins. Such an organizational framework, defining GSL-modulated or GSL-dependent cell adhesion, motility, and growth, is termed a "glycosynapse" (Hakomori and Handa 2002; Hakomori 2004, 2002). Tetraspanins (also called the transmembrane-4 superfamily (TM4SF) because they have four transmembrane domains) clearly play functional roles in cell adhesion, proliferation, differentiation, and motility. Highly ionic detergents such as Triton X-100 are used to separate GEM or rafts from cell membranes, while less polar, nonionic detergents such as Brij 98 are used to separate glycosynapses.

GM3 significantly inhibited haptotactic motility of high-CD9-expressing but not low-CD9-expressing human carcinoma cells. GM1 had no such inhibitory effect. These findings suggested that haptotactic tumor cell motility is inhibited cooperatively by co-expression of CD9 and GM3. This concept was supported by studies using the mutant CHO cell line ldlD14 described above and a CD9-overexpressing variant produced by CD9 gene transfection. Because of the lack of UDP-Gal 4-epimerase, ldlD14 cells are incapable of synthesizing Gal-containing molecules

such as GM3 unless Gal is added to the culture medium. Motility of the transfectant cells was higher in the absence than in the presence of Gal. Follow-up studies using photoactivatable ^{3}H-labeled GM3 confirmed the GM3/CD9 interaction (Ono et al. 2001).

Growth of human embryonal fibroblast WI38 cells, which display clear contact inhibition, is highly dependent on FGF and FGF receptor. Analysis of glycosynapse components showed that confluent, growth-inhibited WI38 cells had a lower degree of FGF-induced MAPK activation than did actively growing cells in sparse culture and that the level of inactive cSrk (detected with Tyr-527 phosphate) was higher in the growth-inhibited cells. These differences were eliminated by preincubation with Et-P4 to deplete GM3. FGF receptor in glycosynapse fraction from WI38 cells bound to GM3-coated polystyrene beads more strongly than to beads coated with GM2, GM1, SPG, or LacCer. Such binding was significantly reduced by pretreatment of GM3 with sialidase (Toledo et al. 2004). A follow-up study of WI38 cells and their oncogenic transformant VA13 showed that tetraspanins CD9/CD81, integrin, and GM3 are jointly involved in cell adhesion and motility induced by FGF activation of FGF receptor (Toledo et al. 2005).

We compared glycosynapse compositions of two human bladder cell lines with high vs. low motility, associated with HGF/Met signaling. Integrins and CD9 were expressed at similar levels in the two cell lines. The low-motility cell line showed much higher GM3 expression and stronger α3 integrin/CD9 interaction. Exogenous GM3 addition converted the high-motility to low-motility phenotype, while GM3 depletion by Et-P4 converted the low-motility to high-motility phenotype, suggesting a ganglioside-based phenotype-modulating function (Mitsuzuka et al. 2005).

A study of HGF/Met signaling showed that GM2 expressed in a human normal bladder cell line interacted with tetraspanin CD82 and that GM2/CD82 complex inhibited HGF-induced Met tyrosine kinase. Depletion of GM2 by preincubation with Et-P4 or reduction of CD82 expression by siRNA significantly enhanced HGF-induced Met tyrosine kinase and cell motility (Todeschini et al. 2007). Our follow-up study showed that GM2/GM3 heterodimer bound more strongly to CD82 than did GM2 alone and that co-culturing of CD82-expressing cells with GM2/GM3 dimer-coated nanoparticles reduced HGF-induced Met phosphorylation and cell motility (Todeschini et al. 2008).

Our recent studies indicate that GM3 interacts with N-linked glycans carrying multi-GlcNAc termini of EGF receptor through carbohydrate-carbohydrate interaction (CCI) (Handa and Hakomori 2012) and that the molecular mechanism underlying the inhibitory effect of GM3 involves such interaction of GM3 with EGF receptor glycans (Yoon et al. 2006; Kawashima et al. 2009). We use the term *cis*-CCI to describe this type of CCI, because CCI occurs on membranes of a same cell. *cis*-CCI may well be involved in the regulatory effects of other GSLs on other GF receptors.

GSLs, particularly gangliosides, play key roles in organization and function of membrane microdomains (Sonnino and Prinetti 2010). The molecular mechanism for the inhibitory effect of GM1 on PDGF receptor activation was studied in Swiss

3 T3 cells by establishing its GM1-expressing lines via transfection of GM2/GD2 synthase and GM1 synthase genes. The study showed the expression of GM1 inhibits PDGF-dependent cell growth and PDGF-induced ERK1/2 activation and reduces the amount of PDGF receptors in GEM where GM1 localizes. This suggests GM1 functions through modulating the distribution of PDGF receptors inside and outside of GEM (Mitsuda et al. 2002). A similar role of GM1 and GM3 was observed in PDGF-induced Src signaling in NIH 3 T3 cells (Veracini et al. 2008) and in insulin resistance induced by TNFα in 3 T3 L-1 adipocytes (Kabayama et al. 2007), respectively.

5.4 Perspectives

Further extensive studies are needed to elucidate the precise molecular mechanism underlying the regulatory effects of GSLs on GFRs in the context of cancer initiation and progression. Numerous recent studies support the concept that cancer cells in tumors are heterogeneous and include a minor subpopulation of cells termed cancer stem cells (CSCs). The characteristic properties of CSCs include self-renewal capability, tumor-initiating ability (assessed by the ability to efficiently form new tumors upon inoculation into recipient immunodeficient mice), enhanced cell motility, and resistance to various chemotherapeutic agents. CSCs have therefore been implicated in cancer metastasis and recurrence following therapeutic treatments. We found recently that certain GSLs are differentially expressed in CSC populations in human breast cancer (Liang et al. 2013). An interesting possibility is that GSLs function to maintain CSC phenotype by modulating specific GFRs.

References

Abercrombie M, Heaysman JE (1954) Observations on the social behaviour of cells in tissue culture. II. Monolayering of fibroblasts. Exp Cell Res 6(2):293–306

Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C (1995) Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature 376 (6543):768–771. doi:10.1038/376768a0

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA (1991) Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 251(4995):802–804

Brady RO, Borek C, Bradley RM (1969) Composition and synthesis of gangliosides in rat hepatocyte and hepatoma cell lines. J Biol Chem 244(23):6552–6554

Bremer EG, Hakomori S (1982) GM3 ganglioside induces hamster fibroblast growth inhibition in chemically-defined medium: ganglioside may regulate growth factor receptor function. Biochem Biophys Res Commun 106(3):711–718

Bremer EG, Hakomori S (1984) Gangliosides as receptor modulators. Adv Exp Med Biol 174:381–394

Bremer EG, Hakomori S, Bowen-Pope DF, Raines E, Ross R (1984) Ganglioside-mediated modulation of cell growth, growth factor binding, and receptor phosphorylation. J Biol Chem 259(11):6818–6825

Bremer EG, Schlessinger J, Hakomori S (1986) Ganglioside-mediated modulation of cell growth. Specific effects of GM3 on tyrosine phosphorylation of the epidermal growth factor receptor. J Biol Chem 261(5):2434–2440

Brown DA, London E (1997) Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem Biophys Res Commun 240(1):1–7. doi:10.1006/bbrc.1997.7575

Brown DA, London E (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem 275(23):17221–17224. doi:10.1074/jbc.R000005200

Cohen S (1965) The stimulation of epidermal proliferation by a specific protein (EGF). Dev Biol 12:394–407

Dohi T, Nores G, Hakomori S (1988) An IgG3 monoclonal antibody established after immunization with GM3 lactone: immunochemical specificity and inhibition of melanoma cell growth in vitro and in vivo. Cancer Res 48(20):5680–5685

Furukawa K, Ohmi Y, Ohkawa Y, Tokuda N, Kondo Y, Tajima O, Furukawa K (2011) Regulatory mechanisms of nervous systems with glycosphingolipids. Neurochem Res 36(9):1578–1586. doi:10.1007/s11064-011-0494-2

Gherardi E, Gray J, Stoker M, Perryman M, Furlong R (1989) Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. Proc Natl Acad Sci USA 86(15):5844–5848

Gospodarowicz D (1974) Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature 249(453):123–127

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73 (7):2424–2428

Hakomori S (1970) Cell density-dependent changes of glycolipid concentrations in fibroblasts, and loss of this response in virus-transformed cells. Proc Natl Acad Sci USA 67(4):1741–1747

Hakomori S (1981) Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Annu Rev Biochem 50:733–764. doi:10.1146/annurev.bi.50.070181.003505

Hakomori S-i (1983) Sphingolipid biochemistry, vol 3, Handbook of lipid research. Springer, New York. doi:10.1007/978-1-4757-0396-2_1

Hakomori S (2002) Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci USA 99(16):10231–10233. doi:10.1073/pnas.172380699

Hakomori S (2003) Structure, organization, and function of glycosphingolipids in membrane. Curr Opin Hematol 10(1):16–24

Hakomori S (2004) Glycosynapses: microdomains controlling carbohydrate-dependent cell adhesion and signaling. An Acad Bras Cienc 76(3):553–572, doi:/S0001-37652004000300010

Hakomori SI (2008) Structure and function of glycosphingolipids and sphingolipids: recollections and future trends. Biochim Biophys Acta 1780(3):325–346. doi:10.1016/j.bbagen.2007.08.015

Hakomori S, Handa K (2002) Glycosphingolipid-dependent cross-talk between glycosynapses interfacing tumor cells with their host cells: essential basis to define tumor malignancy. FEBS Lett 531(1):88–92

Hakomori SI, Murakami WT (1968) Glycolipids of hamster fibroblasts and derived malignant-transformed cell lines. Proc Natl Acad Sci USA 59(1):254–261

Hakomori S, Strycharz GD (1968) Investigations on cellular blood-group substances. I. Isolation and chemical composition of blood-group ABH and Le-b isoantigens of sphingoglycolipid nature. Biochemistry 7(4):1279–1286

Hakomori SI, Siddiqui B, Li YT, Li SC, Hellerqvist CG (1971) Anomeric structure of globoside and ceramide grihexoside of human erythrocytes and hamster fibroblasts. J Biol Chem 246 (7):2271–2277

Hanai N, Nores G, Torres-Mendez CR, Hakomori S (1987) Modified ganglioside as a possible modulator of transmembrane signaling mechanism through growth factor receptors: a preliminary note. Biochem Biophys Res Commun 147(1):127–134

Hanai N, Dohi T, Nores GA, Hakomori S (1988a) A novel ganglioside, de-N-acetyl-GM3 (II3NeuNH2LacCer), acting as a strong promoter for epidermal growth factor receptor kinase and as a stimulator for cell growth. J Biol Chem 263(13):6296–6301

Hanai N, Nores GA, MacLeod C, Torres-Mendez CR, Hakomori S (1988b) Ganglioside-mediated modulation of cell growth. Specific effects of GM3 and lyso-GM3 in tyrosine phosphorylation of the epidermal growth factor receptor. J Biol Chem 263(22):10915–10921

Handa K, Hakomori SI (2012) Carbohydrate to carbohydrate interaction in development process and cancer progression. Glycoconj J 29(8–9):627–637. doi:10.1007/s10719-012-9380-7

Hasegawa T, Yamaguchi K, Wada T, Takeda A, Itoyama Y, Miyagi T (2000) Molecular cloning of mouse ganglioside sialidase and its increased expression in Neuro2a cell differentiation. J Biol Chem 275(11):8007–8015

Ito J, Kato T, Okumura-Noji K, Miyatani Y, Tanaka R, Tsuji S, Nagai Y (1989) Induction of astroglial growth inhibition and differentiation by sialosyl cholesterol. Brain Res 481 (2):335–343

Ito H, Tashiro K, Stroud MR, Orntoft TF, Meldgaard P, Singhal AK, Hakomori S (1992) Specificity and immunobiological properties of monoclonal antibody IMH2, established after immunization with Le(b)/Le(a) glycosphingolipid, a novel extended type 1 chain antigen. Cancer Res 52(13):3739–3745

Kabayama K, Sato T, Saito K, Loberto N, Prinetti A, Sonnino S, Kinjo M, Igarashi Y, Inokuchi J (2007) Dissociation of the insulin receptor and caveolin-1 complex by ganglioside GM3 in the state of insulin resistance. Proc Natl Acad Sci USA 104(34):13678–13683

Kannagi R, Nudelman E, Levery SB, Hakomori S (1982) A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen SSEA-1. J Biol Chem 257(24):14865–14874

Kannagi R, Cochran NA, Ishigami F, Hakomori S, Andrews PW, Knowles BB, Solter D (1983a) Stage-specific embryonic antigens (SSEA-3 and −4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. EMBO J 2(12):2355–2361

Kannagi R, Levery SB, Ishigami F, Hakomori S, Shevinsky LH, Knowles BB, Solter D (1983b) New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3. J Biol Chem 258(14):8934–8942

Kaucic K, Liu Y, Ladisch S (2006) Modulation of growth factor signaling by gangliosides: positive or negative? Methods Enzymol 417:168–185

Kawashima N, Yoon SJ, Itoh K, Nakayama K (2009) Tyrosine kinase activity of epidermal growth factor receptor is regulated by GM3 binding through carbohydrate to carbohydrate interactions. J Biol Chem 284(10):6147–6155. doi:10.1074/jbc.M808171200

Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517):495–497

Lee L, Abe A, Shayman JA (1999) Improved inhibitors of glucosylceramide synthase. J Biol Chem 274(21):14662–14669

Levi-Montalcini R (1987) The nerve growth factor 35 years later. Science 237(4819):1154–1162

Levi-Montalcini R, Cohen S (1956) In vitro and in vivo effects of a nerve growth-stimulating agent isolated from snake venom. Proc Natl Acad Sci USA 42(9):695–699

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 USA 110(13):4968–4973. doi:10.1073/pnas.1302825110

Lingwood CA, Hakomori S (1977) Selective inhibition of cell growth and associated changes in glycolipid metabolism induced by monovalent antibodies to glycolipids. Exp Cell Res 108 (2):385–391

Liu JW, Sun P, Yan Q, Paller AS, Gerami P, Ho N, Vashi N, Le Poole IC, Wang XQ (2009) De-N-acetyl GM3 promotes melanoma cell migration and invasion through urokinase plasminogen activator receptor signaling-dependent MMP-2 activation. Cancer Res 69(22):8662–8669. doi:10.1158/0008-5472.CAN-09-1099

Meuillet EJ, Kroes R, Yamamoto H, Warner TG, Ferrari J, Mania-Farnell B, George D, Rebbaa A, Moskal JR, Bremer EG (1999) Sialidase gene transfection enhances epidermal growth factor receptor activity in an epidermoid carcinoma cell line, A431. Cancer Res 59(1):234–240

Miljan EA, Meuillet EJ, Mania-Farnell B, George D, Yamamoto H, Simon HG, Bremer EG (2002) Interaction of the extracellular domain of the epidermal growth factor receptor with gangliosides. J Biol Chem 277(12):10108–10113. doi:10.1074/jbc.M111669200

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(13):11239–11246

Mitsuzuka K, Handa K, Satoh M, Arai Y, Hakomori S (2005) A specific microdomain ("glycosynapse 3") controls phenotypic conversion and reversion of bladder cancer cells through GM3-mediated interaction of alpha3beta1 integrin with CD9. J Biol Chem 280 (42):35545–35553. doi:10.1074/jbc.M505630200

Miura Y, Kainuma M, Jiang H, Velasco H, Vogt PK, Hakomori S (2004) Reversion of the Jun-induced oncogenic phenotype by enhanced synthesis of sialosyllactosylceramide (GM3 ganglioside). Proc Natl Acad Sci USA 101(46):16204–16209. doi:10.1073/pnas.0407297101

Miyata M, Kambe M, Tajima O, Moriya S, Sawaki H, Hotta H, Kondo Y, Narimatsu H, Miyagi T, Furukawa K, Furukawa K (2011) Membrane sialidase NEU3 is highly expressed in human melanoma cells promoting cell growth with minimal changes in the composition of gangliosides. Cancer Sci 102(12):2139–2149. doi:10.1111/j.1349-7006.2011.02086.x

Mora PT, Brady RO, Bradley RM, McFarland VW (1969) Gangliosides in DNA virus-transformed and spontaneously transformed tumorigenic mouse cell lines. Proc Natl Acad Sci USA 63 (4):1290–1296

Mutoh T, Tokuda A, Guroff G, Fujiki N (1993) The effect of the B subunit of cholera toxin on the action of nerve growth factor on PC12 cells. J Neurochem 60(4):1540–1547

Mutoh T, Tokuda A, Miyadai T, Hamaguchi M, Fujiki N (1995) Ganglioside GM1 binds to the Trk protein and regulates receptor function. Proc Natl Acad Sci USA 92(11):5087–5091

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S (1989) Molecular cloning and expression of human hepatocyte growth factor. Nature 342 (6248):440–443. doi:10.1038/342440a0

Nojiri H, Takaku F, Terui Y, Miura Y, Saito M (1986) Ganglioside GM3: an acidic membrane component that increases during macrophage-like cell differentiation can induce monocytic differentiation of human myeloid and monocytoid leukemic cell lines HL-60 and U937. Proc Natl Acad Sci USA 83(3):782–786

Nojiri H, Kitagawa S, Nakamura M, Kirito K, Enomoto Y, Saito M (1988) Neolacto-series gangliosides induce granulocytic differentiation of human promyelocytic leukemia cell line HL-60. J Biol Chem 263(16):7443–7446

Nores GA, Hanai N, Levery SB, Eaton HL, Salyan EK, Hakomori S (1988) Synthesis and characterization of lyso-GM3 (II3Neu5Ac Lactosyl sphingosine), de-N-acetyl-GM3 (II3NeuNH2 lactosyl Cer), and related compounds. Carbohydr Res 179:393–410

Nores GA, Hanai N, Levery SB, Eaton HL, Salyan ME, Hakomori S (1989) Synthesis and characterization of ganglioside GM3 derivatives: lyso-GM3, de-N-acetyl-GM3, and other compounds. Methods Enzymol 179:242–253

Nudelman ED, Levery SB, Igarashi Y, Hakomori S (1992) Plasmalopsychosine, a novel plasmal (fatty aldehyde) conjugate of psychosine with cyclic acetal linkage. Isolation and characterization from human brain white matter. J Biol Chem 267(16):11007–11016

Ono M, Handa K, Sonnino S, Withers DA, Nagai H, Hakomori S (2001) GM3 ganglioside inhibits CD9-facilitated haptotactic cell motility: coexpression of GM3 and CD9 is essential in the downregulation of tumor cell motility and malignancy. Biochemistry 40(21):6414–6421

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(17):16989–16995. doi:10.1074/jbc.M400881200

Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G (1987) Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci USA 84(18):6379–6383

Paul D, Lipton A, Klinger I (1971) Serum factor requirements of normal and simian virus 40-transformed 3T3 mouse fibroplasts. Proc Natl Acad Sci USA 68(3):645–652

Proia RL (2003) Glycosphingolipid functions: insights from engineered mouse models. Philos Trans R Soc Lond B Biol Sci 358(1433):879–883. doi:10.1098/rstb.2003.1268

Radin NS, Shayman JA, Inokuchi J (1993) Metabolic effects of inhibiting glucosylceramide synthesis with PDMP and other substances. Adv Lipid Res 26:183–213

Robbins PW, Macpherson I (1971) Control of glycolipid synthesis in a cultured hamster cell line. Nature 229(5286):569–570

Rong S, Segal S, Anver M, Resau JH, Vande Woude GF (1994) Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci USA 91(11):4731–4735

Sakakura C, Igarashi Y, Anand JK, Sadozai KK, Hakomori S (1996) Plasmalopsychosine of human brain mimics the effect of nerve growth factor by activating its receptor kinase and mitogen-activated protein kinase in PC12 cells. Induction of neurite outgrowth and prevention of apoptosis. J Biol Chem 271(2):946–952

Sakiyama H, Gross SK, Robbins PW (1972) Glycolipid synthesis in normal and virus-transformed hamster cell lines. Proc Natl Acad Sci USA 69(4):872–876

Shiozaki K, Yamaguchi K, Sato I, Miyagi T (2009) Plasma membrane-associated sialidase (NEU3) promotes formation of colonic aberrant crypt foci in azoxymethane-treated transgenic mice. Cancer Sci 100(4):588–594. doi:10.1111/j.1349-7006.2008.01080.x

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387(6633):569–572. doi:10.1038/42408

Sonnino S, Prinetti A (2010) Gangliosides as regulators of cell membrane organization and functions. Adv Exp Med Biol 688:165–184

Stan RV, Roberts WG, Predescu D, Ihida K, Saucan L, Ghitescu L, Palade GE (1997) Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). Mol Biol Cell 8(4):595–605

Stroud MR, Levery SB, Nudelman ED, Salyan ME, Towell JA, Roberts CE, Watanabe M, Hakomori S (1991) Extended type 1 chain glycosphingolipids: dimeric Lea (III4V4Fuc2Lc6) as human tumor-associated antigen. J Biol Chem 266(13):8439–8446

Stroud MR, Levery SB, Salyan ME, Roberts CE, Hakomori S (1992) Extended type-1 chain glycosphingolipid antigens. Isolation and characterization of trifucosyl-Leb antigen (III4V4VI2Fuc3Lc6). Eur J Biochem/FEBS 203(3):577–586

Taniguchi N, Honke K, Fukuda M (2002) Handbook of glycosyltransferases and related genes. Springer, Tokyo/New York

Tettamanti G, Bonali F, Marchesini S, Zambotti V (1973) A new procedure for the extraction, purification and fractionation of brain gangliosides. Biochim Biophys Acta 296(1):160–170

Todeschini AR, Dos Santos JN, Handa K, Hakomori SI (2007) Ganglioside GM2-tetraspanin CD82 complex inhibits met and its cross-talk with integrins, providing a basis for control of cell motility through glycosynapse. J Biol Chem 282(11):8123–8133. doi:10.1074/jbc.M611407200

Todeschini AR, Dos Santos JN, Handa K, Hakomori SI (2008) Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway. Proc Natl Acad Sci USA 105(6):1925–1930. doi:10.1073/pnas.0709619104

Toledo MS, Suzuki E, Handa K, Hakomori S (2004) Cell growth regulation through GM3-enriched microdomain (glycosynapse) in human lung embryonal fibroblast WI38 and its oncogenic transformant VA13. J Biol Chem 279(33):34655–34664. doi:10.1074/jbc.M403857200

Toledo MS, Suzuki E, Handa K, Hakomori S (2005) Effect of ganglioside and tetraspanins in microdomains on interaction of integrins with fibroblast growth factor receptor. J Biol Chem 280(16):16227–16234. doi:10.1074/jbc.M413713200

Ullrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61(2):203–212

Veracini L, Simon V, Richard V, Schraven B, Horejsi V, Roche S, Benistant C (2008) The Csk-binding protein PAG regulates PDGF-induced Src mitogenic signaling via GM1. J Cell Biol 182(3):603–614

Wada T, Hata K, Yamaguchi K, Shiozaki K, Koseki K, Moriya S, Miyagi T (2007) A crucial role of plasma membrane-associated sialidase in the survival of human cancer cells. Oncogene 26 (17):2483–2490. doi:10.1038/sj.onc.1210341

Watanabe M, Ohishi T, Kuzuoka M, Nudelman ED, Stroud MR, Kubota T, Kodairo S, Abe O, Hirohashi S, Shimosato Y et al (1991) In vitro and in vivo antitumor effects of murine monoclonal antibody NCC-ST-421 reacting with dimeric Le(a) (Le(a)/Le(a)) epitope. Cancer Res 51(8):2199–2204

Weis FM, Davis RJ (1990) Regulation of epidermal growth factor receptor signal transduction. Role of gangliosides. J Biol Chem 265(20):12059–12066

Wiegandt H (1985) Glycolipids. New comprehensive biochemistry, vol 10. Elsevier, Amsterdam/New York

Yang HJ, Hakomori SI (1971) A sphingolipid having a novel type of ceramide and lacto-N-fucopentaose 3. J Biol Chem 246(5):1192–1200

Yarden Y, Ullrich A (1988) Growth factor receptor tyrosine kinases. Annu Rev Biochem 57:443–478. doi:10.1146/annurev.bi.57.070188.002303

Yates AJ, Rampersaud A (1998) Sphingolipids as receptor modulators. An overview. Ann NY Acad Sci 845:57–71

Yoon SJ, Nakayama K, Hikita T, Handa K, Hakomori SI (2006) Epidermal growth factor receptor tyrosine kinase is modulated by GM3 interaction with N-linked GlcNAc termini of the receptor. Proc Natl Acad Sci USA 103(50):18987–18991. doi:10.1073/pnas.0609281103

Yu RK, Tsai YT, Ariga T (2012) Functional roles of gangliosides in neurodevelopment: an overview of recent advances. Neurochem Res 37(6):1230–1244. doi:10.1007/s11064-012-0744-y

Zhou Q, Hakomori S, Kitamura K, Igarashi Y (1994) GM3 directly inhibits tyrosine phosphorylation and de-N-acetyl-GM3 directly enhances serine phosphorylation of epidermal growth factor receptor, independently of receptor-receptor interaction. J Biol Chem 269(3):1959–1965

Chapter 6
Nutrient Regulation of Cancer Cells by O-GlcNAcylation

Xin Liu and Gerald W. Hart

Abstract O-linked β-N-acetylglucosamine (O-GlcNAc) is a ubiquitous and dynamic posttranslational modification that occurs on serine/threonine residues of nuclear and cytoplasmic proteins. This modification is regulated by O-GlcNAc transferase (OGT), which attaches O-GlcNAc to proteins and O-GlcNAcase (OGA), which removes O-GlcNAc. O-GlcNAc serves as a nutrient sensor to regulate virtually all cellular processes, as well as playing roles in various diseases, including Alzheimer's disease, diabetes, and cancer. In this chapter, we present an overview of O-GlcNAcylation in different kinds of cancer.

Keywords O-GlcNAc • OGT • OGA • Cancer • O-GlcNAc transferase • O-GlcNAcase • Nutrients

6.1 O-GlcNAc and Enzymes Controlling Its Cycling

6.1.1 O-GlcNAc

O-GlcNAc is distinct from the other common forms of protein glycosylation in several major respects (Torres and Hart 1984). It occurs both on nuclear and cytoplasmic proteins of the cell (Hart 1997). The GlcNAc is generally not modified to form more complex structures (Comer and Hart 2000). It is attached and removed multiple times in the life of a polypeptide. In terms of its dynamics and functions, O-GlcNAcylation is more similar to protein phosphorylation than it is to classical protein glycosylation.

O-GlcNAc, first characterized in 1983 (Torres and Hart 1984), is an O-linked β-N-acetylglucosamine moiety attached to the side chain hydroxyl of a serine or threonine residue. O-GlcNAc has thus far been reported on over 3000 cytoplasmic and nuclear proteins. The addition of O-GlcNAc to proteins is catalyzed by O-GlcNAc transferase (OGT) (Haltiwanger et al. 1992; Kreppel and Hart 1999;

X. Liu • G.W. Hart (✉)
Department of Biological Chemistry, School of Medicine, Johns Hopkins University, WBSB515, 725N. Wolfe St., Baltimore, MD 21205, USA
e-mail: gwhart@jhmi.edu

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_6

Lubas and Hanover 2000), while the saccharide's removal is catalyzed by O-linked N-acetyl-β-D-glucosaminidase (O-GlcNAcase, OGA (Comtesse et al. 2001; Gao et al. 2001). This dynamic and reversible modification is emerging as a key regulator of various cellular processes, such as signal transduction (Wells et al. 2001), transcription (Ozcan et al. 2010), cell cycle progression (Drougat et al. 2012), and protein-protein interaction (Lim and Chang 2010), documenting its importance in many basic cellular and disease processes.

It has been demonstrated that O-GlcNAc plays important roles in some human diseases, such as cancer (Caldwell et al. 2010; Gu et al. 2010; Slawson et al. 2010; Lynch et al. 2012; Fardini et al. 2013), diabetes (Akimoto et al. 2005; Dias and Hart 2007; Slawson et al. 2010), and neurological disorders (Lefebvre et al. 2003). Several key oncogene and tumor suppressor proteins involved in tumorigenesis and cancer progression have been identified to be O-GlcNAcylated, such as p53 (Shaw et al. 1996) and c-Myc (Chou et al. 1995).

6.1.2 O-GlcNAc Transferase (OGT)

OGT catalyzes the addition of a single GlcNAc moiety to serine or threonine residues on proteins (Haltiwanger et al. 1990). In mammals, OGT is expressed in all cell types, with the highest level of expression in the pancreas followed by the brain (Lubas et al. 1997; Hanover et al. 1999). OGT itself is modified by O-GlcNAc and phosphorylation. OGT exists in three forms: two nucleocytoplasmic forms and one mitochondrial form (Love et al. 2003). In many tissues, OGT is composed of two 110 kDa subunits and one single 78 kDa subunit. However, the ratios of each type of subunit appear to vary depending upon the tissue.

Until now, only one single OGT gene has been identified (Shafi et al. 2000) in mammals, which is highly conserved through evolution. The 110 kDa OGT protein can be divided into two distinct domains, the amino-terminal half of the protein containing multiple tetratricopeptide repeats (TPRs) and the carboxyl-terminal half of the protein containing the catalytic domain of the enzyme. TPRs are found in a large number of proteins of diverse functions, where they serve as protein interaction sites to play a role in modulating a variety of cellular processes, including cell cycle (Hirano et al. 1990; Lamb et al. 1994; Tugendreich et al. 1995), transcription regulation (Schultz et al. 1990; Rameau et al. 1994; Tzamarias and Struhl 1995), and protein transport (Haucke et al. 1996; Goebl and Yanagida 1991).

The mechanism of how OGT recognizes and glycosylates its protein substrates remains largely unknown. However, over the past few years, there are numbers of advances in the study of its structural and kinetic properties that may yield some ideas to us. Two crystal structures of human OGT have been reported, one is a binary complex with UDP (2.8A° resolution) and the other is a ternary complex with UDP and a peptide substrate (1.95A°) which indicated that OGT employs an ordered bi-bi kinetic mechanism where UDP-GlcNAc might bind first followed by the substrate (Lazarus et al. 2011). Posttranslational modifications involving tyrosine kinases, nitrosylation of cysteine residues, and O-GlcNAc modification may

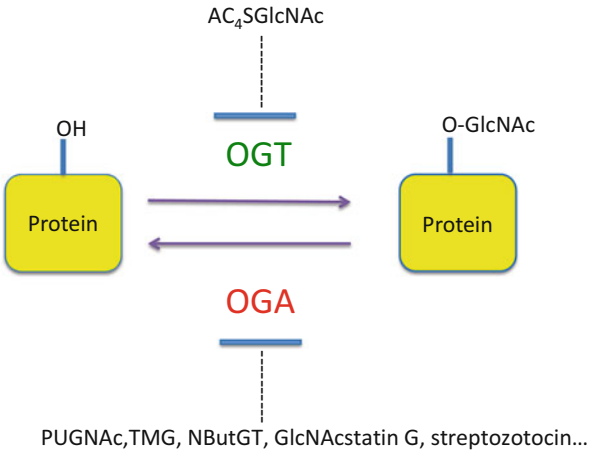

Fig. 6.1 Inhibitors of O-GlcNAc cycling enzymes that have proven useful in biological studies

also regulate OGT activity (Shen et al. 2012). Most recently, it has been found out that host cell factor-1 is cleaved by OGT when the TPR domain of OGT binds to the carboxyl-terminal portion of an HCF-1 proteolytic repeat (Lazarus et al. 2013).

Ac_4-S-GlcNAc (Fig. 6.1), which can penetrate into and be converted to its active form UDP-S-GlcNAc via the GlcNAc pathway, can be used as an OGT inhibitor (Dorfmueller et al. 2011; Gloster et al. 2011).

6.1.3 O-Linked N-Acetyl-β-D-Glucosaminidase (O-GlcNAcase, OGA)

OGA catalyzes the removal of O-GlcNAc from proteins. It is localized mainly to the cytoplasm but is also found within nuclei and mitochondria (Gao et al. 2001; Wells et al. 2002). OGA consists of two main domains: an N-terminal domain with glycoside hydrolase activity and a C-terminal histone acetyltransferase (HAT) domain. These domains flank a region containing a caspase-3 cleavage site (Butkinaree et al. 2008). Analogous to OGT, the highest expression OGA occurs in the pancreas and brain (Dong and Hart 1994; Gao et al. 2001; Whelan and Hart 2003). The HAT domain of OGA likely serves to target the enzyme to transcriptional machinery, but does not appear to have HAT enzymatic activity.

Several OGA inhibitors have been developed to study the biological roles of O-GlcNAc (Fig. 6.1). O-(2-acetamido-2-deoxy-D-glucopyranoseylidene) amino N-phenyl carbamate (PUGNAc), GlcNAcstatin, and Thiamet G are three inhibitors found to effectively limit OGA activity (Banerjee et al. 2013a).

6.2 O-GlcNAc: A Nutrient Sensor

The well-known "Warburg effect" or aerobic glycolysis in which a cancer cell relies mainly on glycolysis instead of oxidative phosphorylation, even when there is high oxygen tension, was first described in 1956 by Otto Warburg (1956a, b). It is now well known that glycolysis is much less efficient in producing energy compared to oxidative phosphorylation. Since cancer cells often have a higher proliferation rate and higher needs for metabolic precursors, the uptake of glucose has to be increased in order to meet the basic needs of the cancer cell.

In most cells, about 2–5 % of glucose is used in the hexosamine biosynthetic pathway (HBP). The end product of the HBP is UDP-GlcNAc, the donor substrate used by OGT in the enzymatic addition of O-GlcNAc. Increased cancer cell glucose uptake likely also drives increased HBP flux that leads to hyper-O-GlcNAcylation. Indeed, increased protein O-GlcNAcylation has been observed in all types of cancer thus far (Shi et al. 2010; Li et al. 2011; Krzeslak 2012a, b; Lynch et al. 2012; Rozanski et al. 2012; Zhu et al. 2012).

6.3 O-GlcNAc and Cancer

Increased O-GlcNAcylation and changes in OGT/OGA expression have been described in many different cancer types including breast, prostate, liver, pancreatic, colorectal, bladder, lung, colon, ovarian, and chronic lymphocytic leukemia (Slawson and Hart 2011; Fardini et al. 2013; Ma and Vosseller 2013) (Table 6.1).

6.3.1 Breast Cancer

Breast cancer remains a major clinical problem worldwide. Most patients succumb to the disease as a result of the metastatic spread of their primary tumor (Chambers et al. 2001; Steeg 2006). Early in the disease process many of these tumors are fueled by estrogen. Estrogen receptors are dynamically modified by O-GlcNAc (Jiang and Hart 1997). Early studies by Slawson et al. documented increased OGA activity in primary breast tumors as compared to matched adjacent breast tissues (Slawson et al. 2001). Donadio et al. showed that glutaminase inhibition greatly reduces glucose:fructose amidotransferase (GFAT) activity, the first committed step in the HBP, and changes the O-GlcNAc pattern of key proteins that control cell proliferation and differentiation (Donadio et al. 2008).

Caldwell et al. showed that OGT and O-GlcNAc levels are elevated in breast cancer cells and that reducing high O-GlcNAcylation inhibits cancer cell growth in vitro and in vivo and also reduces breast cancer cell invasion. They further found that targeted deletion of OGT inhibited the growth of tumor cells and was

Table 6.1 Summary of O-GlcNAc and cancer

Cancer type	O-GlcNAc	OGT	OGA (MGEA5)
Breast cancer	Elevated in cancer, reducing high O-GlcNAcylation inhibits cancer cell growth and invasion; the inhibition of O-GlcNAcylation may improve the sensitivity of some breast cancers to tamoxifen therapy	Elevated in cancer, deletion of OGT inhibited the growth of tumor cell; OGT knockdown inhibition of the anchorage-independent growth in vitro	Increased activity in primary breast tumors, the expression is decreased
Prostate cancer	Elevated in cancer	Elevated in cancer, reducing OGT expression inhibited metastasis to bone; c-MYC is a key target of OGT function	Elevated in cancer
Liver cancer	Elevated in cancer; in vitro assays demonstrated that O-GlcNAcylation plays important roles in migration, invasion, and viability of HCC cells	Not determined	Lower OGA expression level was a prognostic factor for predicting tumor recurrence in HCC
Pancreatic cancer	Hyper-O-GlcNAcylation has also been observed in pancreatic ductal adenocarcinoma cell (PDAC). Reducing O-GlcNAcylation inhibited PDAC cell growth and tumor formation	Not determined	Not determined
Colorectal cancer	Elevated in metastatic colorectal cancer cell line and increased in primary colorectal cancer tissues	OGT levels are increased in primary colorectal cancer tissues	Lower levels of OGA expression in metastatic colorectal cancer cell line, OGA silencing altered the expression of about 1300 genes
Bladder cancer	Not determined	OGT transcript levels were significantly higher in grades II and III in comparison to grade I BC	Poorly differentiated bladder cancer (grade III) showed significantly lower MGEA5 expression than grade I tumors
Other cancers 1. Leukemia	1.Chronic lymphocytic leukemia (CLL) cells expressed high levels of O-GlcNAcylated proteins	2.The migration ability of HO-8910PM cells was significantly inhibited by OGT silencing	2. The migration of OVCAR3 cells was dramatically enhanced by OGA inhibition

(continued)

Table 6.1 (continued)

Cancer type	O-GlcNAc	OGT	OGA (MGEA5)
2. Ovarian 3. Lung	2. O-GlcNAcylation was enhanced in more metastatic human ovarian cancer cell line 3. Elevated in cancer tissues	3. Elevated in cancer tissues	

associated with reduction in the FoxM1 transcriptional target MMP-2 (Caldwell et al. 2010). Additional studies, using immunohistochemistry analysis, observed that the global O-GlcNAcylation levels in breast tumor tissues were significantly elevated compared to the corresponding adjacent normal tissue (Gu et al. 2010). Krzeslak and coworkers showed that the expression of MGEA5 (O-GlcNAcase; OGA) decreased while the expression of OGT increased in higher-grade tumors, suggesting that increased O-GlcNAcylation might be implicated in breast tumor progression and metastasis (Krzeslak et al. 2012a).

By using 2D O-GlcNAc immunoblotting and LC-MS/MS analysis, Champattanachai and colleagues identified 29 proteins (Champattanachai et al. 2013), seven of which are O-GlcNAcylated or associated with O-GlcNAcylation in cancer. Moreover, OGT knockdown revealed that decreasing O-GlcNAcylation was related to inhibition of anchorage-independent growth in vitro. Altogether the results indicate that aberrant protein O-GlcNAcylation is associated with breast cancer. Huang et al. identified that the actin-binding protein cofilin is O-GlcNAcylated at Ser108 and further showed that during three-dimensional invasion, O-GlcNAcylation of cofilin is required for its localization to invadopodia (Huang et al. 2013).

Most recently, Kanwal et al. noticed that increased O-GlcNAcylation protected MCF-7 cells from death induced by tamoxifen; in contrast, inhibition of OGT expression enhanced the ability of tamoxifen to induce cell death. The results indicate that the inhibition of O-GlcNAcylation may improve the sensitivity of some breast cancers to tamoxifen therapy (Kanwal et al. 2013).

6.3.2 Prostate Cancer

Prostate cancer is the most common type of non-cutaneous cancer found in American men and the second leading cause of cancer death behind lung cancer. One in six men will get prostate cancer during his lifetime and one man in 36 will die of this disease. Despite the enormity of these statistics, prostate cancer remains a relatively understudied disease with respect to its biology and molecular mechanisms of action (Chunthapong et al. 2004).

It has been found that OGT is overexpressed in prostate cancer tissue compared to normal prostate epithelium and the expression of OGT and levels of O-GlcNAc

modifications are elevated in prostate cancer cell lines compared to non-transformed prostate cells. In addition, reducing OGT levels inhibits VEGF expression and the angiogenic potential of PC3-ML cells, which is dependent on the transcription factor FoxM1. Finally, reducing OGT expression in human prostate cancer cells inhibited metastasis to bone. Thus, OGT is positioned as a novel target for therapeutic intervention in the treatment of human prostate cancer (Lynch et al. 2012).

Itkonen et al. found that expression of c-MYC and OGT was tightly correlated in human prostate cancer samples. Moreover, they identified c-MYC as an upstream regulator of OGT target genes and OGT inhibition decreased the c-MYC protein level, which suggests that HBP acts as a modulator of prostate cancer growth and c-MYC as a key target of OGT function in prostate cancer cells (Itkonen et al. 2013).

Recently, our lab also found out that the level of O-GlcNAc and its enzymes is increased in prostate cancer cells compared to normal cells. Through in vitro assays, the results indicate that O-GlcNAc and its cycling might be an important factor during the progression of prostate cancer (Liu et al., unpublished).

6.3.3 Liver Cancer

In 2007, it was reported that in human hepatoma cells (HCC) protein O-GlcNAcylation modulates the promoter activities of the transcription factors CRE and activation protein-1 (AP-1) and enhances E-selectin protein expression (Azuma et al. 2007).

Guo et al. observed that O-GlcNAcylation of HSP27 in HCC cells might be a novel regulatory mode of HSP27 function, particularly for its entry into the nucleus. Crosstalk or interplay between glycosylation and phosphorylation of HSP27 could regulate its subcellular localization and biological functions in liver cancer (Guo et al. 2012). Zhu et al. found that global O-GlcNAcylation levels were significantly elevated in HCC tissues compared to that in healthy ones. Global O-GlcNAcylation was also enhanced in the tumor tissues of patients who had suffered from HCC recurrence after liver transplant compared with those who had not. Moreover, in vitro assays demonstrated that O-GlcNAcylation plays important roles in migration, invasion, and viability of HCC cells, partly through regulating E-cadherin, MMP1, MMP2, and MMP3 expression. Most importantly, a lower OGA expression level was a prognostic factor for predicting tumor recurrence in HCC (Zhu et al. 2012).

6.3.4 Pancreatic Cancer

Pancreatic cancer is the fourth most prevalent cancer-related cause of death in the United States. Most pancreatic cancer patients have glucose intolerance or diabetes. Interestingly, the pancreatic β-cells, which secrete insulin, have high levels of O-GlcNAc. The β-cell is unique in containing much more OGT than any other cell type (Konrad and Kudlow 2002). Park et al. revealed that increasing O-GlcNAcylation protein levels were accompanied by enhanced apoptosis in pancreatic β-cells, and they also identified ten new O-GlcNAcylated proteins (Park et al. 2007). By using mass spectrometry, Kang and coworkers found that Ser473 in Akt1 may be modified with O-GlcNAc, and that O-GlcNAc modification and phosphorylation of Ser473 are reciprocally regulated by hyperglycemic treatment in murine β-pancreatic cells (Kang et al. 2008).

Banerjee et al. partially elucidated the mechanism of action of triptolide, a bioactive ingredient in traditional Chinese medicine that has anticancer properties. They showed that triptolide-induced downregulation of HSP70, which leads to cell death, is mediated by impaired O-GlcNAc modification of Sp1 in pancreatic cancer. Triptolide decreases the expression and activity of OGT in these cells, resulting in reduced Sp1 translocation to the nucleus and reduced Sp1 activity. In turn, Sp1 leads to lower expression of HSF1 and other HSPs, finally resulting in tumor cell death (Banerjee et al. 2013b).

Increased HBP flux and hyper-O-GlcNAcylation has also been observed in pancreatic ductal adenocarcinoma cell (PDAC). Reducing O-GlcNAcylation inhibited PDAC cell growth and tumor formation, but did not affect the growth of non-transformed pancreatic epithelial cells. They also found that the NF-κB p65 subunit and kinases IKK α/IKK β were O-GlcNAc modified in PDAC. Reduction of PDAC hyper-O-GlcNAcylation inhibited constitutive NF-κB activity, while elevation of O-GlcNAc activated NF-κB and suppressed apoptosis (Ma et al. 2013).

6.3.5 Colorectal Cancer

Yehezkel and colleagues noticed that the metastatic colorectal cancer cell line, SW620, exhibited higher levels of O-GlcNAcylation and lower levels of OGA expression compared with its parent line, SW480. Elevating O-GlcNAcylation levels through RNA interference of OGA resulted in phenotypic alterations that included acquisition of a fibroblast-like morphology. Microarray analysis revealed that OGA silencing altered the expression of about 1300 genes, most of which are involved in cell movement and growth and specifically affected metabolic pathways of lipids and carbohydrates (Yehezkel et al. 2012).

Very recent studies have documented that O-GlcNAcylation and OGT levels are increased in primary colorectal cancer tissues. Using immunoblotting and LC-MS/MS analysis, 16 proteins were successfully identified and eight proteins showed an

increase in O-GlcNAcylation. Among all the identified proteins, annexin A2 was further confirmed to show increased O-GlcNAcylation in all cancer samples. The results indicate that aberrant O-GlcNAcylation of proteins is associated with colorectal cancer and O-GlcNAc-modified proteins may provide novel biomarkers for cancer.

6.3.6 Bladder Cancer (BC)

Cyclophosphamide-induced cystitis is an established model for the study of bladder injury and wound healing. In 2000, the first study was reported on the alterations in O-GlcNAcylation in bladders with cyclophosphamide-induced cystitis. They concluded that O-GlcNAcylation may have a significant role in the bladder wound healing process (Chung et al. 2010). Rozanski and colleagues analyzed mRNA expression of genes encoding enzymes involved in O-GlcNAcylation using samples in urine obtained from 176 bladder cancer (BC) patients and 143 healthy persons. OGT expression was not detected in the urine of healthy persons but it was found in 51.7 % of BC samples. Positive expression of the MGEA5 gene, encoding OGA, was found in urine of both healthy persons (47.1 %) and BC patients (52.3 %). Poorly differentiated BC (grade III) showed significantly lower MGEA5 expression than grade I tumors. On the contrary, OGT transcript levels were significantly higher in grade II and III in comparison to grade I BC. Moreover, there were significant differences in OGT expression between early bladder cancers and invasive or advanced bladder cancers. These results suggest that analysis of urinary content of OGA and OGT may be useful for bladder cancer diagnostics (Rozanski et al. 2012).

6.3.7 Other Cancers

Changes in O-GlcNAc levels or expression of O-GlcNAc-cycling enzymes have also been described in leukemia and ovarian and lung cancers.

Shi et al. found that chronic lymphocytic leukemia (CLL) cells expressed high levels of O-GlcNAcylated proteins, including p53, c-myc, and Akt compared to normal circulating and tonsillar B cells. Also, high baseline O-GlcNAc levels associated with impaired signaling responses to TLR agonists, chemotherapeutic agents, B-cell receptor cross-linking, and mitogens were observed (Shi et al. 2010). Interestingly, while all CLL cells had higher O-GlcNAcylation, those patients with levels at the lower end of the scale had a poor prognosis, while those with the highest levels of O-GlcNAcylation had a better prognosis because their CLL cells became more indolent.

Recently Jin and coworkers found that O-GlcNAcylation was enhanced in HO-8910PM cells, which is a more metastatic human ovarian cancer cell line

compared to OVCAR3 cells. Additionally, the migration of OVCAR3 cells was dramatically enhanced by OGA inhibition, and the migration ability of HO-8910PM cells was significantly inhibited by OGT silencing. Moreover, E-cadherin, an O-GlcNAcylated protein in ovarian cancer cells, was reduced by OGA inhibition in OVCAR3 cells and elevated by OGT silencing in HO-8910PM cells (Jin et al. 2013).

O-GlcNAcylation levels and the expressions of OGT and OGA in human lung and colon cancer tissues were examined by immunohistochemistry. O-GlcNAcylation as well as OGT expression were significantly elevated in cancer tissues compared with that in the corresponding adjacent tissues. Additionally, the roles of O-GlcNAcylation in the malignancy of lung and colon cancer were investigated in vitro. The results showed that O-GlcNAcylation dramatically enhanced the anchorage-independent growth of lung and colon cancer cells and could also enhance lung and colon cancer invasion. All together, this study suggests that O-GlcNAcylation might play important roles in lung and colon cancer formation and progression and may be a valuable target for diagnosis and therapy of cancer (Li et al. 2011).

In conclusion, it is now clear that altered O-GlcNAcylation occurs in most, if not all, types of cancer. However, very little is known with respect to how O-GlcNAc contributes to the oncogenic phenotype at a mechanistic level. The possible numbers of mechanisms affected by altered O-GlcNAcylation are enormous, including altering signaling cascades, modulation of gene expression at both the transcriptional and translational levels, and by regulation of cytoskeletal dynamics, including mechanisms regulating cell adhesion and epithelial-mesenchymal transitions. Similar to phosphorylation's roles in cancer, elucidation of O-GlcNAc's roles will require focused work of many laboratories, but also these studies will undoubtedly lead to novel and powerful therapeutics which were previously unimagined.

References

Akimoto Y, Hart GW, Hirano H, Kawakami H (2005) O-GlcNAc modification of nucleocytoplasmic proteins and diabetes. Med Mol Morphol 38(2):84–91

Azuma Y, Miura K, Higai K, Matsumoto K (2007) Protein O-N-acetylglucosaminylation modulates promoter activities of cyclic AMP response element and activator protein 1 and enhances E-selectin expression on HuH-7 human hepatoma cells. Biol Pharm Bull 30(12):2284–2289

Banerjee PS, Hart GW, Cho JW (2013a) Chemical approaches to study O-GlcNAcylation. Chem Soc Rev 42(10):4345–4357

Banerjee S, Sangwan V, McGinn O, Chugh R, Dudeja V, Vickers SM, Saluja AK (2013b) Triptolide-induced cell death in Pancreatic cancer is mediated by O-GlcNAc modification of transcription factor sp1. J Biol Chem 288(47):33927–33938

Butkinaree C, Cheung WD, Park S, Park K, Barber M, Hart GW (2008) Characterization of beta-N-acetylglucosaminidase cleavage by caspase-3 during apoptosis. J Biol Chem 283(35):23557–23566

Caldwell SA, Jackson SR, Shahriari KS, Lynch TP, Sethi G, Walker S, Vosseller K, Reginato MJ (2010) Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1. Oncogene 29(19):2831–2842

Chambers AF, Naumov GN, Varghese HJ, Nadkarni KV, MacDonald IC, Groom AC (2001) Critical steps in hematogenous metastasis: an overview. Surg Oncol Clin N Am 10(2): 243–255, vii

Champattanachai V, Netsirisawan P, Chaiyawat P, Phueaouan T, Charoenwattanasatien R, Chokchaichamnankit D, Punyarit P, Srisomsap C, Svasti J (2013) Proteomic analysis and abrogated expression of O-GlcNAcylated proteins associated with primary breast cancer. Proteomics 13(14):2088–2099

Chou TY, Dang CV, Hart GW (1995) Glycosylation of the c-Myc transactivation domain. Proc Natl Acad Sci USA 92(10):4417–4421

Chung S, Kang DO, Yamzon J, Warburton D, Koh CJ (2010) O-GlcNAc mediated glycosylation down-regulation in mice with cyclophosphamide induced cystitis. J Urol 183(1):351–356

Chunthapong J, Seftor EA, Khalkhali-Ellis Z, Seftor RE, Amir S, Lubaroff DM, Heidger PM Jr, Hendrix MJ (2004) Dual roles of E-cadherin in prostate cancer invasion. J Cell Biochem 91(4): 649–661

Comer FI, Hart GW (2000) O-Glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate. J Biol Chem 275(38):29179–29182

Comtesse N, Maldener E, Meese E (2001) Identification of a nuclear variant of MGEA5, a cytoplasmic hyaluronidase and a beta-N-acetylglucosaminidase. Biochem Biophys Res Commun 283(3):634–640

Dias WB, Hart GW (2007) O-GlcNAc modification in diabetes and Alzheimer's disease. Mol Biosyst 3(11):766–772

Donadio AC, Lobo C, Tosina M, de la Rosa V, Martin-Rufian M, Campos-Sandoval JA, Mates JM, Marquez J, Alonso FJ, Segura JA (2008) Antisense glutaminase inhibition modifies the O-GlcNAc pattern and flux through the hexosamine pathway in breast cancer cells. J Cell Biochem 103(3):800–811

Dong DL, Hart GW (1994) Purification and characterization of an O-GlcNAc selective N-acetyl-beta-D-glucosaminidase from rat spleen cytosol. J Biol Chem 269(30):19321–19330

Dorfmueller HC, Borodkin VS, Blair DE, Pathak S, Navratilova I, van Aalten DM (2011) Substrate and product analogues as human O-GlcNAc transferase inhibitors. Amino Acids 40(3):781–792

Drougat L, Olivier-Van Stichelen S, Mortuaire M, Foulquier F, Lacoste AS, Michalski JC, Lefebvre T, Vercoutter-Edouart AS (2012) Characterization of O-GlcNAc cycling and proteomic identification of differentially O-GlcNAcylated proteins during G1/S transition. Biochim Biophys Acta 1820(12):1839–1848

Fardini Y, Dehennaut V, Lefebvre T, Issad T (2013) O-GlcNAcylation: a new cancer hallmark? Front Endocrinol (Lausanne) 4:99

Gao Y, Wells L, Comer FI, Parker GJ, Hart GW (2001) Dynamic O-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic beta-N-acetylglucosaminidase from human brain. J Biol Chem 276(13):9838–9845

Gloster TM, Zandberg WF, Heinonen JE, Shen DL, Deng L, Vocadlo DJ (2011) Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells. Nat Chem Biol 7(3): 174–181

Goebl M, Yanagida M (1991) The TPR snap helix: a novel protein repeat motif from mitosis to transcription. Trends Biochem Sci 16(5):173–177

Gu Y, Mi W, Ge Y, Liu H, Fan Q, Han C, Yang J, Han F, Lu X, Yu W (2010) GlcNAcylation plays an essential role in breast cancer metastasis. Cancer Res 70(15):6344–6351

Guo K, Gan L, Zhang S, Cui FJ, Cun W, Li Y, Kang NX, Gao MD, Liu KY (2012) Translocation of HSP27 into liver cancer cell nucleus may be associated with phosphorylation and O-GlcNAc glycosylation. Oncol Rep 28(2):494–500

Haltiwanger RS, Holt GD, Hart GW (1990) Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins. Identification of a uridine diphospho-N-acetylglucosamine:peptide beta-N-acetylglucosaminyltransferase. J Biol Chem 265(5):2563–2568

Haltiwanger RS, Blomberg MA, Hart GW (1992) Glycosylation of nuclear and cytoplasmic proteins. Purification and characterization of a uridine diphospho-N-acetylglucosamine:polypeptide beta-N-acetylglucosaminyltransferase. J Biol Chem 267(13):9005–9013

Hanover JA, Lai Z, Lee G, Lubas WA, Sato SM (1999) Elevated O-linked N-acetylglucosamine metabolism in pancreatic beta-cells. Arch Biochem Biophys 362(1):38–45

Hart GW (1997) Dynamic O-linked glycosylation of nuclear and cytoskeletal proteins. Annu Rev Biochem 66:315–335

Haucke V, Horst M, Schatz G, Lithgow T (1996) The Mas20p and Mas70p subunits of the protein import receptor of yeast mitochondria interact via the tetratricopeptide repeat motif in Mas20p: evidence for a single hetero-oligomeric receptor. EMBO J 15(6):1231–1237

Hirano T, Kinoshita N, Morikawa K, Yanagida M (1990) Snap helix with knob and hole: essential repeats in S. pombe nuclear protein nuc2+. Cell 60(2):319–328

Huang X, Pan Q, Sun D, Chen W, Shen A, Huang M, Ding J, Geng M (2013) O-GlcNAcylation of cofilin promotes breast cancer cell invasion. J Biol Chem 288(51):36418–25

Itkonen HM, Minner S, Guldvik IJ, Sandmann MJ, Tsourlakis MC, Berge V, Svindland A, Schlomm T, Mills IG (2013) O-GlcNAc transferase integrates metabolic pathways to regulate the stability of c-MYC in human prostate cancer cells. Cancer Res 73(16):5277–5287

Jiang MS, Hart GW (1997) A subpopulation of estrogen receptors are modified by O-linked N-acetylglucosamine. J Biol Chem 272(4):2421–2428

Jin FZ, Yu C, Zhao DZ, Wu MJ, Yang Z (2013) A correlation between altered O-GlcNAcylation, migration and with changes in E-cadherin levels in ovarian cancer cells. Exp Cell Res 319(10): 1482–1490

Kang ES, Han D, Park J, Kwak TK, Oh MA, Lee SA, Choi S, Park ZY, Kim Y, Lee JW (2008) O-GlcNAc modulation at Akt1 Ser473 correlates with apoptosis of murine pancreatic beta cells. Exp Cell Res 314(11–12):2238–2248

Kanwal S, Fardini Y, Pagesy P, N'Tumba-Byn T, Pierre-Eugene C, Masson E, Hampe C, Issad T (2013) O-GlcNAcylation-inducing treatments inhibit estrogen receptor alpha expression and confer resistance to 4-OH-Tamoxifen in human breast cancer-derived MCF-7 cells. PLoS One 8(7):e69150

Konrad RJ, Kudlow JE (2002) The role of O-linked protein glycosylation in beta-cell dysfunction. Int J Mol Med 10(5):535–539

Kreppel LK, Hart GW (1999) Regulation of a cytosolic and nuclear O-GlcNAc transferase. Role of the tetratricopeptide repeats. J Biol Chem 274(45):32015–32022

Krzeslak A, Forma E, Bernaciak M, Romanowicz H, Brys M (2012a) Gene expression of O-GlcNAc cycling enzymes in human breast cancers. Clin Exp Med 12(1):61–65

Krzeslak A, Wojcik-Krowiranda K, Forma E, Bienkiewicz A, Brys M (2012b) Expression of genes encoding for enzymes associated with O-GlcNAcylation in endometrial carcinomas: clinicopathologic correlations. Ginekol Pol 83(1):22–26

Lamb JR, Michaud WA, Sikorski RS, Hieter PA (1994) Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. EMBO J 13(18):4321–4328

Lazarus MB, Nam Y, Jiang J, Sliz P, Walker S (2011) Structure of human O-GlcNAc transferase and its complex with a peptide substrate. Nature 469(7331):564–567

Lazarus MB, Jiang J, Kapuria V, Bhuiyan T, Janetzko J, Zandberg WF, Vocadlo DJ, Herr W, Walker S (2013) HCF-1 is cleaved in the active site of O-GlcNAc transferase. Science 342(6163):1235–1239

Lefebvre T, Caillet-Boudin ML, Buee L, Delacourte A, Michalski JC (2003) O-GlcNAc glycosylation and neurological disorders. Adv Exp Med Biol 535:189–202

Li Y, Zeng Y, Mooney SM, Yin B, Mizokami A, Namiki M, Getzenberg RH (2011) Resistance to paclitaxel increases the sensitivity to other microenvironmental stresses in prostate cancer cells. J Cell Biochem 112(8):2125–2137

Lim K, Chang HI (2010) O-GlcNAc inhibits interaction between Sp1 and sterol regulatory element binding protein 2. Biochem Biophys Res Commun 393(2):314–318

Love DC, Kochan J, Cathey RL, Shin SH, Hanover JA (2003) Mitochondrial and nucleocytoplasmic targeting of O-linked GlcNAc transferase. J Cell Sci 116(Pt 4):647–654

Lubas WA, Hanover JA (2000) Functional expression of O-linked GlcNAc transferase. Domain structure and substrate specificity. J Biol Chem 275(15):10983–10988

Lubas WA, Frank DW, Krause M, Hanover JA (1997) O-Linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats. J Biol Chem 272(14):9316–9324

Lynch TP, Ferrer CM, Jackson SR, Shahriari KS, Vosseller K, Reginato MJ (2012) Critical role of O-Linked beta-N-acetylglucosamine transferase in prostate cancer invasion, angiogenesis, and metastasis. J Biol Chem 287(14):11070–11081

Ma Z, Vosseller K (2013) O-GlcNAc in cancer biology. Amino Acids 45(4):719–733

Ma Z, Vocadlo DJ, Vosseller K (2013) Hyper-O-GlcNAcylation is anti-apoptotic and maintains constitutive NF-kappaB activity in pancreatic cancer cells. J Biol Chem 288(21):15121–15130

Ozcan S, Andrali SS, Cantrell JE (2010) Modulation of transcription factor function by O-GlcNAc modification. Biochim Biophys Acta 1799(5–6):353–364

Park J, Kwon H, Kang Y, Kim Y (2007) Proteomic analysis of O-GlcNAc modifications derived from streptozotocin and glucosamine induced beta-cell apoptosis. J Biochem Mol Biol 40(6): 1058–1068

Rameau G, Puglia K, Crowe A, Sethy I, Willis I (1994) A mutation in the second largest subunit of TFIIIC increases a rate-limiting step in transcription by RNA polymerase III. Mol Cell Biol 14(1):822–830

Rozanski W, Krzeslak A, Forma E, Brys M, Blewniewski M, Wozniak P, Lipinski M (2012) Prediction of bladder cancer based on urinary content of MGEA5 and OGT mRNA level. Clin Lab 58(5–6):579–583

Schultz J, Marshall-Carlson L, Carlson M (1990) The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of Saccharomyces cerevisiae. Mol Cell Biol 10(9): 4744–4756

Shafi R, Iyer SP, Ellies LG, O'Donnell N, Marek KW, Chui D, Hart GW, Marth JD (2000) The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. Proc Natl Acad Sci USA 97(11):5735–5739

Shaw P, Freeman J, Bovey R, Iggo R (1996) Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. Oncogene 12(4): 921–930

Shen DL, Gloster TM, Yuzwa SA, Vocadlo DJ (2012) Insights into O-linked N-acetylglucosamine ([0–9]O-GlcNAc) processing and dynamics through kinetic analysis of O-GlcNAc transferase and O-GlcNAcase activity on protein substrates. J Biol Chem 287(19):15395–15408

Shi Y, Tomic J, Wen F, Shaha S, Bahlo A, Harrison R, Dennis JW, Williams R, Gross BJ, Walker S, Zuccolo J, Deans JP, Hart GW, Spaner DE (2010) Aberrant O-GlcNAcylation characterizes chronic lymphocytic leukemia. Leukemia 24(9):1588–1598

Slawson C, Hart GW (2011) O-GlcNAc signalling: implications for cancer cell biology. Nat Rev Cancer 11(9):678–684

Slawson C, Pidala J, Potter R (2001) Increased N-acetyl-beta-glucosaminidase activity in primary breast carcinomas corresponds to a decrease in N-acetylglucosamine containing proteins. Biochim Biophys Acta 1537(2):147–157

Slawson C, Copeland RJ, Hart GW (2010) O-GlcNAc signaling: a metabolic link between diabetes and cancer? Trends Biochem Sci 35(10):547–555

Steeg PS (2006) Tumor metastasis: mechanistic insights and clinical challenges. Nat Med 12(8): 895–904

Torres CR, Hart GW (1984) Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. J Biol Chem 259(5):3308–3317

Tugendreich S, Tomkiel J, Earnshaw W, Hieter P (1995) CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. Cell 81(2):261–268

Tzamarias D, Struhl K (1995) Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. Genes Dev 9(7):821–831

Warburg O (1956a) On respiratory impairment in cancer cells. Science 124(3215):269–270

Warburg O (1956b) On the origin of cancer cells. Science 123(3191):309–314

Wells L, Vosseller K, Hart GW (2001) Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. Science 291(5512):2376–2378

Wells L, Gao Y, Mahoney JA, Vosseller K, Chen C, Rosen A, Hart GW (2002) Dynamic O-glycosylation of nuclear and cytosolic proteins: further characterization of the nucleocytoplasmic beta-N-acetylglucosaminidase, O-GlcNAcase. J Biol Chem 277(3):1755–1761

Whelan SA, Hart GW (2003) Proteomic approaches to analyze the dynamic relationships between nucleocytoplasmic protein glycosylation and phosphorylation. Circ Res 93(11):1047–1058

Yehezkel G, Cohen L, Kliger A, Manor E, Khalaila I (2012) O-linked beta-N-acetylglucosaminylation (O-GlcNAcylation) in primary and metastatic colorectal cancer clones and effect of N-acetyl-beta-D-glucosaminidase silencing on cell phenotype and transcriptome. J Biol Chem 287(34):28755–28769

Zhu Q, Zhou L, Yang Z, Lai M, Xie H, Wu L, Xing C, Zhang F, Zheng S (2012) O-GlcNAcylation plays a role in tumor recurrence of hepatocellular carcinoma following liver transplantation. Med Oncol 29(2):985–993

Part IV
Regulation of Immune Systems and Cancers by Carbohydrates

Chapter 7
Function of Unique *O*-Glycan Structures in Protecting Gastric Mucosa Against *Helicobacter pylori* Infection and Gastric Cancer Development

Jun Nakayama

Abstract Gastric gland mucin secreted from gland mucous cells located in lower portions of the gastric mucosa contains unique *O*-linked oligosaccharides displaying terminal α1,4-linked *N*-acetylglucosamine (αGlcNAc). αGlcNAc inhibits growth and motility of *Helicobacter pylori*, a microbe causing gastric cancer, by inhibiting biosynthesis of its cell wall component cholesteryl-α-D-glucopyranoside. In addition, αGlcNAc serves as a tumor suppressor for gastric differentiated-type adenocarcinoma, and its loss in gastric cancer cells is associated with progression and poor prognosis of patients with this subtype of gastric cancer. This chapter summarizes protective functions of αGlcNAc against gastric cancer development.

Keywords α1,4-*N*-acetylglucosaminyltransferase • Gastric cancer • *Helicobacter pylori* • Knockout mouse • Mucin • Patient prognosis • Terminal α1,4-linked *N*-acetylglucosamine residue

7.1 Introduction

Gastric cancer ranks fourth in the most commonly diagnosed cancers and second in the most common causes of cancer-related death worldwide and thus remains one of the most common malignancies (Ferlay et al. 2010). On the other hand, gastric mucins play important roles in forming the surface mucous gel layer, which protects tissues from the external environment (Ota and Katsuyama 1992). However, how gastric mucins alter gastric cancer pathogenesis remains unknown. Gastric mucins are divided into surface and gland mucins (Ota et al. 1991). The first are secreted from surface mucous cells lining the gastric mucosa and contain surface mucin-specific glycans such as Lewis-related blood group carbohydrates attached

J. Nakayama (✉)
Department of Molecular Pathology, Shinshu University Graduate School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan
e-mail: jnaka@shinshu-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_7

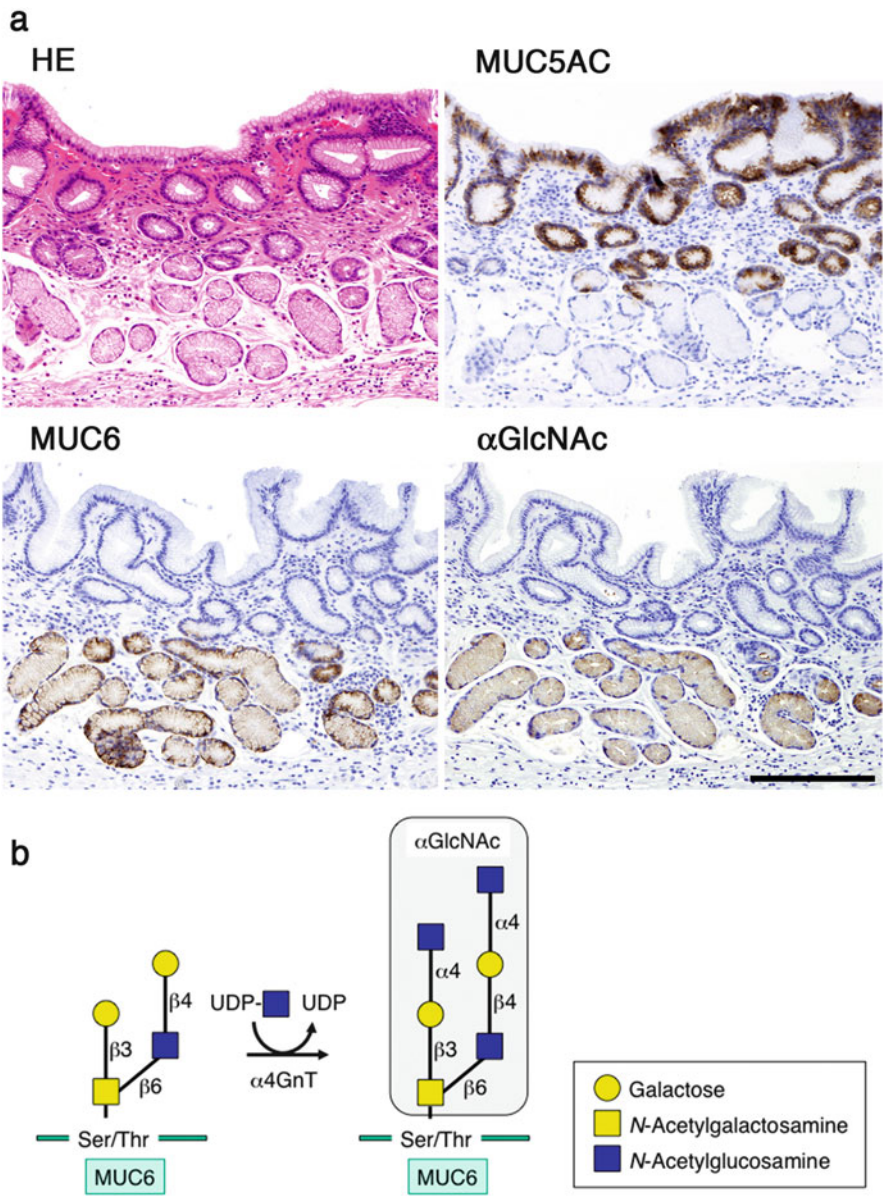

Fig. 7.1 Expression of MUC5AC, MUC6, and αGlcNAc in gastric mucosa and the αGlcNAc biosynthetic pathway. (**a**) MUC5AC is expressed in surface mucous cells, while MUC6 is detected in pyloric gland cells of human gastric mucosa. Note that αGlcNAc is coexpressed with MUC6 in pyloric gland cells. Hematoxylin and eosin (HE) staining (*upper left*) and immunohistochemistry using CLH2 antibody for MUC5AC (*upper right*), CLH5 antibody for MUC6 (*lower left*), and HIK1083 antibody for αGlcNAc (*lower right*). Bar indicates 200 μm. (**b**) α1,4-*N*-Acetylglucosaminyltransferase (α4GnT) forms αGlcNAc primarily attached to MUC6

to the mucin core protein MUC5AC (Nordman et al. 2002) (Fig. 7.1a). By contrast, the latter are secreted from gland mucous cells such as pyloric gland cells and mucous neck cells located in the lower layer of the gastric mucosa and contain gland mucin-specific *O*-glycans exhibiting terminal α1,4-linked *N*-acetylglucosamine residues (hereafter termed αGlcNAc) attached to MUC6 (Ishihara et al. 1996; Zhang et al. 2001; Ferreira et al. 2006). αGlcNAc is a unique *O*-glycan, as its distribution is limited to gastric gland mucous cells and Brunner's glands of the duodenal mucosa (Nakamura et al. 1998).

αGlcNAc biosynthesis is catalyzed by α1,4-*N*-acetylglucosaminyltransferase (α4GnT), which transfers *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to terminal β-linked galactose (Gal) residues present in *O*-glycans with an α1,4-linkage (Fig. 7.1b). Previously, we used an expression cloning to isolate α4GnT cDNA from a human stomach cDNA library (Nakayama et al. 1999). Then, using α4GnT cDNA as a molecular tool, we investigated αGlcNAc function in the pathogenesis of gastric cancer. In this chapter, I describe the roles of αGlcNAc in the gastric mucosa and focus in particular on its protective function against *Helicobacter pylori* (*H. pylori*) infection and gastric cancer development.

7.2 Role of αGlcNAc in *H. pylori* Infection

7.2.1 αGlcNAc Acts as a Natural Antibiotic in Antagonizing H. pylori

H. pylori is a gram-negative bacteria first isolated from gastric mucosa by Marshall and Warren (1984). This microbe is known to cause various gastric diseases, including chronic active gastritis, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (Peek and Blaser 2002). Although *H. pylori* infects over half the world's human population, only a fraction of infected patients develop severe gastric disease. Interestingly, *H. pylori* largely colonizes surface mucins, while it is rarely found in gland mucins (Hidaka et al. 2001) (Fig. 7.2a), suggesting that αGlcNAc protects the gastric mucosa against *H. pylori* infection. To test the hypothesis, we cultured *H. pylori* in the presence of various levels of recombinant soluble CD43 (sCD43) carrying αGlcNAc (hereafter termed αGlcNAc (+)) (Kawakubo et al. 2004), which were secreted from Lec2 cells, a mutant CHO cell line defective in a sialic acid transporter (Deutscher et al. 1984). That line had been cotransfected with three expression vectors encoding α4GnT, core2 β1,6-*N*-acetylglucosaminyltransferase (C2GnT) (Bierhuizen and Fukuda 1992), and sCD43, respectively. In this assay, sCD43 serves as a glycan scaffold, as it contains 80 *O*-glycosylation sites in its extracellular domain (Fukuda 1992). Surprisingly, *H. pylori* growth was suppressed in a dose-dependent manner in the presence of αGlcNAc (+) (Fig. 7.2b). In addition, we observed significantly reduced motility and abnormal morphology, such as elongation and bending in *H. pylori*

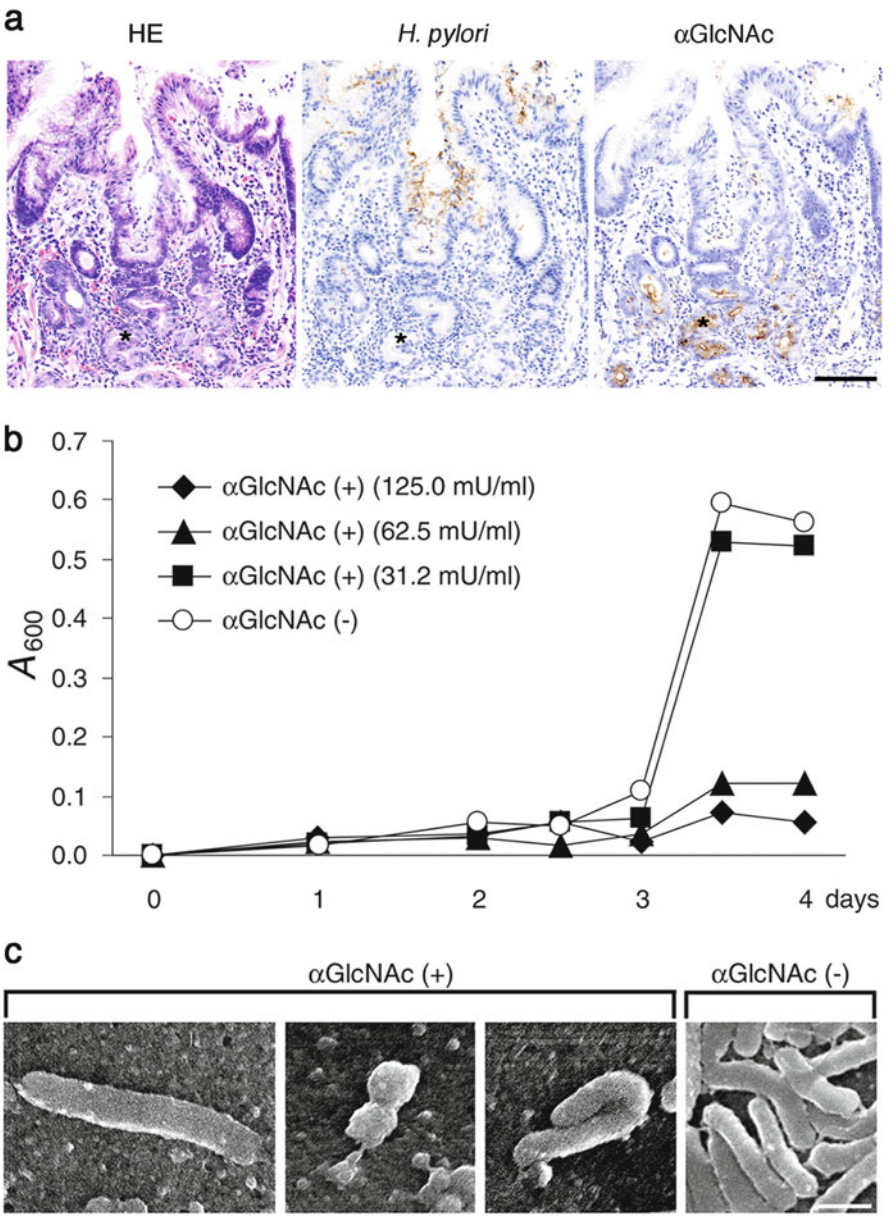

Fig. 7.2 Antimicrobial activity of αGlcNAc against *H. pylori* infection. (**a**) Histopathology of chronic active gastritis of human gastric mucosa caused by *H. pylori* infection. The microbe is rarely found in gland mucin expressing αGlcNAc (*). Hematoxylin and eosin (HE) staining (*left*), immunohistochemistry using anti-*H. pylori* antibody (*middle*), and HIK1083 antibody for αGlcNAc (*right*). Bar indicates 100 μm. (**b**) Growth curves of *H. pylori* cultured in the presence of sCD43 carrying αGlcNAc (αGlcNAc (+)) or sCD43 lacking αGlcNAc (αGlcNAc (−)). One milliunit of αGlcNAc (+) corresponds to 1 μg GlcNAcα–*p*NP. A600: absorbance at 600 nm. (**c**) Scanning electron micrographs showing *H. pylori* incubated with 31.2 mU/ml αGlcNAc (+) or the same concentration of αGlcNAc (−) protein for 3 days. *Bar* indicates 1 μm (Panels **b** and **c** are

cultured with αGlcNAc (+). By contrast, when the microbe was incubated with control sCD43 lacking αGlcNAc (hereafter termed αGlcNAc (−)) secreted from cotransfected Lec2 cells with C2GnT and sCD43 expression vectors, we did not observe these effects, indicating that αGlcNAc antagonizes *H. pylori* growth like a natural antibiotic. Similar antibiotic effects were also obtained when *H. pylori* was incubated with *p*-nitrophenyl-α-*N*-acetylglucosamine (GlcNAcα–*p*NP), recombinant soluble CD34 carrying αGlcNAc, or αGlcNAc-containing gland mucin prepared from human gastric mucosa (Kawakubo et al. 2004).

7.2.2 αGlcNAc Inhibits Cholesterol α-Glucosyltransferase Activity

To define the molecular mechanism underlying αGlcNAc antimicrobial activity, we focused in particular on morphological changes seen in *H. pylori* cultured in the presence of αGlcNAc (Fig. 7.2c) (Kawakubo et al. 2004). We noted that those changes were similar to those seen in *H. pylori* cultured in the presence of β-lactamase inhibitors (Enroth et al. 1999). Thus, we speculated that treatment with αGlcNAc had an effect on the *H. pylori* cell wall. Hirai et al. (1995) previously demonstrated that the *H. pylori* cell wall contains a unique glycolipid, cholesteryl-α-D-glucopyranoside (CGL), as well as its derivatives. CGL biosynthesis is catalyzed by cholesterol α-glucosyltransferase (αCgT), which transfers glucose (Glc) from UDP-Glc to cholesterol with an α1,3-linkage. Molecular mimicking between α1,4-linked GlcNAc in gland mucin and α1,3-linked Glc in CGL suggested that αGlcNAc suppressed αCgT enzymatic activity by an end-product inhibitory mechanism. Thus, we analyzed glycolipid fractions isolated from *H. pylori* cultured in the presence of αGlcNAc (+) or αGlcNAc (−) using mass spectrometry (Kawakubo et al. 2004). We found that CGL levels in *H. pylori* cultured with αGlcNAc (+) were significantly lower than those seen in *H. pylori* cultured with αGlcNAc (−), suggesting that αGlcNAc directly inhibits CGL biosynthesis by *H. pylori* in vivo. Subsequently, we used expression cloning to isolate *αCgT* gene from *H. pylori* (Lee et al. 2006) and proved that αCgT enzymatic activity is inhibited by core2-branched *O*-glycans displaying αGlcNAc in vitro (Lee et al. 2008). We also showed that an active form of αCgT is present in the *H. pylori* membrane fraction, suggesting that bacterial αCgT is likely accessible to αGlcNAc in gland mucin (Hoshino et al. 2011).

Fig. 7.2 (continued) from Kawakubo et al. 2004; Copyright 2004 American Association for the Advancement of Science)

7.2.3 *CGL Is Indispensible for* H. pylori *Survival*

H. pylori requires exogenous cholesterol for CGL biosynthesis. Thus to further define CGL function in *H. pylori*, we created *H. pylori* lacking CGL by culturing the microbe in the absence of cholesterol (Kawakubo et al. 2004). Resultant *H. pylori* exhibited reduced growth and motility, and all microbes died following prolonged (21-day) incubation in cholesterol-free media, indicating that CGL is indispensable for *H. pylori* survival. Overall, these results show that αGlcNAc inhibits CGL biosynthesis by *H. pylori* by suppressing αCgT, thus protecting the gastric mucosa from infection. In fact, αGlcNAc does not exhibit antimicrobial activity against bacteria lacking CGL such as *E. coli* and *S. aureus* (Kawakubo et al. 2004). Most recently, we reported that αCgT enzymatic activity in *H. pylori* was highly correlated with the degree of glandular atrophy in gastric mucosa infected by the bacteria and that the monoacyled form of cholesteryl-6-*O*-phosphatidyl-α-D-glucopyranoside (CPG), a minor constituent of CGL derivatives in the *H. pylori* cell wall, is the most potent antigen for invariant natural killer T cells, thus eliciting an immune response in gastric mucosa (Ito et al. 2013).

7.3 αGlcNAc Serves as a Tumor Suppressor for Gastric Cancer

7.3.1 *αGlcNAc Suppresses Tumorigenesis of Gastric Differentiated-Type Adenocarcinoma*

We then asked whether αGlcNAc had a more general or broader protective function in the gastric mucosa. To address to this question, we generated mice deficient in α4GnT by disrupting the *A4gnt* gene and analyzed αGlcNAc function in vivo (Karasawa et al. 2012). Immunohistochemistry using the αGlcNAc-specific antibody HIK1083 and MALDI-TOF-MS analyses revealed that *A4gnt*-deficient mice showed a complete lack of αGlcNAc expression in gastric gland mucin and duodenal Brunner's gland, formally establishing that α4GnT is the sole enzyme catalyzing addition of αGlcNAc to *O*-glycans in vivo (Fig. 7.3). Surprisingly, *A4gnt*-deficient mice, even in the absence of *H. pylori* infection, spontaneously developed tumor in the antrum as early as 5 weeks of age, and tumor size gradually increased as mice aged (Fig. 7.4a). Histopathology of tumors revealed that the mutant mice exhibited hyperplasia by 5 weeks of age, low-grade dysplasia by 10 weeks, and high-grade dysplasia by 20 weeks in the glandular stomach (Fig. 7.4b). In 30-week-old mice, gastric adenocarcinoma developed in 2 of 6 *A4gnt*-deficient mice, and adenocarcinoma incidence increased by 50 weeks of age. Furthermore, all 50- and 60-week-old mice exhibited gastric adenocarcinoma. These pathologies were consistently seen in the antrum of the glandular stomach, and cancer cells were mostly restricted to the mucosa. No sign of distant metastasis

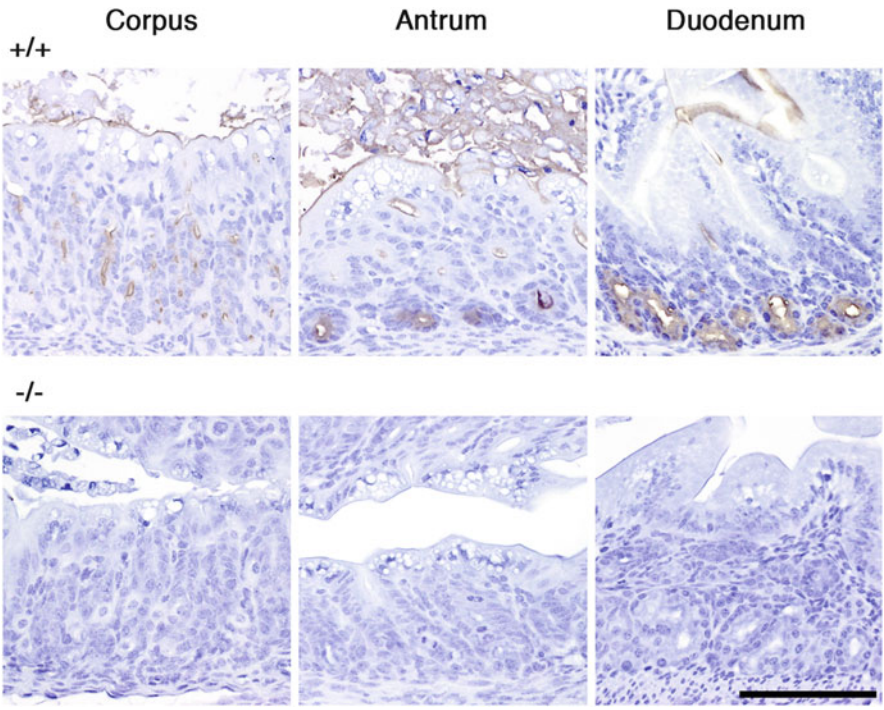

Fig. 7.3 αGlcNAc loss in *A4gnt*-deficient mice. αGlcNAc is expressed in mucous neck cells of the gastric corpus, pyloric gland cells of the gastric antrum, and Brunner's glands of the duodenum of wild-type mouse (+/+), while it is completely absent in these mucous cells of *A4gnt*-deficient mouse (−/−). Shown is immunohistochemistry of gastroduodenal mucosa from 1-week-old mice with αGlcNAc-specific HIK1083 antibody. Bar indicates 100 μm

was noted up to 60 weeks of age. Gastric adenocarcinoma is largely classified into differentiated (or intestinal) or undifferentiated (or diffuse) types, based on tumor cell morphology and histogenesis background (Lauren 1965; Nakamura et al. 1968). Interestingly, the gastric adenocarcinoma seen in *A4gnt*-deficient mice was only of the differentiated type, while undifferentiated-type adenocarcinoma, such as signet ring cell carcinoma, never arose. This indicates that mutant mice develop gastric differentiated-type adenocarcinoma through a hyperplasia-dysplasia-carcinoma sequence in the absence of *H. pylori* infection. Intestinal metaplasia was rarely detected in gastric mucosa of either *A4gnt*-deficient or wild-type mice during the 60-week observation period, indicating that metaplasia is not associated with gastric tumorigenesis in this model. These results establish that αGlcNAc serves as a tumor suppressor for gastric differentiated-type adenocarcinoma.

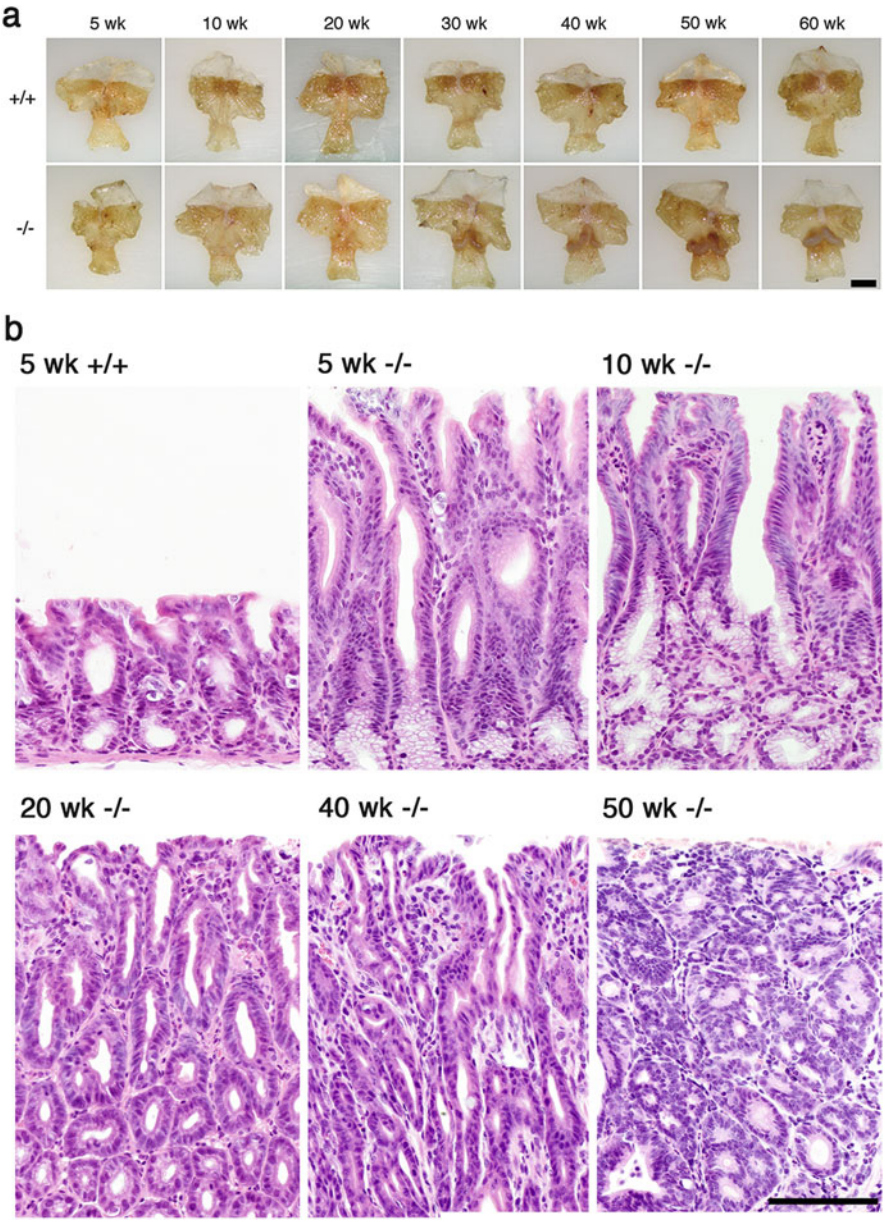

Fig. 7.4 Gastric pathology of *A4gnt*-deficient mice. (**a**) Macroscopic appearance of the stomach removed from wild-type mice (+/+) and *A4gnt*-deficient mice (−/−). Bar indicates 5 mm. (**b**) Representative histopathology showing hyperplasia at 5 weeks, low-grade dysplasia at 10 weeks, high-grade dysplasia at 20 weeks, and differentiated-type adenocarcinoma at 40 and 50 weeks in the antral mucosa of *A4gnt*-deficient mice. For comparison, pyloric mucosa from a 5-week-old wild-type mouse is shown (*upper left*). Bar indicates 100 μm (Panel **a** is from Karasawa et al. 2012; Copyright 2012 The American Society for Clinical Investigation)

7.3.2 *αGlcNAc Suppresses Tumor-Promoting Inflammation in* A4gnt*-Deficient Mice*

It remained unclear why *A4gnt*-deficient mice develop only differentiated-type adenocarcinoma in the gastric mucosa. To clarify molecular mechanisms underlying such a specific tumor suppression function by αGlcNAc, we carried out microarray analysis followed by quantitative RT-PCR using mRNA derived from gastric mucosa of *A4gnt*–deficient and wild-type mice at 5, 10, and 50 weeks of age (Karasawa et al. 2012). Our analysis identified eight genes upregulated in *A4gnt*-deficient mice compared with wild-type mice (Fig. 7.5). Among these genes significantly upregulated in the gastric mucosa of mutant mice older than 10 weeks were those encoding inflammatory chemokine ligands such as Ccl2, Cxcl1, and Cxcl5; proinflammatory cytokines such as Il-11 and Il-1β; and growth factors such as Hgf and Fgf7. In addition, Hgf was upregulated even in 5-week-old mutant mice, indicating that altered gene expression patterns are apparent even at low-grade dysplasia stages, prior to gastric cancer development. Of the altered factors, Ccl2 is of particular interest as it attracts tumor-associated macrophages, which exert pro-tumorigenic immune responses and promote tumor angiogenesis (Grivennikov et al. 2010; Mantovani et al. 2010). In fact, both infiltration of inflammatory cells such as mononuclear cells and neutrophils and angiogenesis increased progressively in the gastric mucosa as *A4gnt*-deficient mice aged. IL-11 is also noteworthy because it functions in progression of inflammation to gastric tumorigenesis via gp130 signaling, followed by STAT3 phosphorylation (Ernst et al. 2008; Howlett et al. 2009). Taken together, our results indicate that αGlcNAc loss triggers gastric carcinogenesis through inflammation-associated pathways in vivo.

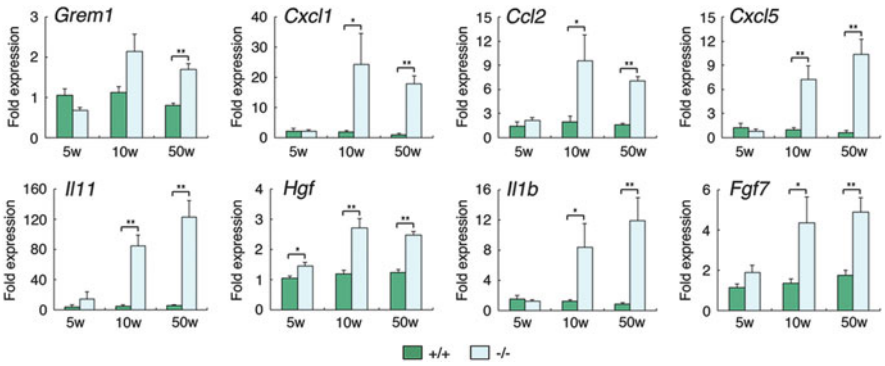

Fig. 7.5 Genes upregulated in the gastric mucosa of *A4gnt*-deficient mice compared with those in age-matched wild-type mice, as determined by quantitative RT-PCR analysis. *Grem1*, Gremlin 1; *Cxcl1*, Chemokine (C-X-C motif) ligand 1; *Ccl2*, Chemokine (C-C motif) ligand 2; *Cxcl5*, Chemokine (C-X-C motif) ligand 5; *Il11*, IL-11; *Hgf*, HGF; *Il1b*, IL-1β; *Fgf7*, FGF7. $^{*}P < 0.05$; $^{**}P < 0.01$ (From Karasawa et al. 2012; Copyright 2012 American Society for Clinical Investigation)

7.3.3 αGlcNAc Loss in Gland Mucin Is Associated with Progression of Human Gastric Differentiated-Type Adenocarcinoma

Lastly, we asked whether αGlcNAc loss occurred in human gastric adenocarcinoma and whether such loss was associated with tumor progression. To do so, we used immunohistochemistry to assess expression of αGlcNAc and its scaffold MUC6 in 214 surgically resected gastric adenocarcinomas and then compared those expression patterns with clinicopathological parameters such as vessel invasion and stage and cancer-specific survival (Shiratsu et al. 2014). MUC6 was detected in gastric cancer cells in 102 (47.6 %) of 214 patients. In differentiated-type adenocarcinoma, 33 (58.9 %) of 54 MUC6-positive cancer lacked αGlcNAc expression, while in undifferentiated-type adenocarcinoma, 22 (45.8 %) of 48 MUC6-positive cancer lacked αGlcNAc expression, indicating that there was no significant difference between absence of αGlcNAc expression in differentiated and undifferentiated tumor types. However, when the comparison was made between a subtype of undifferentiated-type adenocarcinoma, signet ring cell carcinoma, and differentiated-type adenocarcinoma (Fig. 7.6a), only 6 (26.1 %) of 23 signet ring cell carcinoma patients lacked αGlcNAc expression, significantly at lower frequency compared with differentiated-type adenocarcinoma ($P = 0.0049$). Notably, αGlcNAc loss was significantly correlated with depth of invasion, stage, venous invasion, and more importantly, poorer patient prognosis in MUC6-positive differentiated-type adenocarcinoma (Fig. 7.6b). On the other hand, no significant correlation between αGlcNAc loss in tumor cells and any clinicopathological variable or cancer-specific survival of patients with undifferentiated-type adenocarcinoma was observed. Thus, αGlcNAc loss in MUC6-positive cancer cells is significantly associated with progression and poor prognosis in differentiated-type but not undifferentiated-type adenocarcinomas of the stomach, consistent with phenotypes seen in *A4gnt*-deficient mice (Karasawa et al. 2012). As described in Sect. 7.3.2, inflammatory chemokine ligands, proinflammatory cytokines, and growth factors were upregulated in mutant mice, and these molecules are also thought to function in human gastric cancer development. For example, Ohta et al. (2003) reported that CCL2 expression by human gastric carcinoma cells increases with tumor cell invasiveness, and its expression level is positively correlated with angiogenesis and macrophage recruitment. Verbeke et al. (2012) demonstrated that CXC chemokines, including CXCL1/CXCL5, facilitate progression of gastric cancer tumors. Nakayama et al. (2007) observed that IL-11 expression is significantly higher in differentiated compared to undifferentiated types of adenocarcinoma and that IL-11 functions in gastric carcinoma progression. HGF and FGF7 play important roles in gastric epithelial proliferation. Mohri et al. (2012) suggest that HGF expression is an important prognostic factor in gastric cancer. FGF7 is upregulated

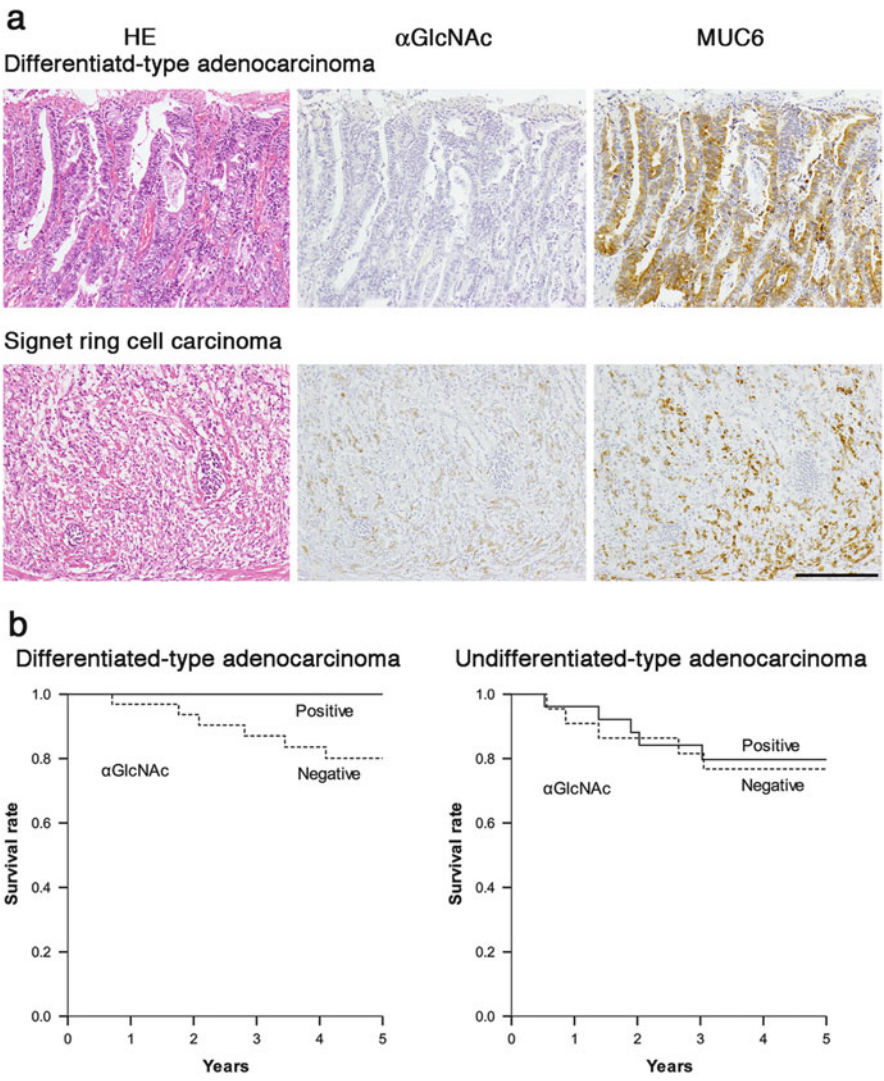

Fig. 7.6 αGlcNAc expression in human gastric adenocarcinoma and correlation with cancer-specific survival of gastric cancer patients. (**a**) Hematoxylin and eosin (HE) staining (*left*) and αGlcNAc (*middle*) and MUC6 (*left*) expression in differentiated-type adenocarcinoma (*upper panels*) and signet ring cell carcinoma (*lower panels*). Bar indicates 200 μm. (**b**) Cancer-specific survival in patients with MUC6-positive gastric cancer. In differentiated-type adenocarcinoma, patients with αGlcNAc-negative tumors had a significant poorer outcome than did patients with αGlcNAc-positive tumors ($P = 0.048$). By contrast, in undifferentiated-type adenocarcinoma, there was no significant difference in survival rate of patients harboring αGlcNAc-positive or αGlcNAc-negative tumors ($P = 0.549$) (Modified from Shiratsu et al. 2014)

by IL-1β (Palmieri et al. 2003). Kai et al. (2005) demonstrated that tumor IL-1β expression levels are elevated more than 50-fold over those seen in normal gastric mucosa and significantly higher in nonscirrhous compared with scirrhous carcinomas. Thus, all of these factors likely promote tumor-promoting inflammation. Accordingly, our results suggest that αGlcNAc loss is correlated with gastric cancer progression in inflammation-related pathways in humans. It remains to be determined how αGlcNAc loss in gastric cancer enhances tumor-promoting inflammation in the stomach. Recently, we demonstrated that reduced αGlcNAc in Barrett's esophagus could also predict its potential to develop into Barrett's adenocarcinoma (Iwaya et al. 2014).

7.4 Conclusion

We conclude that gastric gland mucin-specific αGlcNAc has a protective function against gastric cancer development in two ways: first, as a natural antibiotic against *H. pylori* and second, as a tumor suppressor for gastric differentiated-type adenocarcinoma. Based on these findings, we anticipate future development of new strategies to detect, diagnose, treat, and prevent gastric cancer.

Acknowledgments I am grateful to all collaborators for their contributions to this research. In particular, I wish to thank Dr. Tsutomu Katsuyama, Emeritus Professor of Shinshu University, Dr. Minoru Fukuda, Professor at the Sanford-Burnham Medical Research Institute, and Dr. Michiko N. Fukuda, Professor at the Sanford-Burnham Medical Research Institute, for their encouragement and collaboration, and Dr. Elise Lamar for editing this manuscript.

References

Bierhuizen MF, Fukuda M (1992) Expression cloning of a cDNA encoding UDP-GlcNAc:Gal β1-3-GalNAc-R (GlcNAc to GalNAc) β1-6GlcNAc transferase by gene transfer into CHO cells expressing polyoma large tumor antigen. Proc Natl Acad Sci USA 89:9326–9330

Deutscher SL, Nuwayhid N, Stanley P, Briles EI, Hirschberg CB (1984) Translocation across Golgi vesicle membranes: a CHO glycosylation mutant deficient in CMP-sialic acid transport. Cell 39:295–299

Enroth H, Wreiber K, Rigo R, Risberg D, Uribe A, Engstrand L (1999) In vitro aging of Helicobacter pylori: changes in morphology, intracellular composition and surface properties. Helicobacter 4:7–16

Ernst M, Najdovska M, Grail D, Lundgren-May T, Buchert M, Tye H, Matthews VB, Armes J, Bhathal PS, Hughes NR, Marcusson EG, Karras JG, Na S, Sedgwick JD, Hertzog PJ, Jenkins BJ (2008) STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. J Clin Invest 118:1727–1738

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127:2893–2917

Ferreira B, Marcos NT, David L, Nakayama J, Reis CA (2006) Terminal α1,4-linked N-acetylglucosamine in Helicobacter pylori-associated intestinal metaplasia of the human stomach and gastric carcinoma cell lines. J Histochem Cytochem 54:585–591

Fukuda M (1992) Cell surface carbohydrates in hematopoietic cell differentiation and malignancy. In: Fukuda M (ed) Cell surface carbohydrates and cell development. CRC Press, Boca Raton, pp 127–159

Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. Cell 140:883–899

Hidaka E, Ota H, Hidaka H, Hayama M, Matsuzawa K, Akamatsu T, Nakayama J, Katsuyama T (2001) Helicobacter pylori and two ultrastructurally distinct layers of gastric mucous cell mucins in the surface mucous gel layer. Gut 49:474–480

Hirai Y, Haque M, Yoshida T, Yokota K, Yasuda T, Oguma K (1995) Unique cholesteryl glucosides in Helicobacter pylori: composition and structural analysis. J Bacteriol 177:5327–5333

Hoshino H, Tsuchida A, Kametani K, Mori M, Nishizawa T, Suzuki T, Nakamura H, Lee H, Ito Y, Kobayashi M, Masumoto J, Fujita M, Fukuda M, Nakayama J (2011) Membrane-associated activation of cholesterol α-glucosyltransferase, an enzyme responsible for biosynthesis of cholesteryl-α-D-glucopyranoside in Helicobacter pylori critical for its survival. J Histochem Cytochem 59:98–105

Howlett M, Giraud AS, Lescesen H, Jackson CB, Kalantzis A, Van Driel IR, Robb L, Van der Hoek M, Ernst M, Minamoto T, Boussioutas A, Oshima H, Oshima M, Judd LM (2009) The interleukin-6 family cytokine interleukin-11 regulates homeostatic epithelial cell turnover and promotes gastric tumor development. Gastroenterology 136:967–977

Ishihara K, Kurihara M, Goso Y, Urata T, Ota H, Katsuyama T, Hotta K (1996) Peripheral α-linked N-acetylglucosamine on the carbohydrate moiety of mucin derived from mammalian gastric gland mucous cells: epitope recognized by a newly characterized monoclonal antibody. Biochem J 318:409–416

Ito Y, Vela JL, Matsumura F, Hoshino H, Tyznik A, Lee H, Girardi E, Zajonc DM, Liddington R, Kobayashi M, Bao X, Bugaytsova J, Borén T, Jin R, Zong Y, Seeberger PH, Nakayama J, Kronenberg M, Fukuda M (2013) Helicobacter pylori cholesteryl α-glucosides contribute to its pathogenicity and immune response by natural killer T cells. PLoS ONE 8, e78191

Iwaya Y, Hasebe O, Koide N, Kitahara K, Suga T, Shinji A, Muraki T, Yokosawa S, Yamada S, Arakura N, Tanaka E, Nakayama J (2014) Reduced expression of αGlcNAc in Barrett's oesophagus adjacent to Barrett's adenocarcinoma – a possible biomarker to predict the malignant potential of Barrett's oesophagus. Histopathology 64:536–546

Kai H, Kitadai Y, Kodama M, Cho S, Kuroda T, Ito M, Tanaka S, Ohmoto Y, Chayama K (2005) Involvement of proinflammatory cytokines IL-1β and IL-6 in progression of human gastric carcinoma. Anticancer Res 25:709–713

Karasawa F, Shiota A, Goso Y, Kobayashi M, Sato Y, Masumoto J, Fujiwara M, Yokosawa S, Muraki T, Miyagawa S, Ueda M, Fukuda MN, Fukuda M, Ishihara K, Nakayama J (2012) Essential role of gastric gland mucin in preventing gastric cancer in mice. J Clin Invest 122:923–934

Kawakubo M, Ito Y, Okimura Y, Kobayashi M, Sakura K, Kasama S, Fukuda MN, Fukuda M, Katsuyama T, Nakayama J (2004) Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection. Science 305:1003–1006

Lauren P (1965) The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 64:31–49

Lee H, Kobayashi M, Wang P, Nakayama J, Seeberger PH, Fukuda M (2006) Expression cloning of cholesterol α-glucosyltransferase, a unique enzyme that can be inhibited by natural antibiotic gastric mucin O-glycans, from Helicobacter pylori. Biochem Biophys Res Commun 349:1235–1241

Lee H, Wang P, Hoshino H, Ito Y, Kobayashi M, Nakayama J, Seeberger PH, Fukuda M (2008) α1,4GlcNAc-capped mucin-type O-glycan inhibits cholesterol α-glucosyltransferase from Helicobacter pylori and suppresses *H. pylori* growth. Glycobiology 18:549–558

Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R (2010) The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev 21:27–39

Marshall BJ, Warren JR (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 8390:1311–1315

Mohri Y, Miki C, Tanaka K, Kawamoto A, Ohi M, Yokoe T, Kusunoki M (2012) Clinical correlations and prognostic relevance of tissue angiogenic factors in patients with gastric cancer. Clin Oncol 24:610–616

Nakamura K, Sugano H, Takagi K (1968) Carcinoma of the stomach in incipient phase: its histogenesis and histological appearances. Gann 59:251–258

Nakamura N, Ota H, Katsuyama T, Akamatsu T, Ishihara K, Kurihara M, Hotta K (1998) Histochemical reactivity of normal, metaplastic, and neoplastic tissues to α-linked N-acetylglucosamine residue-specific monoclonal antibody HIK1083. J Histochem Cytochem 46:793–801

Nakayama J, Yeh J-C, Misra AK, Ito S, Katsuyama T, Fukuda M (1999) Expression cloning of a human α1,4-N-acetylglucosaminyltransferase that forms GlcNAcα1 → 4Galβ → R, a glycan specifically expressed in the gastric gland mucous cell-type mucin. Proc Natl Acad Sci USA 96:8991–8996

Nakayama T, Yoshizaki A, Izumida S, Suehiro T, Miura S, Uemura T, Yakata Y, Shichijo K, Yamashita S, Sekine I (2007) Expression of interleukin-11 (IL-11) and IL-11 receptor alpha in human gastric carcinoma and IL-11 upregulates the invasive activity of human gastric carcinoma cells. Int J Oncol 30:825–833

Nordman H, Davies JR, Lindell G, de Bolós C, Real F, Carlstedt I (2002) Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution. Biochem J 364:191–200

Ohta M, Kitadai Y, Tanaka S, Yoshihara M, Yasui W, Mukaida N, Haruma K, Chayama K (2003) Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human gastric carcinomas. Int J Oncol 22:773–778

Ota H, Katsuyama T (1992) Alternating laminated array of two types of mucin in the human gastric surface mucous layer. Histochem J 24:86–92

Ota H, Katsuyama T, Ishii K, Nakayama J, Shiozawa T, Tsukahara Y (1991) A dual staining method for identifying mucins of different gastric epithelial mucous cells. Histochem J 23:22–28

Palmieri C, Roberts-Clark D, Assadi-Sabet A, Coope RC, O'Hare M, Sunters A, Hanby A, Slade MJ, Gomm JJ, Lam EW, Coombes RC (2003) Fibroblast growth factor 7, secreted by breast fibroblasts, is an interleukin-1β-induced paracrine growth factor for human breast cells. J Endocrinol 177:65–81

Peek RM Jr, Blaser MJ (2002) Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer 2:28–37

Shiratsu K, Higuchi K, Nakayama J (2014) Loss of gastric gland mucin-specific O-glycan is associated with progression of differentiated-type adenocarcinoma of the stomach. Cancer Sci 105:126−133

Verbeke H, Geboes K, Van Damme J, Struyf S (2012) The role of CXC chemokines in the transition of chronic inflammation to esophageal and gastric cancer. Biochim Biophys Acta 1825:117–129

Zhang MX, Nakayama J, Hidaka E, Kubota S, Yan J, Ota H, Fukuda M (2001) Immunohistochemical demonstration of α1,4-N-acetylglucosaminyltransferase that forms GlcNAcα1,4Galβ residues in human gastrointestinal mucosa. J Histochem Cytochem 49:587–596

Chapter 8
Sialylation and Immune Surveillance of Cancer by Siglecs

Bindu Mohan and Paul R. Crocker

Abstract Changes in cell surface glycosylation are a key feature of cancer initiation and progression. Sialic acid is a major glycan attached to extracellular proteins and lipids. Altered sialylation in cancer can impact at many levels and may result in improved cancer cell survival and spread. Here we focus on sialic acid-dependent interactions of tumour cells with sialic acid-binding Ig-like lectins (siglecs). These proteins are expressed broadly in the immune system and can modulate cellular functions in diverse ways. We discuss changes in sialylation commonly observed in tumours and the emerging role of siglecs in modulating both host immune responses and tumour responses.

Keywords Sialic acid • Cancer • Glycosylation • Lectins • Siglec • Immune system

8.1 Sialic Acids

Sialic acids (Sias) are a family of nine-carbon-containing acidic sugars that are common to all vertebrates as well as some invertebrates. Cell membranes are covered in a dense glycocalyx which is richly decorated with Sia as terminal capping structures of N- and O-linked glycans and gangliosides. It has been estimated that more than 30 different types of Sia can occur in nature, although in mammals the most common is N-acetylneuraminic acid (NeuAc) (reviewed in Varki and Schauer (2009)). The N-acetyl group can be further modified to form N-glycolylneuraminic acid (NeuGc) or even be de-N-acetylated giving rise to Neu. The four, seven, eight and nine carbons of Sia can also have different substitutions at their hydroxyl group (such as methyl, acetyl, sulphate and phosphate), adding further complexity to this family of sugars. The nature of the Sia expressed is developmentally regulated and also depends on the cell type. Sia can be present in different linkages to the underlying glycans, for example, in α2,3 or α2,6 linkages to Gal and in α2,6 linkage to GalNAc and GlcNAc. It can also link with another Sia residue in α2,8 linkages, giving rise to di-, oligo- and poly-Sia such as seen in the

B. Mohan • P.R. Crocker (✉)
Division of Cell Signalling and Immunology, College of life Sciences, University of Dundee, Dow Street, Dundee DD2 5EH, Scotland, UK
e-mail: p.r.crocker@dundee.ac.uk

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_8

neural cell adhesion molecule (NCAM). The biosynthesis, regulation and turnover of Sia are controlled by a large number of enzymes including ~20 sialyltransferases, CMP-NeuAc hydroxylase, O-acetyltransferases, esterases and sialidases whose expression is often tightly regulated (reviewed in Varki and Schauer (2009)).

The role of Sia on cell surfaces is multifaceted. As acidic sugars carry net negative charge, Sias are important in cell–cell interactions in both physiological and pathological processes. Pathogens can also incorporate or express Sia as a tactic to evade host immune responses through molecular mimicry or subversion of host regulatory pathways. Sias are also target recognition molecules for certain pathogens, for example, the influenza virus' hemagglutinin recognises host Sia linked to Gal in α2,3 or α2,6 linkages.

The major family of Sia-binding lectins in mammals is the Siglecs. Most are expressed in the immune system but some, such as myelin-associated glycoprotein (Siglec-4) and Siglec-6, are mainly expressed in the nervous system and placenta, respectively. In this chapter we discuss various examples of altered sialylation on cancer cells and then consider how recognition of Sia by Siglecs may play a role in cancer cell biology and immune function.

8.2 Glycosylation in Tumours

Glycosylation is an important biosynthetic and post-translational process that is needed for the correct conformation and functioning of many macromolecules (Varki 1993). Aberrant glycosylation is a hallmark of malignant transformation and frequently involves N- and O-linked glycosylation of proteins and biosynthesis of glycolipids. There are numerous examples reported in the literature, covering all classes of cancer, including epithelial carcinomas, leukaemias and melanomas, as recently reviewed (Christiansen et al. 2013; Padler-Karavani V 2013). Although many factors, including changes in metabolic flux, are responsible for altered glycosylation patterns, changes in the expression of enzymes involved in glycan synthesis and degradation pathways are major contributing factors. Two extensively studied enzymes responsible for altered N-linked glycosylation are GlcNAcT-V and ST6GalI. The upregulation of both enzymes has been shown to be associated with increased metastasis and invasiveness of tumour cells and poor patient prognosis. Overexpression of GlcNAcT-V can result in increased β1,6 branching of N-glycans, whereas ST6GalI can enhance α2,6 sialylation of glycoproteins. Gastric, colorectal and breast cancers have all been shown to express high levels of these enzymes (Park and Lee 2013). One important target for both enzymes is the family of β1 integrins (Gu and Taniguchi 2004). Altered glycosylation of β1 integrins either from hypersialylation or increased β1,6 branching of N-glycans has been shown to be associated with cell invasiveness and metastasis. Transfection of colonic epithelial cells with oncogenic Ras resulted in the simultaneous upregulation of ST6GalI and α2,6 sialylation of β1 integrins. These changes affected adhesion of these transformed cells to collagen I, ligand for β1 integrins,

but not to β3 or β5 ligands (Seales et al. 2005) (Seales et al. 2003). Changes in sialylation arising from irregularities in the expression of sialyltransferases are also common to cancers of the colon (Holst et al. 2013; Yang et al. 1994).

Muc-1 is a membrane mucin glycoprotein, expressed by epithelial cells and characterised by extensive O-linked glycosylation of its variable number of tandem repeat (VNTR) region. It is highly expressed in cancers of the breast and is associated with poor prognosis. Apart from being overexpressed, it is also associated with aberrant O-linked glycosylation compared to Muc-1 on untransformed epithelial cells. This leads to truncated O-glycans containing increased amounts of sialylated core 1 structures (Neu5Acα2,3Galβ1,3GalNAc). In addition to this, a common cancer-associated antigen carried by O-linked glycans such as those on Muc-1 is the sialyl-Tn (STn) antigen. It is generated as a result of the transfer of a Sia residue to O-linked glycans by the enzyme ST6GalNAc1, leading to the formation of the epitope Neu5Acα2,6GalNAcα1-O-Ser/Thr. This addition forces the termination of the core O-linked GalNAcα1-O-Ser/Thr structure of the Tn antigen. The expression of STn is common to epithelial cancers such as breast, gastric, pancreatic and ovarian and is associated with poor patient survival (reviewed in Cazet et al. (2010)). Overexpression of this enzyme in the noninvasive, oestrogen-positive breast cancer cell line, T47-D, resulted in decreased adhesion but increased metastatic properties (Julien et al. 2005).

Such aberrantly glycosylated tumour-associated proteins can affect immune interactions in diverse ways. For example, recognition by C-type lectins expressed by dendritic cells may be an important factor in regulating specific T- and B-cell responses to tumour antigens. On the other hand, recognition by inhibitory lectins of the immune system may suppress inflammatory and immune function, leading to anergy and tolerance (reviewed in Rabinovich and Croci (2012)).

Muc-16 is another mucin expressed by the epithelial cells of the ovary, endometrium and trachea and is heavily glycosylated with O- and N-linked glycans. It is surface expressed but is also shed by proteolytic cleavage. The CA125 antigen, carried on Muc-16, is a tumour marker for ovarian carcinomas. Overexpression of this glycoprotein is known to promote tumour growth and metastasis. One of the mechanisms by which Muc-16 exerts its immunosuppressive effects is by its interactions with NK cells. Using Muc-16 knockdown ovarian carcinoma cell line, cell surface Muc-16 was shown to inhibit the formation of immunological synapses between ovarian carcinoma cells and primary NK cells, while the shed glycoprotein inhibited NK cell cytotoxicity (Gubbels et al. 2010).

Other O-linked tetrasaccharide structures commonly associated with cancer phenotypes include the Lewis antigens. Sialyl Lewis^{a} and sialyl Lewis^{x} are ligands for endothelial P- and E-selectins and are important tumour markers in cancers of the gastrointestinal tract such as colon cancer. Their overexpression is associated with enhanced metastasis in these cancer types (Ito et al. 1997). Both antigens have Sia linked to a Gal residue of core 2 O-linked glycans via α2,3 linkage. The sialyl transferases ST3Gal III and ST3Gal IV are responsible for the addition of Sia to type I (Galβ1,3GlcNAc) or type II (Galβ1,4GlcNAc) disaccharide cores resulting in the expression of sialyl Lewis^{a} and sialyl Lewis^{x}, respectively. Overexpression of

ST3Gal IV led to the synthesis of the sialyl Lewis^x antigen in a gastric cell line model. In line with previous findings, this led to an invasive phenotype which was associated with increased activation of c-met and phosphorylation of downstream signalling molecules such as src, FAK, Rac1 and RhoA GTPase (Gomes et al. 2013).

Besides changes in N- and O-linked glycosylation, alterations in glycolipid expression are a hallmark of cancer. In particular, gangliosides are highly expressed during development in the nervous system of humans (Yu et al. 2011). In comparison, gangliosides are minimally expressed in other tissues of the body but they are commonly overexpressed during malignant transformation. Their biosynthesis is controlled at several levels such as regulated expression of different glycosyltransferases and availability and competition for substrates (Daniotti and Iglesias-Bartolome 2011; Lopez and Schnaar 2009). Some gangliosides such as GD3, GM2 and GM3 are highly overexpressed in tumours such as melanomas, neuroblastomas, and breast and renal cell carcinomas. Their expression has been shown to affect various receptors and growth factors, subsequently modulating cell proliferation, metastasis, angiogenesis and immune responses (Handa and Hakomori 2012). GD3 is a marker for melanoma and breast cancer cells. Transfection of a human breast cancer cell line with the enzyme responsible for the conversion of GM3 to GD3, ST8Sia1, resulted in the overexpression of GD3. The accumulation of GD3 correlated with enhanced cell proliferation and migration in the absence of growth factors. Using oestrogen receptor-negative breast cancer cells, a role of NFkB in the transcriptional activation of ST8Sia1 was demonstrated (Bobowski et al. 2013).

The Sia on gangliosides can be modified by 7- or 9-O-acetylation to form Neu5Ac7Ac or Neu5Ac9Ac, respectively. These modified forms are minimally expressed on gangliosides of non-transformed cells but are overexpressed in some tumours (Padler-Karavani V 2013). GD3 and 9-/7-O-acetyl GD3 have roles in cellular development, activation, apoptosis, regulation of lipid raft formation and immunological tolerance. 9-O-Acetyl GD3 is a tumour marker of melanomas, breast cancers as well as tumour cell lines. Intracellular GD3 has been shown to stimulate CD95-mediated apoptosis, while 9-O-acetyl GD3 suppressed this effect. The anti-apoptotic role of 9-O-acetyl GD3 was clearly demonstrated using apoptosis-sensitive tumour T-cell lines (Kniep et al. 2006). Cultured melanoma cell lines showed increased expression of 9-O-acetyl GD3 as well as 9-O-acetyl GD2 (Birks et al. 2011). A critical ratio of GD3 to O-acetylated GD3 in glioblastomas was shown to be responsible for the survival of these cells (Birks et al. 2011). The O-acetylation of Sia is regulated by the balanced activity of O-acetyl transferases and O-acetyl esterases. However, reduction in O-acetylated Sia due to reduced levels of O-acetyl esterases resulted in the formation of sialyl Lewis^x, which is a tumour-associated antigen seen in colon cancer (Shen et al. 2004).

Although humans lack the enzyme CMAH required to make NeuGc, certain tumour cells (primary retinoblastoma and breast cancers) express this modified form of Sia due to its incorporation from dietary sources. An antibody to GM3 (NeuGc) was used in a Phase I/II clinical study of women with stage 2 breast cancer for in vivo imaging. The antibody was found to accumulate in the breast cancer

tissue in a dose-dependent manner and corresponded well with in vitro immunostaining (Oliva et al. 2006). The increased expression of these sialylated molecules and their modifications in tumour tissues are associated not only with phenotypic changes but also enhanced proliferation, metastasis and invasion of the tumour cells.

8.3 Siglecs in Tumour Surveillance

Sialic acid-binding Ig-like lectins (Siglecs) are a major class of mammalian glycan-binding proteins, with 14 members in humans (Fig. 8.1). They are expressed primarily by cells of the immune system and are characterised by a single N-terminal V-set Sia-binding domain, a variable number of C2-set domains, a transmembrane domain and a cytoplasmic region (Crocker et al. 1998). The cytoplasmic regions often contain tyrosine-based inhibitory motifs (ITIMs) and ITIM-like motifs that categorise them as inhibitory receptors. The phosphorylation of these ITIMs can lead to the recruitment of SH2 domain-containing proteins such as SHP-1, SHP-2, SOCS and Cbl. Depending on which of these proteins are recruited, this can result in modulation of cellular functions, including inhibitory signalling and regulation of endocytosis (reviewed in Crocker et al. (2007)). Based on their sequence similarities, this family of lectins has been classified into two

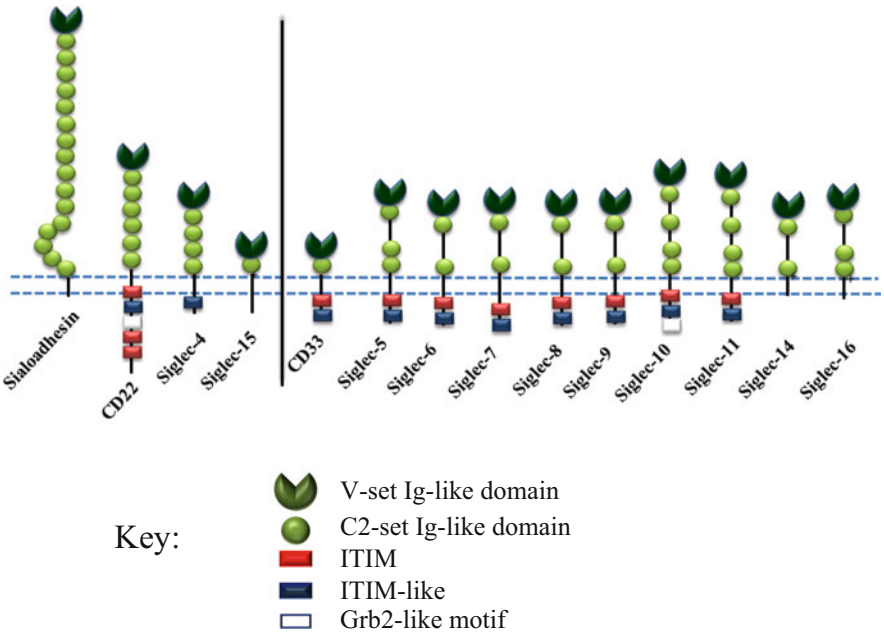

Fig. 8.1 Siglecs expressed in humans

major groups. The first group consists of Siglecs that are very well conserved between mammals – Sialoadhesin (Sn, Siglec-1, CD169), CD22 (Siglec-2), MAG (Siglec-4) and Siglec-15. The second group consists of CD33-related Siglecs, so called because of their high sequence similarity with CD33 (Siglec-3). In humans, the CD33-related group appears to be rapidly evolving and consists of CD33 and Siglec-5, Siglec-7, Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-14 and Siglec-16. Siglec-14, Siglec-15 and Siglec-16 lack ITIMs and form a complex with DAP-12, an adaptor protein that contains a tyrosine-based activation motif (ITAM). Where studied, these receptors deliver activation signals, and one theory is that they have arisen as an evolutionary mechanism to counteract sialylated pathogens which have learnt to exploit ITIM-containing Siglecs to their own advantage (reviewed in Cao and Crocker (2011)). Mice have fewer CD33-related Siglecs which, due to uncertainty with orthology, have been named differently as murine CD33, Siglec-E, Siglec-F, Siglec-G and Siglec-H. Several Siglecs are expressed in a highly cell-specific manner, for example, Sn on macrophages, MAG on myelin-forming cells, CD22 on B cells and Siglec-8 on eosinophils. Others are more diverse, such as Siglec-9 on neutrophils, monocytes, macrophages, dendritic cells, NK cells and minor subsets of T cells.

As a result of the high concentration of Sia within the cellular glycocalyx, many Siglecs are masked in *cis* but can mediate *trans* interactions with Sia on other cells and on pathogens if the ligands are of sufficient density and/or affinity (reviewed in Crocker et al. (2007)). Sn is an exception to this rule since it is thought to extend its Sia-binding site outside of the glycocalyx to enable *trans* interactions and function as a cellular interaction molecule. Ligand recognition by the different members of this family depends on multiple factors, including the nature of Sia (e.g. NeuAc versus NeuGc), its linkage to underlying sugars, the extended structure of the sialoglycan and its presentation on glycoproteins and glycolipids. For example, murine CD22 has strong preference for NeuGc in α2,6 linkages of N-glycans on selected glycoproteins, while Sn prefers α2,3 linkages of NeuAc on N-linked and O-linked glycans and gangliosides. Protein–glycan interactions are typically of low affinity, and stable binding of Siglecs to glycan ligands, *in cis* or *in trans*, normally requires multivalent interactions that can be achieved by ligand clustering on protein or lipid carriers, as well as clustering of counter-receptors on the cell surface.

The use of soluble recombinant proteins with the extracellular domain of the receptor fused to the Fc portion of IgG and clustered with anti-Fc conjugates is a useful way to mimic Siglec presentation on a cell membrane and has led to the identification of higher-affinity ligands and putative counter-receptors for several Siglecs. Some of these ligands and counter-receptors are highly expressed on tumour cells, but more work needs to be done to understand their relevance in a physiological setting. However, one practical application is in the use of Siglecs to discover cancer-related biomarkers. In one study, cancer and noncancer CA125 antigens differing in the sialylation pattern of their N- and O-linked glycans were compared for binding of Siglec-2, Siglec-3, Siglec-7, Siglec-6, Siglec-9 and Siglec-10. While all Siglecs bound both normal and cancer-related CA125, selective binding of certain Siglecs was observed with cancer-related CA125 (Mitic

et al. 2012). This is a good example of how these immunoreceptors can be employed as tools in the identification of tumour markers.

Below we give examples of Siglecs which have been studied in the context of cancer cell recognition and speculate on how these interactions might affect cancer cell behaviour and disease outcome.

8.3.1 Sialoadhesin

Sialoadhesin (Sn) is highly conserved in mammals and is expressed by macrophage subsets with particularly high expression on specialised macrophages of secondary lymphoid tissues involved in antigen handling and host defence. Its expression is upregulated by cytokines, especially type I interferon, in many inflammatory conditions such as rheumatoid arthritis and systemic lupus erythematosus (SLE) (Klaas and Crocker 2012). Sn is characterised by the presence of 17 extracellular Ig domains and no ITIMs in its cytoplasmic domain, and one of its major functions appears to be in regulating cellular interactions with activated T-cell subsets (Kidder et al. 2013). Although it prefers Sia in α2,3 linkages, it can also bind Sia in α2,6 and α2,8 linkages and therefore has the potential to interact with many cell types and soluble glycoproteins including sialylated pathogens and exosomes (Klaas and Crocker 2012; Saunderson et al. 2014). A recent study on the role of Sn in a mouse model of SLE demonstrated that N-linked glycans upregulated on CD4+ T cells serve as potential counter-receptors for this Siglec. mRNA analysis also showed an upregulation of α2,3 ST3Gal IV, an enzyme responsible for the addition of Sia to N-linked glycans (Kidder et al. 2013).

In a study of breast cancer, many Sn-positive macrophages were seen to be present in close association with Muc-1-expressing tumour cells (Nath et al. 1999). Further biochemical analysis using breast cancer cell lines and Sn-Fc identified Muc-1 as a putative counter-receptor for Sn (Nath et al. 1999). More recently, colorectal cancer patients with high expression of Sn/CD169+ sinus macrophages in the regional lymph nodes were associated with infiltration of antigen-specific CD8+ T cells and an overall better prognosis (Ohnishi et al. 2013). Given that many tumour-associated macrophages express Sn and that macrophages play an important role in initiation, development and metastasis of many tumours (reviewed in Richards et al. (2013)), Sn could provide a useful therapeutic target and may be important in local cellular interactions including antigen presentation to the adaptive immune system.

8.3.2 CD22 (Siglec-2)

CD22 (Siglec-2) is an inhibitory receptor expressed on B cells where it plays an important role in the negative regulation of the B-cell receptor signalling. CD22 is

well characterised for specific binding to Neu5Acα2,6Gal-terminating N-linked glycans, but it can also bind the STn structure, Neu5Acα2,6GalNAc, commonly found on tumour-associated mucins. In spite of being *cis* engaged, CD22 is able to redistribute to the site of cell–cell contact and interact in *trans* with adjacent immune cells, carrying high-affinity sialylated glycoprotein ligands (Collins et al. 2004). Mucins, shed from certain cancers that carry a high density of Sia in α2,6 linkages, have the potential to interact with CD22 and modulate B-cell signalling. A variety of mucin preparations were able to bind CD22 on B cells and negatively regulate BCR-induced signal transduction as shown by ERK1/ERK2 phosphorylation signalling (Toda et al. 2008) (Fig. 8.2a). CD22 was also shown to interact with mucins in vivo and modulate B cells. Using mammary adenocarcinoma cells secreting the mucin epiglycanin, a significant reduction in the marginal zone B-cell populations was observed. This was thought to be due to apoptosis triggered by extensive cross-linking of CD22 with epiglycanin (Toda et al. 2009). Such interactions could contribute to immunosuppression in cancer-bearing individuals and should also be considered in vaccination approaches aimed at boosting humoral responses to mucin-type antigens bearing STn.

8.3.3 Siglec-7

Siglec-7 is expressed primarily on NK cells and tissue macrophages and has an unusual specificity, preferring α2,8-linked Sia over α2,6 and α2,3 linkages. Siglec-7 also binds strongly to internally branched α2,6-linked Sia such as found in sialyllacto-N-tetraose b (LSTb) and disialyl Lewis^a structures (Miyazaki et al. 2004). Siglec-7 carries an ITIM and an ITIM-like motif in its cytoplasmic domain and has been shown to be an inhibitory receptor in a variety of different assay systems. Since α2,8-linked Sia is predominant in the nervous system as 'b-series' gangliosides such as GD3 and GT1b, one potential physiological role of Siglec-7 is to protect neuronal cells from NK-mediated damage during inflammation of the nervous system. Consistent with this, when GD3 was overexpressed in the mouse mastocytoma cell line, P815, this altered their sensitivity to peripheral blood NK cell cytotoxicity (Nicoll et al. 2003) (Fig. 8.2b). Likewise, it was shown that expression of the disialosyl globopentaosylceramide (DSGb5) containing an internally branched α2,6-linked Sia could protect renal cancer cells from NK cell cytotoxicity in a Siglec-7-dependent manner (Kawasaki et al. 2010). Even coating cells with artificially high concentrations of Sia using chemical conjugation can lead to Siglec-7-dependent protection against NK cell cytotoxicity (Hudak et al. 2014). This raises the possibility that general increases in Sia on cancer cells regardless of linkage could play a role in tumour protection to NK cell attack.

While the increased expression of Siglec-7 ligands on cancer cells described above is likely to play a role in protection against NK cell attack, the loss of Siglec-7 ligands during the transition to cancer cells has also been proposed to promote tumour development via altered interactions with anti-inflammatory macrophages

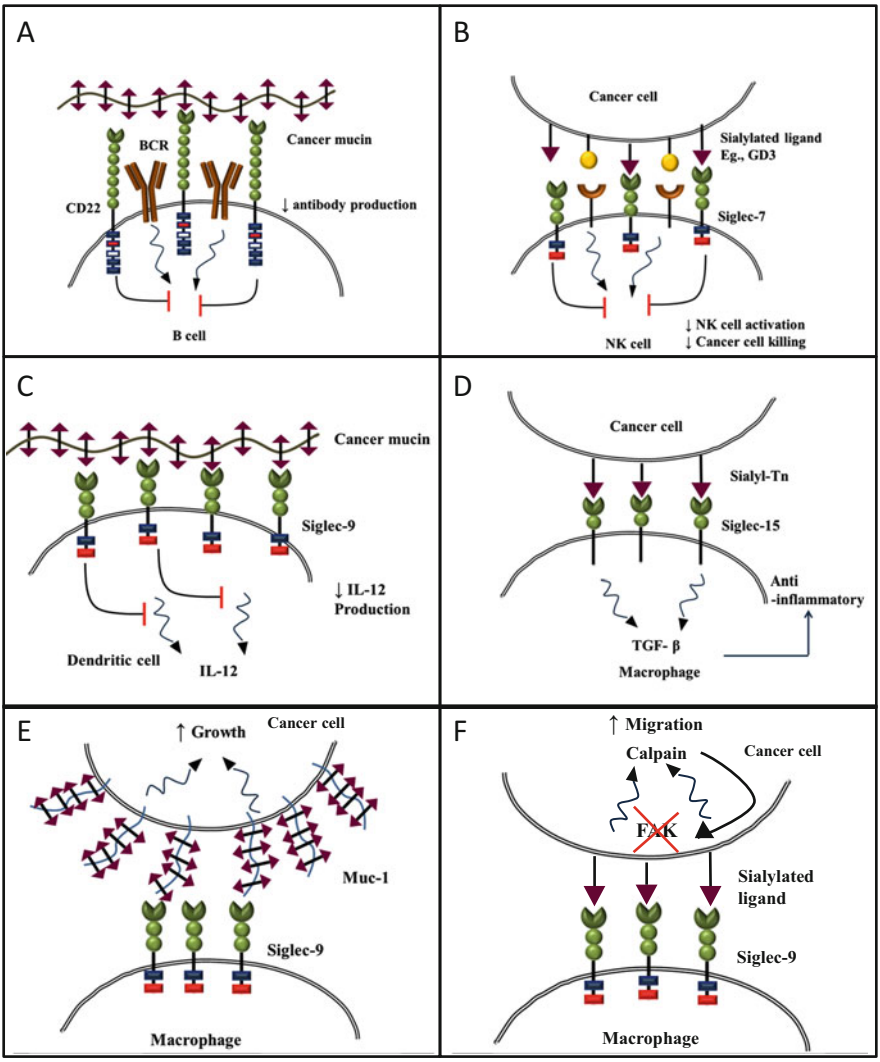

Fig. 8.2 Examples of siglec-dependent interactions with cancer cells and mucins. Panels depict potential outcomes of signalling pathways following Siglec–Sia interactions. (**a**) Negative signal transduction by BCR on B-cells, following ligation of CD22 with mucins. (**b**) Altered cytotoxicity of peripheral blood NK cells, expressing Siglec-7, to the mouse mastocytoma cell line (P815) over-expressing GD3. (**c**) Ligation of Siglec-9, expressed on immature dendritic cells, with mucins negatively regulates IL-2 production. (**d**) Ligation of Siglec-15 with the STn epitope, over-expressed on a leukemic cell line, upregulates the expression of TGF-β. (**e**) Increase in tumour growth following ligation of Siglec-9 to MUC-1, over-expressed on human colon cancer cell line, via recruitment of β-catenin. (**f**) Increased detachment and migration of tumour cells carrying sialylated ligands via calpain dependent proteolytic pathway, following ligation with Siglec-9

(Miyazaki et al. 2012). The disialyl Lewis^a antigen is a strong Siglec-7 ligand and is expressed on non-malignant colonic epithelial cells but downregulated following transformation. This leads to increased expression of its monosialyl counterpart, sialyl Lewis^a, which is not well recognised by Siglec-7. Resident colonic macrophages expressing Siglec-7 were proposed to interact with disialyl Lewis^a on normal epithelial cells and dampen inflammatory responses via reduced PGE_2 production, effectively suppressing tumour development. Hence, the loss of inhibitory signals accompanying the switch from disialyl Lewis^a to sialyl Lewis^a could play an important role in colon cancer development.

8.3.4 Siglec-9

Siglec-9 is an ITIM-containing receptor expressed on neutrophils, monocytes, macrophages, NK cells and a minor subset of CD4 and CD8 T cells and prefers Sia in α2,3 linkages. The binding of cancer mucins and artificial glycopolymers to Siglec-9 expressed on immature dendritic cells was shown to suppress the production of IL-12 but not IL-10, suggesting an immunomodulatory role for this Siglec (Ohta et al. 2010) (Fig. 8.2c). Siglec-9 was also identified as a receptor for the soluble mucin, Muc-16, shed from ovarian cancer cells that bound to a subset of CD16 + CD56dim NK cells, B cells and monocytes. Jurkat cells expressing Siglec-9 bound strongly to the ovarian carcinoma cell line expressing Muc-16 in cell adhesion assays. It was proposed but not demonstrated that this interaction could favour cancer development through suppression of inflammatory responses (Belisle et al. 2010).

8.3.5 Siglec-15

Siglec-15 is expressed on macrophages and osteoclasts and binds STn antigen, a glycan antigen commonly expressed by tumour cells as discussed above. Tumour-associated macrophages (TAMs) from tumour tissue arrays were found to be positive for Siglec-15 expression. TAMs are known to promote tumour progression and metastasis by expressing immunosuppressive cytokines such as TGF-β. Using cocultures of a lung carcinoma cell line, expressing STn antigen, and a monocytic leukaemic cell line, expressing Siglec-15, it was shown that this receptor signals for the increased production of the immunosuppressive cytokine TGF-β, in a DAP-12-SYK pathway-dependent manner (Takamiya et al. 2013) (Fig. 8.2d).

The above examples involve Siglec-dependent signalling to immune cells that modulate cytotoxic and inflammatory responses leading to increased cancer development. However, two recent studies have demonstrated that sialylated ligands expressed on cancer cells can also trigger signalling pathways following engagement with Siglec-9 that can alter tumour cell behaviour. In one case, it was shown

that the binding of Siglec-9 to Muc-1 on a human colon cancer cell line led to the recruitment of β-catenin to the Muc-1 C-terminal domain and promoted tumour growth (Tanida et al. 2013) (Fig. 8.2e). In another system, Siglec-9 binding to sialylated ligands on astrocytoma cancer cell line activated a calpain-dependent proteolysis pathway leading to degradation of focal adhesion kinase and detachment of the tumour cells from the substrate (Sabit et al. 2013) (Fig. 8.2f).

8.4 Conclusions

Taken together, there is growing evidence that Sia presented by many different types of tumour cell interacts with various Siglecs during cancer initiation, expansion and spread. This can occur both through direct cellular interactions and via secreted glycoproteins such as cancer-associated mucins. In some cases it is possible that altered sialylation patterns favour the survival of cancer cells, at least in part due to Siglec-dependent suppression of inflammatory responses and other pathways discussed here. This could lead to broader impact on tumour surveillance by immune cells and mounting of an immune response. Targeting relevant Siglecs and identification of specific ligands and counter-receptors for Siglecs may be useful future areas to consider for developing new immune-based therapies and diagnostic methods.

References

Belisle JA, Horibata S, Jennifer GA, Petrie S, Kapur A, Andre S, Gabius HJ, Rancourt C, Connor J, Paulson JC, Patankar MS (2010) Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes. Mol Cancer 9:118. doi:10.1186/1476-4598-9-118

Birks SM, Danquah JO, King L, Vlasak R, Gorecki DC, Pilkington GJ (2011) Targeting the GD3 acetylation pathway selectively induces apoptosis in glioblastoma. Neuro Oncol 13 (9):950–960. doi:10.1093/neuonc/nor108nor108 [pii]

Bobowski M, Vincent A, Steenackers A, Colomb F, Van Seuningen I, Julien S, Delannoy P (2013) Estradiol represses the G(D3) synthase gene ST8SIA1 expression in human breast cancer cells by preventing NFkappaB binding to ST8SIA1 promoter. PLoS One 8(4):e62559. doi:10.1371/journal.pone.0062559PONE-D-12-26811 [pii]

Cao H, Crocker PR (2011) Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation? Immunology 132(1):18–26. doi:10.1111/j.1365-2567.2010.03368.x

Cazet A, Julien S, Bobowski M, Burchell J, Delannoy P (2010) Tumour-associated carbohydrate antigens in breast cancer. Breast Cancer Res 12(3):204. doi:10.1186/bcr2577

Christiansen MN, Chik J, Lee L, Anugraham M, Abrahams JL, Packer NH (2013) Cell surface protein glycosylation in cancer. Proteomics. doi:10.1002/pmic.201300387

Collins BE, Blixt O, DeSieno AR, Bovin N, Marth JD, Paulson JC (2004) Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact. Proc Natl Acad Sci U S A 101(16):6104–6109. doi:10.1073/pnas.0400851101 [pii]

Crocker PR, Clark EA, Filbin M, Gordon S, Jones Y, Kehrl JH, Kelm S, Le Douarin N, Powell L, Roder J, Schnaar RL, Sgroi DC, Stamenkovic K, Schauer R, Schachner M, van den Berg TK, van der Merwe PA, Watt SM, Varki A (1998) Siglecs: a family of sialic-acid binding lectins. Glycobiology 8(2):v

Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. Nat Rev Immunol 7(4):255–266. doi:10.1038/nri2056, nri2056 [pii]

Daniotti JL, Iglesias-Bartolome R (2011) Metabolic pathways and intracellular trafficking of gangliosides. IUBMB Life 63(7):513–520. doi:10.1002/iub.477

Gomes C, Osorio H, Pinto MT, Campos D, Oliveira MJ, Reis CA (2013) Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. PLoS One 8(6), e66737. doi:10.1371/journal.pone.0066737PONE-D-13-02396 [pii]

Gu J, Taniguchi N (2004) Regulation of integrin functions by N-glycans. Glycoconj 8(4):e62559. doi:10.1023/B:GLYC.0000043741.47559.30, 5277444 [pii]

Gubbels JA, Felder M, Horibata S, Belisle JA, Kapur A, Holden H, Petrie S, Migneault M, Rancourt C, Connor JP, Patankar MS (2010) MUC16 provides immune protection by inhibiting synapse formation between NK and ovarian tumor cells. Mol Cancer 9:11. doi:10.1186/1476-4598-9-111476-4598-9-11 [pii]

Handa K, Hakomori SI (2012) Carbohydrate to carbohydrate interaction in development process and cancer progression. Glycoconj J 29(8–9):627–637. doi:10.1007/s10719-012-9380-7

Holst S, Stavenhagen K, Balog CI, Koeleman CA, McDonnell LM, Mayboroda OA, Verhoeven A, Mesker WE, Tollenaar RA, Deelder AM, Wuhrer M (2013) Investigations on aberrant glycosylation of glycosphingolipids in colorectal cancer tissues using liquid chromatography and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS). Mol Cell Proteomics 12(11):3081–3093. doi:10.1074/mcp.M113.030387

Hudak JE, Canham SM, Bertozzi CR (2014) Glycocalyx engineering reveals a Siglec-based mechanism for NK cell immunoevasion. Nat Chem Biol 10(1):69–75. doi:10.1038/nchembio.1388

Ito H, Hiraiwa N, Sawada-Kasugai M, Akamatsu S, Tachikawa T, Kasai Y, Akiyama S, Ito K, Takagi H, Kannagi R (1997) Altered mRNA expression of specific molecular species of fucosyl- and sialyl-transferases in human colorectal cancer tissues. Int J Cancer 71(4):556–564. doi:10.1002/(SICI)1097-0215(19970516)71:4<556::AID-IJC9>3.0.CO;2-T [pii]

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(1):77–84. doi:10.1007/s10549-004-3137-3

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(11):1373–1379. doi:10.1093/glycob/cwq116

Kidder D, Richards HE, Ziltener HJ, Garden OA, Crocker PR (2013) Sialoadhesin ligand expression identifies a subset of CD4 + Foxp3- T cells with a distinct activation and glycosylation profile. J Immunol 190(6):2593–2602. doi:10.4049/jimmunol.1201172

Klaas M, Crocker PR (2012) Sialoadhesin in recognition of self and non-self. Semin Immunopathol 34(3):353–364. doi:10.1007/s00281-012-0310-3

Klaas M, Oetke C, Lewis LE, Erwig LP, Heikema AP, Easton A, Willison HJ, Crocker PR (2012) Sialoadhesin promotes rapid proinflammatory and type I IFN responses to a sialylated pathogen, Campylobacter jejuni. J Immunol 189(5):2414–2422. doi:10.4049/jimmunol.1200776

Kniep B, Kniep E, Ozkucur N, Barz S, Bachmann M, Malisan F, Testi R, Rieber EP (2006) 9-O-acetyl GD3 protects tumor cells from apoptosis. Int J Cancer 119(1):67–73. doi:10.1002/ijc.21788

Lopez PH, Schnaar RL (2009) Gangliosides in cell recognition and membrane protein regulation. Curr Opin Struct Biol 19(5):549–557. doi:10.1016/j.sbi.2009.06.001S0959-440X(09)00092-X [pii]

Mitic N, Milutinovic B, Jankovic M (2012) Assessment of sialic acid diversity in cancer- and non-cancer related CA125 antigen using sialic acid-binding Ig-like lectins (Siglecs). Dis Markers 32(3):187–194. doi:10.3233/DMA-2011-0872

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(13):4498–4505. doi:10.1158/0008-5472.CAN-03-3614

Miyazaki K, Sakuma K, Kawamura YI, Izawa M, Ohmori K, Mitsuki M, Yamaji T, Hashimoto Y, Suzuki A, Saito Y, Dohi T, Kannagi R (2012) Colonic epithelial cells express specific ligands for mucosal macrophage immunosuppressive receptors siglec-7 and −9. J Immunol 188 (9):4690–4700. doi:10.4049/jimmunol.1100605

Nath D, Hartnell A, Happerfield L, Miles DW, Burchell J, Taylor-Papadimitriou J, Crocker PR (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(2):213–219

Nicoll G, Avril T, Lock K, Furukawa K, Bovin N, Crocker PR (2003) Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. Eur J Immunol 33(6):1642–1648. doi:10.1002/eji.200323693

Ohnishi K, Komohara Y, Saito Y, Miyamoto Y, Watanabe M, Baba H, Takeya M (2013) CD169-positive macrophages in regional lymph nodes are associated with a favorable prognosis in patients with colorectal carcinoma. Cancer Sci 104(9):1237–1244. doi:10.1111/cas.12212

Ohta M, Ishida A, Toda M, Akita K, Inoue M, Yamashita K, Watanabe M, Murata T, Usui T, Nakada H (2010) Immunomodulation of monocyte-derived dendritic cells through ligation of tumor-produced mucins to Siglec-9. Biochem Biophys Res Commun 402(4):663–669. doi:10.1016/j.bbrc.2010.10.079

Oliva JP, Valdes Z, Casaco A, Pimentel G, Gonzalez J, Alvarez I, Osorio M, Velazco M, Figueroa M, Ortiz R, Escobar X, Orozco M, Cruz J, Franco S, Diaz M, Roque L, Carr A, Vazquez AM, Mateos C, Rubio MC, Perez R, Fernandez LE (2006) Clinical evidences of GM3 (NeuGc) ganglioside expression in human breast cancer using the 14F7 monoclonal antibody labelled with (99m)Tc. Breast Cancer Res Treat 96(2):115–121. doi:10.1007/s10549-005-9064-0

Padler-Karavani V (2013) Aiming at the sweet side of cancer: aberrant glycosylation as possible target for personalized-medicine. Cancer Lett. doi:10.1016/j.canlet.2013.10.005

Park JJ, Lee M (2013) Increasing the alpha 2, 6 sialylation of glycoproteins may contribute to metastatic spread and therapeutic resistance in colorectal cancer. Gut Liver 7(6):629–641. doi:10.5009/gnl.2013.7.6.629

Rabinovich GA, Croci DO (2012) Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. Immunity 36(3):322–335. doi:10.1016/j.immuni.2012.03.004

Richards DM, Hettinger J, Feuerer M (2013) Monocytes and macrophages in cancer: development and functions. Cancer Microenviron 6(2):179–191. doi:10.1007/s12307-012-0123-x

Sabit I, Hashimoto N, Matsumoto Y, Yamaji T, Furukawa K (2013) Binding of a sialic acid-recognizing lectin Siglec-9 modulates adhesion dynamics of cancer cells via calpain-mediated protein degradation. J Biol Chem 288(49):35417–35427. doi:10.1074/jbc.M113.513192

Saunderson SC, Dunn AC, Crocker PR, McLellan AD (2014) CD169 mediates the capture of exosomes in spleen and lymph node. Blood 123(2):208–216. doi:10.1182/blood-2013-03-489732

Seales EC, Jurado GA, Singhal A, Bellis SL (2003) Ras oncogene directs expression of a differentially sialylated, functionally altered beta1 integrin. Oncogene 22(46):7137–7145. doi:10.1038/sj.onc.12068341206834 [pii]

Seales EC, Jurado GA, Brunson BA, Wakefield JK, Frost AR, Bellis SL (2005) Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by up-regulating cell motility. Cancer Res 65(11):4645–4652. doi:10.1158/0008-5472.CAN-04-3117, 65/11/4645 [pii]

Shen Y, Kohla G, Lrhorfi AL, Sipos B, Kalthoff H, Gerwig GJ, Kamerling JP, Schauer R, Tiralongo J (2004) O-acetylation and de-O-acetylation of sialic acids in human colorectal carcinoma. Eur J Biochem 271(2):281–290

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(2):178–187. doi:10.1093/glycob/cws139

Tanida S, Akita K, Ishida A, Mori Y, Toda M, Inoue M, Ohta M, Yashiro M, Sawada T, Hirakawa K, Nakada H (2013) Binding of the sialic acid-binding lectin, Siglec-9, to the membrane mucin, MUC1, induces recruitment of beta-catenin and subsequent cell growth. J Biol Chem 288(44):31842–31852. doi:10.1074/jbc.M113.471318

Toda M, Akita K, Inoue M, Taketani S, Nakada H (2008) Down-modulation of B cell signal transduction by ligation of mucins to CD22. Biochem Biophys Res Commun 372(1):45–50. doi:10.1016/j.bbrc.2008.04.175

Toda M, Hisano R, Yurugi H, Akita K, Maruyama K, Inoue M, Adachi T, Tsubata T, Nakada H (2009) Ligation of tumour-produced mucins to CD22 dramatically impairs splenic marginal zone B-cells. Biochem J 417(3):673–683. doi:10.1042/BJ20081241

Varki A (1993) Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3 (2):97–130

Varki A, Schauer R et al (2009) Sialic acids. In: Varki A, Cummings RD, Esko JD (eds) Essentials of glycobiology, 2nd edn. Cold Spring Harbor, New York

Yang JM, Byrd JC, Siddiki BB, Chung YS, Okuno M, Sowa M, Kim YS, Matta KL, Brockhausen I (1994) Alterations of O-glycan biosynthesis in human colon cancer tissues. Glycobiology 4 (6):873–884

Yu RK, Tsai YT, Ariga T, Yanagisawa M (2011) Structures, biosynthesis, and functions of gangliosides--an overview. J Oleo Sci 60(10):537–544, doi:JST.JSTAGE/jos/60.537 [pii]

Part V
Changes in the Organization of Complex Carbohydrates in Cancers

Chapter 9
Expression and Function of Poly-*N*-Acetyllactosamine Type Glycans in Cancer

Motohiro Nonaka and Minoru Fukuda

Abstract Poly-*N*-acetyllactosamine is a polymer consisting of type II lactosamine chain repeats (Galβ1 → 4GlcNAcβ1 → 3). The history of identification and characterization of poly-*N*-acetyllactosamine is largely dependent on the work by many passionate glycobiologists in the early 1970s. They put great efforts to identify the carbohydrate component of human blood cells. Poly-*N*-acetyllactosamine chain has various physiological functions by serving as a scaffold for terminal modifications such as ABO blood group antigens, li antigens, and sialyl Lewis X structure. These modifications sometimes change in cell-type-specific and development-specific manners, resulting in its structural diversity. Moreover, the structure of poly-*N*-acetyllactosamine side chain also changes along with tumor initiation and development. Tumor cells tactfully survive in return for spending a huge energy for the generation of poly-*N*-acetyllactosamine. Tumor cells decorated with newly synthesized poly-*N*-acetyllactosamine facilitate cell–cell interaction and metastasis through interaction with carbohydrate-binding lectins such as E- and P-selectins. The presence of a set of glycosyltransferases is essential for the dramatic changes of poly-*N*-acetyllactosamine chains.

Keywords Poly-*N*-acetyllactosamine • ABO blood group antigen • Li antigens • Development • Glycosyltransferase • Tumor cells • Core2 *O*-glycan • Selectin

M. Nonaka (✉)
Glycobiology Unit, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA

Laboratory for Drug Discovery, National Institute of Advanced Industrial Science and Technology, Umezono 1-1-1, Center 2, Tsukuba-City, Ibaraki 305-8568, Japan
e-mail: moto-nonaka@aist.go.jp

M. Fukuda
Glycobiology Unit, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_9

9.1 History of Isolation and Identification of Poly-*N*-Acetyllactosamine

Poly-*N*-acetyllactosamine is a unique glycan consisting of type 2 LacNAc (Galβ1 → 4GlcNAcβ1 → 3) repeats, which is frequently found on *N*-glycans, *O*-glycans, and glycolipids (Fig. 9.1). Because of hydrophilic nature of the disaccharide, the conformation of poly-*N*-acetyllactosamine is straightly extended and is very conspicuous from extracellular side. Thus poly-*N*-acetyllactosamine often serves as a substrate for additional glycosyltransferases, which gives rise to a huge diversity of the glycan structure. The discovery of poly-*N*-acetyllactosamine was achieved owing to the tremendous efforts of glycobiologists in 1970s, who devoted to characterize carbohydrate components of human erythrocytes containing ABH blood group determinants. The basic components of *O*-glycans had been identified early as di-, tri-, and tetrasaccharides by alkaline borohydride extraction of erythrocyte-derived glycopeptides. The "alkali-stable" substances containing *N*-glycans were then isolated and analyzed. The description of the disaccharide structure [galactose → *N*-acetylglucosamine] in the erythrocyte was first appeared in the report from Kornfeld's group (Kornfeld and Kornfeld 1970). They found PHA-bound, alkali-stable fraction contained the disaccharide structure, which was the same sequence seen in secreted fetuin and α-acid glycoprotein (Spiro 1969) and human chronic gonadotropin (Bahl 1969). However, the sequence identified at that time was a monomer and was different from later-identified, repeating *N*-acetyllactosamine unit.

The presence of repeated type of *N*-acetyllactosamine chain was initially proposed by Tanner and Boxer (1972). They found that protein fractions purified from human erythrocyte membrane (equivalent to Band 3) carried a high amount of

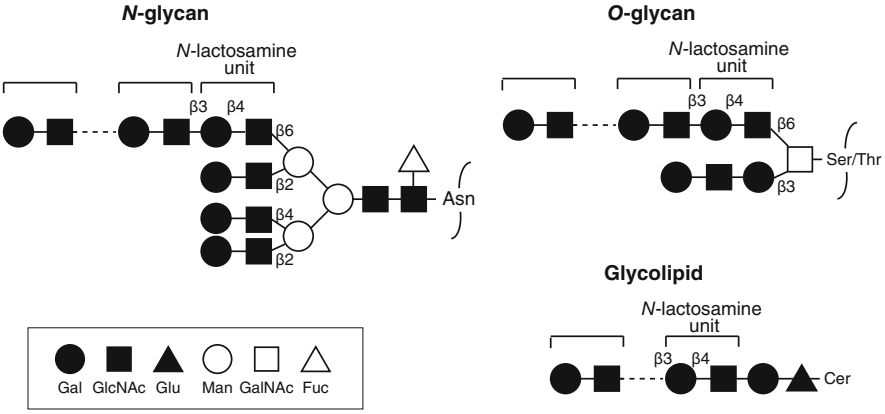

Fig. 9.1 Examples of poly-*N*-acetyllactosamine chains extended from *N*-glycan, *O*-glycan, and glycolipid. Note that poly-*N*-acetyllactosamine can also be initiated from GlcNAcβ1-2Manα-R by the action of β4GalT1. Core2-branched *O*-glycans usually contain fewer and shorter poly-N-acetyllactosamines than *N*-glycans in many cells

carbohydrate (7–9 %) consisting high proportions of galactose and *N*-acetylglucosamine. Those fractions contained certain amount of *N*-acetylgalactosamine when purified from blood type A, but not from blood type O individuals, showing that those glycoproteins carry the ABO determinants. Fukuda et al. improved the purification method for Band 3 and showed that the Band 3 carbohydrate component is rich in galactose and *N*-acetylglucosamine and is distinct from other glycoproteins (Fukuda et al. 1978). Finne et al. also found similar galactose/*N*-acetylglucosamine-rich components from blood group B erythrocyte by *Bandeiraea simplicifolia* lectin (BS I) column (Finne et al. 1978). Meanwhile, Jarnefelt et al. first demonstrated that the disaccharide structure of (Galβ1 → 4GlcNAcβ1 → 3) was indeed repeating and forming poly-*N*-acetyllactosamine (Jarnefelt et al. 1978). Partial purification and characterization of a protein-linked Band 3 polysaccharide, which was called "erythroglycan" at that time, demonstrated that the high molecular weight glycopeptides of erythroglycan were susceptible to endo-β-galactosidase digestion. Interestingly, those disaccharide chains were branched at 50 % of the galactose residues with additional 30–50 sugars. Structural analysis following chemical and enzymatic digestion revealed that the carbohydrate chain of Band 3 consists of poly-*N*-acetyllactosamine extended from $(Man)_3(GlcNAc)_2$ core (Jarnefelt et al. 1978; Krusius et al. 1978; Fukuda et al. 1979a). The poly-*N*-acetyllactosamine chain of Band 3 was soon recognized as functionally important component in erythrocytes because it carries Ii antigens (Childs et al. 1978; Fukuda et al. 1979b) as well as ABO blood group determinants. By early 1980, various studies have revealed that the repeating disaccharide sequence was expressed in embryonal carcinoma (Muramatsu et al. 1979), Chinese hamster ovary (CHO) (Li et al. 1980), Ehrlich ascites tumor (Eckhardt and Goldstein 1983), and lymphocyte (Childs et al. 1983) cell surface glycoproteins and keratan sulfate (Roden 1980).

9.2 Linear and Branched Poly-*N*-Acetyllactosamine Structure (I and i Antigens)

Poly-*N*-acetyllactosamine often plays as a scaffold backbone for additional modifications, which results in high molecular weight structures. Those modifications entirely depend on the cell-type and developmental phases, which ensure its structural heterogeneity. Developmentally regulated carbohydrate epitopes were first found by the study based on monoclonal antibody technique, which was cutting-edge technology at that time. The monoclonal anti-I antibody was found in sera of patients with an autoimmune hemolytic disorder (Wiener et al. 1956). They found that anti-I antibodies react with I antigen expressed on erythrocytes derived from normal adults except of 5 out of 22,000 individuals who solely expressed i antigen and this i phenotype is inherited as an autosomal recessive trait. Then Marsh et al. found cold agglutinin that reacts with i antigen of

erythrocytes (Marsh 1961). The anti-i antibody was shown to recognize human fetal and umbilical cord erythrocytes. The level of i antigen gradually decreases and abolishes in children by 18 months. In contrast, the I antigen level reaches a peak by the time of adulthood (Marsh 1961). Therefore, it was proposed that these antigenic determinants might be classified into several groups (Feizi et al. 1971a). For mouse embryogenesis, the i antigen is the first detected in the 5-day embryo, whereas the I antigen can be found throughout the preimplantation period. The distinct expression pattern of the i antigen is more prominent in the primary endoderm; the expression of the i antigen is inversely correlated with that of the I antigen (Kapadia et al. 1981; Knowles et al. 1982). Structural analysis of Ii antigens was first conducted by way of enzymatic digestion (Marcus et al. 1963). Normal erythrocytes treated with β-galactosidase and β-*N*-acetylglucosaminidase decreased anti-I antibody-dependent agglutination, indicating that galactose/*N*-acetylglucosamine is a carrier of I antigen. Feizi et al. conducted oligosaccharide inhibition assays for anti-I antibody and found that a terminal nonreducing Galβ1 → 4GlcNAcβ1 → 6 structure had the best inhibitory effect (Feizi et al. 1971b).

It took more than two decades to finally assign li antigen structures since the original antibodies were found. The i antigen turned out to be a linear repeating of [Galβ1 → 4GlcNAcβ1 → 3Gal] structure (Niemann et al. 1978), and the I antigen turned out to be a branched [Galβ1 → 4GlcNAcβ1 → 3(Galβ1 → 4GlcNAcβ1 → 6) Gal] structure (Watanabe et al. 1979).

In fact, branching of I antigen on adult human erythrocytes allows modifications in two terminal ends (e.g., ABO antigens). When both branches are modified, antibodies that react with carbohydrate antigens such as ABO will exhibit a greater affinity than single antigens on a linear poly-*N*-acetyllactosamine (Romans et al. 1980). Therefore, a linear structure on fetal type i antigen may help to minimize the immune response in maternal–fetal incompatibility of blood group antigens.

9.3 Developmental Changes of Li Antigens

In parallel with the structural analyses of each antigen, attempts have also been made to obtain the direct evidence of li antigen conversion corresponding to developmental stage. In those days, erythrocyte Band 3 glycoprotein was believed to be a major carrier for Ii antigens in erythrocytes that are susceptible to endo-β-galactosidase (Fukuda et al. 1978, 1979b; Childs et al. 1978). According to this, Fukuda et al. (1979a) compared the carbohydrate structure of erythrocyte Band 3 from normal adult, fetus, and adult I variant erythrocyte and demonstrated that one-third of galactose on i antigen is branched by β1 → 6 linkage, yielding I antigen during development. This paper was the first report of the chemical-based analysis which demonstrates direct structural change of cell surface carbohydrate during development. Moreover, Fukuda et al. determined the structure of poly-*N*-acetyllactosamine prepared from umbilical cord blood erythrocytes. The Band

3 carbohydrate fraction that was released by endo-β-galactosidase contained linear lactosamine chain (Galβ1 → 4GlcNAcβ1 → 3)n with the average 4 or 5 of repeating units (Fukuda et al. 1984). Notably, fetal poly-*N*-acetyllactosamine contained NeuNacα2 → 8 NeuNAcα2 → 3Gal structure, of which the structure is absent in adult. In addition to glycoproteins, the Ii antigens were also identified in glycolipids. It was reported that the amount of branched glycolipids was lower in fetal or neonatal erythrocytes than that in adult erythrocytes (Watanabe and Hakomori 1976). Koschielak et al. isolated large glycolipids "polyglycosyl ceramides" from both adult and neonatal erythrocytes and found that the lack of highly branched glycolipids in cord and fetal erythrocytes was due to inadequate biosynthetic process of GlcNAcβ1 → 6 formation (Koscielak et al. 1979). The amount of gangliosides with *N*-acetyllactosamine unit was much higher in neonatal cord blood cells than in adult cells, although there was no difference in the total amount of glycolipids between neonatal and adult cells (Fukuda and Levery 1983). Taken together, Ii antigen structures indeed change from linear to branched poly-*N*-acetyllactosamine during development of erythrocytes and both glycoproteins and glycolipids can be carriers for Ii antigens.

9.4 Enzymes Responsible for the Synthesis of Poly-*N*-Acetyllactosamine, i and I Antigens

*9.4.1 β1-6*N*-Acetylglucosaminyltransferase (IGnT)*

Considering the branching configuration, the enzyme responsible for Ii antigenic conversion had been supposed to be a β1-6*N*-acetylglucosaminyltransferase, of which activity should be regulated during development (Fig. 9.2). At first, activities of *N*-acetylglucosaminyltransferase in various tissues were examined using nonspecific acceptors with ^{3}H-labeled terminal galactose residues (van den Eijnden

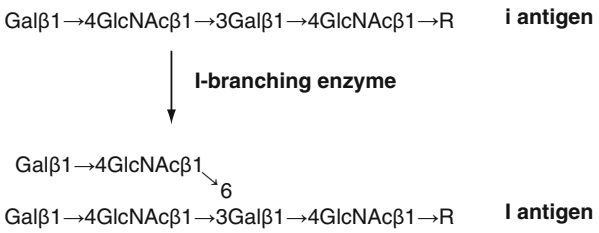

Fig. 9.2 Developmental conversion of linear i to branching I antigen. In fetal erythrocytes, [Galβ1 → 4GlcNAcβ1 → 3]n repeats serve as i antigen. The level of i antigen decreases and abolishes in children by 18 months. With the expression of β1,6-N-acetylglucosaminyltransferase (I-branching enzyme), i antigen is branched into I antigen, forming [Galβ1 → 4GlcNAcβ1 → 3 (Galβ1 → 4GlcNAcβ1 → 6)Gal] (Fukuda et al. 1979a). It is known that both glycoproteins and glycolipids can be carriers for Ii antigens

et al. 1983; Zielenski and Koscielak 1983). Among them, it was found that certain amounts of radioactive galactose residue were indeed substituted in position 6. In 1984, Piller et al. identified the responsible β1-6*N*-acetylglucosaminyltransferase by showing evidence of its enzymatic activity in hog gastric mucosa, using trisaccharide acceptor GlcNAcβ1-3Galβ1-4Glcβ-OMe. This enzyme was found to form GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-3GalNAcα1-R from GlcNAcβ1-3Galβ1-3GalNAcα1-R, acting as distal I enzyme (dIGnT) (see below). On the other hand, the presence of central IGnT (cIGnT) was first described in 1992 (Gu et al. 1992). Later, cDNA encoding *IGnT1* (also known as IGnT or GCNT2), which is the responsible gene for cIGnT, was cloned from human embryonal carcinoma cells, PA-1 (Bierhuizen et al. 1993). Newly developed system of the expression cloning method was successfully applied in this cloning. Since CHO cells originally do not express branched I antigen, forced expression of IGnT1 cDNA theoretically enables to distinguish cells with I phenotype from others. Based on this, the cDNA expression library derived from PA-1 cells was transfected into the CHO cell line that expresses polyoma virus large T-antigen. Then, another enzyme having I-branching activity was identified as C2GnT2 (also known as GCNT3) (Yeh et al. 1999). C2GnT2 was found in the process of cloning for a novel core2 β1-6 *N*-acetylglucosaminyltransferase for *O*-glycans by expressed sequence tag (EST) homology search with C2GnT1. Interestingly, C2GnT2, having a weak enzymatic activity for I-branching, was proven to be the responsible enzyme for dIGnT. Now two synthetic pathways of poly-*N*-acetyllactosamine, which are carried out by IGnT and C2GnT2, have been well characterized (Fig. 9.3). IGnT requires terminal galactose in the substrate to exhibit its enzymatic activity, producing [Galβ1 → 4GlcNAcβ1 → 3(GlcNAcβ1 → 6)Galβ1 → 4GlcNAc], whereas C2GnT2 does not require terminal galactose, producing [GlcNAcβ1 → 3(GlcNAcβ1 → 6) Galβ1 → 4GlcNAc]. The third and fourth enzymes related to IGnT1 were then cloned and designated as IGnT2 and IGnT3 (Inaba et al. 2003). Therefore, four enzymes to date are reported to have I-branching activity. Among them, IGnT1

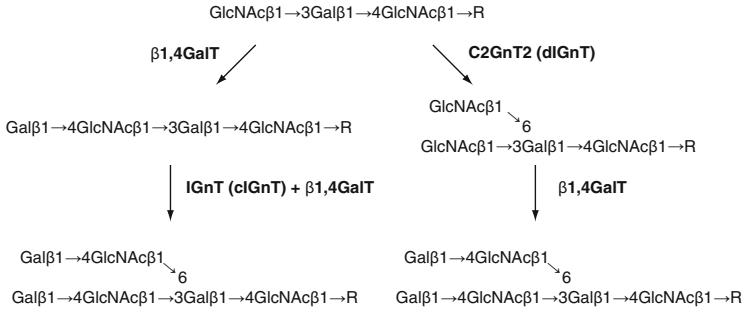

Fig. 9.3 Two distinct synthetic pathways of I-branching in poly-*N*-acetyllactosamine. When galactose is present at the terminal of linear poly-*N*-acetyllactosamine chain, IGnT (cIGnT) acts on the inner galactose residue, forming I branch. When GlcNAcβ1 → 3Galβ1 → R or GlcNAcβ1 → 3GalNAcβ1 → R is available, distal galactose or *N*-acetylgalactosamine residue is utilized by C2GnT2 (dIGnT), forming distal I branch (Yeh et al. 1999)

dysfunction had been long believed to be responsible for the i phenotype. It was reported that individuals with the i phenotype have missense mutations in the exon 3 of IGnT1 gene (Yu et al. 2001). Those mutations were indeed linked to lack of branching activity, resulting in a loss of erythrocyte I antigen. However, when the genomic sequence of the coding regions of three IGnT (IGnT1-3) genes were determined in adult I and i phenotypes, there were two additional types of point mutations in the exon 2. Now it is widely accepted that IGnT3 is the most feasible candidate for the blood group I gene causing i phenotype (Inaba et al. 2003). The adult i phenotype is also reportedly associated with congenital cataracts in Asians (Lin-Chu et al. 1991; Ogata et al. 1979; Yu et al. 2001, 2003) but less associated in Caucasian populations (Macdonald et al. 1983; Marsh and DePalma 1982). Interestingly, IGnT1-deficient mice did not develop cataracts earlier than wild-type mice (Chen et al. 2005). This result may be reflecting the observation that the onset of i phenotype is more attributable to IGnT3 mutation than to IGnT1 mutation.

*9.4.2 β1-3*N*-Acetylglucosaminyltransferases (B3GNTs)*

For the synthesis of backbone of poly-*N*-acetyllactosamine, the presence of β-1,3-N-acetylglucosaminyltransferase is essential. While isolation of IGnT1 cDNA was successful by expression cloning as described above, it took several more years to finally clone the cDNA encoding iGnT (also known as B3GNT1). This is largely because iGnT is ubiquitously present in mammalian cells and it was extremely difficult to obtain the cells entirely deficient in the i antigen. However, by increasing the expression of cDNAs to a comparable level, they could finally obtain enriched cells containing iGnT cDNA and then succeeded in the cDNA cloning (Sasaki et al. 1997). Eventually, seven additional β-1,3-N-acetylglucosaminyltransferases have been so far cloned and their substrate specificities have been studied (Sasaki et al. 1997; Shiraishi et al. 2001; Togayachi et al. 2001; Yeh et al. 2001; Iwai et al. 2002; Ishida et al. 2005). All B3GNTs, except B3GNT6, were shown to have capacity to initiate and elongate poly-*N*-acetyllactosamine chains though they exhibit a different substrate specificity depending on the length of the lactosamine chain (Sasaki et al. 1997; Shiraishi et al. 2001; Ishida et al. 2005). Importantly, B3GNT2-8 shares structural similarity with each other, whereas iGnT is structurally distinct from others. Therefore, it was proposed that iGnT and others might have different functions (Fukuda 2002; Zhou et al. 1999; Shiraishi et al. 2001). Indeed, an enzyme was identified as a responsible gene for meningioma, and one of two conserved domains of the enzyme is similar to iGnT and then called LARGE (like-acetylglucosaminyltransferase) (Peyrard et al. 1999). LARGE was now considered as the key enzyme forming laminin-binding glycans that are attached to α-dystroglycan (Grewal et al. 2001; Kanagawa et al. 2004). Deficiency in LARGE causes muscular dystrophy, and overexpression of LARGE attenuated the phenotype of muscular dystrophy (Barresi and Campbell 2006). It is noteworthy that co-expression of LARGE with iGnT, but not with other B3GNT

members, is crucial for LARGE activity. In fact, LARGE and iGnT were associated with each other in cells to form laminin-binding glycans (Bao et al. 2009). Moreover, only iGnT was identified most recently as the causative gene of Walker–Warburg syndrome that is a genetically heterogeneous form of congenital muscular dystrophy (Shaheen et al. 2013) (Buysse et al. 2013). Collectively, iGnT is functionally as well as structurally distinct from other B3GNT members.

9.4.3 β1-4 Galactosyltransferases (β4GalTs)

So far, seven β4GalTs have been identified and characterized (Almeida et al. 1997, 1999; Narimatsu et al. 1986; Shaper et al. 1988; Masri et al. 1988; Sato et al. 1998; Nomura et al. 1998; Schwientek et al. 1998; Okajima et al. 1999). Among them, β4GalT1 was proven to be crucial for synthesis of poly-*N*-acetyllactosamine in *N*-glycans. In *N*-glycans, poly-*N*-acetyllactosamines are found mainly in tetraantennary and triantennary *N*-glycans which contain a side chain linked to α1,6-linked mannose through a GlcNAcβ1-6 linkage in GlcNAcβ1-6(GlcNAcβ1-2)Manα1-6Manβ-R. It was shown that β4GalT1 prefers GlcNAcβ1-6Manα-R chain rather than GlcNAcβ1-2Manα-R chain, yielding Galβ1-4GlcNAcβ1-6Manα-R (Ujita et al. 1999). This galactosylation gives next two possible pathways. One is that poly-*N*-acetyllactosamine extension occurs on Galβ1-4GlcNAcβ1-6Manα-R chain by iGnT. The other is that β4GalT1 acts on GlcNAcβ1-2Manα-R, producing Galβ1-4GlcNAcβ1-2Manα-R, then poly-*N*-acetyllactosamine extension occurs on Galβ1-4GlcNAcβ1-2Manα-R chain by iGnT. In fact, poly-*N*-acetyllactosamine extension was detected equally between two branches (Ujita et al. 1999). In this study, iGnT level was a rate-limiting factor. Those observations were consistent with structural analysis obtained by granulocytes (Fukuda et al. 1984; Mizoguchi et al. 1984) and erythropoietin (Sasaki et al. 1987; Takeuchi et al. 1988).

By contrast, when poly-*N*-acetyllactosamine was tried to be synthesized on the core2-branched oligosaccharides, the combination of iGnT and milk-derived β4Gal-T1 failed to form the repeating unit (Ujita et al. 1998). There were N-acetylglucosamine residues at most of nonreducing ends, suggesting insufficient galactosylation by β4Gal-TI and the requirement of other members of β4Gal-Ts. Then β4Gal-T4 was identified as the responsible enzyme required for the poly-*N*-acetyllactosamine extension of core2-branched oligosaccharides (Ujita et al. 1998). Indeed, β4Gal-T4 was demonstrated to be a rate-limiting factor for poly-*N*-acetyllactosamine extension in core2-branched *O*-glycans. By contrast, β4Gal-T1 was proven to be the most efficient enzyme for poly-*N*-acetyllactosamine in *N*-glycans. Moreover, β4Gal-T4, but not β4Gal-T1, drastically reduced its enzymatic activity as the poly-*N*-acetyllactosamine extends longer. These findings nicely explain the observation that core2-branched *O*-glycans contain fewer and shorter poly-*N*-acetyllactosamines than *N*-glycans in many cells. It was also reported that β4Gal-T4 decreases its activity on longer glycolipids (Schwientek et al. 1998).

9.5 Poly-*N*-Acetyllactosamine in Cancer

*9.5.1 Poly-*N*-Acetyllactosamine Expressed in* N*-Glycans in Cancer*

The number of *N*-acetyllactosamine repeat and the structure of its side chain are likely to change upon malignant transformation. This was first demonstrated by using polyoma-transformed cells (Yamashita et al. 1984). They showed that expression of poly-*N*-acetyllactosamine and sialyl Lewis X in multiantennary *N*-glycans was correlated with transformation of baby hamster kidney cell. Similar changes were reported in hepatic carcinogenesis (Yamashita et al. 1989) and granulocyte differentiation (Lee et al. 1990). All those studies were focused on the poly-*N*-acetyllactosamine on *N*-glycans. The biosynthesis of poly-*N*-acetyllactosamine in *N*-glycan largely depends on the enzyme Mgat5, which initiates β-1,6-branching from bi-antennary core mannose (van den Eijnden et al. 1988; Shoreibah et al. 1993). A number of reports have shown that the increased activity of Mgat5 is associated with phenotype of oncogenic transformation: baby hamster kidney cells transformed by polyoma virus (Yamashita et al. 1985), rat 2 fibroblast and mouse mammary carcinoma SP1 carrying H-Ras or v-FPS (Dennis et al. 1987, 1989), and mouse fibroblast NIH3T3 cells transformed by Ras (Easton et al. 1991; Lu and Chaney 1993). Mgat5 mRNA levels are increased in Her-2/Neu-transformed cells (Chen et al. 1998) and in tumors of LEC rat model that develop hereditary hepatocarcinomas with age (Miyoshi et al. 1993). Two papers showed Mgat5-deficient tumor cells lost poly-*N*-acetyllactosamine on *N*-glycan (Cummings and Kornfeld 1984; Yousefi et al. 1991), providing us an important insight that Mgat5 activity is rate limiting for poly-*N*-acetyllactosamine biosynthesis in *N*-glycans of tumor cells (Dennis et al. 1999).

*9.5.2 Poly-*N*-Acetyllactosamine Expressed in* O*-Glycans in Cancer*

It is also true that poly-*N*-acetyllactosamine chain can be synthesized from *O*-glycans. For most of the cells, core1 structure, Galβ1 → 3GalNAc, is the major component of *O*-glycans. On the other hand, core2 oligosaccharides, Galβ1 → 3 (GlcNAcβ1 → 6)GalNAc, are converted from core1 oligosaccharides when core2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT) is present. As far as *O*-glycan is concerned, it is believed that poly-*N*-acetyllactosamine can be formed only from core2-branched oligosaccharides. Particularly, poly-*N*-acetyllactosamine extended from core2 *O*-glycan carries sialyl Lewis X, NeuNAcα2 → 3Galβ1 → 4 (Fucα1 → 3)GlcNAc → R. This type of carbohydrate is constitutively expressed on neutrophils, monocytes, and certain T lymphocytes (Fukuda et al. 1984; Spooncer et al. 1984; Asada et al. 1991; Mizoguchi et al. 1984). Sialyl Lewis X

attaches to E- and P-selectin expressed on activated endothelial cells, which allows free-flowing leukocytes to roll, tether, and extravasate into inflammatory site (Rosen and Bertozzi 1996; Forster et al. 2008). In regard to cancer, there is close link between the expression of poly-*N*-acetyllactosamine in *O*-glycan and tumor malignancy. Upon tumor transformation, some types of cells newly express core2 *O*-glycan. In this case, core2 *O*-glycan can serve as a scaffold for newly synthesized poly-*N*-acetyllactosamine in cancer cells (Maemura and Fukuda 1992; Lofling and Holgersson 2009; Stone et al. 2009). Tumor malignancy is closely correlated with terminal modification by sialic acid and fucose moieties (Gilbert 2009; Hedlund et al. 2008; Wang et al. 2009; Cohen et al. 2010).

*9.5.3 Cancer-Dependent Changes of Expression of the Glycosyltransferases Involved in Poly-*N*-Acetyllactosamine Synthesis*

Early studies indicated that poly-*N*-acetyllactosamine repeats in *O*-glycans are almost exclusively extended from the core2 glycans, which are formed by β1 → 6 GlcNAc (GlcNAc to GalNAc) transferase (Fukuda et al. 1986). The enzymatic aspect of core2-branching glycans associated with tumor malignancy was first examined by comparing activities among metastatic murine tumor cell lines (Yousefi et al. 1991). In this report, the branching activity of C2GnT1 (also known as GCNT1) was elevated up to 70 % in the malignant rat 2 and SP1 cells, whereas other glycosyltransferase activities did not significantly change. They also demonstrated that the activity of C2GnT1 is indeed a rate-limiting factor for poly-*N*-acetyllactosamine levels. Soon after, the responsible transferase, C2GnT1, was cloned using CHO cell line that expresses polyoma virus large T-antigen (Bierhuizen and Fukuda 1992). CHO cells were transfected with leukosialin (CD43), a carrier protein for Core2 *O*-glycosylation, in this experiment. Correlation between the expression of C2GnT1 and tumor progression was extensively studied (Saitoh et al. 1991; Shimodaira et al. 1997; Machida et al. 2001; Hagisawa et al. 2005; Hatakeyama et al. 2010; Tsuboi et al. 2011). Another enzyme, C2GnT2, was found to be abundantly expressed in the digestive tract. Interestingly, this enzyme has less stringent substrate specificity and can initiate core4 and I-branches as well as core2 branch (Bierhuizen et al. 1993; Yeh et al. 1999). It was reported that C2GnT3 has also core2-branching activity but not I-branching activity (Schwientek et al. 2000). The physiological significance of C2GnT2 and C2GnT3 in cancer remains to be characterized.

The role of IGnT expression in cancer progression was first proposed by the reports that sera of breast cancer patients contained high level of I antigen and the I antigen levels were correlated with the breast cancer stages (Burchell et al. 1984; Dube et al. 1984, 1987). Recently, the expression profile of IGnT during carcinogenesis was studied more directly using tissue microarray of human breast tumor

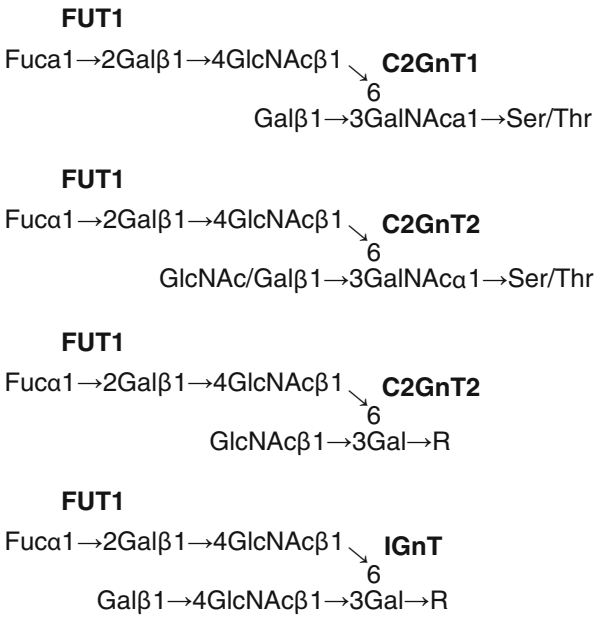

Fig. 9.4 Proposed structure of F77 epitope. Cotransfection analysis suggests that FUT1 plus either C2GnT1, C2GnT2, or IGnT synthesizes Fucα1–2Galβ1–4GlcNAc terminal structure extended from Gal or GalNAc through a GlcNAcβ1–6 linkage

(Zhang et al. 2011). In this experiment, the expression of IGnT was closely related to its basal-like and metastatic phenotypes. In addition, IGnT expression was regulated by TGF-β1 and this enzyme was involved in epithelial-to-mesenchymal transition (EMT) (Zhang et al. 2011). Poly-*N*-acetyllactosamine branching in the breast cancer cells presumably induces further glycan modification, ensuring its highly metastatic potential.

Recent reports showed that poly-*N*-acetyllactosamine chain having terminal α1-2 fucose is recognized by prostate cancer-specific F77 monoclonal antibody (Gao et al. 2014; Nonaka et al. 2014a), which was originally raised against prostate cancer cell line PC-3 and showed strong cytotoxicity to prostate cancer cells in vivo (Zhang et al. 2010). A series of cotransfection studies clearly demonstrated that FUT1 plus either C2GnT1, C2GnT2, or IGnT were required for robust stain by F77 antibody (Fig. 9.4). The study also showed that hypoxia, which is one of the conditions associated with tumor malignancy, elevated FUT1, C2GNT2, and IGnT levels (Nonaka et al. 2014a). Since F77 epitope is synthesized only in the cancer condition, poly-*N*-acetyllactosamine chain containing terminal α1-2 fucose would become a novel marker for prostate cancer.

9.5.4 *Glycosignals of Poly-*N*-Acetyllactosamine in Cancer Cells*

The functional aspect of poly-*N*-acetyllactosamine, particularly with respect to cell signaling, has been extensively studied. In thymus, galectin-1 induces apoptosis of the immature thymocytes which expresses poly-*N*-acetyllactosamine synthesized by C2GnT1 (Galvan et al. 2000; Nguyen et al. 2001). Studies have shown that galectin-1, galectin-3, and galectin-9 bind to T cells that express *N*-lactosamine unit, resulting in suppression of antitumor responses by regulating T cell function (Rubinstein et al. 2004; Demotte et al. 2008; Dardalhon et al. 2010; Peng et al. 2008). T lymphocytes constitutively express poly-*N*-acetyllactosamine chain on CD28 and CD19 costimulatory molecules. Reduction of poly-*N*-acetyllactosamine chain in those molecules elevated intracellular Ca^{2+} flux, resulting in rapid proliferation of the cell (Togayachi et al. 2007).

In contrast to the case of expression in normal cells as described above, it is known that some cancer cells newly obtain poly-*N*-acetyllactosamine chain. Given that the generation of poly-*N*-acetyllactosamine requires a huge energy investment by tumor cells, it is predicted that cells, in return, acquire some advantages that are beneficial for their survival. Oligosaccharide structure that is newly exposed to the outside of the cells plays the role by binding to carbohydrate-binding lectins such as selectin, resulting in promotion of cell–cell interaction and metastasis. Terminal sialyl Lewis X of poly-*N*-acetyllactosamine allows cells to attach to endothelial cells and thus promote tumor metastasis through E-selectin and P-selectin (Laubli and Borsig 2010; St Hill 2011; Sozzani et al. 2008; Yusa et al. 2010; Sawada et al. 1994). Moreover, it was recently reported that colorectal cancer-associated Lewis glycans that are extended from linear poly-*N*-acetyllactosamine chain can be recognized by mannose-binding protein (MBP), a C-type lectin (Terada et al. 2005; Nonaka et al. 2014b). It is interesting that the core structure of the MBP-ligand glycan was found to be multiantennary *N*-glycans and the nonreducing ends consisted of Lewis B–Lewis A or tandem repeats of the Lewis A. Thus it is plausible that MBP recognizes terminal type 1 Lewis glycans. Since MBP reportedly showed antitumor effect in colon cancer cells in vivo (Ma et al. 1999), such glycans are supposedly involved in apoptotic signaling cascade. Furthermore, signaling of poly-*N*-acetyllactosamine in cancer cell is involved in evasion of immune cell attack. In the circulation, rejection of cancer cell occurs by host NK cells. NK cells are activated through the interaction of natural killer group 2 member D (NKG2D) receptor with MHC class I-related chain A (MICA) molecule on tumor cells. This interaction stimulates apoptosis signaling and promotes secretion of granzyme B and perforin. However, once cancer cells acquire poly-*N*-acetyllactosamine chain on MICA molecule, galectin-3 masks NKG2D-binding site of MICA, leading to silencing of NK cells and thereby promoting immune escape of tumor cells (Fig. 9.5) (Tsuboi et al. 2011).

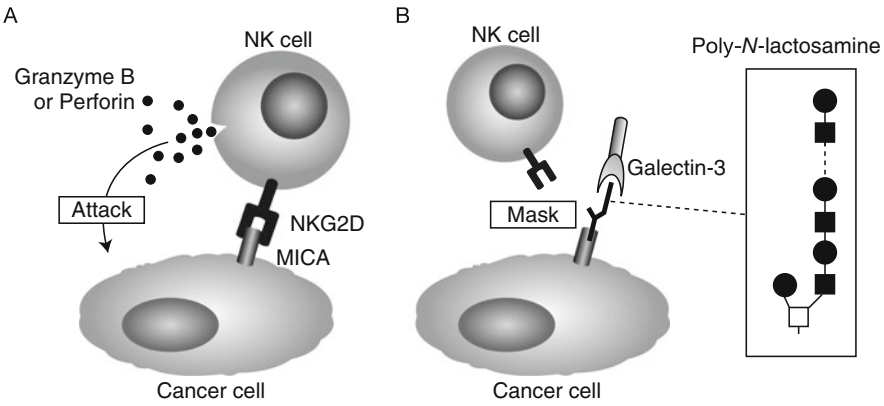

Fig. 9.5 Immune-escaping signals by tumor cells which express poly-*N*-acetyllactosamine chains. (**a**) NK cells secrete cytotoxic granules through interaction with NK cell receptor NKG2D and tumor-derived MICA molecule. (**b**) When tumor cells express poly-*N*-acetyllactosamine glycans on MICA molecule, galectin-3 masks the binding site of MICA. This blocks interaction between NKG2D and MICA, leading to evasion from NK cell immunity

9.6 Concluding Remarks

The glycobiology of poly-*N*-acetyllactosamine has been developed together with the evolution of genetics, immunology, developmental biology, and molecular biology. The expression of poly-*N*-acetyllactosamine chain is regulated by cell-type-specific manner, and it can be further modified by a wide variety of glycosyltransferases or other enzymes. As a result, poly-*N*-acetyllactosamine has multiple functions. The functions of poly-*N*-acetyllactosamine can be conceptionally divided into three categories: (1) poly-*N*-acetyllactosamine serves as backbone for additional modifications such as ABO and Lewis blood type determinants, (2) it behaves as a ligand for antibodies and lectins and actively modulates the function of its carrier proteins or even the cell fates, or (3) it sometimes masks original carbohydrate structures and helps cancer cells to escape from recognition by immune surveillance. The future challenge will be to clarify the whole picture of expression profile of related glycosyltransferases during carcinogenesis, which may lead to discovery of new mechanism for cancer malignancy.

Acknowledgments The authors thank Dr. Michiko N. Fukuda for critical contribution on the early studies. Particularly, the work regarding developmental conversion of i to I antigen could not have been done without the help of her endo-β-galactosidase. This work was supported by National Institutes of Health (NIH) grant no. U01 CA168925 (M. Fukuda).

References

Almeida R, Amado M, David L, Levery SB, Holmes EH, Merkx G, van Kessel AG, Rygaard E, Hassan H, Bennett E, Clausen H (1997) A family of human beta4-galactosyltransferases. Cloning and expression of two novel UDP-galactose:beta-n-acetylglucosamine beta1, 4-galactosyltransferases, beta4Gal-T2 and beta4Gal-T3. J Biol Chem 272(51):31979–31991

Almeida R, Levery SB, Mandel U, Kresse H, Schwientek T, Bennett EP, Clausen H (1999) Cloning and expression of a proteoglycan UDP-galactose:beta-xylose beta1,4-galactosyltransferase I. A seventh member of the human beta4-galactosyltransferase gene family. J Biol Chem 274(37):26165–26171

Asada M, Furukawa K, Kantor C, Gahmberg CG, Kobata A (1991) Structural study of the sugar chains of human leukocyte cell adhesion molecules CD11/CD18. Biochemistry 30 (6):1561–1571

Bahl OP (1969) Human chorionic gonadotropin. II. Nature of the carbohydrate units. J Biol Chem 244(4):575–583

Bao X, Kobayashi M, Hatakeyama S, Angata K, Gullberg D, Nakayama J, Fukuda MN, Fukuda M (2009) Tumor suppressor function of laminin-binding alpha-dystroglycan requires a distinct beta3-N-acetylglucosaminyltransferase. Proc Natl Acad Sci USA 106(29):12109–12114. doi:10.1073/pnas.0904515106, 0904515106 [pii]

Barresi R, Campbell KP (2006) Dystroglycan: from biosynthesis to pathogenesis of human disease. J Cell Sci 119(Pt 2):199–207, doi:119/2/199 [pii]10.1242/jcs.02814

Bierhuizen MF, Fukuda M (1992) Expression cloning of a cDNA encoding UDP-GlcNAc:Gal beta 1-3-GalNAc-R (GlcNAc to GalNAc) beta 1-6GlcNAc transferase by gene transfer into CHO cells expressing polyoma large tumor antigen. Proc Natl Acad Sci USA 89(19):9326–9330

Bierhuizen MF, Mattei MG, Fukuda M (1993) Expression of the developmental I antigen by a cloned human cDNA encoding a member of a beta-1,6-N-acetylglucosaminyltransferase gene family. Genes Dev 7(3):468–478

Burchell J, Wang D, Taylor-Papadimitriou J (1984) Detection of the tumour-associated antigens recognized by the monoclonal antibodies HMFG-1 and 2 in serum from patients with breast cancer. Int J Cancer 34(6):763–768

Buysse K, Riemersma M, Powell G, van Reeuwijk J, Chitayat D, Roscioli T, Kamsteeg EJ, van den Elzen C, van Beusekom E, Blaser S, Babul-Hirji R, Halliday W, Wright GJ, Stemple DL, Lin YY, Lefeber DJ, van Bokhoven H (2013) Missense mutations in beta-1,3-N-acetylglucosaminyltransferase 1 (B3GNT1) cause Walker-Warburg syndrome. Hum Mol Genet 22 (9):1746–1754. doi:10.1093/hmg/ddt021

Chen L, Zhang W, Fregien N, Pierce M (1998) The her-2/neu oncogene stimulates the transcription of N-acetylglucosaminyltransferase V and expression of its cell surface oligosaccharide products. Oncogene 17(16):2087–2093. doi:10.1038/sj.onc.1202124

Chen GY, Muramatsu H, Kondo M, Kurosawa N, Miyake Y, Takeda N, Muramatsu T (2005) Abnormalities caused by carbohydrate alterations in Ibeta6-N-acetylglucosaminyltransferase-deficient mice. Mol Cell Biol 25(17):7828–7838. doi:10.1128/MCB.25.17.7828-7838.2005

Childs RA, Feizi T, Fukuda M, Hakomori SI (1978) Blood-group-I activity associated with band 3, the major intrinsic membrane protein of human erythrocytes. Biochem J 173(1):333–336

Childs RA, Dalchau R, Scudder P, Hounsell EF, Fabre JW, Feizi T (1983) Evidence for the occurrence of O-glycosidically linked oligosaccharides of poly-*N*-acetyllactosamine type on the human leucocyte common antigen. Biochem Biophys Res Commun 110(2):424–431

Cohen M, Elkabets M, Perlmutter M, Porgador A, Voronov E, Apte RN, Lichtenstein RG (2010) Sialylation of 3-methylcholanthrene-induced fibrosarcoma determines antitumor immune responses during immunoediting. J Immunol 185(10):5869–5878. doi:10.4049/jimmunol.1001635

Cummings RD, Kornfeld S (1984) The distribution of repeating [Gal beta 1,4GlcNAc beta 1,3] sequences in asparagine-linked oligosaccharides of the mouse lymphoma cell lines BW5147 and PHAR 2.1. J Biol Chem 259(10):6253–6260

Dardalhon V, Anderson AC, Karman J, Apetoh L, Chandwaskar R, Lee DH, Cornejo M, Nishi N, Yamauchi A, Quintana FJ, Sobel RA, Hirashima M, Kuchroo VK (2010) Tim-3/galectin-9 pathway: regulation of Th1 immunity through promotion of CD11b + Ly-6G+ myeloid cells. J Immunol 185(3):1383–1392. doi:10.4049/jimmunol.0903275

Demotte N, Stroobant V, Courtoy PJ, Van Der Smissen P, Colau D, Luescher IF, Hivroz C, Nicaise J, Squifflet JL, Mourad M, Godelaine D, Boon T, van der Bruggen P (2008) Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. Immunity 28(3):414–424. doi:10.1016/j.immuni.2008.01.011

Dennis JW, Laferte S, Waghorne C, Breitman ML, Kerbel RS (1987) Beta 1–6 branching of Asn-linked oligosaccharides is directly associated with metastasis. Science 236 (4801):582–585

Dennis JW, Kosh K, Bryce DM, Breitman ML (1989) Oncogenes conferring metastatic potential induce increased branching of Asn-linked oligosaccharides in rat2 fibroblasts. Oncogene 4 (7):853–860

Dennis JW, Granovsky M, Warren CE (1999) Glycoprotein glycosylation and cancer progression. Biochim Biophys Acta 1473(1):21–34

Dube VE, Haid M, Chmiel JS, Anderson B (1984) Serum cold agglutinin and IgM levels in breast carcinoma. Breast Cancer Res Treat 4(2):105–108

Dube VE, Kallio P, Chmiel JS, Haid M, Hakim A (1987) An immunosorbent assay for blood group I antigens in breast carcinoma. Clin Immunol Immunopathol 45(2):196–207

Easton EW, Bolscher JG, van den Eijnden DH (1991) Enzymatic amplification involving glycosyltransferases forms the basis for the increased size of asparagine-linked glycans at the surface of NIH 3T3 cells expressing the N-ras proto-oncogene. J Biol Chem 266 (32):21674–21680

Eckhardt AE, Goldstein IJ (1983) Isolation and characterization of a family of alpha-D-galactosyl-containing glycopeptides from Ehrlich ascites tumor cells. Biochemistry 22(23):5290–5297

Feizi T, Kabat EA, Vicari G, Anderson B, Marsh WL (1971a) Immunochemical studies on blood groups. XLVII. The I antigen complex--precursors in the A, B, H, Lea, and leb blood group system--hemagglutination-inhibition studies. J Exp Med 133(1):39–52

Feizi T, Kabat EA, Vicari G, Anderson B, Marsh WL (1971b) Immunochemical studies on blood groups.XLIX. The I antigen complex: specificity differences among anti-I sera revealed by quantitative precipitin studies; partial structure of the I determinant specific for one anti-I serum. J Immunol 106(6):1578–1592

Finne J, Krusius T, Rauvala H, Kekomaki R, Myllyla G (1978) Alkali-stable blood group A- and B-active poly(glycosyl)-peptides from human erythrocyte membrane. FEBS Lett 89 (1):111–115

Forster R, Davalos-Misslitz AC, Rot A (2008) CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 8(5):362–371. doi:10.1038/nri2297

Fukuda M (2002) β1,3-N-acetylglucosaminyltransferase (iGnT). In: Taniguchi N, Honke K, Fukuda M (eds) Handbook of Glycosyltransferase and Related Genes. Springer, Berlin, pp 114–124

Fukuda MN, Levery SB (1983) Glycolipids of fetal, newborn, and adult erythrocytes: glycolipid pattern and structural study of H3-glycolipid from newborn erythrocytes. Biochemistry 22 (21):5034–5040

Fukuda M, Eshdat Y, Tarone G, Marchesi VT (1978) Isolation and characterization of peptides derived from the cytoplasmic segment of band 3, the predominant intrinsic membrane protein of the human erythrocyte. J Biol Chem 253(7):2419–2428

Fukuda M, Fukuda MN, Hakomori S (1979a) Developmental change and genetic defect in the carbohydrate structure of band 3 glycoprotein of human erythrocyte membrane. J Biol Chem 254(10):3700–3703

Fukuda MN, Fukuda M, Hakomori S (1979b) Cell surface modification by endo-beta-galactosidase. Change of blood group activities and release of oligosaccharides from glycoproteins and glycosphingolipids of human erythrocytes. J Biol Chem 254(12):5458–5465

Fukuda M, Spooncer E, Oates JE, Dell A, Klock JC (1984) Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes. J Biol Chem 259(17):10925–10935

Fukuda M, Carlsson SR, Klock JC, Dell A (1986) Structures of O-linked oligosaccharides isolated from normal granulocytes, chronic myelogenous leukemia cells, and acute myelogenous leukemia cells. J Biol Chem 261(27):12796–12806

Galvan M, Tsuboi S, Fukuda M, Baum LG (2000) Expression of a specific glycosyltransferase enzyme regulates T cell death mediated by galectin-1. J Biol Chem 275(22):16730–16737. doi:10.1074/jbc.M001117200

Gao C, Liu Y, Zhang H, Zhang Y, Fukuda MN, Palma AS, Kozak RP, Childs RA, Nonaka M, Li Z, Siegel DL, Hanfland P, Peehl DM, Chai W, Greene MI, Feizi T (2014) Carbohydrate sequence of the prostate cancer-associated antigen F77 assigned by a mucin O-glycome designer array. J Biol Chem 289(23):16462–16477. doi:10.1074/jbc.M114.558932

Gilbert SF (2009) Ageing and cancer as diseases of epigenesis. J Biosci 34(4):601–604

Grewal PK, Holzfeind PJ, Bittner RE, Hewitt JE (2001) Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. Nat Genet 28(2):151–154. doi:10.1038/88865, 88865 [pii]

Gu J, Nishikawa A, Fujii S, Gasa S, Taniguchi N (1992) Biosynthesis of blood group I and i antigens in rat tissues. Identification of a novel beta 1-6-N-acetylglucosaminyltransferase. J Biol Chem 267(5):2994–2999

Hagisawa S, Ohyama C, Takahashi T, Endoh M, Moriya T, Nakayama J, Arai Y, Fukuda M (2005) Expression of core 2 beta1,6-N-acetylglucosaminyltransferase facilitates prostate cancer progression. Glycobiology 15(10):1016–1024. doi:10.1093/glycob/cwi086

Hatakeyama S, Kyan A, Yamamoto H, Okamoto A, Sugiyama N, Suzuki Y, Yoneyama T, Hashimoto Y, Koie T, Yamada S, Saito H, Arai Y, Fukuda M, Ohyama C (2010) Core 2 N-acetylglucosaminyltransferase-1 expression induces aggressive potential of testicular germ cell tumor. Int J Cancer 127(5):1052–1059. doi:10.1002/ijc.25117

Hedlund M, Ng E, Varki A, Varki NM (2008) alpha 2-6-Linked sialic acids on *N*-glycans modulate carcinoma differentiation in vivo. Cancer Res 68(2):388–394. doi:10.1158/0008-5472.CAN-07-1340

Inaba N, Hiruma T, Togayachi A, Iwasaki H, Wang XH, Furukawa Y, Sumi R, Kudo T, Fujimura K, Iwai T, Gotoh M, Nakamura M, Narimatsu H (2003) A novel I-branching beta-1,6-N-acetylglucosaminyltransferase involved in human blood group I antigen expression. Blood 101(7):2870–2876. doi:10.1182/blood-2002-09-2838

Ishida H, Togayachi A, Sakai T, Iwai T, Hiruma T, Sato T, Okubo R, Inaba N, Kudo T, Gotoh M, Shoda J, Tanaka N, Narimatsu H (2005) A novel beta1,3-N-acetylglucosaminyltransferase (beta3Gn-T8), which synthesizes poly-*N*-acetyllactosamine, is dramatically upregulated in colon cancer. FEBS Lett 579(1):71–78. doi:10.1016/j.febslet.2004.11.037

Iwai T, Inaba N, Naundorf A, Zhang Y, Gotoh M, Iwasaki H, Kudo T, Togayachi A, Ishizuka Y, Nakanishi H, Narimatsu H (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(15):12802–12809. doi:10.1074/jbc.M112457200

Jarnefelt J, Rush J, Li YT, Laine RA (1978) Erythroglycan, a high molecular weight glycopeptide with the repeating structure [galactosyl-(1 leads to 4)-2-deoxy-2-acetamido-glucosyl(1 leads to 3)] comprising more than one-third of the protein-bound carbohydrate of human erythrocyte stroma. J Biol Chem 253(22):8006–8009

Kanagawa M, Saito F, Kunz S, Yoshida-Moriguchi T, Barresi R, Kobayashi YM, Muschler J, Dumanski JP, Michele DE, Oldstone MB, Campbell KP (2004) Molecular recognition by LARGE is essential for expression of functional dystroglycan. Cell 117(7):953–964. doi:10.1016/j.cell.2004.06.003, S0092867404005434 [pii]

Kapadia A, Feizi T, Evans MJ (1981) Changes in the expression and polarization of blood group I and i antigens in post-implantation embryos and teratocarcinomas of mouse associated with cell differentiation. Exp Cell Res 131(1):185–195

Knowles BB, Rappaport J, Solter D (1982) Murine embryonic antigen (SSEA-1) is expressed on human cells and structurally related human blood group antigen I is expressed on mouse embryos. Dev Biol 93(1):54–58

Kornfeld R, Kornfeld S (1970) The structure of a phytohemagglutinin receptor site from human erythrocytes. J Biol Chem 245(10):2536–2545

Koscielak J, Zdebska E, Wilczynska Z, Miller-Podraza H, Dzierzkowa-Borodej W (1979) Immunochemistry of Ii-active glycosphingolipids of erythrocytes. Eur J Biochem 96(2):331–337

Krusius T, Finne J, Rauvala H (1978) The poly(glycosyl) chains of glycoproteins. Characterisation of a novel type of glycoprotein saccharides from human erythrocyte membrane. Eur J Biochem 92(1):289–300, FEBS

Laubli H, Borsig L (2010) Selectins promote tumor metastasis. Semin Cancer Biol 20(3):169–177. doi:10.1016/j.semcancer.2010.04.005

Lee N, Wang WC, Fukuda M (1990) Granulocytic differentiation of HL-60 cells is associated with increase of poly-*N*-acetyllactosamine in Asn-linked oligosaccharides attached to human lysosomal membrane glycoproteins. J Biol Chem 265(33):20476–20487

Li E, Gibson R, Kornfeld S (1980) Structure of an unusual complex-type oligosaccharide isolated from Chinese hamster ovary cells. Arch Biochem Biophys 199(2):393–399

Lin-Chu M, Broadberry RE, Okubo Y, Tanaka M (1991) The i phenotype and congenital cataracts among Chinese in Taiwan. Transfusion 31(7):676–677

Lofling J, Holgersson J (2009) Core saccharide dependence of sialyl Lewis X biosynthesis. Glycoconj J 26(1):33–40. doi:10.1007/s10719-008-9159-z

Lu Y, Chaney W (1993) Induction of N-acetylglucosaminyltransferase V by elevated expression of activated or proto-Ha-ras oncogenes. Mol Cell Biochem 122(1):85–92

Ma Y, Uemura K, Oka S, Kozutsumi Y, Kawasaki N, Kawasaki T (1999) Antitumor activity of mannan-binding protein in vivo as revealed by a virus expression system: mannan-binding proteindependent cell-mediated cytotoxicity. Proc Natl Acad Sci USA 96(2):371–375

Macdonald EB, Douglas R, Harden PA (1983) A Caucasian family with the i phenotype and congenital cataracts. Vox Sang 44(5):322–325

Machida E, Nakayama J, Amano J, Fukuda M (2001) Clinicopathological significance of core 2 beta1,6-N-acetylglucosaminyltransferase messenger RNA expressed in the pulmonary adenocarcinoma determined by in situ hybridization. Cancer Res 61(5):2226–2231

Maemura K, Fukuda M (1992) Poly-N-acetyllactosaminyl *O*-glycans attached to leukosialin. The presence of sialyl Le(x) structures in *O*-glycans. J Biol Chem 267(34):24379–24386

Marcus DM, Kabat EA, Rosenfield RE (1963) The action of enzymes from Clostridium tertium on the I antigenic determinant of human erythrocytes. J Exp Med 118:175–194

Marsh WL (1961) Anti-i: a cold antibody defining the Ii relationship in human red cells. Br J Haematol 7:200–209

Marsh WL, DePalma H (1982) Association between the Ii blood group and congenital cataract. Transfusion 22(4):337–338

Masri KA, Appert HE, Fukuda MN (1988) Identification of the full-length coding sequence for human galactosyltransferase (beta-N-acetylglucosaminide: beta 1,4-galactosyltransferase). Biochem Biophys Res Commun 157(2):657–663

Miyoshi E, Nishikawa A, Ihara Y, Gu J, Sugiyama T, Hayashi N, Fusamoto H, Kamada T, Taniguchi N (1993) N-acetylglucosaminyltransferase III and V messenger RNA levels in LEC rats during hepatocarcinogenesis. Cancer Res 53(17):3899–3902

Mizoguchi A, Takasaki S, Maeda S, Kobata A (1984) Changes in asparagine-linked sugar chains of human promyelocytic leukemic cells (HL-60) during monocytoid differentiation and myeloid differentiation. Decrease of high-molecular-weight oligosaccharides in acidic fraction. J Biol Chem 259(19):11949–11957

Muramatsu T, Gachelin G, Damonneville M, Delarbre C, Jacob F (1979) Cell surface carbohydrates of embryonal carcinoma cells: polysaccharidic side chains of F9 antigens and of receptors to two lectins, FBP and PNA. Cell 18(1):183–191

Narimatsu H, Sinha S, Brew K, Okayama H, Qasba PK (1986) Cloning and sequencing of cDNA of bovine N-acetylglucosamine (beta 1–4)galactosyltransferase. Proc Natl Acad Sci USA 83 (13):4720–4724

Nguyen JT, Evans DP, Galvan M, Pace KE, Leitenberg D, Bui TN, Baum LG (2001) CD45 modulates galectin-1-induced T cell death: regulation by expression of core 2 *O*-glycans. J Immunol 167(10):5697–5707

Niemann H, Watanabe K, Hakomori S (1978) Blood group i and I activities of "lacto-N-norhexaosylceramide" and its analogues: the structural requirements for i-specificities. Biochem Biophys Res Commun 81(4):1286–1293

Nomura T, Takizawa M, Aoki J, Arai H, Inoue K, Wakisaka E, Yoshizuka N, Imokawa G, Dohmae N, Takio K, Hattori M, Matsuo N (1998) Purification, cDNA cloning, and expression of UDP-Gal: glucosylceramide beta-1,4-galactosyltransferase from rat brain. J Biol Chem 273 (22):13570–13577

Nonaka M, Fukuda MN, Gao C, Li Z, Zhang H, Greene MI, Peehl DM, Feizi T, Fukuda M (2014a) Determination of carbohydrate structure recognized by prostate-specific F77 monoclonal antibody through expression analysis of glycosyltransferase genes. J Biol Chem 289 (23):16478–16486. doi:10.1074/jbc.M114.559047

Nonaka M, Imaeda H, Matsumoto S, Yong Ma B, Kawasaki N, Mekata E, Andoh A, Saito Y, Tani T, Fujiyama Y, Kawasaki T (2014b) Mannan-binding protein, a C-type serum lectin recognizes primary colorectal carcinomas through tumor-associated Lewis glycans. J Immunol. doi:10.4049/jimmunol.1203023

Ogata H, Okubo Y, Akabane T (1979) Phenotype i associated with congenital cataract in Japanese. Transfusion 19(2):166–168

Okajima T, Yoshida K, Kondo T, Furukawa K (1999) Human homolog of Caenorhabditis elegans sqv-3 gene is galactosyltransferase I involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans. J Biol Chem 274(33):22915–22918

Peng W, Wang HY, Miyahara Y, Peng G, Wang RF (2008) Tumor-associated galectin-3 modulates the function of tumor-reactive T cells. Cancer Res 68(17):7228–7236. doi:10.1158/0008-5472.CAN-08-1245

Peyrard M, Seroussi E, Sandberg-Nordqvist AC, Xie YG, Han FY, Fransson I, Collins J, Dunham I, Kost-Alimova M, Imreh S, Dumanski JP (1999) The human LARGE gene from 22q12.3-q13.1 is a new, distinct member of the glycosyltransferase gene family. Proc Natl Acad Sci USA 96(2):598–603

Roden L (1980) In: Lennarz WJ (ed) The biochemistry of glycoproteins and proteoglycans. Plenum, New York, pp 267–371

Romans DG, Tilley CA, Dorrington KJ (1980) Monogamous bivalency of IgG antibodies. I. Deficiency of branched ABHI-active oligosaccharide chains on red cells of infants causes the weak antiglobulin reactions in hemolytic disease of the newborn due to ABO incompatibility. J Immunol 124(6):2807–2811

Rosen SD, Bertozzi CR (1996) Two selectins converge on sulphate. Leukocyte adhesion. Curr Biol 6(3):261–264

Rubinstein N, Alvarez M, Zwirner NW, Toscano MA, Ilarregui JM, Bravo A, Mordoh J, Fainboim L, Podhajcer OL, Rabinovich GA (2004) Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. Cancer Cell 5(3):241–251

Saitoh O, Piller F, Fox RI, Fukuda M (1991) T-lymphocytic leukemia expresses complex, branched O-linked oligosaccharides on a major sialoglycoprotein, leukosialin. Blood 77 (7):1491–1499

Sasaki H, Bothner B, Dell A, Fukuda M (1987) Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. J Biol Chem 262 (25):12059–12076

Sasaki K, Kurata-Miura K, Ujita M, Angata K, Nakagawa S, Sekine S, Nishi T, Fukuda M (1997) Expression cloning of cDNA encoding a human beta-1,3-N-acetylglucosaminyltransferase that

is essential for poly-*N*-acetyllactosamine synthesis. Proc Natl Acad Sci USA 94 (26):14294–14299

Sato T, Furukawa K, Bakker H, Van den Eijnden DH, Van Die I (1998) Molecular cloning of a human cDNA encoding beta-1,4-galactosyltransferase with 37% identity to mammalian UDP-Gal:GlcNAc beta-1,4-galactosyltransferase. Proc Natl Acad Sci USA 95(2):472–477

Sawada R, Tsuboi S, Fukuda M (1994) Differential E-selectin-dependent adhesion efficiency in sublines of a human colon cancer exhibiting distinct metastatic potentials. J Biol Chem 269 (2):1425–1431

Schwientek T, Almeida R, Levery SB, Holmes EH, Bennett E, Clausen H (1998) Cloning of a novel member of the UDP-galactose:beta-N-acetylglucosamine beta1,4-galactosyltransferase family, beta4Gal-T4, involved in glycosphingolipid biosynthesis. J Biol Chem 273 (45):29331–29340

Schwientek T, Yeh JC, Levery SB, Keck B, Merkx G, van Kessel AG, Fukuda M, Clausen H (2000) Control of *O*-glycan branch formation. Molecular cloning and characterization of a novel thymus-associated core 2 beta1, 6-n-acetylglucosaminyltransferase. J Biol Chem 275 (15):11106–11113

Shaheen R, Faqeih E, Ansari S, Alkuraya FS (2013) A truncating mutation in B3GNT1 causes severe Walker-Warburg syndrome. Neurogenetics 14(3–4):243–245. doi:10.1007/s10048-013-0367-8

Shaper NL, Hollis GF, Douglas JG, Kirsch IR, Shaper JH (1988) Characterization of the full length cDNA for murine beta-1,4-galactosyltransferase. Novel features at the 5'-end predict two translational start sites at two in-frame AUGs. J Biol Chem 263(21):10420–10428

Shimodaira K, Nakayama J, Nakamura N, Hasebe O, Katsuyama T, Fukuda M (1997) Carcinoma-associated expression of core 2 beta-1,6-N-acetylglucosaminyltransferase gene in human colorectal cancer: role of *O*-glycans in tumor progression. Cancer Res 57(23):5201–5206

Shiraishi N, Natsume A, Togayachi A, Endo T, Akashima T, Yamada Y, Imai N, Nakagawa S, Koizumi S, Sekine S, Narimatsu H, Sasaki K (2001) Identification and characterization of three novel beta 1,3-N-acetylglucosaminyltransferases structurally related to the beta 1,3-galactosyltransferase family. J Biol Chem 276(5):3498–3507. doi:10.1074/jbc.M004800200, M004800200 [pii]

Shoreibah M, Perng GS, Adler B, Weinstein J, Basu R, Cupples R, Wen D, Browne JK, Buckhaults P, Fregien N, Pierce M (1993) Isolation, characterization, and expression of a cDNA encoding N-acetylglucosaminyltransferase V. J Biol Chem 268(21):15381–15385

Sozzani P, Arisio R, Porpiglia M, Benedetto C (2008) Is Sialyl Lewis x antigen expression a prognostic factor in patients with breast cancer? Int J Surg Pathol 16(4):365–374. doi:10.1177/1066896908324668

Spiro RG (1969) Glycoproteins: their biochemistry, biology and role in human disease (first of two parts). N Engl J Med 281(18):991–1001. doi:10.1056/NEJM196910302811806

Spooncer E, Fukuda M, Klock JC, Oates JE, Dell A (1984) Isolation and characterization of polyfucosylated lactosaminoglycan from human granulocytes. J Biol Chem 259(8):4792–4801

St Hill CA (2011) Interactions between endothelial selectins and cancer cells regulate metastasis. Front Biosci (Landmark Ed) 16:3233–3251

Stone EL, Ismail MN, Lee SH, Luu Y, Ramirez K, Haslam SM, Ho SB, Dell A, Fukuda M, Marth JD (2009) Glycosyltransferase function in core 2-type protein O glycosylation. Mol Cell Biol 29(13):3770–3782. doi:10.1128/MCB.00204-09

Takeuchi M, Takasaki S, Miyazaki H, Kato T, Hoshi S, Kochibe N, Kobata A (1988) Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. J Biol Chem 263 (8):3657–3663

Tanner MJ, Boxer DH (1972) Separation and some properties of the major proteins of the human erythrocyte membrane. Biochem J 129(2):333–347

Terada M, Khoo KH, Inoue R, Chen CI, Yamada K, Sakaguchi H, Kadowaki N, Ma BY, Oka S, Kawasaki T, Kawasaki N (2005) Characterization of oligosaccharide ligands expressed on

SW1116 cells recognized by mannan-binding protein. A highly fucosylated polylactosamine type *N*-glycan. J Biol Chem 280(12):10897–10913. doi:10.1074/jbc.M413092200, M413092200 [pii]

Togayachi A, Akashima T, Ookubo R, Kudo T, Nishihara S, Iwasaki H, Natsume A, Mio H, Inokuchi J, Irimura T, Sasaki K, Narimatsu H (2001) Molecular cloning and characterization of UDP-GlcNAc:lactosylceramide beta 1,3-N-acetylglucosaminyltransferase (beta 3Gn-T5), an essential enzyme for the expression of HNK-1 and Lewis X epitopes on glycolipids. J Biol Chem 276(25):22032–22040. doi:10.1074/jbc.M011369200

Togayachi A, Kozono Y, Ishida H, Abe S, Suzuki N, Tsunoda Y, Hagiwara K, Kuno A, Ohkura T, Sato N, Sato T, Hirabayashi J, Ikehara Y, Tachibana K, Narimatsu H (2007) Polylactosamine on glycoproteins influences basal levels of lymphocyte and macrophage activation. Proc Natl Acad Sci USA 104(40):15829–15834. doi:10.1073/pnas.0707426104

Tsuboi S, Sutoh M, Hatakeyama S, Hiraoka N, Habuchi T, Horikawa Y, Hashimoto Y, Yoneyama T, Mori K, Koie T, Nakamura T, Saitoh H, Yamaya K, Funyu T, Fukuda M, Ohyama C (2011) A novel strategy for evasion of NK cell immunity by tumours expressing core2 *O*-glycans. Embo J 30(15):3173–3185. doi:10.1038/emboj.2011.215

Ujita M, McAuliffe J, Schwientek T, Almeida R, Hindsgaul O, Clausen H, Fukuda M (1998) Synthesis of poly-*N*-acetyllactosamine in core 2 branched *O*-glycans. The requirement of novel beta-1,4-galactosyltransferase IV and beta-1,3-n-acetylglucosaminyltransferase. J Biol Chem 273(52):34843–34849

Ujita M, McAuliffe J, Hindsgaul O, Sasaki K, Fukuda MN, Fukuda M (1999) Poly-*N*-acetyllactosamine synthesis in branched *N*-glycans is controlled by complemental branch specificity of I-extension enzyme and beta1,4-galactosyltransferase I. J Biol Chem 274(24):16717–16726

van den Eijnden DH, Winterwerp H, Smeeman P, Schiphorst WE (1983) Novikoff ascites tumor cells contain N-acetyllactosaminide beta 1 leads to 3 and beta 1 leads to 6 N-acetylglucosaminyltransferase activity. J Biol Chem 258(6):3435–3437

van den Eijnden DH, Koenderman AH, Schiphorst WE (1988) Biosynthesis of blood group i-active polylactosaminoglycans. Partial purification and properties of an UDP-GlcNAc:N-acetyllactosaminide beta 1–3-N-acetylglucosaminyltransferase from Novikoff tumor cell ascites fluid. J Biol Chem 263(25):12461–12471

Wang FL, Cui SX, Sun LP, Qu XJ, Xie YY, Zhou L, Mu YL, Tang W, Wang YS (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(5–6):437–443. doi:10.1016/j.cdp.2009.01.001

Watanabe K, Hakomori SI (1976) Status of blood group carbohydrate chains in ontogenesis and in oncogenesis. J Exp Med 144(3):644–653

Watanabe K, Hakomori SI, Childs RA, Feizi T (1979) Characterization of a blood group I-active ganglioside. Structural requirements for I and i specificities. J Biol Chem 254(9):3221–3228

Wiener AS, Unger LJ, Cohen L, Feldman J (1956) Type-specific cold auto-antibodies as a cause of acquired hemolytic anemia and hemolytic transfusion reactions: biologic test with bovine red cells. Ann Intern Med 44(2):221–240

Yamashita K, Ohkura T, Tachibana Y, Takasaki S, Kobata A (1984) Comparative study of the oligosaccharides released from baby hamster kidney cells and their polyoma transformant by hydrazinolysis. J Biol Chem 259(17):10834–10840

Yamashita K, Tachibana Y, Ohkura T, Kobata A (1985) Enzymatic basis for the structural changes of asparagine-linked sugar chains of membrane glycoproteins of baby hamster kidney cells induced by polyoma transformation. J Biol Chem 260(7):3963–3969

Yamashita K, Totani K, Iwaki Y, Takamisawa I, Tateishi N, Higashi T, Sakamoto Y, Kobata A (1989) Comparative study of the sugar chains of gamma-glutamyltranspeptidases purified from human hepatocellular carcinoma and from human liver. J Biochem 105(5):728–735

Yeh JC, Ong E, Fukuda M (1999) Molecular cloning and expression of a novel beta-1, 6-N-acetylglucosaminyltransferase that forms core 2, core 4, and I branches. J biol chem 274 (5):3215–3221

Yeh JC, Hiraoka N, Petryniak B, Nakayama J, Ellies LG, Rabuka D, Hindsgaul O, Marth JD, Lowe JB, Fukuda M (2001) Novel sulfated lymphocyte homing receptors and their control by a Core1 extension beta 1,3-N-acetylglucosaminyltransferase. Cell 105(7):957–969

Yousefi S, Higgins E, Daoling Z, Pollex-Kruger A, Hindsgaul O, Dennis JW (1991) Increased UDP-GlcNAc:Gal beta 1-3GaLNAc-R (GlcNAc to GaLNAc) beta-1, 6-N-acetylglucosaminyltransferase activity in metastatic murine tumor cell lines Control of polylactosamine synthesis. J Biol Chem 266(3):1772–1782

Yu LC, Twu YC, Chang CY, Lin M (2001) Molecular basis of the adult i phenotype and the gene responsible for the expression of the human blood group I antigen. Blood 98(13):3840–3845

Yu LC, Twu YC, Chou ML, Reid ME, Gray AR, Moulds JM, Chang CY, Lin M (2003) The molecular genetics of the human I locus and molecular background explain the partial association of the adult i phenotype with congenital cataracts. Blood 101(6):2081–2088. doi:10.1182/blood-2002-09-2693

Yusa A, Miyazaki K, Kimura N, Izawa M, Kannagi R (2010) Epigenetic silencing of the sulfate transporter gene DTDST induces sialyl Lewisx expression and accelerates proliferation of colon cancer cells. Cancer Res 70(10):4064–4073. doi:10.1158/0008-5472.CAN-09-2383

Zhang G, Zhang H, Wang Q, Lal P, Carroll AM, de la Llera-Moya M, Xu X, Greene MI (2010) Suppression of human prostate tumor growth by a unique prostate-specific monoclonal antibody F77 targeting a glycolipid marker. Proc Natl Acad Sci USA 107(2):732–737. doi:10.1073/pnas.0911397107

Zhang H, Meng F, Wu S, Kreike B, Sethi S, Chen W, Miller FR, Wu G (2011) Engagement of I-branching {beta}-1, 6-N-acetylglucosaminyltransferase 2 in breast cancer metastasis and TGF-{beta} signaling. Cancer Res 71(14):4846–4856. doi:10.1158/0008-5472.CAN-11-0414

Zhou D, Dinter A, Gutierrez Gallego R, Kamerling JP, Vliegenthart JF, Berger EG, Hennet T (1999) A beta-1,3-N-acetylglucosaminyltransferase with poly-*N*-acetyllactosamine synthase activity is structurally related to beta-1,3-galactosyltransferases. Proc Natl Acad Sci USA 96 (2):406–411

Zielenski J, Koscielak J (1983) Sera of i subjects have the capacity to synthesize the branched GlcNAc beta (1 leads to 6)[GlcNAc(beta 1 leads to 3)]Gal ... structure. FEBS Lett 163 (1):114–118

Chapter 10
Role of Glycans in Cancer Cell Death: A Deadly Relationship

Inka Brockhausen

Abstract Cancer cells often rapidly proliferate and are resistant to the induction of cell death (apoptosis). The field of apoptosis control by glycans is still in the infant stages and requires much more research and knowledge of the biological functions of cell surface receptors. Extrinsic apoptosis is the cell death cascade initiated at the cell surface involving specific receptors, followed by recruitment of protein complexes and activation of proteins intracellularly that eventually lead to DNA breakage and cell death. The cell surface receptors that bind apoptosis-inducing ligands are glycoproteins, and a number of studies show that the glycans play a role in the expression, transport, oligomerization, or function of the receptors. Glycans can also be directly recognized by apoptosis-inducing lectins. Cancer cells often have specific alterations in these glycan structures. These abnormal glycans, and the enzymes that synthesize these glycans, may be partly responsible for the malfunctioning of apoptosis pathways. In particular, sialic acids and specific sialyltransferases are potential therapeutic targets to increase cell death.

However, it is necessary to consider the glycosylation potentials of the specific cancer cells expressing the glycoproteins involved in apoptosis, such as Fas and receptors for tumor necrosis factor (TNF)α and TNF-related apoptosis-inducing ligand (TRAIL). Cells that undergo apoptosis also show altered expression and activities of glycosyltransferases. Technologies to alter cellular glycosylation may be successful in restoring apoptosis in cancer cells or in maintaining the populations of immune cells that eliminate cancer cells.

Keywords Apoptosis • Fas • TNFα • TRAIL • Receptors • Galectin • *O*-glycosylation • *N*-glycosylation • Glycosyltransferases

List of Abbreviations

AGE	Advanced glycosylation end products
C1GalT	Core 1 β3Gal-transferase

I. Brockhausen (✉)
Department of Biomedical and Molecular Sciences, Queen's University, 18 Stuart Street, Kingston, ON K7L3N6, Canada
e-mail: brockhau@queensu.ca

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_10

C2GnT	Core 2 β6GlcNAc-transferase
C3GnT6	Core 3 β3GlcNAc-transferase
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing nonintegrin
DISC	Death-inducing signaling complex
DR	Death receptor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FADD	Fas-associated death domain adapter protein
FUT	Fucosyltransferase
GalT	Gal-transferase
GlcNAcT	GlcNAc-transferase
NF-κB	Nuclear factor κB
NK	Natural killer cell
ppGalNAcT	Polypeptide GalNAc-transferase
RAGE	Receptor for advanced glycosylation end products
ST3Gal	α3-Sialyltransferase acting on Gal
ST6Gal	α6-Sialyltransferase acting on Gal
ST6GalNAc	α6-Sialyltransferase acting on GalNAc
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

10.1 Introduction

The problem with uncontrolled tumor growth lies in mechanisms that allow rapid proliferation and/or decrease cell death. Many of these processes are initiated by glycoproteins located on the cell surface, and the glycan structures are closely linked to cell death. Cell death can occur by many different mechanisms, including necrosis (leading to inflammation), the homeostatic autophagy process, or the activation of apoptosis pathways (Nikoletopoulou et al. 2013). This review deals mainly with receptor-mediated extrinsic pathways of apoptosis which utilize external signals (Fig. 10.1). Intrinsic apoptosis pathways involve mitochondrial signaling pathways. As a result of either type of induction, cysteine aspartyl proteases (caspases) are cleaved and activated, ultimately leading to DNA fragmentation, cellular changes, and cell death (Nikoletopoulou et al. 2013; Jin et al. 2004).

Glycans play critical roles in the induction of apoptosis. Cancer cell glycoproteins are often altered, and this can potentially impact on the ability of the cell to undergo apoptosis. Cell surface glycans are functionally important in the regulation of receptor functions, cell signaling, cell adhesion, migration, and metastasis. Although many studies suggest a link between glycosylation and apoptosis, few studies have elucidated the specific role of glycans in the initiation and propagation of apoptosis. Many of the cell surface receptors involved in regulating cell growth, proliferation, induction, and regulation of apoptosis have been identified as

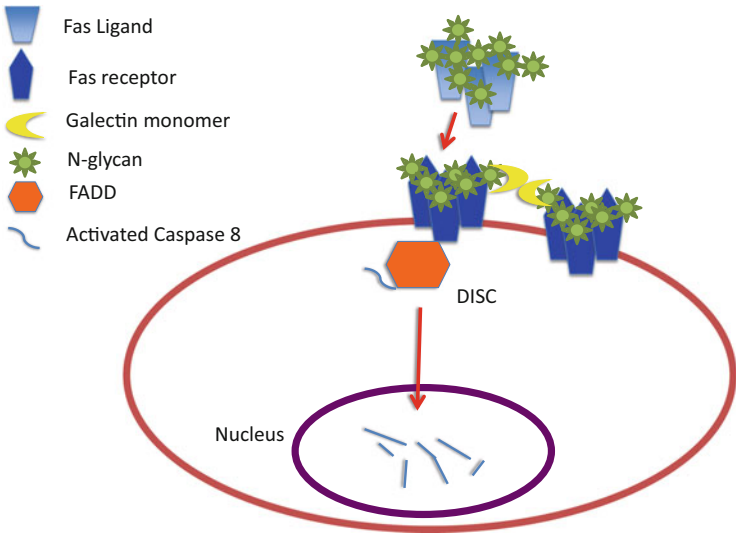

Fig. 10.1 Example of extrinsic apoptosis. In one example of extrinsic apoptosis pathways, trimeric Fas ligand on cell surfaces or in soluble forms binds to its receptor, trimeric Fas. Fas ligand is a glycoprotein with three *N*-glycans, and Fas is a glycoprotein with two *N*-glycans per monomer, thus forming a layer of carbohydrates over a substantial part of the proteins. Interaction between ligand and receptor that has an intracellular death domain induces signaling, including the recruitment of FADD (Fas-associated death domain adapter protein), the formation of death-inducing signaling complex (DISC), and the activation of caspases that eventually lead to DNA fragmentation, morphological changes, and cell death. The cascades are regulated by a multitude of proteins and enzymes intracellularly (not shown), including caspases. Extracellularly, complex glycans have been shown to regulate apoptosis, and apoptotic cells have altered expression of glycosyltransferases and glycans. Multimeric galectins can also induce apoptosis by binding to cell surface Gal residues that may be attached to mucin-type *O*-glycans or receptors, forming glycoprotein lattices

glycoproteins. Upon induction of apoptosis, cells have been shown to exhibit different glycosylation patterns and expression of glycosyltransferases.

However, the functions of posttranslational modifications have received minimal attention, and our knowledge of the role of glycans in regulating the activities of specific receptors is limited. There are many possibilities for carbohydrates to carry out their regulatory functions on the cell surface. This includes roles in expression and stability of glycoproteins, conformational properties of receptors, exposure of ligand-binding sites and regulation of ligand binding, receptor oligomerization, uptake, and signaling. The biological roles of glycans seem to depend on their structures and patterns, their attachment sites, and the specific glycoprotein that carries them.

Apoptosis can be induced by several different mechanisms that appear to be controlled by the presence of glycoprotein-bound glycans. We expect that the altered biosynthesis of glycans in cancer cells leads to functional changes of cell surface receptors (Barthel et al. 2008, 2009; Brockhausen 2006; Brockhausen and

Gao 2012; Picco et al. 2010; Julien et al. 2005, 2006; Pinho et al. 2007). The intracellular signaling pathways leading to apoptosis also include proteins that carry Ser/Thr-linked *O*-GlcNAc residues which appear to be critical for the functions of these proteins (Wells et al. 2003; Lazarus et al. 2009). Gal-binding lectins have critical roles, both in extracellular (extrinsic) and intracellular (intrinsic) apoptosis pathways. A better understanding of the relationship between glycosylation and apoptosis could therefore lead to new therapeutic strategies in cancer.

10.2 *O*- and *N*-glycan Structures Related to Cancer and Cell Death

N- and *O*-glycan structures of glycoproteins are often altered in tissues and cells undergoing apoptosis or oncogenic transformation (Adamczyk et al. 2012; Brockhausen 2006; Brockhausen and Gao 2012; Hiraishi et al. 1993; Minamide et al. 1995; Rapoport and Le Pendu 1999; Russell et al. 1998). For example, Lewis-type glycan structures are associated with the apoptotic phenotype, and specific glycans or glycosylation phenotypes have been shown to contribute to receptor signaling and the induction of apoptosis or proliferation (Gabius 2001; Orlinick et al. 1997; Patsos et al. 2007; Wagner et al. 2007; Woronowicz et al. 2004). Glycans potentially regulate receptor functions such as receptor oligomerization, uptake, and signaling.

Many glycoproteins have been shown to require *N*-glycans for stability, secretion, conformation, and function. Sialic acids are commonly found on the terminal position of an oligosaccharide chain and can modulate adhesive interactions. Short sialylated *O*-glycan chains (sialyl-Tn and sialyl-Thomsen-Friedenreich (TF) antigens) as well as their unsialylated versions are ubiquitous in cancer cells, e.g., in prostate, breast, and colon cancer cells (Kim et al. 1996; Springer et al. 1995) (Fig. 10.2). In contrast, the *N*-glycans of cancer glycoproteins are often highly branched and extended and carry an excess of sialic acid residues, in particular with sialylα2-6 linkages (Dall'Olio et al. 1989; Lise et al. 2000; Pinho et al. 2009; Wang et al. 2009). The amount of sialic acid on cancer cells is usually increased in cancer, in metastatic cells, and in leukemia, and this may contribute to the survival of cells (Bresalier et al. 1996; Brockhausen and Kuhns 1997; Keppler et al. 1999). Changes of glycan structures are expected to have a significant impact on the cell surface functions, adhesive properties, and survival of cancer cells in the blood (Picco et al. 2010). Sialylated short *O*-glycans as well as sialylated *N*-glycans (Fig. 10.3) appear to lead to a decrease in cell adhesion and an increase in migration, invasion, and survival of cancer cells that could form metastases (Brockhausen 2006; Julien et al. 2005, 2006; Pinho et al. 2007; Wang et al. 2009). Sialylation provides favorable conditions for tumor dissemination and survival of tumor cells in the blood and protects cells in the blood from recognition by glycan-binding proteins and uptake.

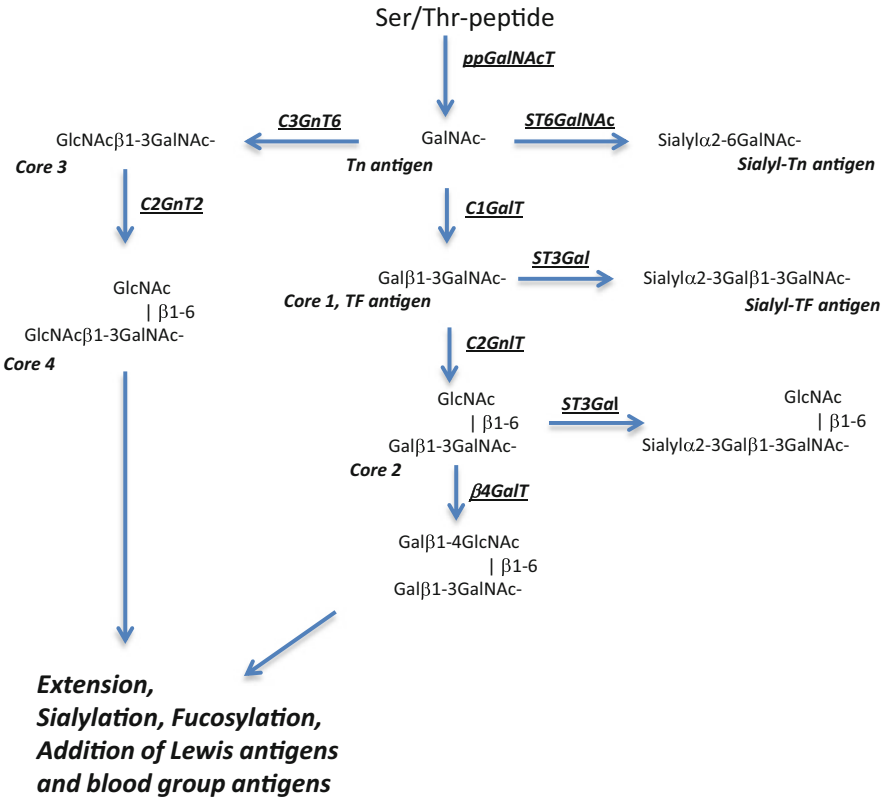

Fig. 10.2 Main *O*-glycosylation biosynthetic pathways in apoptotic cancer cells. *O*-glycans in cancer cells often have shorter structures, compared to normal cells, but complex branched and Lewis-type structures are also found on aggressively growing cancer cells. All mucin-type *O*-glycans have GalNAcα linked to Ser or Thr (the Tn antigen) which is transferred by members of related polypeptide GalNAc-transferases (ppGalNAcT). ppGalNAcT3, ppGalNAcT6 and ppGalNAcT14 are involved in the regulation of apoptosis, possibly by acting on different glycoprotein substrates. Subsequently, β3-Gal-transferase C1GalT adds a Gal residue to GalNAc, to synthesize core 1, the TF antigen. The unmodified Tn and TF antigens are often associated with a poor prognosis in cancer. Alternatively, core 3 is synthesized by core 3 β3-GlcNAc-transferase C3GnT6. The α3-sialyltransferases ST3Gal synthesize the sialyl-TF antigens, while α6-sialyltransferases ST6GalNAc add sialic acid to GalNAc and synthesize sialyl-Tn and sialyl-T antigens. In the absence of core sialylation, a branch can be introduced by β6-GlcNAc-transferases C2GnT to form core 2 from core 1 and by C2GnT2 to form core 4 from core 3. All of these core structures can be extended, and many different antigens and terminal structures can be added, depending on the expression levels of a multitude of glycosyltransferases. Specific members of the extending Gal-transferase family (β4GalT) have been shown to control cell proliferation and apoptosis, but the roles of many more glycosyltransferases in apoptosis remain to be defined. The activities of glycosyltransferases can vary significantly among cancer cells and are often different from those in the corresponding normal cell types. Activities are also affected when cells undergo apoptosis

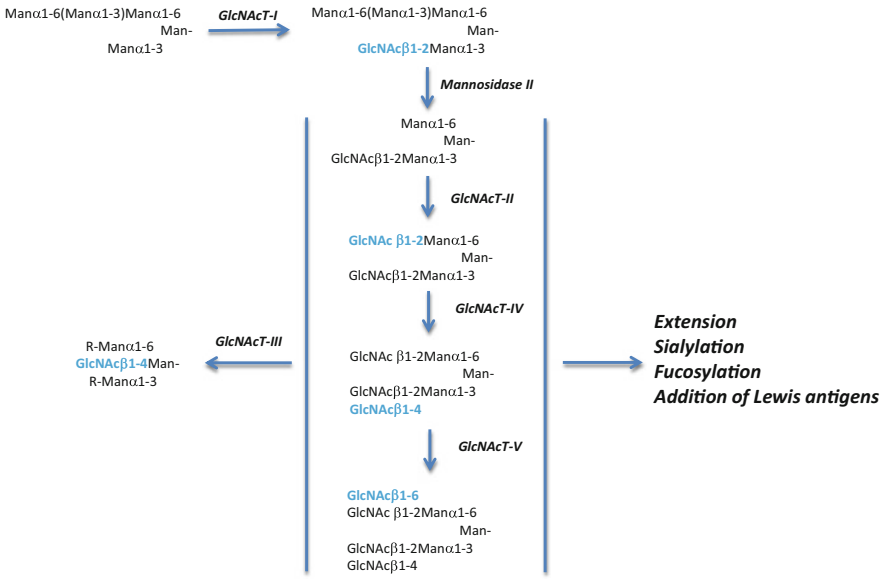

Fig. 10.3 Main biosynthetic pathways of complex *N*-glycans in cancer cells. Complex branched *N*-glycans are often found in cancer cells, and the overall structures as well as specific terminal structures appear to be involved in the biological properties of cancer cells and the metastatic process, as well as in controlling apoptosis. GlcNAc-transferase I (GlcNAcT-I) synthesizes the first antenna and is the critical enzyme for the formation of complex *N*-glycans. The addition of the first GlcNAcβ1-2 residue to the *N*-glycan core is followed by the removal of Man residues by mannosidase II, allowing GlcNAcT-II to act. GlcNAcT-III adds the bisecting residue which appears to distort the *N*-glycan conformation resulting in decreased further processing of *N*-glycan chains. This appears to reduce the metastatic properties of cancer cells. Alternatively, GlcNAcT-IV and GlcNAcT-V add additional antennae which can all be extended by alternating Gal and GlcNAc residues and terminated and further processed in similar pathways as for *O*-glycans. GlcNAcT-V is often associated with metastatic cells and decreased cell-cell adhesion. Some of the extending enzymes, e.g., sialyltransferases, prefer to act on *O*-glycans and some prefer *N*-glycans. For example, α6-sialyltransferase ST6Gal acts on *N*-glycans of Fas and can protect cells from apoptosis

10.2.1 Role of O-Glycan Structures in Cancer and Cell Death

The major *O*-glycan structures of glycoproteins and mucins are based on four core structures, with cores 1 and 2 being the most common (Brockhausen 2010; Gao et al. 2013a). Cores can be extended and terminated with a great variety of glycan structures (Fig. 10.2). Alterations of *O*-glycans in cancer have been summarized in previous reviews (Brockhausen and Kuhns 1997; Brockhausen 1999). Sialyl-Tn expression has been shown to play an important role in the development of malignant carcinoma cell types as well as regulation of apoptosis (Pinho et al. 2007) and may protect cancer cells from natural killer (NK) cell cytotoxicity (Blottière et al. 1992; Ogata et al. 1992). The sialyl-Tn, Tn, and TF antigens have been used as cancer vaccines with moderate success (Hakomori 2001; Julien

et al. 2009). The hope is to use these cancer-associated antigens to target therapeutic antibodies or drugs to tumors.

The branched core 2 structure can be extended by Gal and other sugar residues and carry a number of sialic acid residues and other epitopes such as Lewis antigens and has been associated with an aggressive and invasive cancer phenotype (Hagisawa et al. 2005; Hatakeyama et al. 2010; Shimodaira et al. 1997). Lewis antigens are often present on *O*-glycans and found in apoptotic tissue (Fujita et al. 2011; Nakamori et al. 1993). These antigens play an important role in the interactions of cancer cell glycoproteins with carbohydrate-binding molecules (selectins) of the immune system and trafficking of cancer cells (Läubli and Borsig 2010). Extensive efforts have been made to synthesize analogs of Lewis antigens to block cell adhesion involving selectins. Studies on death receptors suggest that O-glycans are important in their functions (see Sect. 10.5.3).

10.2.2 Role of N-Glycan Structures in Cancer and Cell Death

N-glycans are important in the regulation of cell adhesion, protein structure, and ligand binding and are found on many cell surface receptors and their ligands. Changes in the ratios of bi-, tri-, and tetra-antennary *N*-glycan structures commonly occur on cancer glycoproteins. Multivalent lectins that bind to multiple *N*-glycans on glycoproteins can form lattices that regulate cell adhesion, cell growth, and apoptosis (Lagana et al. 2006).

The GlcNAcβ1-6Man antennae appear to have anti-adhesive properties and often but not always correlate with metastasis (Guo et al. 2000; Przybylo and Lityńska 2011). While bisected GlcNAcβ1-4Man-linked *N*-glycans cause increased cell adhesion of mammary tumor cells possibly through E-cadherin interactions which suppress metastases, the GlcNAcβ1-6 antennae contribute to decreased cell adhesion and tumor cell invasiveness (Pinho et al. 2009). In B16 melanoma cells, bisected GlcNAc residues reduced integrin $\alpha_5\beta_1$-mediated adhesion to fibronectin, and this may be the reason why decreased numbers of cancer cells appear in the circulation (Isaji et al. 2004).

Sialic acids are ligands for sialic acid-binding lectins (siglecs) as regulators of immune cell activation (Läubli and Borsig 2010) and mask the Gal receptor ligands for galectins or ligands for other lectins (Stillman et al. 2006). Highly α6-sialylated colon cancer cells were shown to be more invasive through the extracellular matrix (Zhu et al. 2001). In addition, glycans having α3-linked sialic acid were shown to be prevalent in gastric tumors with a high metastatic potential, and this correlated with invasive depth and lymph node metastasis (Wang et al. 2009). The biological activities of cell surface glycoproteins that control cell growth and cell death in cancer cells often involve *N*-glycan-bound sialic acids (Arnold et al. 2011; Dall'Olio and Chiricolo 2001).

10.3 Biosynthesis of Glycoproteins in Cancer and Apoptotic Cells

Abnormal glycan structures of cancer glycoproteins are the result of the altered activities of glycosyltransferases (Dalziel et al. 2001; Kudo et al. 1998; Petretti et al. 2000; Brockhausen 1999; Brockhausen and Gao 2012). We are just beginning to understand the complex control of glycosyltransferase gene expression, in different growth conditions and after cytokine treatments (Brockhausen et al. 2002; Delmotte et al. 2002; Garcia-Vallejo et al. 2006; Higai et al. 2006; Yang et al. 2004, 2007, 2008). Cultured cells derived from prostate or colonic tumors can show significant differences in glycosylation potential (Gao et al. 2012; Vavasseur et al. 1995) indicating that glycosylation is not tumor specific but can be variable and depend on cell type, growth condition, and the biological activity of the cell.

10.3.1 Abnormal O-Glycan Biosynthesis in Cancer

The *O*-glycan chains of glycoproteins are initiated by the addition of GalNAcα to Ser or Thr by members of a large family of polypeptide GalNAc-transferases (ppGalNAcT), forming the cancer-associated Tn antigen (Fig. 10.2). Specific members of this transferase family have been associated with cancer and increased cell growth. For example, ppGalNAcT3 has been found to be overexpressed in human pancreatic cancer tissues. Knockdown of the enzyme expression in pancreatic cancer cells attenuated growth and increased the number of cells undergoing apoptosis (Taniuchi et al. 2011). The expression of another GalNAc-transferase, ppGalNAcT6, is also upregulated in pancreatic tissues, as well as in the majority of breast cancers (Li et al. 2011; Park et al. 2010, 2011). When the expression of the enzyme was suppressed in metastatic breast cancer cells, cell adhesion was enhanced and cell proliferation was suppressed. The high expression of another enzyme of this family, ppGalNAcT14, in patient's breast tumors (Wu et al. 2010) was found to be associated with invasiveness, mucinous adenocarcinomas, and ductal carcinomas in situ. Although it is not clear which glycoprotein substrates these enzymes act on, these results suggest that excessive *O*-glycosylation may affect the activities of cell surface receptors involved in proliferation or cell death.

The sialyltransferase ST6GalNAc-1 is the main enzyme that converts the Tn to the sialyl-Tn antigen (Brockhausen 2010). Alternatively, *O*-glycan core 1 (TF antigen) is synthesized by core 1 β3-Gal-transferase (C1GalT) that is related to T-cell apoptosis (Chen et al. 2012). The activity is often absent in cancer cells (Brockhausen 1999) due to lack of necessary co-expression of the unique chaperone Cosmc (Ju et al. 2008). Core 1 is a substrate for a multitude of elongating, branching, or terminally acting glycosyltransferases.

In selected cell types such as colonic cells, core 3 β3-GlcNAc-transferase (core 3 synthase, C3GnT6) is expressed, the enzyme that competes with C1GalT to synthesize *O*-glycan core 3 from GalNAc-R (Gao et al. 2012; Vavasseur et al. 1995). Core 3 may be functionally very significant and appears to have a protective function. In prostate cancer cells, core 3 synthase reduced the ability of cell migration and invasion through extracellular matrix components and suppressed tumor formation and metastasis in mice (Lee et al. 2009). In human pancreatic cancer cells, core 3 synthase expression is lost, and forced re-expression suppressed tumor growth and metastases in mice (Radhakrishnan et al. 2013).

O-glycan core 1 can be branched by members of the β6-GlcNAc-transferase (C2GnT) family to form core 2 (Brockhausen 2010; Gao et al. 2013a). C2GnT1 is variably active in cancer cells (Gao et al. 2012; Vavasseur et al. 1995) and forms a scaffold for the addition of sialyl-Lewis^x. Therefore, C2GnT1 can regulate cancer cell adhesion to the endothelium, and its expression correlates with aggressiveness and invasiveness of cancer cells (Hagisawa et al. 2005; St. Hill et al. 2009). C2GnT1 also regulates apoptosis in T cells by forming a scaffold that presents Gal residues, the receptors for apoptosis-inducing galectins (Nguyen et al. 2001; Valenzuela et al. 2007). The MUC1 mucin, which is usually overexpressed in epithelial cancers, can carry the core 2 structure that shields cells from cytotoxicity induced by NK cells. In bladder cancer metastasis, C2GnT1 provides protection of cancer cells against NK cell-induced cell death which prolongs cancer cell survival (Okamoto et al. 2013; Suzuki et al. 2012), possibly by displaying extended and branched glycans that interfere with NK cell binding. C2GnT2 is a homologous enzyme that synthesizes core 2 from core 1 and also core 4 from core 3 (Fig. 10.2). In contrast to C2GnT1, C2GnT2 appears to have a protective function, and the expression of C2GnT2 in colon cancer cells causes growth inhibition (Huang et al. 2006).

Sialylation of cancer cells is mediated by a large family of sialyltransferases (Cazet et al. 2010; Dall'Olio and Chiricolo 2001). Inhibition of high sialyltransferase activities in cancer may increase galectin-induced apoptosis by exposing underlying Gal residues. The expression levels and activities of ST3Gal-I that synthesizes the sialyl-TF antigen (sialylα2-3Galβ1-3GalNAc-) are high in many different types of cancer cells and tissues (Brockhausen and Gao 2012; Burchell et al. 1999). Picco et al. (2010) found that a high expression level of ST3Gal-I promotes tumorigenesis of breast cancer cells, likely by prolonging the life span of circulating tumor cells displaying sialyl-T on the surface. ST3Gal-I also contributes to resistance to galectin-1-induced apoptosis (Valenzuela et al. 2007). This suggests that ST3Gal-I is a potential target for anticancer treatment.

10.3.2 Altered Biosynthesis of N-Glycans in Cancer

The antennae of *N*-glycans are initiated by GlcNAc-transferases-I to GlcNAc-transferases-V followed by extension by Gal-transferases and GlcNAc-transferases

and termination by sialyltransferases and Fuc-transferases (Brockhausen 2010; Taniguchi and Korekane 2011) (Fig. 10.3). Changes in these enzyme activities affect the number of antennae and functional glycan epitopes, the bulkiness of glycans, and adhesive properties of glycans and could alter the functions of glycoproteins.

In a number of cancer models studied, GlcNAcT-III caused a decrease in cell migration, and GlcNAcT-V promoted cell motility. GlcNAcT-V clearly plays a critical role in tumorigenesis and metastasis (Demetriou et al. 1995; Granovsky et al. 2000; Zhou et al. 2011). GlcNAcT-V contributes to the bulkiness of *N*-glycans by synthesizing an additional antenna and contributes to loss of contact inhibition and reduced cell-extracellular matrix interactions of cancer cells and may correlate with malignant potential (Ohyama 2008). Thus, increased expression of GlcNAcT-V has been found in many metastatic cells (Gao et al. 2012; Petretti et al. 2000).

In contrast, GlcNAcT-III synthesizes bisected *N*-glycan structures that block further processing and extension of chains, and the enzyme appears to have anti-metastatic properties (Yoshimura et al. 1995; Zhao et al. 2008). In mice, GlcNAcT-III inhibited breast tumor growth, possibly by reducing platelet-derived growth factor signaling (Song et al. 2010). In human gastric carcinoma cells, GlcNAcT-V caused increased cell migration on laminin, while GlcNAcT-III had the opposite effect (Kariya et al. 2008). Thus, additional carbohydrate epitopes created by GlcNAcT-V could promote lattice formation of lectins and regulate cell adhesion, motility, cell growth, and apoptosis (Guo et al. 2000; Lagana et al. 2006; Tsui et al. 2008). However, the biological effects of highly branched *N*-glycans can vary among tumor types and are a function of the individual glycoproteins involved in the metastatic process (Ohyama 2008).

Colon cancer and many other cancers and metastatic cells exhibit a high expression of α6-sialyltransferase (ST6Gal) (Dall'Olio et al. 1989; Dall'Olio and Chiricolo 2001; Swindall and Bellis 2011). ST6Gal is the major enzyme responsible for sialylation of *N*-glycans and may protect cancer cells from the induction of apoptosis. ST6Gal substrates include the TNFR1 receptor and Fas (Swindall and Bellis 2011), and receptor sialylation prevents the induction of apoptosis. The sialyl residues added by ST6Gal may extend the lifetime of cancer cells and confer the ability to migrate through extracellular matrix (Zhu et al. 2001).

Cancer-selective or cancer-specific antibacterial peptides can be cytotoxic to cancer cells (Han et al. 2013). Cationic antibacterial peptides may interact with negatively charged sialic acid on cell surfaces which play a role in cytotoxicity. Cationic peptides bound less to breast cancer cells after treatment of cells with α2-3,6,8-specific sialidase, tunicamycin, GalNAc-benzyl, and ganglioside biosynthesis inhibitor, suggesting a role of sialic acids and O-glycosylation or *N*-glycosylation in cationic peptide binding (Han et al. 2013). Upon alteration of glycosylation, the lethal effects of cationic peptides were reduced. In contrast, when Chinese hamster ovary cells were transfected with ST6Gal, the lethal effect of cationic peptides was significantly increased, presumably since more binding sites were available. Similar results were obtained from a breast cancer cell xenograft mouse model where the cationic peptide buforin IIb induced extensive apoptosis in tumor cells. Buforin

Table 10.1 Abnormal *N*- and *O*-glycan structures and glycosyltransferase levels in apoptotic cells

Abnormality	Cell/tissue	Reference
Altered biosynthesis		
Expression levels of many GT	Endothelial cells (TNFα)	Garcia-Vallejo et al. (2006)
		Brockhausen et al. (2002)
Activities of GT	Bone and cartilage cells (TNFα and TGFβ)	Yang et al. (2004, 2007, 2008)
β4GalTII	HeLa cells (cisplatin, adriamycin)	Zhou et al. (2008)
Altered structures		
Sialyl-Tn	Gastric cancer cells	Pinho et al. (2007)
Sialyl-Lewis^x	Colorectal cancer	Nakamori et al. (1993)
	Breast cancer	Fujita et al. (2011)
Lewis^y	Skin cells	Hiraishi et al. (1993)
Truncated *O*-glycans (GalNAc-benzyl)	HL60 cells	Li et al. (2007)
	Colon cancer cells	Patsos et al. (2009)
Fewer *N*-glycans (tunicamycin)	Many cell types	Shiraishi et al. (2005)

When cells or tissues undergo apoptosis, expression and activities of several glycosyltransferase (GT) are affected, and thus the structures of glycoprotein-bound glycans are altered

IIb induces caspase-dependent apoptosis in MCF-7 or Jurkat cells likely through electrostatic interactions. Thus, the negative charges of *N*-glycan-bound sialic acids have diverse effects on apoptosis.

10.3.3 Glycosyltransferases in Apoptotic Cells

The inflammatory cytokine TNFα has been shown to induce apoptosis while altering the gene expression and activities of glycosyltransferases in a number of cell types, including endothelial cells and bone cancer cells (Brockhausen et al. 2002; Garcia-Vallejo et al. 2006; Yang et al. 2004, 2007, 2008). This suggests that downstream events of TNFα effects regulate many different genes (Table 10.1).

A family of Gal-transferases adds Gal residues to glycoproteins and glycolipids with slightly different substrate specificities (Figs. 10.2 and 10.3). The Golgi enzyme β4-Gal-transferase (β4GalTI) extends *N*- and *O*-glycan chains, and the expression of β4GalTI regulates apoptosis. The long isoform of β4GalTI has been found to distribute to the cell surface in several cell types including mammary gland, where it can act as a GlcNAc-binding lectin and participate in cell adhesion events. Cell surface β4GalTI was shown to inhibit the autophosphorylation of the epidermal growth factor receptor (EGFR) and thus proliferation in human hepatocarcinoma cells. Overexpression of the enzyme increased

cycloheximide-induced cell death (Li et al. 2006). The enzyme is a receptor for extracellular matrix components on the cell surface of murine mammary glands (de la Cruz et al. 2004) where it promotes apoptosis as well as morphological changes. In Schwann cells, β4GalTI expression controls both proliferation and apoptosis induced by TNFα (Yuan et al. 2012). Altered expression levels of β4-GalT affect several different pathways mediated by TNFα.

Another enzyme involved in *N*-glycan chain extension is β4GalTII. The enzyme is regulated in cancer and in neural development and was shown to also have a pro-apoptotic function in HeLa cells (Jiang et al. 2007). Other members of this glycosyltransferase family, β4GalTI to β4GalTV and β4GalTVII, have various effects on apoptosis suggesting that Gal residues on different glycoproteins have distinct biological functions. The chemotherapeutic agent cisplatin induced DNA damage and apoptosis in HeLa cells as well as an increased expression of β4GalTII. The Golgi localization of the enzyme was critical for this effect. The β4GalTII gene appears to be a target for the p53 transcription factor. Adriamycin which causes DNA damage and apoptosis through the p53 transcription factor also induced the overexpression of β4GalTII in HeLa cells (Zhou et al. 2008). When the expression of β4GalTII was decreased by short inhibitory RNA (iRNA) interference, p53 transcription factor-mediated apoptosis induced by adriamycin was reduced. This suggests a close link between apoptosis and Gal-transferase expression and emphasizes the importance of Gal residues (Table 10.2).

Changes in sialylation status often accompany dying cells. For example, during camptothecin-induced apoptosis in rat cortical neurons, significantly higher amounts of sialylα2-3 residues were expressed on the cells' surface (Kim et al. 2007). ST3Gal-III is involved in sialyl-Lewis^x synthesis. Mice deficient in the ST3Gal-III gene have less siglec ligands in their airways but more intense allergic eosinophilic inflammation. This indicates that pro-apoptotic pathways that control eosinophilic inflammation may require sialic acid (Kiwamoto et al. 2014). The commonly used chemotherapeutic agent taxol causes an arrest of the cell cycle and upregulates ST3Gal-III expression in human ovarian cancer cells. A high expression of ST3Gal-III was found to downregulate caspase-8, thus inhibiting taxol-induced apoptosis and providing a negative feedback mechanism (Huang et al. 2009). In K562 cells, the cytotoxicity of phenol derivatives (but not phenol itself) was accompanied by increased cell surface expression of Fas and FasL, increased mRNA expression of Neu3 sialidase, and decreased expression of sialyltransferase ST3Gal-III, indicating that sialic acid metabolism is critically important in apoptosis (Wang 2012).

10.4 Glycosylation and Cell Death

Apoptosis is a natural process that helps to eliminate excess of senescing and cytotoxic cells. In non-cancerous cells, apoptosis is critical for homeostasis, for the control of the immune system, and in the development. This process is often

Table 10.2 Glycosyltransferases involved in the regulation of apoptosis

Glycosyltransferase	Cell/tissue	Effect	Reference
ppGalNAcT3	Pancreatic cancer cells	Increases apoptosis	Taniuchi et al. (2011)
ppGalNAcT6	Breast cancer cells	Suppresses proliferation	Park et al. (2010)
ppGalNAcT14	Colon cancer cells	Increases TRAIL apoptosis	Wagner et al. (2007)
β4GalT-I	Mammary cells	Decreases proliferation	Li et al. (2006)
		Increases apoptosis	De la Cruz et al. (2004)
	Schwann cells	Controls apoptosis and proliferation	Yuan et al. (2012)
β4GalT-II	HeLa cells	Pro-apoptotic	Jiang et al. (2007) Zhou et al. (2008)
β4GalT-III	Ovarian cancer cells	Inhibits apoptosis	Huang et al. (2009)
C2GnT1	T cells	Supports galectin apoptosis	Valenzuela et al. (2007) Nguyen et al. (2001)
	Bladder cancer cells	Blocks NK cell apoptosis	Suzuki et al. (2012) Okamoto et al. (2013)
α3-Sialyltransferases (ST3Gal-I)	cells	Block galectin apoptosis	Valenzuela et al. (2007) Toscano et al. (2007)
α3-Sialyltransferase (ST3Gal-III)	Ovarian cancer cells	Blocks apoptosis	Huang et al. (2009)
α6-Sialyltransferase (ST6Gal)	T cells	Blocks galectin apoptosis	Clark and Baum (2012)
	Colon cancer cells	Blocks Fas apoptosis	Swindall and Bellis (2011)
	Monocytic cells	Blocks TNF apoptosis	Liu et al. (2011)

deregulated in cancer, thus resulting in proliferation rather than removal of cancer cells. The hope is to induce apoptosis specifically in tumor cells but not normal cells. It is crucial therefore that we understand how apoptosis is controlled and how cancer cells can be distinguished from normal cells and targeted in this process. There are many and significant glycosylation differences between cancer and normal cells that are potential targets for the delivery of apoptosis-inducing agents to tumor cells.

A number of tools are available to study the role of glycosylation. For example, the UDP-GlcNAc analog tunicamycin blocks *N*-glycosylation. Since many glycoproteins are *N*-glycosylated, tunicamycin treatment has a number of complex biological effects and can lead to apoptosis. The cell surface expression and

secretion of *N*-glycosylated proteins can be affected by tunicamycin treatment (Li et al. 2007; Shiraishi et al. 2005). Tunicamycin leads to underglycosylated and sometimes misfolded proteins which can cause endoplasmic reticulum (ER) stress and apoptosis due to accumulation of unfolded proteins (Table 10.1) (Kim et al. 2013; Longas et al. 2012; Lopez et al. 2013; Shiraishi et al. 2005). Treatment of cancer cells with GalNAcα-benzyl (Li et al. 2007; Patsos et al. 2007, 2009) which competes with the natural glycoprotein substrates in the early pathways of *O*-glycan biosynthesis reduces the amount of extended and sialylated *O*-glycans. GalNAc-benzyl can also block cell growth and induce apoptosis (Li et al. 2007; Patsos et al. 2009), possibly also due to accumulation of underglycosylated glycoproteins. Other glycosylation inhibitors may have similar effects, but the mechanisms are not yet known (Gao et al. 2013b). The impact of specific glycan changes on the ability of cancer cells to undergo apoptosis needs a much more detailed understanding.

Interestingly, the *N*-glycosylated glycodelin has apoptotic activity toward T cells (Mukhopadhyay et al. 2004) which depends on the glycoform of glycodelin and its sialylation status. While the presence of sialic acid often blocks the induction of apoptosis, the sialylated glycodelin is more apoptotic than the undersialylated form. Thus, sialic acids have multiple roles in the induction and prevention of apoptosis, and the role of these sugars needs to be considered with respect to their effect on functionally active domains in each protein.

10.4.1 Glycosylation Plays Important Roles in the Immune System

Glycoprotein-bound glycans are involved in cell adhesion, cell differentiation and maturation, cell activation, and the invasion of immune cells through the endothelium. They also regulate apoptosis and cytotoxicity which affects the immune responses. Specific glycans containing sialic acids have been shown to be involved in cell adhesion, proliferation, and apoptosis (de Freitas et al. 2011; Liu et al. 2011; Radhakrishnan et al. 2009; Wang et al. 2010). Carbohydrate-binding proteins (lectins) found on cell surfaces and in body fluids play an important role in the innate immune system by binding to carbohydrates on cell surfaces and to polysaccharides of microbes (Mazurek et al. 2012; Nakahara and Raz 2008; Sukhithasria et al. 2013; Wagener et al. 2012). For example, galectins bind to Gal on microbial cells and participate in the development of innate immune responses (Gabius 2001; Galvan et al. 2000; Rabinovich and Gruppi 2005). Because lectins form multimeric complexes, thus having a number of carbohydrate recognition domains, lectin interactions can cross-link molecules and cells and activate or induce signaling, differentiation of cells, or apoptosis (Fig. 10.1).

Immature human dendritic cells express a Gal/GalNAc-binding receptor that plays a role in the removal of desialylated glycoproteins (Valladeau et al. 2001).

DC-SIGN (dendritic cell-specific ICAM-3-grabbing nonintegrin) is a C-type lectin expressed on dendritic cells involved in antigen presentation to T cells (Aarnoudse et al. 2006). The carbohydrate recognition domain of DC-SIGN recognizes high mannose structures of glycoproteins as well as Lewis epitopes which promotes the adhesion of dendritic cells to endothelial cells (García-Vallejo et al. 2008).

Advanced glycation end products (AGE) are formed from glucosylated proteins that are nonenzymatically formed, especially at high blood glucose levels such as those found in diabetes. Several cell surface receptors have been identified that bind AGE, including RAGE, an *N*-glycosylated glycoprotein (Srikrishna et al. 2002). Binding of AGE to its receptor RAGE induces signaling and activation of nuclear factor NF-κB, resulting in a pro-inflammatory and cell survival effects, including the production of cytokines, growth factors, and cell adhesion molecules (Pullerits et al. 2005; Sunahori et al. 2006). Continuous expression of RAGE prevents apoptosis and supports inflammation and cancer growth (Kang et al. 2011; Sparvero et al. 2009).

10.4.2 Anti- and Pro-apoptotic Galectins

Galectins (Liu 2002) are immunomodulatory soluble lectins that are expressed in antigen-presenting T cells, activated B cells, macrophages, and other cell types, such as cancer cells with a broad range of biological activities (Bidon-Wagner and Le Pennec 2004). Galectins often form noncovalent homodimers or multimers that can bind to Gal determinants on cell surfaces or in the extracellular matrix and cross-link glycoproteins. Galectins bind to receptors having terminal Gal residues and form lattices, thus facilitating dimerization or trimerization of receptors which then leads to signaling (Johnson et al. 2013) (Fig. 10.1). Both *N*-glycans and *O*-glycans have receptors for galectins and regulate galectin apoptosis. Sialylation masks Gal receptors and precludes the apoptotic-inducing effect of galectins (Toscano et al. 2007). By inducing apoptosis in T-cell subsets, galectin-1 contributes to the escape of tumor cells from the immune system and to increased tumor survival and aggressive metastases (Stillman et al. 2006; Stannard et al. 2010).

The individual members of the galectin family have characteristic binding specificities toward structures underlying terminal Gal residues with different biological effects (Ochieng et al. 2004; Xue et al. 2013). T cells undergo apoptosis (Clark and Baum 2012) via binding of galectins to mucin-like glycoproteins CD43 and CD45, abundant on T-cell surfaces, and glycosylation is an important control factor in this process. In the extracellular domains, CD43 and CD45 have a large number of *O*-glycan chains with sialylated core 1 and core 2 structures that regulate galectin binding and signaling. In addition, *N*-glycans that lack sialylα2-6 modifications on CD45 may be involved in T-cell apoptosis. The intracellular phosphatase domain of CD45 is required for signaling. Sialic acids reduce the binding of multivalent lectins and thus block phosphatase activity and clustering or oligomerization of CD45,

resulting in decreased signaling (Clark et al. 2012). CD45 plays a major role in the galectin-3-induced intrinsic pathway of cell death in Jurkat cells.

The apoptosis-inducing galectin-1 may eliminate immune cells that control tumor growth. Galectin-1 induces apoptosis in prostate cancer cells LNCaP but not in a galectin-1-resistant cell line that has reduced expression of C2GnT1. This suggests that *O*-glycan core 2 structures may be responsible for galectin-induced apoptosis (Valenzuela et al. 2007). The susceptibility to galectin-1-induced apoptosis in LNCaP cells is also regulated by ST3Gal-I that sialylates both core 1 and core 2 structures (Brockhausen 2010).

Galectin-3 differs from other galectins in having both anti- and pro-apoptotic effects; it regulates the survival of T cells and has a wide range of effects including control of cell growth and differentiation, cell adhesion, angiogenesis, and apoptosis (Chen et al. 2005; Takenaka et al. 2004). Galectin-3 can induce apoptosis by binding to Gal residues extracellularly, forming lattices that restrict receptor mobility (Hsu et al. 2009). The expression of galectin-3 is ubiquitous among cancer cell types and in the blood and correlates with tumor progression and metastasis in a number but not all cancer types (Takenaka et al. 2004; Yu et al. 2007). In Jurkat cells, galectin-3 regulates apoptosis by binding to both *N*-glycans and *O*-glycans on CD45 (Clark et al. 2012; Xue et al. 2013). Galectin-3 also binds to the cancer-associated TF antigen as well as to the Gal residues of *N*-glycans (Srinivasan et al. 2009) which promotes the adhesion of B16 melanoma cells to fibronectin as well as lung metastasis in mice (Zhang et al. 2002).

At least ten different membrane-bound highly *O*-glycosylated mucins form complexes at the cell membrane. MUC1 on cancer cell surfaces interacts with circulating galectin-3 which increases cancer cell adhesion to the endothelium and promotes metastases (Zhao et al. 2009). MUC1 interacts with the death domain of Fas-associated death domain adapter protein (FADD) at the cell surface of breast cancer cells and has multiple downstream effects. MUC1 is usually overexpressed in cancer cells and can protect the cell from the oxidative stress-induced apoptosis (Yin et al. 2003). Both MUC1 and MUC4 promote cell proliferation and suppress the induction of apoptosis by a multitude of mechanisms (Bafna et al. 2010).

10.5 Role of Glycans in Receptor Functions

Glycans containing sialic acids are involved in cell proliferation and apoptosis of cancer cells and may control receptor endocytosis and signaling (Woronowicz et al. 2004). Sialic acids influence the physical and chemical properties of a glycoprotein, its bioavailability, interactions, and functions. Thus, sialic acids may prevent receptor oligomerization and regulate cell signaling, proliferation, and apoptosis (Schultz et al. 2012). However, the specific roles of glycans at each glycosylation site must be seen in context with the individual protein and its functions. Sialidases (neuraminidases) found on the cell surface appear to be important regulators of receptor functions (Lillehoj et al. 2012). The stimulation

of cell surface receptors is also regulated by soluble receptors (decoy receptors) that bind the ligand and thus reduce its concentration at the receptor site, preventing receptor signaling. Blocking ligand-receptor interactions could be beneficial to decrease cell numbers in cancer and autoimmune diseases.

Glycolipids also have critical roles in receptor signaling. Cell surface-bound ganglioside GM3 controls EGFR signaling by decreasing receptor phosphorylation. Neuraminidase Neu3 is thought to reside on the cell surface where it reduces the amount of GM3 ganglioside and relieves the inhibition of EGFR phosphorylation by GM3. Neu3 cleaves sialic acid preferentially from gangliosides such as GM3 and is activated by hypoxia. Neu3 expression therefore enhances cell survival and proliferation of skeletal muscle cells through the EGFR pathway and protects cells from hypoxic stress, while silencing of Neu3 results in increased apoptosis (Scaringi et al. 2013).

The superfamily of apoptosis-inducing cell surface receptors includes the tumor necrosis factor-α (TNFα) death receptor family (TNFR1, DR4, DR5, Fas) (Li et al. 2013). These receptors are glycoproteins involved in apoptosis by binding to homotrimeric members of the TNFα family, e.g., TNFα, TNF-related apoptosis-inducing ligand (TRAIL), or Fas ligand (FasL) (Nikoletopoulou et al. 2013), respectively. Many types of human cancers are resistant to FasL or TRAIL-induced apoptosis, but other components of the complex apoptotic pathways could also be dysfunctional in cancer cells. The trimeric ligands bind to the extracellular domain of the TNF receptor, recruiting three receptor molecules. This is followed by binding receptor-interacting proteins, including activating or inhibiting factors, and activation of caspases.

The *N*-glycans of receptor glycoproteins have been shown to play an important role in apoptosis induction; they serve in controlling the cell surface expression, conformation, or ligand binding of receptors and may regulate the induction of signaling cascades, i.e., receptor internalization, DISC formation, and caspase-8 activation (Amith et al. 2010; Li et al. 2013). In contrast, *O*-glycans of death receptor 4 (DR4) play a central role in the sensitivity to TRAIL in non-small cell lung carcinoma and melanoma cells (Wagner et al. 2007).

10.5.1 Fas Glycoprotein

Fas glycoprotein, the receptor for Fas ligand (FasL), is ubiquitously expressed in epithelia and endothelia and many cancer cells. In breast cancer (Bebenek et al. 2013), the expression levels of Fas/FasL have a prognostic value. Fas-deficient breast tumors are more aggressive, likely due to poor induction of Fas-mediated apoptosis. Colorectal carcinoma cells express Fas, but the expression is relatively low in the tissues (Mann et al. 1999). Interestingly, FasL is expressed in metastasizing carcinomas and in liver metastases, while metastatic tissues have fewer infiltrating lymphocytes compared to nonmetastatic tissues. It is possible that FasL expression could give metastatic cells a growth advantage by inducing

apoptosis in lymphocytes and keeping the population of tumor infiltrating lymphocytes low (Mann et al. 1999).

FasL is a glycoprotein present mainly on the surfaces of many leukocytes. When trimeric FasL binds to Fas, trimeric Fas associates and induces the complex cascade of apoptosis (Orlinick et al. 1997) (Fig. 10.1). Soluble FasL or anti-Fas antibodies also have the ability to induce Fas association and apoptosis (Schneider et al. 1997). This type of apoptosis is important in T-cell cytotoxicity and the selection of T cells. Fas is a type I membrane glycoprotein with an extracellular glycosylated domain with 2 *N*-glycans, a transmembrane domain, and a cytoplasmic death domain. Since FasL binds to Fas near the *N*-glycosylation sites, the *N*-glycans may control ligand binding and receptor oligomerization. Although the antibody-binding epitope is distinct from that of FasL, it is also in the vicinity of *N*-glycosylation sites. *N*-glycans also control the stability and secretion of the soluble extracellular domain expressed in HeLa cells (Li et al. 2007). Altered *N*-glycosylation patterns in cancer may thus have an impact on receptor cell surface expression and function. The soluble extracellular domain (sFasL) that can be secreted from certain cancer cells can induce apoptosis but can also block interactions of Fas with membrane-bound FasL (Abrahams et al. 2003). For example, hyperglycosylated larger, secreted FasL is present in the ascites fluid of ovarian cancer patients but is not found in secretions of normal ovarian cells. Removal of sialic acid did not change the ability of FasL to induce apoptosis in human T-lymphocytic Jurkat cells. The *N*-glycans of FasL, however, control the expression levels of FasL (Orlinick et al. 1997) as well as the secretion of soluble FasL from human embryonic kidney cells (Schneider et al. 1997).

The sialylation status of a cell is closely linked to Fas-mediated apoptosis, and cancer cells may protect themselves from apoptosis by adding an excess of sialic acids (Table 10.2) (Shatnyeva et al. 2011). The cell surface sialylation status of lymphoma cells has been suggested to be linked to the apoptotic potential of cells, and sialic acids may provide protection from Fas-mediated cell death (Keppler et al. 1999). Thus, desialylation of Burkitt's lymphoma cells increased their sensitivity to Fas apoptosis. When Fas is hypersialylated with α2-6-linked sialic acid residues by overexpression of ST6Gal-1 in human colon cancer cells, Fas trimerization and induction of apoptosis are blocked (Swindall and Bellis 2011). The mechanisms underlying the control of apoptosis by Fas sialylation may involve a role in the overall receptor conformation and repulsion of negative charges preventing receptor trimerization. It is not known if specific sialic acid linkages or the total numbers of these large acidic sugar residues in the receptor glycans are important in this process.

10.5.2 TNFα Receptors

TNFα is a growth factor that can induce cell proliferation and also necrotic or apoptotic cell death. The TNFα trimer binds to its trimeric receptor which then

forms signaling complexes of different protein compositions and functions. There are two TNF receptors (the ubiquitous TNFR1 and TNFR2 that has a more restricted expression) having a broad spectrum of biological responses (Cabal-Hierro and Lazo 2012). While TNFR2 is mainly involved in survival mechanisms, TNFR1 can induce either proliferation or apoptosis through caspase-8 activation.

TNFR2 has an *O*-glycosylated domain which may serve to present the receptor to its ligand and to ensure stability or transport to the cell surface (Liu et al. 2013). Cell surface sialylα2-6 linkages have been shown to protect monocytic cells from apoptosis through TNFR1 (Liu et al. 2011). This is similar to the effect of α2-6-linked sialic acid on Fas and suggests a common mechanism of apoptosis regulation by sialic acids. Forced overexpression of ST6Gal in human monocytic U937 cells inhibited apoptosis induced by TNFα as well as phorbol-12-myristate-13-acetate. Similarly, macrophages from ST6Gal transgenic mice showed reduced TNF-α-induced apoptosis. Reduction in sialylα2-6-linked residues by neuraminidase treatment or knockdown of ST6Gal enhanced TNFα-induced apoptosis. This indicates that sialylα2-6 linkages are involved in the functions of these receptors and control apoptosis.

10.5.3 TRAIL-Induced Apoptosis

TRAIL is expressed and functional in NK cells and other cells of the immune system and can induce apoptosis primarily in cancer but not in normal cells (Jin et al. 2004; Zinonos et al. 2009). It is therefore an important component of the immune surveillance against cancer. Homotrimeric TRAIL ligand binds to death receptor glycoproteins DR4 and DR5 on cancer cells, recruiting FADD and forming the multiprotein DISC, which then activates caspase-8, caspase-3, and caspase-7, involving both the extrinsic and intrinsic apoptotic pathways. Monoclonal antibodies to DR5 also activate the extrinsic apoptotic pathway (Zinonos et al. 2009).

DR4 and DR5 are modified by *N*-glycans and *O*-glycans (Yoshida et al. 2007). It is possible that the cancer-associated *N*-glycans (Jin et al. 2004; Zinonos et al. 2009) affect the induction of apoptosis. Resistance of human colon cancer cells to TRAIL-induced apoptosis was relieved by tunicamycin treatment which enhanced the transport of death receptor DR4 to the cell surface. An anti-DR5 antibody (apomab) has been developed and shown to have potent antitumor activity in metastatic breast cancer (Zinonos et al. 2009). The antibody was effective in inducing apoptosis in breast tumors in mice and in breast cancer cells but not in normal cells which suggests a potent therapeutic potential for these antibodies. The difference in effects between normal and tumor-derived DR5 may be based on the abnormal glycosylation of the cancer glycoproteins.

TRAIL also binds soluble decoy receptors 1 and 2 as well as osteoprotegerin which inhibits apoptosis (Jin et al. 2004). Conversely, soluble TRAIL has therapeutic potential since it can bind to its receptors and induce apoptosis in various

cancer cell lines. The utility of soluble TRAIL has been shown in the treatment of breast cancer in mice (Chinnaiyan et al. 2000).

In TRAIL-resistant metastatic colon cancer cells LS-LIM6, death receptors co-express and bind to galectin-3 produced in the cancer cells. The presence of galectin-3 blocked apoptosis by preventing the endocytosis of the receptor complex and recruitment of caspase-8 (Mazurek et al. 2012). However, it is not clear whether TRAIL apoptosis requires the internalization of the receptor for forming DISC. TRAIL resistance caused by galectin-3 can be overcome by knockdown of galectin-3 which suggests a mechanism of apoptosis control by glycan recognition.

Resistance to TRAIL is also associated with decreased fucosylation (Moriwaki et al. 2010). Treatment of colonic and other cancer cells with the DNA methyltransferase inhibitor zebularine caused increased fucosylation, probably due to altered expression of a number of genes involved in the fucosylation pathway. The remodeling of cell surface glycans was associated with increased sensitivity to TRAIL-induced apoptosis.

A new member of the TNFR family (death receptor 6, DR6) has been shown to regulate apoptosis. DR6 is expressed in various cell types, including lymphocytes and tumor cell lines where it can be overexpressed. Deletion of DR6 in B lymphocytes promotes cell proliferation. The extracellular domain of DR6 has six *N*-glycosylation sites, and there are multiple *O*-glycosylation sites in the stem region near the membrane domain (Klima et al. 2009). The cleaved extracellular portion of death receptor 6 (DR6) can downregulate the effect of DR6 by binding to TNFα. *N*-glycans seem to play a role in localizing DR6 in the cell membrane.

O-glycans appear to be required for the proper functions of death receptors DR4 and DR5. The four *O*-glycosylation sites in DR5 contain one to four sialylated core 1 chains, depending on the cell type expressing the receptor. These *O*-glycosylation sites are not directly within the ligand-binding site but may promote ligand-induced receptor clustering by affecting the overall conformation or presentation of the receptors or specific peptide epitopes. The *O*-glycan core 2 structure appears to be specifically involved in the regulation of TRAIL apoptosis. C2GnT-expressing prostate cancer cells are more resistant to TRAIL-induced apoptosis as well as NK cytotoxicity than cells that do not carry core 2 on the cell surface (Okamoto et al. 2013). This is possibly due to the abundance of poly-*N*-acetyllactosamine chains on core 2 glycans that may interfere with cell surface interactions.

The numbers of *O*-glycosylation sites occupied also have a role in apoptosis. The GalNAc-transferases ppGalNAcT3 and ppGalNAcT14 were identified as the enzymes that *O*-glycosylate TRAIL receptors in colon cancer cells. The expression of ppGalNAcT14 in pancreatic and lung cancers as well as in many other cancer cell lines is higher in the TRAIL-sensitive cells compared to resistant cells. Overexpression of ppGalNAcT14 appeared to increase the amount of sialylated core 1 *O*-glycans on DR5 and increased the sensitivity of colon cancer cells to TRAIL-induced apoptosis through both DR4 and DR5 (Wagner et al. 2007), promoting DISC formation and caspase-8 activation. Knockdown of ppGalNAcT14 expression or mutations of the *O*-glycosylation sites reduced TRAIL sensitivity and receptor clustering and attenuated apoptosis (Wagner et al. 2007). Similarly,

ppGalNAcT3 expression is associated with TRAIL sensitivity in colon cancer cells. It is possible that *O*-glycosylation stabilizes the receptor in the membrane, enhancing its ligand-binding properties.

Fucosylation also plays a role in apoptosis. The α3-Fuc-transferases FUT3 and FUT6 involved in Lewis antigen synthesis were found to be associated with TRAIL-induced apoptosis (Wagner et al. 2007). The GDP-Man 4,6-dehydratase is required for the synthesis of GDP-Fuc, the donor substrate for Fuc-transferases. Defects in fucosylation due to mutations in the 4,6-dehydratase gene have been shown to produce resistance of human colon cancer cells to TRAIL-induced apoptosis (Moriwaki et al. 2009). Restoring the synthesis of GDP-Fuc led to susceptibility to apoptosis and suppressed tumor growth and metastases in mice.

10.6 Conclusions and Tools to Restore Apoptosis in Cancer Cells

These combined studies support the theory that both *N*-glycans and *O*-glycans are involved in the induction of apoptosis through glycoprotein receptors. However, the roles of different glycan structures at individual glycosylation sites need to be examined, as well as their specific roles in apoptosis induction which may vary between cell types.

Since the abnormal cancer cell glycosylation and expression of glycosyltransferases in part control apoptosis, a modification of glycosylation could be an effective therapy for cancer. A number of potential therapeutic targets have been identified, including ppGalNAcT, β4GalT, and sialyltransferases. Because of the complex role of sialic acids which are often overexpressed in cancer and modify the structures and functions of apoptosis receptors, specific sialyltransferases are new targets for tumor therapy. Plasma membrane-bound sialidases can control both apoptosis and proliferation pathways. Thus, sialidase inhibitors are also potential therapeutic targets for cancer (Scaringi et al. 2013).

The delivery of therapeutic drugs specifically to tumors and metastatic cells remains to be explored. Cancer cells may have characteristic structural glycan features and cancer-associated antigens (such as Lewis or T/Tn antigens) that could be exploited to target therapeutic antibodies or drugs to tumors. Inhibitors of glycosylation are powerful tools to alter glycosylation patterns and can be delivered into cells as modified precursors of active inhibitors. This could lead to a reduction in proliferation or in the induction of apoptosis (Fuster et al. 2003). Since cancer cells derived from the same tumor may have a quite different glycosylation potential, stable packaging technology of anticancer drugs targeting characteristic glycan structures should be developed for a cell-specific delivery. As we learn more about the role of glycan structures and the mechanisms of their functions, new targets will emerge. Control of the expression and activities of

biosynthetic enzymes will be an important field in the future that can be applied in removing cancer cells in vivo.

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

Abrahams VM, Straszewski SL, Kamsteeg M, Hanczaruk B, Schwartz PE, Rutherford TJ, Mor G (2003) Epithelial ovarian cancer cells secrete functional Fas ligand. Cancer Res 63:5573–5581

Adamczyk B, Tharmalingam T, Rudd PM (2012) Glycans as cancer biomarkers. Biochim Biophys Acta 1820:1347–1353

Amith SR, Jayanth P, Franchuk S, Finlay T, Seyrantepe V, Beyaert R, Pshezhetsky AV, Szewczuk MR (2010) Neu1 desialylation of sialyl alpha-2,3-linked beta-galactosyl residues of TOLL-like receptor 4 is essential for receptor activation and cellular signaling. Cell Signal 22:314–324

Arnold JN, Saldova R, Galligan MC, Murphy TB, Mimura-Kimura Y, Telford JE, Godwin AK, Rudd PM (2011) Novel glycan biomarkers for the detection of lung cancer. J Proteome Res 10:1755–1764

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

Barthel SR, Gavino JD, Wiese GK, Jaynes JM, Siddiqui J, Dimitroff CJ (2008) Analysis of glycosyltransferase expression in metastatic prostate cancer cells capable of rolling activity on microvascular endothelial (E)-selectin. Glycobiology 18:806–817

Barthel SR, Wiese GK, Cho J, Opperman MJ, Hays DL, Siddiqui J, Pienta KJ, Furie B, Dimitroff CJ (2009) Alpha 1,3 fucosyltransferases are master regulators of prostate cancer cell trafficking. Proc Natl Acad Sci 106:19491–19496

Bębenek M, Duś D, Koźlak J (2013) Prognostic value of the Fas/Fas ligand system in breast cancer. Contemp Oncol (Pozn) 17:120–122

Bidon-Wagner N, Le Pennec JP (2004) Human galectin-8 isoforms and cancer. Glycoconj J 19:557–563

Blottière HM, Burg C, Zennadi R, Perrin P, Blanchardie P, Bara J, Meflah K, Le Pendu J (1992) Involvement of histo-blood-group antigens in the susceptibility of colon carcinoma cells to natural killer-mediated cytotoxicity. Int J Cancer 52:609–618

Bresalier RS, Ho SB, Schoeppner HL, Kim YS, Sleisenger MH, Brodt P, Byrd JC (1996) Enhanced sialylation of mucin-associated carbohydrate structures in human colon cancer metastasis. Gastroenterol 110:1354–1367

Brockhausen I (1999) Pathways of O-glycan biosynthesis in cancer cells. Biochim Biophys Acta 1473:67–95

Brockhausen I (2006) Glycosyltransferases involved in the synthesis of mucin type O-glycans in human cancer cells. EMBO Rep 7:599–604

Brockhausen I (2010) Biosynthesis of complex mucin-type O-glycans. In: Mander L, Lui H-W, Wang PG (eds) Comprehensive natural products II chemistry and biology. Elsevier, Oxford, Carbohydrates, nucleosides and nucleic acids, vol 6. 11:315–350

Brockhausen I, Gao Y (2012) Structural glycobiology: applications in cancer research. Chapter 8. In Structural Glycobiology. Yurevicz E (ed) CRC Press/Taylor & Francis, Abingdon UK, pp 177–213

Brockhausen I, Kuhns W (1997) Glycoproteins and human disease. Medical Intelligence Unit/ CRC Press/Mosby-Year Book/Chapman & Hall, New York, R.G. Landes Company, Georgetown

Brockhausen I, Lehotay M, Yang JM, Qin W, Young D, Lucien J, Coles J, Paulsen H (2002) Glycoprotein biosynthesis in porcine aortic endothelial cells and changes in the apoptotic cell population. Glycobiology 12:33–45

Burchell J, Poulsom R, Hanby A, Whitehouse C, Cooper L, Clausen H, Miles D, Taylor-Papadimitriou J (1999) An alpha 2,3 sialyltransferase (ST3Gal I) is elevated in primary breast carcinomas. Glycobiology 9:1307–1311

Cabal-Hierro L, Lazo PS (2012) Signal transduction by tumor necrosis factor receptors. Cell Signal 24:1297–1305

Cazet A, Julien S, Bobowski M, Krzewinski-Recchi MA, Harduin-Lepers A, Groux-Degroote S, Delannoy P (2010) Consequences of the expression of sialylated antigens in breast cancer. Carbohydr Res 345:1377–1383

Chen HY, Liu FT, Yang RY (2005) Roles of galectin-3 in immune responses. Arch Immunol Ther Exp (Warsz) 53:497–504

Chen HD, Zhou X, Yu G, Zhao YL, Ren Y, Zhou YD, Li Q, Zhang XL (2012) Knockdown of core 1 beta 1, 3-galactosyltransferase prolongs skin allograft survival with induction of galectin-1 secretion and suppression of CD8 + T cells. T synthase knockdown effects on galectin-1 and CD8 + T cells. J Clin Immunol 32:820–836

Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD, Rehemtulla A (2000) Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci USA 97:1754–1759

Clark MC, Baum LG (2012) T cells modulate glycans on CD43 and CD45 during development and activation, signal regulation, and survival. Ann NY Acad Sci 1253:58–67

Clark MC, Pang M, Hsu DK, Liu FT, de Vos S, Gascoyne RD, Said J, Baum LG (2012) Galectin-3 binds to CD45 on diffuse large B-cell lymphoma cells to regulate susceptibility to cell death. Blood 120:4635–4644

Dall'Olio F, Chiricolo M (2001) Sialyltransferases in cancer. Glycoconj J 18:841–850

Dall'Olio F, Malagolini N, di Stefano G, Minni F, Marrano D, Serafini-Cessi F (1989) Increased CMP-NeuAc: Gal beta 1,4GlcNAc-R alpha 2,6 sialyltransferase activity in human colorectal cancer tissues. Int J Cancer 44:434–439

Dalziel M, Whitehouse C, McFarlane I, Brockhausen I, Gschmeissner S, Schwientek T, Clausen H, Burchell J, Taylor-Papadimitriou J (2001) The relative activities of the C2GnT1 and ST3Gal-I glycosyltransferases determine O-glycan structure and expression of a tumor-associated epitope on MUC1. J Biol Chem 276:11007–11015

de Freitas Junior JCM, Du Rocher B, Silva DA, de Souza WF, de Arau jo WM, Abdelhay ESFW, Morgado-Diaz JA (2011) Inhibition of N-linked glycosylation by tunicamycin induces E-cadherin-mediated cell–cell adhesion and inhibits cell proliferation in undifferentiated human colon cancer cells. Cancer Chemother Pharmacol 68:227–238

de la Cruz L, Steffgen K, Martin A, McGee C, Hathaway H (2004) Apoptosis and involution in the mammary gland are altered in mice lacking a novel receptor, beta1,4-Galactosyltransferase I. Dev Biol 272:286–309

Delmotte P, Degroote S, Lafitte JJ, Lamblin G, Perini JP, Roussel P (2002) Tumor necrosis factor alpha increases the expression of glycosyltransferases and sulfotransferases responsible for the biosynthesis of sialylated and sulfated Lewis x epitopes in the human bronchial mucosa. J Biol Chem 277:424–431

Demetriou M, Nabi IR, Coppolino M, Dedhar S, Dennis JW (1995) Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. J Cell Biol 130:383–392

Fujita T, Murayama K, Hanamura T, Okada T, Ito T, Harada M, Komatsu A, Koyama H, Kanai T, Maeno K, Mochizuki Y, Hama Y, Ito K, Amano J, Fujimori M (2011) CSLEX (Sialyl Lewis X) is a useful tumor marker for monitoring of breast cancer patients. Jpn J Clin Oncol 41:394–399

Fuster MM, Brown JR, Wang L, Esko JD (2003) A disaccharide precursor of sialyl Lewis X inhibits metastatic potential of tumor cells. Cancer Res 63:2775–2781

Gabius HJ (2001) Probing the cons and pros of lectin-induced immunomodulation: case studies for the mistletoe lectin and galectin-1. Biochimie 83:659–666

Galvan M, Tsuboi S, Fukuda M, Baum LG (2000) Expression of a specific glycosyltransferase enzyme regulates T cell death mediated by galectin-1. J Biol Chem 275:16730–16737

Gao Y, Chachadi V, Chen PW, Brockhausen I (2012) Glycosylation potential of human prostate cancer cell lines. Glycoconj J 9:525–537

Gao Y, Aryal RP, Ju T, Cummings RD, Gahlay G, Jarvis DL, Matta KL, Vlahakis JZ, Szarek WA, Brockhausen I (2013a) Acceptor specificities and selective inhibition of recombinant human Gal- and GlcNAc-transferases that synthesize O-glycan core structures 1, 2, 3 and 4 of O-glycans. Biochim Biophys Acta 1830:4274–4281

Gao Y, Vlahakis JZ, Szarek WA, Brockhausen I (2013b) Selective inhibition of glycosyltransferases by bivalent imidazolium salts. Bioorg Med Chem 21:1305–1311

Garcia-Vallejo JJ, Van Dijk W, Van Het Hof B, Van Die I, Engelse MA, Van Hinsbergh VW, Gringhuis SI (2006) Activation of human endothelial cells by tumor necrosis factor-alpha results in profound changes in the expression of glycosylation-related genes. J Cell Physiol 206:203–210

García-Vallejo JJ, van Liempt E, da Costa MP, Beckers C, van het Hof B, Gringhuis SI, Zwaginga JJ, van Dijk W, Geijtenbeek TB, van Kooyk Y, van Die I (2008) DC-SIGN mediates adhesion and rolling of dendritic cells on primary human umbilical vein endothelial cells through LewisY antigen expressed on ICAM-2. Mol Immunol 45:2359–2369

Granovsky M, Fata J, Pawling J, Muller WJ, Khokha R, Dennis JW (2000) Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nat Med 6:306–312

Guo HB, Zhang QS, Chen HL (2000) Effects of H-ras and v-sis overexpression on N-acetylglucosaminyltransferase V and metastasis-related phenotypes in human hepatocarcinoma cells. J Cancer Res Clin Oncol 126:263–270

Hagisawa S, Ohyama C, Takahashi T, Endoh M, Moriya T, Nakayama J, Arai Y, Fukuda M (2005) Expression of core 2 β1,6-N-acetylglucosaminyltransferase facilitates prostate cancer progression. Glycobiology 15:1016–1024

Hakomori S (2001) Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol 491:369–402

Han YY, Liu HY, Han DJ, Zong XC, Zhang SQ, Chen YQ (2013) Role of glycosylation in the anticancer activity of antibacterial peptides against breast cancer cells. Biochem Pharmacol 86:1254–1262

Hatakeyama S, Kyan A, Yamamoto H, Okamoto A, Sugiyama N, Suzuki Y, Yoneyama T, Hashimoto Y, Koie T, Yamada S, Saito H, Arai Y, Fukuda M, Ohyama C (2010) Core 2 N-acetylglucosaminyltransferase-1 expression induces aggressive potential of testicular germ cell tumor. Int J Cancer 127:1052–1059

Higai K, Ishihara S, Matsumoto K (2006) NFkappaB-p65 dependent transcriptional regulation of glycosyltransferases in human colon adenocarcinoma HT-29 by stimulation with tumor necrosis factor alpha. Biol Pharm Bull 29:2372–2377

Hiraishi K, Suzuki K, Hakomori S, Adachi M (1993) Le(y) antigen expression is correlated with apoptosis (programmed cell death). Glycobiology 3:381–390

Hsu DK, Chen HY, Liu FT (2009) Galectin-3 regulates T-cell functions. Immun Rev 230:114–127

Huang MC, Chen HY, Huang HC, Huang J, Liang JT, Shen TL, Lin NY, Ho CC, Cho IM, Hsu SM (2006) C2GnT-M is downregulated in colorectal cancer and its re-expression causes growth inhibition of colon cancer cells. Oncogene 25:3267–3276

Huang S, Day TW, Choi MR, Safa AR (2009) Human β-galactoside α-2,3-sialyltransferase (ST3Gal III) attenuated Taxol-induced apoptosis in ovarian cancer cells by downregulating caspase-8 activity. Mol Cell Biochem 331:81–88

Isaji T, Gu J, Nishiuchi R, Zhao Y, Takahashi M, Miyoshi E, Honke K, Sekiguchi K, Taniguchi N (2004) Introduction of bisecting GlcNAc into integrin alpha5beta1 reduces ligand binding and down-regulates cell adhesion and cell migration. J Biol Chem 279:19747–19754

Jiang J, Zhou J, Wei Y, Shen J, Liu D, Chen X, Zhang S, Kong X, Yun X, Gu J (2007) Beta4GalT-II increases cisplatin-induced apoptosis in HeLa cells depending on its Golgi localization. Biochem Biophys Res Commun 358:41–46

Jin Z, McDonald ER III, Dicker DT, El-Deiry WS (2004) Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. J Biol Chem 279:35829–35839

Johnson JL, Jones MB, Ryan SO, Cobb BA (2013) The regulatory power of glycans and their binding partners in immunity. Trends Immunol 34:290–296

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

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

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

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

Kang R, Tang D, Livesey KM, Schapiro NE, Lotze MT, Zeh HJ III (2011) The receptor for advanced glycation end-products (RAGE) protects pancreatic tumor cells against oxidative injury. Antioxid Redox Signal 15:2175–2184

Kariya Y, Kato R, Itoh S, Fukuda T, Shibukawa Y, Sanzen N, Sekiguchi K, Wada Y, Kawasaki N, Gu J (2008) N-glycosylation of laminin-332 regulates its biological functions. A novel function of the bisecting GlcNAc. J Biol Chem 283:33036–33045

Keppler OT, Peter ME, Hinderlich S, Moldenhauer G, Stehling P, Schmitz I, Schwartz-Albiez R, Reutter W, Pawlita M (1999) Differential sialylation of cell surface glycoconjugates in a human B lymphoma cell line regulates susceptibility for CD95 (APO-1/Fas)-mediated apoptosis and for infection by a lymphotropic virus. Glycobiology 9:557–569

Kim YS, Gum J, Brockhausen I (1996) Mucin glycoproteins in neoplasia. Glycoconj J 13:693–707

Kim SM, Lee JS, Lee YH, Kim WJ, Do SI, Choo YK, Park YI (2007) Increased alpha2,3-sialylation and hyperglycosylation of N-glycans in embryonic rat cortical neurons during camptothecin-induced apoptosis. Mol Cells 24:416–423

Kim SY, Hwang JS, Han IO (2013) Tunicamycin inhibits Toll-like receptor-activated inflammation in RAW264.7 cells by suppression of NF-κB and c-Jun activity via a mechanism that is independent of ER-stress and N-glycosylation. Eur J Pharmacol 721:294–300

Kiwamoto T, Brummet ME, Wu F, Motari MG, Smith DF, Schnaar RL, Zhu Z, Bochner BS (2014) Mice deficient in the St3gal3 gene product α2,3 sialyltransferase (ST3Gal-III) exhibit enhanced allergic eosinophilic airway inflammation. J Allergy Clin Immunol 133:240–247

Klíma M, Zájedová J, Doubravská L, Anděra L (2009) Functional analysis of the posttranslational modifications of the death receptor 6. Biochim Biophys Acta 1793:1579–1587

Kudo T, Ikehara Y, Togayachi A, Morozumi K, Watanabe M, Nakamura M, Nishihara S, Narimatsu H (1998) Up-regulation of a set of glycosyltransferase genes in human colorectal cancer. Lab Investig 78:797–811

Lagana A, Goetz JG, Cheung P, Raz A, Dennis JW, Nabi IR (2006) Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells. Mol Cell Biol 26:3181–3193

Läubli H, Borsig L (2010) Selectins promote tumor metastasis. Semin Cancer Biol 20:169–177

Lazarus BD, Love DC, Hanover JA (2009) O-GlcNAc cycling: implications for neurodegenerative disorders. Int J Biochem Cell Biol 41:2134–2146

Lee SH, Hatakeyama S, Yu SY, Bao X, Ohyama C, Khoo KH, Fukuda MN, Fukuda M (2009) Core3 O-glycan synthase suppresses tumor formation and metastasis of prostate carcinoma PC3 and LNCaP cells through down-regulation of α2β1 integrin complex. J Biol Chem 284:17157–17169

Li Z, Zong H, Kong X, Zhang S, Wang H, Sun Q, Gu J (2006) Cell surface beta 1, -4-galactosyltransferase1 promotes apoptosis by inhibiting epidermal growth factor receptor pathway. Mol Cell Biochem 291:69–76

Li Y, Yang X, Nguyen A, Brockhausen I (2007) Requirement of N-glycosylation for the secretion of recombinant extracellular domain of human Fas in HeLa cells. Internat J Biochem Cell Biol 39:1625–1636

Li Z, Yamada S, Inenaga S, Imamura T, Wu Y, Wang KY, Shimajiri S, Nakano R, Izumi H, Kohno K, Sasaguri Y (2011) Polypeptide N-acetylgalactosaminyltransferase 6 expression in pancreatic cancer is an independent prognostic factor indicating better overall survival. Br J Cancer 104:1882–1889

Li J, Yin Q, Wu H (2013) Structural basis of signal transduction in the TNF receptor superfamily. Adv Immunol 119:135–153

Lillehoj EP, Hyun SW, Feng C, Zhang L, Liu A, Guang W, Nguyen C, Luzina IG, Atamas SP, Passaniti A, Twaddell WS, Puché AC, Wang LX, Cross AS, Goldblum SE (2012) NEU1 sialidase expressed in human airway epithelia regulates epidermal growth factor receptor (EGFR) and MUC1 protein signaling. J Biol Chem 287:8214–8231

Lise M, Belluco C, Perera SP, Patel R, Thomas P, Ganguly A (2000) Clinical correlations of alpha 2,6-sialyltransferase expression in colorectal cancer patients. Hybridoma 19:281–286

Liu FT (2002) Galectins: novel anti-inflammatory drug targets. Expert Opin Ther Targets 6:461–468

Liu Z, Swindall AF, Kesterso RA, Schoeb TR, Bullard DC, Bellis SL (2011) ST6Gal-I regulates macrophage apoptosis via α2-6 sialylation of the TNFR1 death receptor. J Biol Chem 286:39654–39662

Liu L, Gomathinayagam S, Hamuro L, Prueksaritanont T, Wang W, Stadheim TA, Hamilton SR (2013) The impact of glycosylation on the pharmacokinetics of a TNFR2:Fc fusion protein expressed in glycoengineered pichia pastoris. Pharm Res 30:803–812

Longas MO, Kotapati A, Prasad KPVRK, Banerjee A, Santiago J, Baksi K, Banerjee DK (2012) Balancing life with glycoconjugates: monitoring unfolded protein response-mediated anti-angiogenic action of tunicamycin by Raman Spectroscopy. Pure Appl Chem 84:1907–1918

Lopez JJ, Palazzo A, Chaabane C, Albarran L, Polidano E, Lebozec K, Dally S, Nurden P, Enouf J, Debili N, Bobe R (2013) Crucial role for endoplasmic reticulum stress during megakaryocyte maturation. Arterioscler Thromb Vasc Biol 33:2750–2758

Mann B, Gratchev A, Böhm C, Hanski ML, Foss HD, Demel G, Trojanek B, Schmidt-Wolf I, Stein H, Riecken EO, Buhr HJ, Hanski C (1999) FasL is more frequently expressed in liver metastases of colorectal cancer than in matched primary carcinomas. Br J Cancer 79:1262–1269

Mazurek N, Byrd JC, Sun Y, Hafley M, Ramirez K, Burks J, Bresalier RS (2012) Cell-surface galectin-3 confers resistance to TRAIL by impeding trafficking of death receptors in metastatic colon adenocarcinoma cells. Cell Death Diff 19:523–533

Minamide S, Naora H, Adachi M, Okano A, Naora H (1995) Apoptosis as a mechanism of skin renewal: Le(y)-antigen expression is involved in an early event of a cell's commitment to apoptosis. Histochem Cell Biol 103:339–343

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. Gastroenterol 137:188–198

Moriwaki K, Narisada M, Imai T, Shinzaki S, Miyoshi E (2010) The effect of epigenetic regulation of fucosylation on TRAIL-induced apoptosis. Glycoconj J 27:649–659

Mukhopadhyay D, SundarRaj S, Alok A, Karande AA (2004) Glycodelin A, not glycodelin S, is apoptotically active. Relevance of sialic acid modification. J Biol Chem 279:8577–8584

Nakahara S, Raz A (2008) Biological modulation by lectins and their ligands in tumor progression and metastasis. Anticancer Agents Med Chem 8:22–36

Nakamori S, Kameyama M, Imaoka S, Furukawa H, Ishikawa O, Sasaki Y, Kabuto T, Iwanaga T, Matsushita Y, Irimura T (1993) Increased expression of sialyl Lewis x antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study. Cancer Res 53:3632–3637

Nguyen JT, Evans DP, Galvan M, Pace KE, Leitenberg D, Bui TN, Baum LG (2001) CD45 modulates galectin-1-induced T cell death: regulation by expression of core 2 O-glycans. J Immunol 167:5697–5707

Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N (2013) Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta 1833:3448–3459

Ochieng J, Furtak V, Lukyanov P (2004) Extracellular functions of galectin-3. Glycoconj J 19:527–535

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

Ohyama C (2008) Glycosylation in bladder cancer. Int J Clin Oncol 13:308–313

Okamoto T, Yoneyama MS, Hatakeyama S, Mori K, Yamamoto H, Koie T, Saitoh H, Yamaya K, Funyu T, Fukuda M, Ohyama C, Tsuboi S (2013) Core2 O-glycan-expressing prostate cancer cells are resistant to NK cell immunity. Mol Med Rep 7:359–364

Orlinick JR, Elkon KB, Chao MV (1997) Separate domains of the human Fas ligand dictate self-association and receptor binding. J Biol Chem 272:32221–32229

Park JH, Nishidate T, Kijima K, Ohashi T, Takegawa K, Fujikane T, Hirata K, Nakamura Y, Katagiri T (2010) Critical roles of mucin 1 glycosylation by transactivated polypeptide N-acetylgalactosaminyltransferase 6 in mammary carcinogenesis. Cancer Res 70:2759–2769

Park JH, Katagiri T, Chung S, Kijima K, Nakamura Y (2011) Polypeptide n-acetylgalactosaminyltransferase 6 disrupts mammary acinar morphogenesis through o-glycosylation of fibronectin. Neoplasia 13:320–326

Patsos G, Robbe-Masselot C, Klein A, Hebbe-Viton V, San Martin R, Masselot D, Graessmann M, Paraskeva C, Gallagher T, Corfield A (2007) O-glycan regulation of apoptosis and proliferation in colorectal cancer cell lines. Biochem Soc Trans 35:1372–1374

Patsos G, Hebbe-Viton V, Robbe-Masselot C, Masselot D, San Martin R, Greenwood R, Paraskeva C, Klein A, Graessmann M, Michalski JC, Gallagher T, Corfield A (2009) O-glycan inhibitors generate aryl-glycans, induce apoptosis and lead to growth inhibition in colorectal cancer cell lines. Glycobiology 19:382–398

Petretti T, Kemmner W, Schulze B, Schlag PM (2000) Altered mRNA expression of glycosyltransferases in human colorectal carcinomas and liver metastases. Gut 46:359–366

Picco G, Julien S, Brockhausen I, Beatson R, Antonopoulos A, Haslam S, Mandel U, Dell A, Pinder S, Taylor-Papadimitriou J, Burchell J (2010) Over-expression of ST3Gal-I promotes mammary tumorigenesis. Glycobiology 20:1241–1250

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

Pinho SS, Osório H, Nita-Lazar M, Gomes J, Lopes C, Gärtner F, Reis CA (2009) Role of E-cadherin N-glycosylation profile in a mammary tumor model. Biochem Biophys Res Commun 379:1091–1096

Przybylo M, Lityńska A (2011) Glycans in melanoma screening. Part 1. The role of β1,6-branched N-linked oligosaccharides in melanoma. Biochem Soc Trans 39:370–373

Pullerits R, Bokarewa M, Dahlberg L, Tarkowski A (2005) Decreased levels of soluble receptor for advanced glycation end products in patients with rheumatoid arthritis indicating deficient inflammatory control. Arthritis Res Ther 7:R817–R824

Rabinovich GA, Gruppi A (2005) Galectins as immunoregulators during infectious processes: from microbial invasion to the resolution of the disease. Parasite Immunol 27:103–114

Radhakrishnan P, Lin MF, Cheng PW (2009) Elevated expression of L-selectin ligand in lymph node-derived human prostate cancer cells correlates with increased tumorigenicity. Glycoconj J 26:75–81

Radhakrishnan P, Grandgenett PM, Mohr AM, Bunt SK, Yu F, Chowdhury S, Hollingsworth MA (2013) Expression of core 3 synthase in human pancreatic cancer cells suppresses tumor growth and metastasis. Int J Cancer 133:2824–2833

Rapoport E, Le Pendu J (1999) Glycosylation alterations of cells in late phase apoptosis from colon carcinomas. Glycobiology 9:1337–1345

Russell L, Waring P, Beaver JP (1998) Increased cell surface exposure of fucose residues is a late event in apoptosis. Biochem Biophys Res Commun 250:449–453

Scaringi R, Piccoli M, Papini N, Cirillo F, Conforti E, Bergante S, Tringali C, Garatti A, Gelfi C, Venerando B, Menicanti L, Tettamanti G, Anastasia L (2013) NEU3 sialidase is activated under hypoxia and protects skeletal muscle cells from apoptosis through the activation of the epidermal growth factor receptor signaling pathway and the Hypoxia-inducible Factor (HIF)-1. J Biol Chem 288:3153–3162

Schneider P, Bodmer JL, Holler N, Mattmann C, Scuderi P, Terskikh A, Peitsch MC, Tschopp J (1997) Characterization of Fas (Apo-1, CD95)-Fas ligand interaction. J Biol Chem 272:18827–18833

Schultz MJ, Swindall AF, Bellis SL (2012) Regulation of the metastatic cell phenotype by sialylated Glycans. Cancer Metastasis Rev 31:501–518

Shatnyeva OM, Kubarenko AV, Weber CEM, Pappa A, Schwartz-Albiez R, Weber ANR, Krammer PH, Lavrik IN (2011) Modulation of the CD95-induced apoptosis: the role of CD95 N-glycosylation. PLoS One 6:e19927

Shimodaira K, Nakayama J, Nakamura N, Hasebe O, Katsuyama T, Fukuda M (1997) Carcinoma-associated expression of core 2 beta-1,6-N-acetylglucosaminyltransferase gene in human colorectal cancer: role of O-glycans in tumor progression. Cancer Res 57:5201–5206

Shiraishi T, Yoshida T, Nakata S, Horinaka M, Wakada M, Mizutani Y, Miki T, Sakai T (2005) Tunicamycin enhances tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis in human prostate cancer cells. Cancer Res 65:6364–6370

Song Y, Aglipay JA, Bernstein JD, Goswami S, Stanley P (2010) The bisecting GlcNAc on N-glycans inhibits growth factor signaling and retards mammary tumor progression. Cancer Res 70:3361–3371

Sparvero LJ, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, Rutledge R, Lin B, Amoscato AA, Zeh HJ, Lotze MT (2009) RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. J Translat Med 7:17

Springer GF, Desai PR, Spencer BD, Tegtmeyer H, Carlstedt SC, Scanlon EF (1995) T/Tn antigen vaccine is effective and safe in preventing recurrence of advanced breast carcinoma. Cancer Detect Prev 19:374–380

Srikrishna G, Huttunen HJ, Johansson L, Weigle B, Yamaguchi Y, Rauvala H, Freeze HH (2002) N-glycans on the receptor for advanced glycation end products influence amphoterin binding and neurite outgrowth. J Neurochem 80:998–1008

Srinivasan N, Bane SM, Ahire SD, Ingle AD, Kalraiya RD (2009) Poly N-acetyllactosamine substitutions on N- and not O-oligosaccharides or Thomsen-Friedenreich antigen facilitate lung specific metastasis of melanoma cells via galectin-3. Glycoconj J 26:445–456

St Hill CA, Farooqui M, Mitcheltree G, Gulbahce HE, Jessurun J, Cao Q, Walcheck B (2009) The high affinity selectin glycan ligand C2-O-sLex and mRNA transcripts of the core 2 beta-1,6-N-acetylglucosaminyltransferase (C2GnT1) gene are highly expressed in human colorectal adenocarcinomas. BMC Cancer 9:79

Stannard KA, Collins PM, Ito K, Sullivan EM, Scott SA, Gabutero E, Darren Grice I, Low P, Nilsson UJ, Leffler H, Blanchard H, Ralph SJ (2010) Galectin inhibitory disaccharides promote tumour immunity in a breast cancer model. Cancer Lett 299:95–110

Stillman BN, Hsu DK, Pang M, Brewer CF, Johnson P, Liu FT, Baum LG (2006) Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. J Immunol 176:778–789

Sukhithasria V, Nishaa N, Biswasa L, Kumarb VA, Biswasa R (2013) Innate immune recognition of microbial cell wall components and microbial strategies to evade such recognitions. Microbiol Res 168:396–406

Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Makino H (2006) Increased expression of receptor for advanced glycation end products by synovial tissue macrophages in rheumatoid arthritis. Arthr Rheum 54:97–104

Suzuki Y, Sutoh M, Hatakeyama S, Mori K, Yamamoto H, Koie T, Saitoh H, Yamaya K, Funyu T, Habuchi T, Arai Y, Fukuda M, Ohyama C, Tsuboi S (2012) MUC1 carrying core 2 O-glycans functions as a molecular shield against NK cell attack, promoting bladder tumor metastasis. Int J Oncol 40:1831–1838

Swindall AF, Bellis SL (2011) Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells. J Biol Chem 286:22982–22990

Takenaka Y, Fukumori T, Raz A (2004) Galectin-3 and metastasis. Glycoconj J 19:543–549

Taniguchi N, Korekane H (2011) Branched N-glycans and their implications for cell adhesion, signaling and clinical applications for cancer biomarkers and in therapeutics. BMB Rep 44:772–781

Taniuchi K, Cerny RL, Tanouchi A, Kohno K, Kotani N, Honke K, Saibara T, Hollingsworth MA (2011) Overexpression of GalNAc-transferase GalNAc-T3 promotes pancreatic cancer cell growth. Oncogene 30:4843–4854

Toscano MA, Bianco GA, Ilarregui JM, Croci DO, Correale J, Hernandez JD, Zwirner NW, Poirier NW, Riley EM, Baum LG, Rabinovich GA (2007) Differential glycosylation of TH1, TH2 and TH17 effector cells selectively regulates susceptibility to cell death. Nature Immunol 8:825–834

Tsui KH, Chang PL, Feng TH, Chung LC, Sung HC, Juang HH (2008) Evaluating the function of matriptase and N-acetylglucosaminyltransferase V in prostate cancer metastasis. Anticancer Res 28:1993–2000

Valenzuela HF, Pace KE, Cabrera PV, White R, Porvari K, Kaija H, Vihko P, Baum LG (2007) O-glycosylation regulates LNCaP prostate cancer cell susceptibility to apoptosis induced by galectin-1. Cancer Res 67:6155–6162

Valladeau J, Duvert-Frances V, Pin JJ, Kleijmeer MJ, Ait-Yahia S, Ravel O, Vincent C, Vega F Jr, Helms A, Gorman D, Zurawski SM, Zurawski G, Ford J, Sem Saeland S (2001) Immature human dendritic cells express asialoglycoprotein receptor isoforms for efficient receptor-mediated endocytosis. J Immunol 167:5767–5774

Vavasseur F, Yang J, Dole K, Paulsen H, Brockhausen I (1995) Synthesis of O-glycan core 3: characterization of UDP-GlcNAc: GalNAc-R β3-N-acetyl-glucosaminyltransferase activity from colonic mucosal tissues and lack of the activity in human cancer cell lines. Glycobiology 5:351–357

Wagener J, Weindl G, de Groot PWJ, de Boer AD, Kaesler S, Thavaraj S, Bader O, Mailaender-Sanchez D, Borelli C, Weig M, Biedermann T, Naglik JR, Korting HC, Schaller M (2012) Glycosylation of Candida albicans cell wall proteins is critical for induction of innate immune responses and apoptosis of epithelial cells. PLoS One 7:11le50518

Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, Huw L, Katta V, Cavet G, Hymowitz SG, Amler L, Ashkenazi A (2007) Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nature Med 13:1070–1077

Wang ECY (2012) On death receptor 3 and its ligands. . .. Immunology 137:114–116

Wang FL, Cui SX, Sun LP, Qu XJ, Xie YY, Zhou L, Mu YL, Tang W, Wang YS (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

Wang C, Li Z, Yang Z, Zhao H, Yang Y, Chen K, Cai X, Wang L, Shi Y, Qiu S, Fan J, Zha X (2010) The effect of receptor protein tyrosine phosphatase kappa on the change of cell adhesion and proliferation induced by N-acetylglucosaminyltransferase V. J Cell Biochem 109:113–123

Wells L, Whelan SA, Hart GW (2003) O-GlcNAc: a regulatory post-translational modification. Biochem Biophys Res Commun 302:435–441

Woronowicz A, De Vusser K, Laroy W, Contreras R, Meakin SO, Ross GM, Szewczuk MR (2004) Trypanosome trans-sialidase targets TrkA tyrosine kinase receptor and induces receptor internalization and activation. Glycobiology 14:987–998

Wu C, Guo X, Wang W, Wang Y, Shan Y, Zhang B, Song W, Ma S, Ge J, Deng H, Zhu M (2010) N-acetylgalactosaminyltransferase-14 as a potential biomarker for breast cancer by immunohistochemistry. BMC Cancer 10:123

Xue J, Gao X, Fu C, Cong Z, Jiang H, Wang W, Chen T, Wei Q, Qin C (2013) Regulation of galectin-3-induced apoptosis of Jurkat cells by both O-glycans and N-glycans on CD45. FEBS Lett 587:3986–3994

Yang X, Lehotay M, Anastassiades T, Harrison M, Brockhausen I (2004) The effect of TNFalpha on glycosylation pathways in bovine synoviocytes. Biochem Cell Biol 82:559–568

Yang X, Yip J, Anastassiades T, Harrison M, Brockhausen I (2007) The action of TNFalpha and TGFbeta include specific alterations of the glycosylation of bovine and human chondrocytes. Biochim Biophys Acta 1773:264–272

Yang X, Yip J, Harrison M, Brockhausen I (2008) Primary human osteoblasts and bone cancer cells as models to study glycodynamics in bone. Internat J Biochem Cell Biol 40:471–483

Yin L, Li Y, Ren J, Kuwahara H, Kufe D (2003) Human MUC1 carcinoma antigen regulates intracellular oxidant levels and the apoptotic response to oxidative stress. J Biol Chem 278:35458–35464

Yoshida T, Shiraishi T, Horinaka M, Wakada M, Sakai T (2007) Glycosylation modulates TRAIL-R1/death receptor 4 protein: different regulations of two pro-apoptotic receptors for TRAIL by tunicamycin. Oncol Rep 18:1239–1242

Yoshimura M, Nishikawa A, Ihara Y, Taniguchi S, Taniguchi N (1995) Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection. Proc Natl Acad Sci USA 92:8754–8758

Yu LG, Andrews N, Zhao Q, McKean D, Williams JF, Connor LJ, Gerasimenko OV, Hilkens J, Hirabayashi J, Kasai K, Rhodes JM (2007) Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion. J Biol Chem 282:773–781

Yuan Q, Yang H, Cheng C, Li C, Wu X, Huan W, Sun H, Zhou Z, Wang Y, Zhao Y, Lu X, Shen A (2012) β-1,4-Galactosyltransferase I involved in Schwann cells proliferation and apoptosis induced by tumor necrosis factor-alpha via the activation of MAP kinases signal pathways. Mol Cell Biochem 365:149–158

Zhang J, Nakayama J, Ohyama C, Suzuki M, Suzuki A, Fukuda M, Fukuda MN (2002) Sialyl Lewis X-dependent lung colonization of B16 melanoma cells through a selectin-like endothelial receptor distinct from E-or P-selectin. Cancer Res 62:4194–4198

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 99:1304–1310

Zhao Q, Guo X, Nash GB, Stone PC, Hilkens J, Rhodes JM, Yu LG (2009) Circulating galectin-3 promotes metastasis by modifying MUC1 localization on cancer cell surface. Cancer Res 69:6799–6806

Zhou J, Wei Y, Liu D, Ge X, Zhou F, Yun X, Jiang J, Gu J (2008) Identification of b1,4GalT II as a target gene of p53-mediated HeLa cell apoptosis. J Biochem 143:547–554

Zhou X, Chen H, Wang Q, Zhang L, Zhao J (2011) Knockdown of Mgat5 inhibits CD133+ human pulmonary adenocarcinoma cell growth in vitro and in vivo. Clin Invest Med 34:E155–E162

Zhu Y, Srivatana U, Ullah A, Gagneja H, Berenson CS, Lance P (2001) Suppression of a sialyltransferase by antisense DNA reduces invasiveness of human colon cancer cells in vitro. Biochim Biophys Acta 1536:148–160

Zinonos I, Labrinidis A, Lee M, Liapis V, Hay S, Ponomarev V, Diamond P, Zannettino ACW, Findlay DM, Evdokiou A (2009) Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer. Mol Cancer Ther 8:2969–2980

Part VI
Implication of Sugars in Cancer Diagnostics and Therapeutics

Chapter 11
Targeting Glycans for Immunotherapy of Human Cancers

Jung-Tung Hung and Alice L. Yu

Abstract Surface carbohydrate-containing molecules, such as glycoproteins and glycolipids, have been shown to play crucial regulatory roles in the normal physiological process as well as in pathological conditions including tumor progression. Those glycans which are overexpressed on the surface of tumor cells, but not detected or only weakly expressed in some limited normal tissues, are designated as tumor-associated carbohydrate antigens (TACAs). These TACAs may serve as potential targets for immunotherapy. The biological functions of TACAs and therapeutic strategies against TACAs will be addressed in this review.

Keywords Cancer immunotherapy • GD2 • Globo H • Sialyl-Tn • GM2 • Immune checkpoints • Angiogenesis

11.1 Introduction

Glycosylation is an important posttranslational modification process to produce diverse glycans that are frequently attached to proteins and lipids. These glycoconjugates play a key role in cells, including receptor activation, cell adhesion, signal transduction, endocytosis, molecular trafficking, and clearance (Ohtsubo and Marth 2006). Altered glycosylation on glycoproteins and glycolipids is a prominent feature of cancer cells (Reis et al. 2010). These abnormal glycoconjugates are involved in tumor proliferation, invasion, angiogenesis, and metastasis. Patient with altered glycoconjugates in tumor tissue usually has poor prognosis (Miyake et al. 1992). Changes in glycosylation, including over-, under-,

J.-T. Hung
Institute of Stem Cell and Translational Cancer Research, Chang Gung Memorial Hospital at Linkou, No.5, Fu-Shin St. Kuei Shang, 333 Taoyuan, Taiwan

A.L. Yu (✉)
Institute of Stem Cell and Translational Cancer Research, Chang Gung Memorial Hospital at Linkou, No.5, Fu-Shin St. Kuei Shang, 333 Taoyuan, Taiwan

University of California in San Diego Medical Center, 200 W Arbor Drive, San Diego 92103, CA, USA
e-mail: aliceyu@ucsd.edu

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes*, DOI 10.1007/978-4-431-55939-9_11

and neo-expression of sugar moieties, might result from the upregulation/downregulation of some glycosyltransferases and glycosidases. Increased N-glycosylation, such as 1,6-branched N-glycans, was observed in breast cancer and colon cancer (Dennis et al. 1987; Fernandes et al. 1991; Seelentag et al. 1998; Murata et al. 2004), which was mediated by GnT-V. On the other hand, O-glycosylation is often reduced resulting in the accumulation of core 1-based O-glycan during the tumorigenesis. The most common O-glycan epitopes were TF (Galβ1, 3GalNAc), Tn (GalNAc), Lewis^x, Lewis^a, and their sialylated counterparts (Springer 1984; Yuan et al. 1986; Itzkowitz et al. 1989; Itzkowitz et al. 1986; Tozawa et al. 2005). They were reported to enhance the intravasation of cancer cells, binding of circulating cancer cells to endothelium, and extravasation and colonization at the distant sites (Rosen and Bertozzi 1994; Borsig et al. 2001, 2002). For instance, increased expression of sialyl-Le^x (sLe^x) and sialyl-Le^a (sLe^a) was shown to assist the invasion and metastasis of tumor (Hoff et al. 1989; Kannagi 1997) and was associated with poor survival of patients (Makino et al. 2001). So far, a long list of TACAs has been identified, such as Tn, sialyl-Tn, TF, Lewis^y, sialyl Lewis^x, sialyl Lewis^a, Lewis^x, Globo H, stage-specific embryonic antigen-3 (SSEA-3), GD2, GD3, GM2, fucosyl GM1, Neu5Gc GM3, and polysialic acid. Some of these have been exploited as targets for immunotherapy of cancers.

Conventional treatments of cancer, including radiation, surgery, and chemotherapy, are not cancer-specific. Immunotherapy, on the other hand, provides a strategy to target specific cancer cells. Cancer immunotherapy can be categorized into active and passive immunotherapy. An active cancer immunotherapy is to activate the immune system of patients to attack cancer cells, which can trigger immunological memory. On the other hand, passive immunotherapy is to deliver tumor antigen-specific monoclonal antibodies to kill cancer cells through complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC). Since the initial approval of anti-CD20 (rituximab) for the treatment of lymphoma in 1994, more than 10 monoclonal antibodies have been approved for passive immunotherapy of cancer and all of them target protein antigens. On the other hand, the first and only approved active immunotherapy is sipuleucel-T (*Provenge*, Dendreon), for the treatment of metastatic prostate cancer (Kantoff et al. 2010). Sipuleucel-T is an autologous cellular vaccine activated ex vivo by recombinant prostate acid phosphatase (PAP) fused to GM-CSF. Although a number of clinical trials of cancer immunotherapy targeting TACAs have been conducted over the past two decades, majority of the trials did not proceed beyond early phase I/II studies. Only three TACAs have reached clinical phase III development: sialyl-Tn, GM2, and GD2. Unfortunately, randomized phase III clinical trials of sialyl-Tn-KLH vaccine (Theratope) in metastatic breast cancer (Miles et al. 2011a) and GM2-KLH vaccine in melanoma (Eggermont et al. 2013) failed to demonstrate any benefit of the vaccine, although subsequent subgroup analysis did demonstrate survival benefit of Theratope in metastatic breast cancer patients on endocrine therapy (Ibrahim et al. 2013). On the other hand, passive immunotherapy with dinutuximab, a chimeric anti-GD2, has demonstrated a significant improvement in event-free survival and overall survival in patients with high-risk neuroblastoma

(Yu et al. 2010) which led to regulatory approval in the USA and Europe in 2015. Thus, GD2 is the first TACA proven to be an effective target antigen for cancer immunotherapy.

The approval of ipilimumab (anti-CTLA-4) for the treatment of melanoma in 2011 as the first monoclonal antibody targeting an immune checkpoint molecule (Hodi et al. 2010) heralded a new era of cancer immunotherapy. The success of ipilimumab was closely followed by the development of additional immune checkpoint inhibitors, including nivolumab (Robert et al. 2015) and pembrolizumab (Robert et al. 2014), which target PD-1 and ensure the emergence of many more immune checkpoint blockers on the horizon. Such breakthroughs beg the question whether TACAs may act as immune checkpoint molecules. Indeed, several cancer-associated gangliosides were shown to inhibit immune cell responses, including antigen processing and presentation (Peguet-Navarro et al. 2003), T-cell proliferation (Biswas et al. 2009; Chu and Sharom 1993; Morioka et al. 1991), and cytokine production, such as IFN-γ and IL-4 (Biswas et al. 2006; Irani et al. 1996). Purified gangliosides from cancer cells displayed immunosuppressive activities which aided cancers to escape from host immune surveillance (Ladisch et al. 1992; Wolfl et al. 2002), which were mediated by hampering the interaction of IL-2 with its receptor (Lu and Sharom 1996), inducing apoptotic cell death (Das et al. 2008) and deviation toward Th2 response (Crespo et al. 2006). Such ganglioside-induced T-cell dysfunction involved NF-kappa B inhibition (Uzzo et al. 1999) through degradation of RelA and p50 proteins (Thornton et al. 2004). In contrast, there are relatively few studies on the functions of Globo-series TACAs. The core structure of Globo-series glycosphingolipids is Galα1,4-Galβ1,4Glc-ceramide (Gb3), which is catalyzed by α1,4-galactosyltransferase (A4galt) through the transfer of a galactose to lactosylceramide (Kojima et al. 2000). Gb3 has been found in Burkitt lymphoma (Wiels et al. 1981) and germ cell-derived tumors (Murray et al. 1985) and also in a subpopulation of B cells in germinal centers (Klein et al. 1983) and on kidney proximal tubules and intestinal epithelial cells (Fujii et al. 2005). In addition, Gb3 could be induced on the surface of human monocytes by LPS (van Setten et al. 1996) or on endothelial cells by interleukin-1 and tumor necrosis factor-alpha (van de Kar et al. 1992). Besides, Gb3 could serve as a receptor on endothelial cells for verotoxins produced by *Escherichia coli* O157 (Jacewicz et al. 1986), which was further confirmed by increased sensitivity to LPS-induced lethal shock in A4galt knockout mice (Okuda et al. 2006) (Kondo et al. 2013). Intriguingly, in wild-type mice, injection of LPS did not increase the expression of Gb3 on the surface of endothelial cells although it induced the expression of A4galt RNA and globotetraosylceramide (Gb4), one of the Globo-series glycolipids generated by b1,3-N-acetylgalactosaminyltransferase (B3galnt1) through the transfer of galactosamine to Gb3, suggesting that Gb4, but not Gb3, might play a role in LPS-induced lethal shock. Indeed, administration of Gb4 increased the survival rate of mice injected with LPS. The protective effect of Gb4 on LPS-challenged mice was mediated by the binding of Gb4 to the complex of toll-like receptor-4 and myeloid differentiation factor 2 on the endothelia, thereby interfering with the binding of LPS to this complex (Kondo et al. 2013).

However, the effects of Globo-series glycolipids on immune cells have remained unclear until Tsai et al. reported the immunosuppressive activity of Globo H ceramide (Tsai et al. 2013). Globo H ceramides released from the surface of tumor cells were taken up by T and B lymphocytes, with ensuing inhibition of activation of lymphocytes. Tumor-infiltrating lymphocytes in close proximity to the Globo H-expressing tumor cells showed positive staining of anti-Globo H antibody by IHC, consistent with the in vitro observation of uptake of Globo H ceramide released from tumor cells by lymphocytes. Treatment of lymphoid cells with Globo H ceramide did not induce apoptosis nor expand regulatory T cells. The molecular mechanisms of Globo H ceramide induced immunosuppression involved upregulation of id3 and itch via upregulation of egr2/3, leading to diminished expression of Notch, which is crucial for T-cell activation (Palaga et al. 2003). These results provide the first evidence that Globo H ceramide acts as an immune checkpoint molecule to facilitate the escape of cancer cells from immune surveillance.

In addition to the function of TACAs as immune checkpoints, several gangliosides have been reported to exhibit angiogenic activities. Tumor cells with GM2 synthase/GM3 synthase deficiency formed avascular tumor on mice (Liu et al. 2014a), whereas upregulation of GM1, GM2, and GD1a enhanced blood vessel density in tumors (Manfredi et al. 1999). On the other hand, GM3 blocked the dimerization of vascular endothelial growth factor receptor 2 (VEGFR2) to inhibit the signaling transduced by VEGF (Chung et al. 2009). The effects of Globo-series TACA on angiogenesis were first addressed in a report showing angiogenic activity of Globo H ceramide (Cheng et al. 2014). Globo H ceramide induced tube formation of endothelial cell in vitro and angiogenesis in vivo. When Globo H-positive tumor cells were sorted into two subpopulations based on Globo H expression, the Globo H^{hi} tumor cells grew faster with greater vessel density than Globo H^{low} tumor cells in vivo. Consistent with this was the observation of higher vessel density in Globo H^{+} than Globo H^{-} breast cancer specimens. Mechanistic investigations linked the angiogenic effects of Globo H ceramide to its endocytosis and binding to TRAX, with consequent release of PLCβ1 from TRAX to trigger Ca^{2+} mobilization. This is the first globoside shown to display angiogenic activity, along with elucidation of its mechanisms. On the other hand, Globo H has been identified as one of the glycans that bind human RNase 1, facilitating the internalization of the RNase 1 which induced cell death. Blocking the interaction of Globo H and RNase 1 with anti-Globo H antibody partially rescued the cells from RNase-induced cell lysis (Eller et al. 2015). These findings suggested multifaceted roles of Globo H in tumor biology.

The findings of certain TACAs acting as immune checkpoint molecules and angiogenic factors further strengthen the scientific rationales for immunotherapy targeting TACAs. The following sections will address various strategies for developing TACA-targeted cancer immunotherapies.

11.2 Disialoganglioside (GD2)-Targeted Cancer Immunotherapies

Disialoganglioside (GD2), a b-series ganglioside, is a sialic acid-containing surface glycolipid that generated from precursor GM2 by GD3 synthase and GD2 synthase. It is expressed by neuroblastoma (>98 %), melanoma, glioma, small-cell lung cancer, sarcomas (Schulz et al. 1984; Cheung et al. 1987), breast cancer stem cell (Liang et al. 2013; Battula et al. 2012), as well as some normal neuroectodermal (Yanagisawa et al. 2011), and mesenchymal stem cells (MSCs) (Martinez et al. 2007; Jin et al. 2010). GD2 plays an important role in the proliferation and invasiveness of tumor cells (Yoshida et al. 2001; Shibuya et al. 2012). It could directly induce activation of the proto-oncogene c-Met to enhance proliferation of triple-negative breast cancer cells (Cazet et al. 2012). It upregulates integrin α2β1-mediated tyrosine phosphorylation of p125FAK, which enhances platelet adhesion to extracellular matrix collagen, thereby promoting metastasis of neuroblastoma cells (Chen et al. 2013). Furthermore, $GD2^+$ murine bone marrow MSCs (mBM-MSC) possessed not only much greater clonogenic and proliferative capabilities but also stronger differentiation potential to adipocytes and osteoblasts, as compared to unsorted mBM-MSCs (Xu et al. 2013). Moreover, in human osteosarcoma cell lines, a murine anti-GD2 antibody, mAb 14G2a, effectively inhibits cell invasiveness, MMP-2 activity, and cell viability (Liu et al. 2014b). On the other hand, in human tissues, only weak expression of GD2 is observed in neurons, skin melanocytes, and peripheral pain fibers (Svennerholm et al. 1994). Therefore, GD2 is an ideal glycan antigen target for immunotherapy. Three immunotherapeutic strategies have been developed so far, including GD2-specific monoclonal antibodies, GD2-specific chimeric antigen receptor T cells, and GD2 vaccines.

11.2.1 GD2-Specific Monoclonal Antibodies

11.2.1.1 3F8

3F8 is a murine IgG3 monoclonal antibody which binds to GD2-expressing tumor cells and mediates cytotoxicity by activating human complement system (Cheung et al. 1985). ^{131}I-labeled 3F8 has been used for neuroblastoma imaging (Miraldi et al. 1986) and shown to eradicate human NB xenografts (Cheung et al. 1986). A phase I clinical trial of 3F8 was conducted in 17 patients with relapsed or refractory neuroblastoma. Significant toxicities including neuropathic pain, tachycardia, hypotension, hypertension, fever, and urticaria were observed. Antitumor activities were noted in some patients, ranging from complete clinical remissions to mixed responses. All patients developed human anti-mouse antibodies (HAMA) to 3F8. A phase II study in 16 patients with stage 4 neuroblastoma showed clinical responses in bony lesions and marrow diseases (Cheung et al. 1998a). Subsequently, the effect

of 3F8 on minimal residual disease of stage 4 neuroblastoma was evaluated in 34 patients in first or subsequent response, and 13/34 patients remained progression-free for 53–143 months (Cheung et al. 2000). A series of sequential phase II studies in 139 patients showed an overall 5-year EFS of 62 % for stage 4 patients in first remission who received 3F8 + GM-CSF + cis-retinoic acid (Cheung et al. 2012) and a correlation of a better outcome for patients with the FCGR2A (R/R) genotype which favored the binding of the IgG3 antibody (Cheung et al. 2006). Moreover, a better survival correlated with a transient anti-mouse response or completion of 4 cycles of 3F8 treatment (Cheung et al. 1998b). Humanized 3F8 has been generated along with the generation of hu3F8/IL-2 and hu3F8/GM-CSF, which are undergoing phase I clinical trials in patients with high-risk neuroblastoma: hu3F8 (NCT01419834) and hu3F8/IL-2 (NCT01662804 and NCT01757626).

11.2.1.2 14G2a

MAb 14G2a was generated from murine 14.18 IgG3 anti-GD2 by class switch to IgG2a antibody (Mujoo et al. 1987, 1989). Three phase I trials of mAb 14G2a were conducted in patients with melanoma, neuroblastoma, and osteosarcoma. The dose of mAb 14G2a was escalated up to 500 mg/m^2/course, with significant dose and infusion rate-dependent toxicities, including pain, tachycardia, hypotension, hypertension, fever, hyponatremia, and urticaria (Uttenreuther-Fischer et al. 1995a; Murray et al. 1994; Saleh et al. 1992a). Pain was thought to be due to binding of antibody to peripheral nerve fibers expressing GD2 (Svennerholm et al. 1994). Clinical benefits were observed in some patients even in these early phase trials. To enhance the ADCC effect, 14G2a was combined with IL-2 and a maximum tolerated dose (MTD) of 14G2a plus IL-2 was 15 mg/m^2/day. Similar side effects were observed although IL-2 might have contributed to some of the toxicities, such as fever. One patient with neuroblastoma had a partial response (PR), and one patient with osteosarcoma had a complete response (CR) (Frost et al. 1997).

11.2.1.3 Ch14.18

A human-mouse chimeric anti-GD2 monoclonal antibody, ch14.18, was constructed by combining the variable regions of 14G2a and the constant regions of human IgG1-k (Gillies et al. 1989). MAb ch14.18 could activate complement system (Zeng et al. 2005) and mediate ADCC through neutrophils, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells (Barker et al. 1991) with an efficiency 50–100 times greater than the murine mAb 14G2a (Mueller et al. 1990). Investigational New Drug (IND) application for ch14.18 was filed in 1989, marking the first IND application for mAb generated by recombinant DNA technology. Two phase I clinical trials of ch14.18 in relapsed/refractory neuroblastoma revealed similar toxicity profile as 14G2a (Yu et al. 1998; Handgretinger

et al. 1995). As expected, the half-life of ch14.18 was longer than 14G2a, with a beta t 1/2 of 66.6 ± 27.4 h for ch14.18 and 18.3 ± 11.8 h for 14G2a (Handgretinger et al. 1995; Uttenreuther-Fischer et al. 1995b). Among a total of 19 neuroblastoma patients, 2 CR and 3 PR were observed, although another phase I trial in 13 adult patients with metastatic melanoma showed no clinical responses (Saleh et al. 1992b). Based on the in vitro findings that GM-CSF not only raised the number of leukocytes but also enhanced their anti-GD2-mediated ADCC (Barker et al. 1991), a pilot study of ch14.18 + GM-CSF was conducted, which showed 5 CRs and 3 stable diseases (SDs) in 17 refractory/recurrent neuroblastoma (Yu et al. 1995). This was subsequently confirmed by a phase II Pediatric Oncology Group study showing 2 CR, 2 PR, and 1 mixed response in 32 neuroblastoma patients (Yu et al. 1997). In these early phase trials, most clinical responses occurred in patients with small disease burden, esp. bone marrow metastasis. Thus, anti-GD2 immunotherapy was subsequently developed to target neuroblastoma in the setting of minimal residual disease (MRD). The feasibility of administering ch14.18 in combination with GM-CSF, IL-2, and isotretinoin after high-dose chemotherapy and stem cell transplant period was demonstrated in 2 pilot phase I studies, and the maximum tolerated dose (MTD) of ch14.18 in combination with cytokines was 25 mg/m^2/d for 4 days (Gilman et al. 2009; Ozkaynak et al. 2000). These studies paved the way for the pivotal phase III randomized clinical trial of ch14.18 + IL-2/GM-CSF. Patients with high-risk neuroblastoma who achieved at least PR to induction therapy and received stem cell transplantation and posttransplant radiotherapy were randomly assigned, in a 1:1 ratio, to receive standard therapy with six cycles of isotretinoin or immunotherapy with six cycles of isotretinoin and five concomitant cycles of ch14.18 in combination with alternating GM-CSF and IL-2. Randomization was stopped early because interim analysis of 226 eligible patients revealed a significant 2-year overall survival (86 ± 4 % versus 75 ± 5 %, p = 0.02 without adjustment for interim analyses) and event-free survival (66 ± 5 % versus 46 ± 5 % at 2 years, p = 0.01) advantage for 113 patients receiving immunotherapy versus those 113 receiving standard therapy (ClinicalTrials.gov number NCT00026312) (Yu et al. 2010). This major breakthrough has now been considered as a standard treatment for high-risk neuroblastoma. It also marks the first successful immunotherapy to target a nonprotein antigen.

11.2.1.4 Hu14.18K322A

Ch14.18 was further humanized by CDR grafting of 14.18 V regions to generate humanized 14.18 (hu14.18) antibody (Metelitsa et al. 2002). Since anti-GD2-induced neuropathic pain is complement-dependent, a K322A mutation of the C region of IgG1 in hu14.18 was made to limit the ability of complement fixation of hu14.18. Preclinical studies in rats confirmed that hu14.18K322A elicited significantly less allodynia than ch14.18 while maintaining its ADCC activity (Sorkin et al. 2010). A phase I clinical trial of hu14.18K322A in 38 neuroblastoma showed

the MTD, and recommended phase II dose, of hu14.18K322A to be 60 mg/m^2 per day for 4 days (Navid et al. 2014). Adverse effects, predominately pain, were manageable and improved with subsequent courses. Median hu14.18K322A α (initial phase) and β (terminal phase) half-lives were 1.74 and 21.1 days, respectively. Objective responses (four complete responses; two partial responses) were noted in 6 of 31 patients evaluable for response by iodine-123 metaiodobenzylguanidine score. Several early phase trials in patients with GD2$^+$ tumors are in progress (ClinicalTrials.gov numbers NCT01576692 and NCT00743496).

11.2.1.5 Hu14.18-IL-2

Another strategy to enhance the antitumor efficacy of an antibody is to link the antibody with cytokine to generate immunocytokine fusion proteins that accumulate high cytokine concentrations in the tumor microenvironment and thereby stimulate cellular immune responses against cancer cell.

Hu14.18-IL-2 is a fusion protein of hu14.18 and IL-2 (Neal et al. 2004a, b). A phase I trial of hu14.18-IL-2 in recurrent/refractory neuroblastoma ($n = 27$) and melanoma ($n = 1$) patients showed the MTD to be 12 mg/m^2/day, with similar toxicities as anti-GD2 combined with IL-2. No measurable CRs or PRs to hu14.18-IL-2 were observed; however, evidence of antitumor activity was noted in three neuroblastoma patients (Osenga et al. 2006). A phase II study showed 5 CR in 23 neuroblastoma patients with evaluable disease only by MIBG and/or bone marrow histology, but no responses for patients with measurable disease (Shusterman et al. 2010). In this study, patients with KIR-ligand mismatch seemed to be associated with better clinical response (Delgado et al. 2010). Another phase I trial of hu14.18-IL-2 in adults with melanoma ($n = 33$) showed MTD to be 7.5 mg/m^2/day, with the dose-limiting toxicities of hypoxia, hypotension, and elevations of AST and ALT, which were reversible (King et al. 2004). Subsequently, a phase II study was conducted in metastatic melanoma patients ($n = 14$) who received hu14.18-IL-2 at 6 mg/m^2/day as 4-h intravenous infusions on days 1, 2, and 3 of each 28-day cycle. All patients received 2 cycles of treatment, and one patient had a PR (7.1 %, 1/14) and 4 patients had SD (28.5 %, 4/14). The toxicities were reversible, including grade 3 hypotension ($n = 2$) and grade 2 renal insufficiency with oliguria ($n = 1$). The accrual was held due to limited availability of hu14.18-IL-2 (Albertini et al. 2012).

11.2.1.6 Anti-O-Acetyl GD2 Monoclonal Antibody 8B6

Although the therapeutic efficacy of anti-GD2 has been well-documented, neuropathic pain can limit its application. O-acetyl GD2 is an analog of GD2 with an acetyl group linked to oxygen at the 9 position of NeuAc. An O-acetyl GD2-specific antibody 8B6 was shown to bind to neuroblastoma and other neuroectodermal tumors, but not peripheral pain fibers (Alvarez-Rueda et al. 2011). Thus, antibodies

against O-acetyl GD2 may have the advantage over anti-GD2 which is dose-limited by neuropathic pain. Indeed, a mouse-human chimeric antibody c.8B6 was reported to display potent antitumor activity without inducing allodynia in preclinical studies (Terme et al. 2014). Clinical trials of mAbc.8B6 are eagerly awaited.

11.2.1.7 Bispecific Antibody

Bispecific antibody which binds to two different types of antigen by combining fragments of two different monoclonal antibodies is an attractive alternative to immunocytokine. A GD2-targeting bispecific antibody 3F8 × CD3 (3F8BiAb) has been developed. It could redirect activated T cells to GD2-expressing murine neuroblastoma (Yankelevich et al. 2012). A phase I/II clinical trial of 3F8BiAb in children and young adults with neuroblastoma and osteosarcoma is under development (NCT02173093).

11.2.2 GD2 Chimeric Antigen Receptor

T lymphocytes can be engineered to express chimeric antigen receptors (CARs), which can bind to tumor antigens, leading to antitumor activity in an MHC-independent manner. CARs are generated by joining a single-chain variable fragment (scFv) of monoclonal antibody with the transmembrane and cytoplasmic portions of T-cell receptor (TCR) ζ-chain, via a flexible hinge region, to form a functional CAR (Savoldo and Dotti 2013). Louis et al. generated GD2-CAR-expressing T lymphocytes for the treatment of 19 patients with neuroblastoma. Persistence of GD2-CAR T lymphocytes beyond 6 weeks was associated with better clinical outcome, and three patients with active disease achieved complete remission. Thus, the GD2-CAR T lymphocytes might provide an alternative strategy for immunotherapy of neuroblastoma (Louis et al. 2011).

11.2.3 GD2-Specific Vaccines

11.2.3.1 GD2-KLH

The main challenge for developing carbohydrate vaccines is their poor immunogenicity. Chemical conjugation of glycans to a highly immunogenic protein scaffold, such as keyhole limpet hemocyanin (KLH), may enhance the immune responses to glycans. GD2-KLH is a synthetic GD2 conjugated to KLH. A phase I clinical trial of GD2-KLH using monophosphoryl lipid A (MPL-A) as an adjuvant in seven patients with recurrent or progressive gliomas showed no adverse effects. However, neither anti-GD2 antibody nor clinical response was observed (Becker et al. 2002).

Another phase I clinical trial of GD2-KLH using OPT-821 combined with oral beta-glucan as adjuvants was conducted in neuroblastoma. Anti-GD2 antibody was induced in 12 of 15 patients. Importantly, disappearance of MRD was observed in 6 of 10 patients (Kushner et al. 2014). A subsequent phase I study of combined GM2-KLH and GD2-KLH mixed with QS-21 adjuvant in 31 patients with melanoma or sarcoma showed successful induction of IgM/IgG anti-GM2 and anti-GD2 in 97 % and 73 % of patients, respectively (Chapman et al. 2000). These encouraging findings suggest that adjuvants may play an important role in glycan-based vaccine.

11.2.3.2 Anti-GD2 Idiotype Monoclonal Antibody 1A7

mAb1A7 is an anti-idiotype antibody mimicking GD2 antigen which was generated by immunizing mice with anti-GD2, mAb 14G2a (Saleh et al. 1993). Active immunotherapy with anti-idiotype antibody is anticipated to induce a gradual release of anti-GD2 via humoral antibody response, which may be beneath the threshold of anti-GD2-induced toxicities. In preclinical study, immunization of C57BL/6 mice and rabbits with mAb1A7 induced anti-GD2 antibodies of IgG isotype that recognized GD2 by ELISA and flow cytometry. These antisera specifically lysed GD2-positive target cells in an ADCC assay (Sen et al. 1998). Foon et al. initiated a clinical trial for anti-GD2 idiotype antibody (1A7) in patients with advanced melanoma. Patients ($n = 47$) received 1A7 (TriGem) at dose of 1, 2, 4, or 8 mg mixed with QS-21 (100 μg) weekly for 4 weeks and then monthly until disease progression. A majority of patients (40/47, 85.1 %) generated an anti-1A7 response. The isotypic specificity of the anti-1A7 antibody was predominantly IgG, with minimal IgM, and these antibodies reacted specifically with tumor cells expressing GD2 by flow cytometry. Immune sera from five patients tested displayed ADCC activity. Complete response lasting for 24 months was noted in one patient and stable disease (14+ to 37+ months) in 12 patients. Disease progression occurred in 32 patients (1–17 months) and 21 had died (1–16 months). The Kaplan-Meier-derived overall median survival was not reached. Toxicities were mild, including local reaction at the site of the injection, with mild fever and chills (Foon et al. 1998; Foon et al. 2000). In addition, a clinical trial of mAb1A7 as a GD2 vaccine was conducted in high-risk neuroblastoma patients ($n = 31$, 26 stage IV, 5 stage III) who achieved first or subsequent complete remission or very good partial remission (Yu et al. 2001). Patients received subcutaneous injection of 1A7 mixed with QS-21 as adjuvant every 2 weeks for 4 weeks and then monthly for 11 months thereafter and switched to 1A7 in aluminum hydroxide gel during the second year. After treatment, all patients had local reactions, four developed transient fever and chills, and one patient had serum sickness. All patients generated anti-1A7 antiserum, and immune sera from some patients displayed CDC and ADCC activities against neuroblastoma. At a median of 6.8 years from study entry, 76.1 % (16/21) patients who enrolled during first remission have no evidence of disease progression, whereas only one of ten patients who enrolled during second

or subsequent remission remains progression-free. Thus, active immunotherapy with anti-idiotypic antibody-based GD2 vaccine may offer therapeutic advantage over passive immunotherapy with reduced infusion-related toxicities.

11.3 Sialyl-Tn-Targeted Cancer Vaccine

Sialyl-Tn, Neu5Acα2,6-N-acetylgalactosamine (STn), is a carcinoma-associated carbohydrate determinant expressed on cancer-associated mucins, while it is weakly expressed in fetal and restricted normal adult tissues (Kjeldsen et al. 1988). Circulating STn has been detected in patients with gastrointestinal (Motoo et al. 1991) and ovarian (Kobayashi et al. 1992) malignancies. Expression of STn in colorectal carcinoma (Itzkowitz et al. 1990), gastric carcinoma (Ma et al. 1993), and breast cancer (Leivonen et al. 2001) correlates with poor prognosis and predicts a poor response to chemotherapy (Miles et al. 1994). In endometrial cancer, overexpression of STn correlated with overexpression of cyclooxygenase 2 (Ohno et al. 2006), which is linked to angiogenesis, tumor growth (Ohno et al. 2005a), and inhibition of the infiltration of CD8 T cell (Ohno et al. 2005b). STn has been reported to be involved in cell-cell aggregation, ECM adhesion, and migration and invasion of tumor cells, as shown in STn-overexpressing gastric cancer cells transfected with ST6Gal I transferase (Pinho et al. 2007). Moreover, STn on the tumor cells could interact with Siglec-15 expressed on tumor-associated macrophages to enhance the production of transforming growth factor-β through spleen tyrosine kinase (Syk) pathway (Takamiya et al. 2013). These findings suggest that STn may be a good candidate target for cancer immunotherapy.

A synthetic STn-keyhole limpet hemocyanin (KLH) vaccine (Theratope) was evaluated in clinical trials as an active specific immunotherapy in the treatment of advanced cancer. One of the first studies of Theratope was conducted by MacLean and colleagues in patients with metastatic breast cancer, ovarian cancer, and colon cancer (MacLean et al. 1996). They reported that 51 patients who produced anti-STn + mucin IgG titers higher than the median value survived longer than 46 patients who generated lower titers. Based on promising results of STn-KLH vaccine in early clinical trials, a phase III randomized trial was conducted in patients with metastatic breast cancer who had nonprogressive disease after first-line chemotherapy. A total of 1028 patients were randomly assigned to either STn-KLH plus Detox as adjuvant or KLH plus Detox (control group). The vaccine was well tolerated, with mild to moderate injection-site reactions and reversible flu-like symptoms. Specific IgG and IgM antibodies were detected at week 12. Unfortunately, there were no significant differences in the time to progression (TTP) and overall survival (OS) between STn-KLH vaccine group (3.4 and 23.1 months, respectively) and control group (3 and 22.3 months, respectively) (Miles et al. 2011b), although a post hoc analysis suggested benefit of concurrent endocrine therapy and STn-KLH vaccine for women with metastatic breast cancer

(Ibrahim et al. 2013). Several factors may have contributed to the lack of overall clinical efficacy of this vaccine. First, STn is not expressed uniformly in all breast cancer specimens. It ranges from low 20 % to high 80 % in various reports (Julien et al. 2012). In this phase III study, STn expression was not determined nor used as enrollment criteria, which might mask any benefit from the vaccine due to heterogeneity in STn expression among patients. Second, significant titers of anti-KLH IgM and IgG antibodies were observed in control group, which may have conferred some anticancer benefits. Nonetheless, lessons learned from this failed large randomized clinical trial may serve as stepping stones to the ultimate success by modifying the clinical design and patient selection.

11.4 GM2-Targeted Cancer Vaccines

While GM3 is the predominant ganglioside in normal melanocytes (Carubia et al. 1984), in malignant melanoma, activation of glycosylating enzymes leads to increased expression of GD3, GD2, GM2, and 9-O-acetyl GD3 (Tsuchida et al. 1987). GM2 is also expressed on metastatic prostate cancer specimens (Zhang et al. 1998) and adult T-cell leukemia (Suzuki et al. 1987). Antibodies against GM2 were able to induce apoptosis (Retter et al. 2005; Nakamura et al. 1999) or necrosis (Bjerkvig et al. 1991) of GM2-expressing cancer cell lines. Furthermore, GM2 was found to inhibit immunoglobulin production of human B cell lines through impeding the production of IL-10 and TNF-α (Kimata and Yoshida 1996). In addition, complex of GM2 and GM3 was shown to associate with cMet-CD82 to regulate hepatocyte growth factor-induced motility of HCV29 cells (Todeschini et al. 2008). These findings suggest that GM2 is an attractive target for immunotherapy.

In 1994, 122 patients with stage III melanoma ($N = 122$) were treated with unconjugated GM2 and bacillus Calmette-Guerin (BCG) or BGC alone. The OS and DFS were not statistically significant between patients treated with GM2/BCG and BCG, although DFS was greater in patients producing anti-GM2 antibody (Livingston et al. 1994a). Most anti-GM2 antibodies induced by GM2/BCG vaccine in patients were IgM, suggesting that BCG adjuvant in glycan vaccine could not efficiently trigger antibody isotype switch to GM2-specific IgG antibody, which is an important mediator of ADCC. Subsequently, potent carrier protein, KLH, and adjuvant, QS-21, were used to generate GM2-KLH/QS-21 vaccine which induced higher titers of IgM anti-GM2 antibody and more IgG anti-GM2 antibody responses than GM2/BCG vaccine (Helling et al. 1995). A phase I trial of GM2-KLH vaccine plus QS-21 as an adjuvant in 22 patients with AJCC stage III/IV melanoma showed the induction of IgM and IgG antibodies against GM2 in patients treated with 100 or 200 μg of QS-21 (Livingston et al. 1994b). This led to two randomized phase III trials. One was conducted in 880 patients with resected high-risk melanoma (AJCC stages IIB and III) comparing the therapeutic efficacy of GM2-KLH/QS-21 (GMK) vaccine with standard therapy, high-dose interferon alfa-2b (HDI) (Kirkwood

et al. 2001). The trial was closed after interim analysis showing inferiority of GMK compared with HDI, although patients with higher antibody responses to GM2 had a trend toward improved RFS and OS ($p = 0.068$ at day 29). Another phase III trial was conducted in 1314 patients with stage II melanoma to evaluate the efficacy and toxicity of GMK vaccine as compared to observation. Unfortunately, GM2-KLH/QS-21 failed to improve RFS, distant metastasis-free survival, and overall survival (Eggermont et al. 2013). In view of the impressive response of melanoma to immune checkpoint blockade therapy (Hodi et al. 2010), it is possible that clinical benefit of GM2 vaccine may become evident when combined with inhibitors of immune checkpoint.

11.5 Globo H-Targeted Cancer Vaccines

Globo H, a hexasaccharide (Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1), was initially identified as a ceramide-linked glycolipid in human breast cancer cell line MCF-7 (Kannagi et al. 1983) and subsequently found to be expressed on a variety of epithelial cancers including breast, colon, ovarian, gastric, pancreatic, lung, and prostate cancers (Zhang et al. 1997, 1998).

Examination of Globo H expression in breast cancer stem cells (BCSCs) by flow cytometry revealed Globo H expression in 61 % (25/41) of breast cancer specimens and in 20 % (8/40) of BCSC-enriched subpopulation ($CD44^+/CD24^-$). The expression of Globo H precursor, stage-specific embryonic antigen 3 (SSEA3), was 77.5 % (31/40) in breast cancer tissues and 62.5 % (25/40) in BCSCs. Like Globo H, SSEA3 expression in normal tissues was predominately at the secretory borders of epithelium, where access to the immune system is restricted. Immunization of mice with Globo H-KLH and alpha-GalCer induced antibodies reactive with Globo H and SSEA3, suggesting that a Globo H-based vaccine will target tumor cells expressing Globo H or SSEA3, including BCSCs (Chang et al. 2008).

The overexpression of Globo H in cancer with limited expression in normal tissues makes Globo H a potential target for cancer immunotherapy. The findings of Globo H ceramide as stem cell markers (Chang et al. 2008), immune checkpoint molecules (Tsai et al. 2013), and angiogenic factors (Cheng et al. 2014) provide further impetus for Globo H-targeted immunotherapy (Sabbatini et al. 2007). Two phase I clinical trials of Globo H-KLH/QS-21 vaccine were conducted in patients with relapsed prostate cancer ($n = 18$) (Slovin et al. 1999) and metastatic breast cancer ($n = 27$), respectively (Gilewski et al. 2001). The treatment schedules consisted of injection of Globo H-KLH at weeks 1, 2, 3, 7, and 19. In general, the vaccine was well tolerated, with only local reactions and occasional fever and chills. Humoral responses to Globo H-KLH vaccine were observed. In the trial of relapsed prostate cancer, the highest median IgM antibody titer was around 300 at the dose level of 10, 30, and 100 μg of Globo H-KLH, and peak response was observed at weeks 34, 3, and 9, respectively. For the dose level of 3 μg of Globo H-KLH, the peak titer was 150 at week 7. Interestingly, the production of Globo

H-specific IgG antibody showed a different pattern from IgM responses. There were two obvious peak titers at the dose level of 10 μg and 30 μg of Globo H-KLH. At 30 μg Globo H-KLH, the IgG antibody titer reached to the maximal titer of 160 at weeks 9 and 34. At 10 μg Globo H-KLH, the peak IgG antibody titer was 80 at week 3 and week 35. In patients treated with 100 μg or 3 μg of Globo H-KLH, the titers of Globo H-specific IgG was only 100 (around week 31) or less than 20 (week 26), respectively. In the trial of metastatic breast cancer, Globo H-specific IgM peaked around weeks 5–7. Antisera in several patients of both trials displayed CDC activity. Both trials demonstrated that the Globo H-KLH vaccine was safe and effective in inducing humoral antibody response with moderate Globo H-specific IgM antibody titers in most patients, but only minimal IgG antibody. Recently, a multinational randomized phase II/III clinical trial of Globo H-KLH vaccine vs. placebo in patients with metastatic breast cancer has completed accrual of 349 patients and is awaiting further follow-up for outcome analysis (NCT01516307). Another phase II clinical trial of this vaccine in ovarian cancer is ongoing. In addition, a new generation of Globo H vaccine consisting of Globo H conjugated to diphtheria toxin as a carrier protein was shown to elicit more desirable IgG anti-Globo H, when combined with a novel analog of NKT-stimulatory alpha-galactosylceramide (α-GalCer) as an adjuvant. The efficacy of this promising Globo H vaccine awaits clinical trial in the near future (Huang et al. 2013).

11.6 Lewis^y-Targeted Cancer Vaccine

In ovarian cancer, Lewis^y is overexpressed which promotes metastasis through epididymis protein 4 (Zhuang et al. 2013, 2014). The expression of Lewis^y antigen was considered as an independent, drug resistance-related risk factors (Gao et al. 2014). A clinical trial of Lewis^y pentasaccharide conjugated with KLH together with immunological adjuvant QS-21 in ovarian cancer patients ($n = 25$) showed that the majority of the patients (16/24) produced anti-Lewis^y antibodies with significant antitumor cell reactivity as assessed by CDC in some patients. The vaccine was well tolerated without any gastrointestinal, hematologic, renal, or hepatic toxicity (Sabbatini et al. 2000). Another phase II trial was conducted with a doxorubicin-conjugated chimeric variant of anti-Lewis^y monoclonal antibody, BMS-182248-01, in patients ($n = 15$) with advanced gastric carcinoma (Ajani et al. 2000). However, BMS-182248-01 vaccine appeared to be ineffective in patients with gastric carcinoma with 10 patients progressed on study.

11.7 Polysialic Acid-Targeted Cancer Vaccine

Polysialic acid (polySA), a carbohydrate polymer of negatively charged sialic acid attached to the neural cell adhesion molecule (NCAM), is overexpressed on the surface of various cancers including small-cell lung cancer (SCLC) (Tanaka et al. 2001), Wilms' tumor (Roth et al. 1988a, b), neuroblastoma (Gluer et al. 1998), and neuroectodermal tumors (Figarella-Branger et al. 1990). A clinical trial of polySA-KLH (30 μg) vaccine in small-cell lung cancer ($n = 13$) did not induce immune response, but N-propionylated (NP)-polySA (30 μg) developed high-titer anti-SA antibody along with peripheral neuropathy and ataxia in several patients (Krug et al. 2004). Another trial of lower dose of NP-polySA vaccine (10 μg) resulted in the induction of IgM antibodies against polySA antigen in all 18 patients, with self-limited grade 3 ataxia of unclear etiology in 1 of 18 patients (Krug et al. 2012).

11.8 Polyvalent Glycan Vaccine

A hexavalent vaccine, including GM2, Globo H, Lewis^y, glycosylated MUC-1-32mer, Tn, and TF in a clustered formation conjugated to KLH, mixed with QS-21 was administered in a phase II setting to 30 patients with relapsed prostate cancer. All 30 patients showed increased antibody titers to at least two of the six antigens, but these serologic responses were lower than those seen previously with the respective monovalent vaccines (Slovin et al. 2007). In another study, GPI-0100, a semisynthetic low toxicity saponin, was used as adjuvant at doses ranging between 100 and 5000 μg for a bivalent vaccine containing the Globo H and the mucin MUC2 conjugated to KLH with in groups of five prostate cancer patients who had no evidence of disease except for rising PSA levels. All doses of GPI-0100 were well tolerated with dose-dependent increases in antibody titers against Globo H and MUC2. At the 5000 μg dose level, toxicity remained minimal with only occasional grade II local toxicity at vaccination sites and occasional sporadic grade I elevations in ALT. Compared with a subsequent trial with the same bivalent vaccine plus QS-21 at the maximal tolerated dose of 100 μg, the 5000 μg dose of GPI-0100 induced comparable antibody titers (Slovin et al. 2005).

11.9 Conclusion

Tumor-associated carbohydrate antigens are attractive targets for cancer therapy. Glycan-targeted immunotherapy holds the promise to have less side effects and greater specificity compared to conventional cancer therapy. To date, passive immunotherapy with anti-GD2 antibody in patients with neuroblastoma is the

first successful glycan-targeted immunotherapy, which has documented that targeting TACA is a feasible strategy for cancer immunotherapy. On the other hand, carbohydrate-based vaccines for active immunotherapy have yet to be proven effective in phase III randomized trials, although encouraging results were noted in early clinical trials. New strategies are needed for enhancing the potency of carbohydrate-based cancer vaccine by improving the design of vaccine. Designs with better adjuvants that effectively boost IgG humoral and/or cellular immune response against TACAs are also critically needed.

References

Ajani JA et al (2000) A multi-institutional phase II study of BMS-182248-01 (BR96-doxorubicin conjugate) administered every 21 days in patients with advanced gastric adenocarcinoma. Cancer J 6(2):78–81

Albertini MR et al (2012) Phase II trial of hu14.18-IL2 for patients with metastatic melanoma. Cancer Immunol Immunother 61(12):2261–2271

Alvarez-Rueda N et al (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(9), e25220

Barker E et al (1991) Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. Cancer Res 51(1):144–149

Battula VL et al (2012) Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. J Clin Invest 122(6):2066–2078

Becker R et al (2002) Phase I clinical trial on adjuvant active immunotherapy of human gliomas with GD2-conjugate. Br J Neurosurg 16(3):269–275

Biswas K et al (2006) GM2 expression in renal cell carcinoma: potential role in tumor-induced T-cell dysfunction. Cancer Res 66(13):6816–6825

Biswas S et al (2009) Elevated levels of select gangliosides in T cells from renal cell carcinoma patients is associated with T cell dysfunction. J Immunol 183(8):5050–5058

Bjerkvig R et al (1991) Anti-GM2 monoclonal antibodies induce necrosis in GM2-rich cultures of a human glioma cell line. Cancer Res 51(17):4643–4648

Borsig L et al (2001) Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci USA 98 (6):3352–3357

Borsig L et al (2002) Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci USA 99(4):2193–2198

Carubia JM et al (1984) Gangliosides of normal and neoplastic human melanocytes. Biochem Biophys Res Commun 120(2):500–504

Cazet A et al (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 (6):806–816

Chang WW et al (2008) Expression of Globo H and SSEA3 in breast cancer stem cells and the involvement of fucosyltransferases 1 and 2 in Globo H synthesis. Proc Natl Acad Sci USA 105 (33):11667–11672

Chapman PB et al (2000) Vaccination with a bivalent G(M2) and G(D2) ganglioside conjugate vaccine: a trial comparing doses of G(D2)-keyhole limpet hemocyanin. Clin Cancer Res 6 (12):4658–4662

Chen YX et al (2013) Effect of tumor gangliosides on tyrosine phosphorylation of p125FAK in platelet adhesion to collagen. Oncol Rep 29(1):343–348
Cheng JY et al (2014) Globo-H ceramide shed from cancer cells triggers translin-associated factor X-dependent angiogenesis. Cancer Res 74:6856–6866
Cheung NK et al (1985) Monoclonal antibodies to a glycolipid antigen on human neuroblastoma cells. Cancer Res 45(6):2642–2649
Cheung NK et al (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(3):739–745
Cheung NK et al (1987) Ganglioside GD2 specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. J Clin Oncol 5(9):1430–1440
Cheung NK et al (1998a) 3F8 monoclonal antibody treatment of patients with stage 4 neuroblastoma: a phase II study. Int J Oncol 12(6):1299–1306
Cheung NK et al (1998b) Anti-G(D2) antibody treatment of minimal residual stage 4 neuroblastoma diagnosed at more than 1 year of age. J Clin Oncol 16(9):3053–3060
Cheung NK et al (2000) Induction of Ab3 and Ab3' antibody was associated with long-term survival after anti-G(D2) antibody therapy of stage 4 neuroblastoma. Clin Cancer Res 6 (7):2653–2660
Cheung NK et al (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(18):2885–2890
Cheung NK et al (2012) 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(26):3264–3270
Chu JW, Sharom FJ (1993) Gangliosides inhibit T-lymphocyte proliferation by preventing the interaction of interleukin-2 with its cell surface receptors. Immunology 79(1):10–17
Chung TW et al (2009) Ganglioside GM3 inhibits VEGF/VEGFR-2-mediated angiogenesis: direct interaction of GM3 with VEGFR-2. Glycobiology 19(3):229–239
Crespo FA et al (2006) The immunoregulatory effects of gangliosides involve immune deviation favoring type-2 T cell responses. J Leukoc Biol 79(3):586–595
Das T et al (2008) GM1 and tumor necrosis factor-alpha, overexpressed in renal cell carcinoma, synergize to induce T-cell apoptosis. Cancer Res 68(6):2014–2023
Delgado DC et al (2010) Genotypes of NK cell KIR receptors, their ligands, and Fcgamma receptors in the response of neuroblastoma patients to Hu14.18-IL2 immunotherapy. Cancer Res 70(23):9554–9561
Dennis JW et al (1987) Beta 1–6 branching of Asn-linked oligosaccharides is directly associated with metastasis. Science 236(4801):582–585
Eggermont AM et al (2013) Adjuvant ganglioside GM2-KLH/QS-21 vaccination versus observation after resection of primary tumor > 1.5 mm in patients with stage II melanoma: results of the EORTC 18961 randomized phase III trial. J Clin Oncol 31(30):3831–3837
Eller CH et al (2015) Human cancer antigen globo H is a cell-surface ligand for human ribonuclease 1. ACS Cen Sci 1(4):181–190
Fernandes B et al (1991) Beta 1–6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. Cancer Res 51(2):718–723
Figarella-Branger DF, Durbec PL, Rougon GN (1990) Differential spectrum of expression of neural cell adhesion molecule isoforms and L1 adhesion molecules on human neuroectodermal tumors. Cancer Res 50(19):6364–6370
Foon KA et al (1998) Antibody responses in melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. Clin Cancer Res 4(5):1117–1124
Foon KA et al (2000) Clinical and immune responses in advanced melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. J Clin Oncol 18(2):376–384

Frost JD et al (1997) A phase I/IB trial of murine monoclonal anti-GD2 antibody 14.G2a plus interleukin-2 in children with refractory neuroblastoma: a report of the Children's Cancer Group. Cancer 80(2):317–333

Fujii Y et al (2005) Murine glycosyltransferases responsible for the expression of globo-series glycolipids: cDNA structures, mRNA expression, and distribution of their products. Glycobiology 15(12):1257–1267

Gao J et al (2014) Expression of CD147 and Lewis y antigen in ovarian cancer and their relationship to drug resistance. Med Oncol 31(5):920

Gilewski T et al (2001) Immunization of metastatic breast cancer patients with a fully synthetic globo H conjugate: a phase I trial. Proc Natl Acad Sci USA 98(6):3270–3275

Gillies SD, Lo KM, Wesolowski J (1989) High-level expression of chimeric antibodies using adapted cDNA variable region cassettes. J Immunol Methods 125(1–2):191–202

Gilman AL et al (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 (1):85–91

Gluer S et al (1998) Polysialylated neural cell adhesion molecule in childhood ganglioneuroma and neuroblastoma of different histological grade and clinical stage. Langenbecks Arch Surg 383(5):340–344

Handgretinger R et al (1995) A phase I study of human/mouse chimeric antiganglioside GD2 antibody ch14.18 in patients with neuroblastoma. Eur J Cancer 31A(2):261–267

Helling F et al (1995) GM2-KLH conjugate vaccine: increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. Cancer Res 55 (13):2783–2788

Hodi FS et al (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363(8):711–723

Hoff SD et al (1989) Increased expression of sialyl-dimeric LeX antigen in liver metastases of human colorectal carcinoma. Cancer Res 49(24 Pt 1):6883–6888

Huang YL et al (2013) Carbohydrate-based vaccines with a glycolipid adjuvant for breast cancer. Proc Natl Acad Sci USA 110(7):2517–2522

Ibrahim NK et al (2013) Survival advantage in patients with metastatic breast cancer receiving endocrine therapy plus sialyl Tn-KLH vaccine: post Hoc analysis of a large randomized trial. J Cancer 4(7):577–584

Irani DN, Lin KI, Griffin DE (1996) Brain-derived gangliosides regulate the cytokine production and proliferation of activated T cells. J Immunol 157(10):4333–4340

Itzkowitz SH et al (1986) Lewisx- and sialylated Lewisx-related antigen expression in human malignant and nonmalignant colonic tissues. Cancer Res 46(5):2627–2632

Itzkowitz SH et al (1989) Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. Cancer Res 49(1):197–204

Itzkowitz SH et al (1990) Sialosyl-Tn. A novel mucin antigen associated with prognosis in colorectal cancer patients. Cancer 66(9):1960–1966

Jacewicz M et al (1986) Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. J Exp Med 163(6):1391–1404

Jin HJ et al (2010) GD2 expression is closely associated with neuronal differentiation of human umbilical cord blood-derived mesenchymal stem cells. Cell Mol Life Sci 67(11):1845–1858

Julien S, Videira PA, Delannoy P (2012) Sialyl-tn in cancer: (how) did we miss the target? Biomolecules 2(4):435–466

Kannagi R (1997) Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. Glycoconj J 14(5):577–584

Kannagi R et al (1983) New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3. J Biol Chem 258(14):8934–8942

Kantoff PW et al (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363(5):411–422
Kimata H, Yoshida A (1996) Inhibition of spontaneous immunoglobulin production by ganglioside GM2 in human B cells. Clin Immunol Immunopathol 79(2):197–202
King DM et al (2004) Phase I clinical trial of the immunocytokine EMD 273063 in melanoma patients. J Clin Oncol 22(22):4463–4473
Kirkwood JM et al (2001) High-dose interferon alfa-2b significantly prolongs relapse-free and overall survival compared with the GM2-KLH/QS-21 vaccine in patients with resected stage IIB-III melanoma: results of intergroup trial E1694/S9512/C509801. J Clin Oncol 19 (9):2370–2380
Kjeldsen T et al (1988) Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl-2----6 alpha-N-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res 48(8):2214–2220
Klein G et al (1983) Expression of the BLA antigen, defined by the monoclonal 38.13 antibody, on Burkitt lymphoma lines, lymphoblastoid cell lines, their hybrids and other B-cell lymphomas and leukemias. Int J Cancer 31(5):535–542
Kobayashi H, Terao T, Kawashima Y (1992) Serum sialyl Tn as an independent predictor of poor prognosis in patients with epithelial ovarian cancer. J Clin Oncol 10(1):95–101
Kojima Y et al (2000) Molecular cloning of globotriaosylceramide/CD77 synthase, a glycosyltransferase that initiates the synthesis of globo series glycosphingolipids. J Biol Chem 275 (20):15152–15156
Kondo Y et al (2013) TLR4-MD-2 complex is negatively regulated by an endogenous ligand, globotetraosylceramide. Proc Natl Acad Sci USA 110(12):4714–4719
Krug LM et al (2004) Vaccination of small cell lung cancer patients with polysialic acid or N-propionylated polysialic acid conjugated to keyhole limpet hemocyanin. Clin Cancer Res 10 (3):916–923
Krug LM et al (2012) Immunization with N-propionyl polysialic acid-KLH conjugate in patients with small cell lung cancer is safe and induces IgM antibodies reactive with SCLC cells and bactericidal against group B meningococci. Cancer Immunol Immunother 61(1):9–18
Kushner BH et al (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 (5):1375–1382
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 (2):180–188
Leivonen M et al (2001) STn and prognosis in breast cancer. Oncology 61(4):299–305
Liang YJ et al (2013) Differential expression profiles of glycosphingolipids in human breast cancer stem cells vs. cancer non-stem cells. Proc Natl Acad Sci USA 110(13):4968–4973
Liu Y et al (2014a) Tumor gangliosides accelerate murine tumor angiogenesis. Angiogenesis 17 (3):563–571
Liu B et al (2014b) Endothelin A receptor antagonism enhances inhibitory effects of anti-ganglioside GD2 monoclonal antibody on invasiveness and viability of human osteosarcoma cells. PLoS ONE 9(4), e93576
Livingston PO et al (1994a) Improved survival in stage III melanoma patients with GM2 antibodies: a randomized trial of adjuvant vaccination with GM2 ganglioside. J Clin Oncol 12(5):1036–1044
Livingston PO et al (1994b) Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine 12(14):1275–1280
Louis CU et al (2011) Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood 118(23):6050–6056
Lu P, Sharom FJ (1996) Immunosuppression by YAC-1 lymphoma: role of shed gangliosides. Cell Immunol 173(1):22–32

Ma XC et al (1993) Expression of sialyl-Tn antigen is correlated with survival time of patients with gastric carcinomas. Eur J Cancer 29A(13):1820–1823
MacLean GD et al (1996) Antibodies against mucin-associated sialyl-Tn epitopes correlate with survival of metastatic adenocarcinoma patients undergoing active specific immunotherapy with synthetic STn vaccine. J Immunother Emphasis Tumor Immunol 19(1):59–68
Makino T et al (2001) Carbohydrate antigens as a risk factor for hematogenous recurrence of esophageal squamous cell carcinoma patients. Oncol Rep 8(5):981–985
Manfredi MG et al (1999) Gangliosides influence angiogenesis in an experimental mouse brain tumor. Cancer Res 59(20):5392–5397
Martinez C et al (2007) Human bone marrow mesenchymal stromal cells express the neural ganglioside GD2: a novel surface marker for the identification of MSCs. Blood 109 (10):4245–4248
Metelitsa LS et al (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(11):4166–4173
Miles DW et al (1994) Expression of sialyl-Tn predicts the effect of adjuvant chemotherapy in node-positive breast cancer. Br J Cancer 70(6):1272–1275
Miles D et al (2011) Phase III multicenter clinical trial of the sialyl-TN (STn)-keyhole limpet hemocyanin (KLH) vaccine for metastatic breast cancer. Oncologist 16(8):1092–1100
Miraldi FD et al (1986) Diagnostic imaging of human neuroblastoma with radiolabeled antibody. Radiology 161(2):413–418
Miyake M et al (1992) Correlation of expression of H/Le(y)/Le(b) antigens with survival in patients with carcinoma of the lung. N Engl J Med 327(1):14–18
Morioka N et al (1991) Gangliosides inhibit the proliferation of human T cells stimulated with interleukin-4 or interleukin-2. J Dermatol 18(8):447–453
Motoo Y et al (1991) Serum sialyl-Tn antigen levels in patients with digestive cancers. Oncology 48(4):321–326
Mueller BM et al (1990) Enhancement of antibody-dependent cytotoxicity with a chimeric anti-GD2 antibody. J Immunol 144(4):1382–1386
Mujoo K et al (1987) Disialoganglioside GD2 on human neuroblastoma cells: target antigen for monoclonal antibody-mediated cytolysis and suppression of tumor growth. Cancer Res 47 (4):1098–1104
Mujoo K et al (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(11):2857–2861
Murata K et al (2004) Attachment of human colon cancer cells to vascular endothelium is enhanced by N-acetylglucosaminyltransferase V. Oncology 66(6):492–501
Murray LJ et al (1985) Expression of Burkitt lymphoma-associated antigen (defined by the monoclonal antibody 38.13) on both normal and malignant germinal-centre B cells. Int J Cancer 36(5):561–565
Murray JL 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 (1):184–193
Nakamura K et al (1999) Apoptosis induction of human lung cancer cell line in multicellular heterospheroids with humanized antiganglioside GM2 monoclonal antibody. Cancer Res 59 (20):5323–5330
Navid F et al (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(14):1445–1452
Neal ZC et al (2004a) Enhanced activity of hu14.18-IL2 immunocytokine against murine NXS2 neuroblastoma when combined with interleukin 2 therapy. Clin Cancer Res 10(14):4839–4847

Neal ZC et al (2004b) NXS2 murine neuroblastomas express increased levels of MHC class I antigens upon recurrence following NK-dependent immunotherapy. Cancer Immunol Immunother 53(1):41–52

Ohno S et al (2005a) Multiple roles of cyclooxygenase-2 in endometrial cancer. Anticancer Res 25 (6A):3679–3687

Ohno Y et al (2005b) Role of cyclooxygenase-2 in immunomodulation and prognosis of endometrial carcinoma. Int J Cancer 114(5):696–701

Ohno S et al (2006) Expression of Tn and sialyl-Tn antigens in endometrial cancer: its relationship with tumor-produced cyclooxygenase-2, tumor-infiltrated lymphocytes and patient prognosis. Anticancer Res 26(6A):4047–4053

Ohtsubo K, Marth JD (2006) Glycosylation in cellular mechanisms of health and disease. Cell 126 (5):855–867

Okuda T et al (2006) Targeted disruption of Gb3/CD77 synthase gene resulted in the complete deletion of globo-series glycosphingolipids and loss of sensitivity to verotoxins. J Biol Chem 281(15):10230–10235

Osenga KL et al (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(6):1750–1759

Ozkaynak MF et al (2000) Phase I study of chimeric human/murine anti-ganglioside G (D2) monoclonal antibody (ch14.18) with granulocyte-macrophage colony-stimulating factor in children with neuroblastoma immediately after hematopoietic stem-cell transplantation: a children's cancer group study. J Clin Oncol 18(24):4077–4085

Palaga T et al (2003) TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. J Immunol 171(6):3019–3024

Peguet-Navarro J et al (2003) Gangliosides from human melanoma tumors impair dendritic cell differentiation from monocytes and induce their apoptosis. J Immunol 170(7):3488–3494

Pinho S et al (2007) Biological significance of cancer-associated sialyl-Tn antigen: modulation of malignant phenotype in gastric carcinoma cells. Cancer Lett 249(2):157–170

Reis CA et al (2010) Alterations in glycosylation as biomarkers for cancer detection. J Clin Pathol 63(4):322–329

Retter MW et al (2005) Characterization of a proapoptotic antiganglioside GM2 monoclonal antibody and evaluation of its therapeutic effect on melanoma and small cell lung carcinoma xenografts. Cancer Res 65(14):6425–6434

Robert C et al (2015) Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med 372(4):320–330

Robert C et al (2014) Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. Lancet 384(9948):1109–1117

Rosen SD, Bertozzi CR (1994) The selectins and their ligands. Curr Opin Cell Biol 6(5):663–673

Roth J et al (1988a) Presence of the long chain form of polysialic acid of the neural cell adhesion molecule in Wilms' tumor. Identification of a cell adhesion molecule as an oncodevelopmental antigen and implications for tumor histogenesis. Am J Pathol 133(2):227–240

Roth J et al (1988b) Reexpression of poly(sialic acid) units of the neural cell adhesion molecule in Wilms tumor. Proc Natl Acad Sci USA 85(9):2999–3003

Sabbatini PJ et al (2000) Immunization of ovarian cancer patients with a synthetic Lewis(y)-protein conjugate vaccine: a phase 1 trial. Int J Cancer 87(1):79–85

Sabbatini PJ et al (2007) Pilot study of a heptavalent vaccine-keyhole limpet hemocyanin conjugate plus QS21 in patients with epithelial ovarian, fallopian tube, or peritoneal cancer. Clin Cancer Res 13(14):4170–4177

Saleh MN et al (1992a) Phase I trial of the murine monoclonal anti-GD2 antibody 14G2a in metastatic melanoma. Cancer Res 52(16):4342–4347

Saleh MN et al (1992b) Phase I trial of the chimeric anti-GD2 monoclonal antibody ch14.18 in patients with malignant melanoma. Hum Antibodies Hybridomas 3(1):19–24

Saleh MN et al (1993) Generation of a human anti-idiotypic antibody that mimics the GD2 antigen. J Immunol 151(6):3390–3398
Savoldo B, Dotti G (2013) Chimeric antigen receptors (CARs) from bench-to-bedside. Immunol Lett 155(1–2):40–42
Schulz G et al (1984) Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. Cancer Res 44(12 Pt 1):5914–5920
Seelentag WK et al (1998) Prognostic value of beta1,6-branched oligosaccharides in human colorectal carcinoma. Cancer Res 58(23):5559–5564
Sen G et al (1998) Induction of IgG antibodies by an anti-idiotype antibody mimicking disialoganglioside GD2. J Immunother 21(1):75–83
Shibuya H et al (2012) Enhancement of malignant properties of human osteosarcoma cells with disialyl gangliosides GD2/GD3. Cancer Sci 103(9):1656–1664
Shusterman S et al (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 (33):4969–4975
Slovin SF et al (1999) Carbohydrate vaccines in cancer: immunogenicity of a fully synthetic globo H hexasaccharide conjugate in man. Proc Natl Acad Sci USA 96(10):5710–5715
Slovin SF et al (2005) A bivalent conjugate vaccine in the treatment of biochemically relapsed prostate cancer: a study of glycosylated MUC-2-KLH and Globo H-KLH conjugate vaccines given with the new semi-synthetic saponin immunological adjuvant GPI-0100 OR QS-21. Vaccine 23(24):3114–3122
Slovin SF et al (2007) A polyvalent vaccine for high-risk prostate patients: "are more antigens better?". Cancer Immunol Immunother 56(12):1921–1930
Sorkin LS et al (2010) Anti-GD(2) with an FC point mutation reduces complement fixation and decreases antibody-induced allodynia. Pain 149(1):135–142
Springer GF (1984) T and Tn, general carcinoma autoantigens. Science 224(4654):1198–1206
Suzuki Y et al (1987) Aberrant expression of ganglioside and asialoglycosphingolipid antigens in adult T-cell leukemia cells. Jpn J Cancer Res 78(10):1112–1120
Svennerholm L et al (1994) Gangliosides and allied glycosphingolipids in human peripheral nerve and spinal cord. Biochim Biophys Acta 1214(2):115–123
Takamiya R et al (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(2):178–187
Tanaka F et al (2001) Prognostic significance of polysialic acid expression in resected non-small cell lung cancer. Cancer Res 61(4):1666–1670
Terme M et al (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(2):e87210
Thornton MV et al (2004) Degradation of NF-kappa B in T cells by gangliosides expressed on renal cell carcinomas. J Immunol 172(6):3480–3490
Todeschini AR et al (2008) Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway. Proc Natl Acad Sci USA 105(6):1925–1930
Tozawa K et al (2005) Positive correlation between sialyl Lewis X expression and pathologic findings in renal cell carcinoma. Kidney Int 67(4):1391–1396
Tsai YC et al (2013) A prevalent cancer associated glycan, globo H ceramide, induces immunosuppression by reducing Notch1 signaling. J Cancer Sci Ther 5(7):264–270
Tsuchida T et al (1987) Gangliosides of human melanoma. J Natl Cancer Inst 78(1):45–54
Uttenreuther-Fischer MM et al (1995a) Pharmacokinetics of anti-ganglioside GD2 mAb 14G2a in a phase I trial in pediatric cancer patients. Cancer Immunol Immunother 41(1):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(6):331–338

Uzzo RG et al (1999) Renal cell carcinoma-derived gangliosides suppress nuclear factor-kappaB activation in T cells. J Clin Invest 104(6):769–776
van de Kar NC et al (1992) Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: implications for the pathogenesis of the hemolytic uremic syndrome. Blood 80(11):2755–2764
van Setten PA et al (1996) Effects of verocytotoxin-1 on nonadherent human monocytes: binding characteristics, protein synthesis, and induction of cytokine release. Blood 88(1):174–183
Wiels J, Fellous M, Tursz T (1981) Monoclonal antibody against a lymphoma-associated antigen. Proc Natl Acad Sci USA 78(10):6485–6488
Wolfl M et al (2002) Gangliosides inhibit the development from monocytes to dendritic cells. Clin Exp Immunol 130(3):441–448
Xu J et al (2013) Neural ganglioside GD2(+) cells define a subpopulation of mesenchymal stem cells in adult murine bone marrow. Cell Physiol Biochem 32(4):889–898
Yanagisawa M, Yoshimura S, Yu RK (2011) Expression of GD2 and GD3 gangliosides in human embryonic neural stem cells. ASN Neuro 3(2):e00054
Yankelevich M et al (2012) Anti-CD3 x anti-GD2 bispecific antibody redirects T-cell cytolytic activity to neuroblastoma targets. Pediatr Blood Cancer 59(7):1198–1205
Yoshida S et al (2001) Ganglioside G(D2) in small cell lung cancer cell lines: enhancement of cell proliferation and mediation of apoptosis. Cancer Res 61(10):4244–4252
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 et al (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 et al (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 (6):2169–2180
Yu AL, Eskenazi A, Strother D (2001) A pilot study of anti-idiotype monoclonal antibody as tumor vaccine in patients with high risk neuroblastoma. Proc Am Soc Clin Oncol 20:1470
Yu AL et al (2010) Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med 363(14):1324–1334
Yuan M et al (1986) Comparison of T-antigen expression in normal, premalignant, and malignant human colonic tissue using lectin and antibody immunohistochemistry. Cancer Res 46 (9):4841–4847
Zeng Y et al (2005) Anti-neuroblastoma effect of ch14.18 antibody produced in CHO cells is mediated by NK-cells in mice. Mol Immunol 42(11):1311–1319
Zhang S et al (1997) Selection of tumor antigens as targets for immune attack using immunohistochemistry: II. Blood group-related antigens. Int J Cancer 73(1):50–56
Zhang S et al (1998) Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. Clin Cancer Res 4(2):295–302
Zhuang H et al (2013) Co-expression of Lewis y antigen with human epididymis protein 4 in ovarian epithelial carcinoma. PLoS ONE 8(7), e68994
Zhuang H et al (2014) Overexpression of Lewis y antigen promotes human epididymis protein 4-mediated invasion and metastasis of ovarian cancer cells. Biochimie 105:91–98