Glycoconjugate Journal 20, 189–198, 2004

Sialidase and malignancy: A minireview

Taeko Miyagi, Tadashi Wada, Kazunori Yamaguchi and Keiko Hata

Division of Biochemistry, Research Institute, Miyagi Prefectural Cancer Center, Natori, Miyagi 981-1293, Japan

Aberrant sialylation in cancer cells is thought to be a characteristic feature associated with malignant properties including invasiveness and metastatic potential. Sialidase which catalyzes the removal of sialic acid residues from glycoproteins and glycolipids, has been suggested to play important roles in many biological processes through regulation of cellular sialic acid contents. The altered expression of sialidase observed in cancer would, therefore, suggest its involvement in the malignant process. In mammalian cells, three types of sialidase cloned and characterized to date were found to behave in different manners during carcinogenesis. Recent progress in molecular cloning of these sialidases has facilitated elucidation of the molecular mechanisms and significance of these alterations. Herein we briefly describe our own studies on sialidase changes associated with malignant transformation and summarize the topic from both a retrospective and a prospective viewpoint. Sialidases are indeed closely related to malignancy and are thus potential targets for cancer diagnosis and therapy.
Published in 2004.

Keywords: **sialidase, sialic acid, cancer, metastasis, glycosylation, sialylation, glycoprotein, ganglioside**

Introduction

Sialic acids are generally found in the non-reducing terminus of most glycoproteins and glycolipids. The subject of cell surface sialic acids in malignant cells received attention in the 1960's and early 1970's [1,2]. A number of studies suggested the increase in negative surface charge determined by electrophoretic mobility to be correlated with reduced adhesiveness of tumor cells; and incubation with bacterial sialidase resulted in decreased surface charge followed by suppression of malignancy, probably due to the increased immunogenicity of the cells. However, no definite conclusions could be drawn because of some controversial experimental results. Investigations into biochemical properties of the cell surface were then pursued extensively, and characteristic features of the changes in cancer cells were identified [3–8]. Carbohydrate portions of glycoproteins and glycolipids undergo neoplastic alterations, and the changes in glycoprotein carbohydrates include an increase in branched asparagine-linked and polylactosamine sugar chains as well as in sialylation. In particular, alteration of sialic acids is associated with cancer cell behavior, such as invasiveness and metastasis [9–13] . Altered glycosylation of functionally important membrane glycoproteins may affect tumor cell adhesion or motility, resulting in invasion and metastasis. A general increase in sialylation is often found in cell surface glycoproteins of malignant cells, and altered sialylation of glycolipids [14,15] is also observed as a ubiquitous phenotype, leading to the appearance of tumor-associated antigens, aberrant adhesion, and blocking of transmembrane signaling [6,7]. Despite the number of reports describing involvement of sialic acids in cancer, it is still uncertain what the causes of such aberrant sialylation are and what the consequences of these changes are. Cellular sialic acid contents are mainly controlled metabolically by sialyltransferase and sialidase. In fact, sialidase activity levels consistently fluctuate with cell differentiation, cell growth, and malignant transformation, but little is known about the mechanisms and significance of such sialidase alterations. Herein, we focus on endogenous sialidases in mammalian cells in connection with malignancy.

To whom correspondence should be addressed: Taeko Miyagi, Division of Biochemistry, Research Institute, Miyagi Prefectural Cancer Center, Natori, Miyagi 981-1293, Japan. Tel: +81-22-384-3151; Fax: +81-22-381-1195; E-mail: tmiyagi@mcc.pref.miyagi.jp

Multiple forms of mammalian sialidase

The sialidase reaction is an initial step in the degradation of glycoproteins and gangliosides. Sialidases of microorganism origin have been suggested to possibly play roles in nutrition and pathogenesis [16]. Sialidases of mammalian origin, on the other hand, have been implicated not only in lysosomal catabolism but also in modulation of functional molecules involved in many biological processes [17–19] although their functional aspects are not fully understood, probably due to their instability and low activity. Unlike microbial sialidasaes, biochemical characterization of mammalian sialidase has thus far demonstrated the

Table 1. Comparison of three types of mammalian sialidase

Abbreviation	*Major subcellular location*	*Catalytic properties*		*Total amino acids (human)*	*Chromosome location (human)*	*References*
		Good substrate	*Optimal pH*			
Neu1	Lysosomal sialidase	Sialyllactose 4MU-Neu5Ac	4.4–4.6	415	6p 21.3	[42–47]
Neu2	Cytosolic sialidase	Sialyllactose 4MU-Neu5Ac Gangliosides Glycoprotein	6.0–6.5	380	2q 37	[34–39]
Neu3	Plasma-membrane sialidase	Gangliosides	4.6–4.8	428	11q 13.5	[86,50,52–54]

existence of multiple forms. Sialidase activity in higher organisms was described for the first time in 1960 by Warren and Spearing [20]. The enzyme activity was found in commercial preparations of bovine and human glycoproteins in Cohn Fraction VI. In the following years numerous papers demonstrated the presence of sialidase in mammalian tissues. Carubelli *et al.* [21] detected the activity in the soluble fractions isolated from different tissues of rats, and then several reports described its lysosomal occurrence [22–24]. Subsequently, it was detected in plasma membranes [25–28], Golgi fractions [29], and recently in nuclear membranes [30]. However, it remained uncertain whether the activities originated from the same or different types of sialidase.

Our previous reports on biochemical isolation and characterization of murine sialidase presented evidence of four types of sialidase differing in subcellular localization and in enzymatic properties. They are classified according to their major intracellular location as intralysosomal [31], cytosolic [32], lysosomal membrane and plasma membrane-associated sialidases [33]. Several rat tissues including liver and brain and even isolated hepatocytes were found to contain the four forms of sialidase. Intralysosomal sialidase possesses a narrow substrate specificity such that only oligosaccharides, glycopeptides, and a synthetic substrate, 4-methylumbelliferyl-*N*-acetylneuraminic acid (4MU-Neu5Ac) are good substrates. Sialidase found in the cytosol, in contrast, can also hydrolyze glycoproteins and gangliosides at near neutral pH. These two sialidases are distinct from membrane-associated sialidases in that the latter required detergents for solubilization and hydrolyzed gangliosides preferentially. Plasma membrane-associated sialidase barely hydrolyzes other substrates including oligosaccharides and glycoproteins, while lysosomal membrane sialidase also acts on oligosaccharides, glycoproteins and even on gangliosides GM1 and GM2. Our findings that these sialidases are distinct proteins support our hypothesis that each one has a unique role depending on its subcellular location and catalytic properties. Intralysosomal sialidase, for example, may participate mainly in glycoprotein catabolism by collaborating with lysosomal proteases or endoglycosidase since fragmentation into glycopeptides or oligosaccharide chains is required prior to cleavage by this sialidase. In contrast to lysosomal sialidase, cytosolic sialidase may participate in regulatory desialylation because of its ability to act on native glycoproteins at neutral pH. Plasma membrane-associated sialidase may be involved in the regulation of cell growth and cell differentiation via modulation of cell surface gangliosides, since gangliosides are known to function as mediators in cell phenomena including signal transduction.

Three types of sialidases have been cloned and characterized to date, as shown in Table 1, and they are now abbreviated to Neu1 (Lysosome), Neu2 (cytosol), and Neu3 (plasma membrane). The molecular cloning results confirmed our aforementioned hypothesis that sialidases are products of distinct genes with different localization signals although, in humans, two types of lysosomal sialidase located in the matrix and membranes seem to be encoded by a single gene. Neu2 was the first example of cDNA for mammalian sialidase [34], and the primary structures of Neu1 and then Neu3 were successively reported. The *Neu2* gene encodes an open reading frame of 379 amino acids with a molecular mass of 42,381 Da. Although the primary sequence is not particularly similar to those of bacterial and parasite sialidases, it contains two Asp boxes (-Ser-X-Asp-X-Gly-X-Thr-Trp-), the conserved sequences being found in the sialidases from these microorganisms. The amino acid residue distances between the two Asp boxes are similar to the distance between Asp box II and III, which is highly conserved in bacterial sialidases. Other residues similar to the sequences often found in bacterial sialidases, Arg-Ileu-Pro and (Val)-Gly-Pro-Gly, are also present in the N-terminal region of the sialidase protein and in the C-terminal side of the first Asp box, respectively. In addition, their distances from the first Asp box were close to those from block III in bacteria. The fact that a long stretch of hydrophobic amino acid residues representing the transmembrane domain or targeting signal to some membranes was not found in the sequence is compatible with recovery mainly from the tissue cytosol. When the cDNA was transfected into COS cells, sialidase activity appeared mainly in the supernatant of the cell. In addition to location in cytosol, the sialidase was also found in nucleoplasma of muscle fibers by immuno-histochemical analysis on electron microscopy, probably due to the presence of a nuclear localization signal near the

N-terminus. The sialidase could be completely immunoprecipitated by antiserum against the cytosolic sialidase of rat skeletal muscle. Like the skeletal muscle sialidase, the expressed sialidase showed an optimum pH of about 6.5 and could desialylate fetuin and gangliosides as well as 4MU-Neu5Ac. The homologues were cloned from libraries of CHO [35], human skeletal muscle [36], and mouse brain [37,38] and thymus [39], respectively, showing high amino acid identity (98–70%) to the rat gene.

Lysosomal sialidase was investigated extensively as a target gene for sialidosis, and was found to be associated with a protective protein (carboxypeptidase A) and β-galactosidase as a complex in lysosomes [40], and dissosiation of the complex led to sialidase inactivation [41]. In 1996–1998, the *Neu1* gene was identified by three groups in humans [42–44] and mice [45–47] as major histocompatibility complex (MHC)–related sialidase genes, by analyzing of MHC class III region or searching the expressed sequence tags database based on the conserved sequence. The cDNA encodes a protein of 415 amino acids with an Arg-Ileu-Pro sequence, five Asp boxes and the potential N-linked glycosylation sites. The human sialidase was suggested to be located in lysosomes because of the presence of the lysosomal C-terminal targeting motif. Evidence of a protective protein transporting it to lysosomes has been provided [48]. However, recent observations revealed that the intracellular distribution of sialidase encoded by the *Neu1* gene is regulated by the signal sequence at the cytoplasmic tail, and that the sialidase can be detected within the lysosome matrix as well as in the plasma membrane under conditions of cell stimulation [49]. Examination of sialidosis patients, who have a sialidase deficiency, revealed mutations in genomic DNA including frameshift insertion and missense mutations. Transfection with the cDNA restores sialidase activity toward 4MU-Neu5Ac in human sialidosis fibroblasts to normal levels, indicating that it is a target gene for sialidosis. Cotransfection of the protective protein cDNA with the sialidase cDNA further increases sialidase activity. In SM/J mice which have been characterized by their altered sialylation of several lysosomal glycoproteins, a point mutation of sialidase encoded by the *Neu1* locus in the *S* region of histocompatibility-2-complex was found to be responsible for the sialidase deficiency.

A plasma membrane-associated sialidase was then cloned from a bovine brain library [50], based on the peptide sequence information from the purified enzyme protein [51]. This cDNA encodes a 428 amino acid protein containing a putative transmembrane domain and the three Asp-boxes, sharing 19–38% sequence identity with other sialidases. In COS-7 cells transiently expressing the sialidase, the hydrolysis was essentially specific to gangliosides other than GM1 and GM2, in the presence of Triton X-100, as observed in the purified enzyme from bovine brain. The major subcellular localization of the expressed sialidase was assessed to be plasma membrane by Percoll density gradient centrifugation of cell homogenates and by immunofluorescence staining of transfected COS-7 cells. Analysis of the membrane topology by protease protection assay suggested that this sialidase has a type I membrane orientation with its amino-terminus facing the extracytoplasmic side and lacking a signal sequence. Northern blot analysis showed a 7.4 kb transcript for the bovine sialidase, and the same size transcript was observed in human tissues using the bovine cDNA probe. The primary sequences covering the entire coding region of the corresponding human [52] and mouse [38] genes displayed an 83% and 79% overall identity with the bovine gene, respectively. The identical human gene was also cloned afterward based on information about the expressed sequence tags database [53] and the rat homologue from the rat brain cDNA library [54]. The highest homology (38%) was found with *Neu2*, and 24%, 21%, and 19% with human MHC-related *Clostridium perfringens* [55] and *Salmonella typhimurium* [56] sialidases, respectively. Despite the low identity in primary structure with bacterial sialidases, this enzyme likewise had a high content of cysteine residues (21 cysteins) and β-sheet structures. Alignment of this enzyme with *S. typhimurium* sialidase, whose three-dimensional structure has been determinined by X-ray crystallography [57], revealed a strikingly similar spatial arrangement of the catalytic amino acid residues: 8 of the 13 active site residues are conserved in the bovine and human sialidases. The three active site residues forming the hydrophobic pocket are identical to the corresponding residues in the rat cytosolic sialidase, and all the four residues differ from those of human MHC-sialidase probably reflecting differences in substrate specificity. Site-directed mutagenesis of these amino-acid residues, in fact, resulted in alterations of substrate specificity [58]. Taken together, our observations indicate that these mammmalian sialidases share certain characteristic features including Arg-Ileu-Pro and (Val)-Gly-Pro-Gly sequences and Asp boxes, and they seem to have a three-dimentional structure similar to that of bacterial and viral sialidases.

Possible functions of mammalian sialidase

Although the physiological functions of mammalian sialidases are not fully understood at present, cDNAs have facilitated the disclosure of some of their functions. Using the cytosolic sialidase gene, we investigated its expression during skeletal muscle cell differentiation. The rat cytosolic sialidase gene was found to be highly enriched in skeletal muscle and to contain two E-box pairs, known to be consensus binding sites for muscle-specific transcription factors, in the 5′-flanking enhancer/promotor region of the gene. This region exhibits transcriptional activity in rat L6 myogenic cells, and the activity is increased during myotube formation. During L6 myoblast differentiation induced by serum depletion, cytosolic sialidase showed increased activity and mRNA level [59]. Sialidase activity was essentially lacking in untreated myoblasts but appears concomitantly with myotube formation after induction of differentiation. The mRNA was detectable after 3 days when the mRNA of myogenin, a member of the MyoD family, reached a maximum level.

Myotube formation could, in fact, be blocked by addition of the antisense oligonucleotide [60]. These altered expressions of cytosolic sialidase indicate that the enzyme plays a critical role in muscle cell differentiation.

With regard to lysosomal sialidase, it is generally accepted that the major role of lysosomal sialidase is in glycoconjugate catabolism as described above. In addition to this important function, sialidase has been suggested to be involved in cellular signaling during immune responses. Neu1 sialidase showed increased activity during mitogen-activation of T lymphocytes [61], and its participation in the regulation of allogenic Ia and the production of cytokine IL4 by activated T cells was observed [62,63]. It was also suggested that T cell sialidase was required to convert vitamin D3 binding protein to macrophage activating factor in combination with β-galactosidase [64], which did not occur in T cells from SM/J mice [65].

Ganglioside sialidases have also been suggested to play important roles in various cellular functions. Although sufficient information as to what types of ganglioside sialidase were involved was not available, activity levels consistently fluctuate with cell differentiation and cell growth. The observations of Usuki *et al.* [66] showed that inhibition of ganglioside sialidase by 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (NeuAc2en) leads to growth retardation in cultured human fibroblasts, suggesting the participation of ganglioside sialidase in cell growth regulation. Kopitz *et al.* reported that NeuAc2en abolishes increases in differentiation markers in human neuroblastoma cells [67,68] and that a cell surface sialidase may take part in growth inhibition and neural differentiation of the cells by providing the reaction product GM1 as a ligand for galectin 1 [69] without affecting cell apoptosis [70]. After success in cloning, further studies on the molecular basis were performed to elucidate these cell phenomena. Membrane-bound Neu3 sialidase was proved to indeed participate in neurite formation of mice [38] and human neuroblastoma cells [71], and in regulation and regeneration in rat hippocampal neurons [72]. From another aspect, a possible involvement of human NEU3 in signal transduction was recently described. The sialidase was proved to be located in a raft of neuroblastoma cells [73] and in caveolae of Hela cells, closely associated with caveolin-1 [74]. In addition, ganglioside depletion via the introduction of NEU3 resulted in activation of integrin-linked kinase/Akt with inhibition of caspase-9 [75] in a human keratinocyte-derived SCC12 cell line. These data suggest that Neu3 sialidase indeed plays critical roles in signal transduction by modulating gangliosides intracellularly.

Alterations of sialidase expression and their significance in cancer

As mentioned earlier, it was observed in the 1960s that an electrophoretic mobility shift of the cancer cells, following treatment with bacterial sialidases, was accompanied by reduced malignancy. Microbial sialidases were also used to investigate the functional actions of sialic acids on gangliosides and glycoproteins. However, to obtain a better understanding of the physiological and pathological significance of these sialic acid changes, it is necessary to pay attention to the endogenous sialidases, responsible for sialic acid hydrolysis inside of cells. In this context, we have focused on endogenous sialidases in cancer, and discussed the significance of their altered expressions.

In the period when observations on cancer cells by bacterial sialidase treatment began, several reports on alteration of endogenous sialidase activity in cancer were published suggesting that sialidase might be related to tumorigenic transformation and tumor invasiveness. For example, Schengrund *et al.* described sialidase activity toward gangliosides as being increased in BHK-transformed cells [76], and Bosmann *et al.* observed increased sialidase activity in human cancer tissues with fetuin as a substrate [77]. Loss of cell density-dependent suppression of a membrane-bound sialidase activity for gangliosides was observed in 3T3-transformed cells [78]. In the human promyelocytic leukemia cell line HL-60, stimulation of sialidase activity toward 4MU-Neu5Ac occurred during cell differentiation into granulocytes by retinoic acid or DMSO [79]. After these pioneering studies, our attempts at isolation and characterization of mammalian sialidases provided some evidence for the presence of multiple forms of sialidase. Based on sialidase multiplicity, using a differential assay procedure for each form of sialidase, we observed that intra-lysosomal and membrane-bound sialidase activities were elevated, but cytosolic sialidase activity was reduced in rat hepatomas as compared with normal liver [80,81]. In mouse epidermal JB6 cells exposed to TPA and in the anchorage-independent transformants, we also found lysosomal sialidase activity to be decreased while plasma membrane-associated sialidase activity was increased as compared with that in untreated JB6 cells [82]. The activity decrease in lysosomal sialidase also occurred in rat 3Y1 fibroblasts after *src*-transformation [83]. Martinez-Zorzano *et al.* recently described that sialidase activity toward 4MU-Neu5Ac was increased in human colon cancer compared to normal mucosa [84]. However, the molecular mechanisms underlying these observations remained to be elucidated.

Increased sialylation has been proposed to be intimately related not only with tumorigenicity but also with invasiveness and metastatic ability. When the levels of sialidase activity were assayed in transformed rat 3Y1 cells, a lysosomal-type sialidase was found to be inversely correlated with the metastatic potential of the cells [83]. As compared with control 3Y1 cells, *src*-transformed cells exhibited decreased lysosomal-type sialidase activity, and v-*fos* transfer to these cells induced an even more severe decrease in the sialidase activity with acquisition of high lung metastatic ability (Figure 1, left). Various lysosomal enzymes other than sialidase were barely affected by the transformation, suggesting that the alterations occur specifically in sialidase. Since their metastatic potential did not parallel the sialic acid levels or the levels of various sialyltransferases

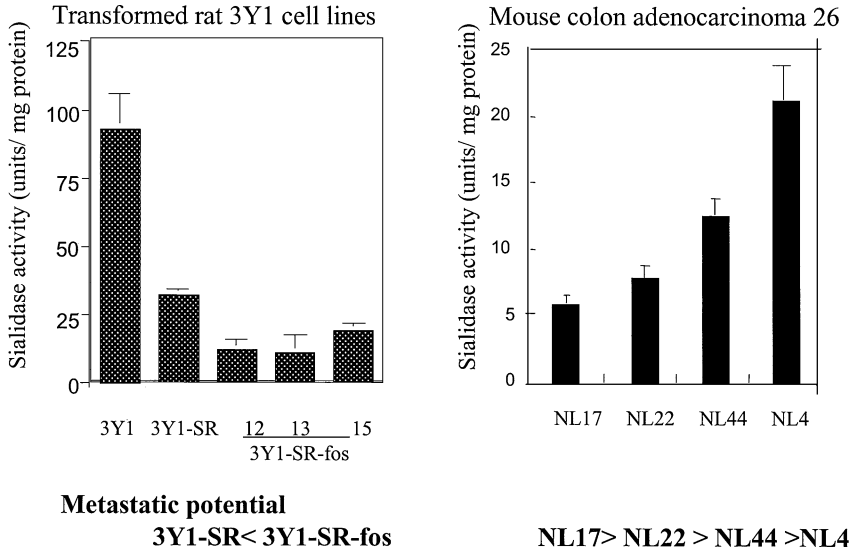

Figure 1. Inverse correlation of lysosomal sialidase activity with metastatic potential.

responsible for glycoprotein and ganglioside formation, it is likely that altered sialidase expression is more important for metastasis in transformed cells. The relationships of the sialidase activity and the mRNA level with metastatic potential were confirmed in mouse adenocarcinoma colon 26 cells of different metastatic potential [85] (Figure 1, right).

To investigate how sialidase expression influences metastasis, we first introduced a cytosolic sialidase cDNA, which was found to have broad substrate specificity, acting on both glycoproteins and gangliosides, into a B16-BL6 mouse melanoma cell line known to be highly invasive and metastatic [86]. Intravenous injection of these stable transfectants into syngeneic mice resulted in a marked decrease in experimental pulmonary metastasis (Figure 2a), in invasiveness in collagen gels and in cell motility on colloidal gold-coated glass plates (Figure 2b), but no change in cell growth or cell attachment to fibronectin, collagen type VI or laminin. Analysis of the mechanisms revealed that sialidase overexpression did not lead to any significant change in cell surface carbohydrates or the intracellular glycoproteins by lectin flow cytometry and lectin blotting, respectively, while there was a decrease in the ganglioside GM3 and an increase in lactosylceramide based on thin layer chromatography results. Since there is evidence for glycosphingolipids being localized not only in the outer leaflet of the plasma membrane but also in intracellular organelles [87], and gangliosides have been demonstrated to be associated with cytoskeletal components such as microtubules and intermediate filaments [88,89], it is interesting to note that the cytosolic sialidase was demonstrated to be involved in the differentiation of rat skeletal muscle cells in our previous studies. These findings suggest that cellular events in the positive transfectants are the following: intracellular GM3 associated with the cytoskeleton may be desialylated by the expressed sialidase resulting in alteration of cytoskeletal functions and subsequently cell motility, an important factor for invasiveness. Although a direct relationship between cytosolic sialidase and the cytoskeleton remains to be proved, the results provide an indication that desialylation of gangliosides by this type of sialidase can regulate cytoskeleton-related functions including metastatic potential. When the sialidase gene was transfected into a highly metastatic cell line of mouse colon 26 adenocarcinoma cells, changes in the sialyl Le^x level were observed in addition to marked suppression of metastasis and ganglioside alterations similar to those in BL6 cells [85]. Compared to low metastatic NL4 and NL44 cell

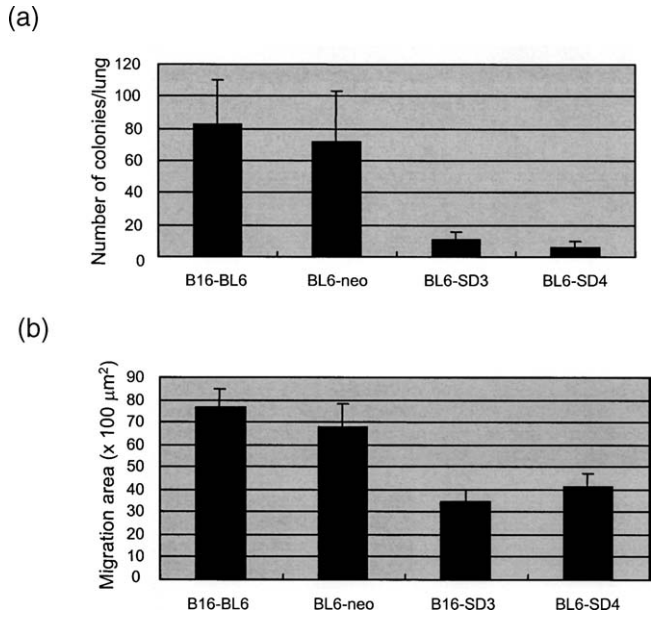

Figure 2. Suppression of metastasis and cell motility by transfection of sialidase gene. The numbers of lung metastatic nodules and phagokinetic tracks on the gold coverslips were markedly decreased with the stable transfectants (BL6-SD3,-SD4) compared with the control cells (BL6, Bl6-neo).

lines, the highly metastatic NL17 and NL 22 cells exhibit low expression of sialidases, accompanied by higher levels of sialyl Le^x and GM3. NL17 stable transfectants showed marked inhibition of lung metastasis, invasion and cell motility with a concomitant decrease in sialyl Le^x and GM3 levels, in line with the case of spontaneously low metastatic sublines having a relatively high level of endogenous sialidase. Treatment of the cells with antibodies against sialyl Le^x and GM3 affected cell adhesion and/or cell motility, providing evidence that desialylation of these molecules, as targets of sialidase, is involved in the suppression of metastasis. It should be noted that the highly metastatic cells exhibited rather lower sialic acid contents, both total and cell surface, as compared to the low metastatic cells, which was not consistent with the sialidase activity. The results together indicate that the sialidase level is a determining factor affecting metastatic ability, at least of murine origin, irrespective of sialic acid contents. Another line of the experiments with cytosolic sialidase reported by Meuillet *et al.* demonstrated that transfection of cytosolic sialidase gene into a human epidermoid carcinoma cell line (A431) reduced GM3 level and enhanced cell growth and tyrosine autophosphorylation of EGF receptor at low EGF concentration [90].

To investigate whether overexpression of lysosomal sialidase can reverse metastatic ability, we then introduced a rat lysosomal sialidase gene into Bl6 melanoma cells [91]. As expected, sialidase-overexpressing cells showed suppression of experimental pulmonary metastasis and tumor progression. In contrast to the cytosolic sialidase case, the transfectants exhibited diminished cell growth and anchorage-independent growth and increased sensitivity to apoptosis, induced by suspension culture or serum depletion *in vitro*, but no significant alterations in invasiveness, cell motility, or cell attachment to fibronectin, collagen IV, and laminin in this cell system. Although the target molecule for the lysosomal sialidase has not been specified, the results indicate that lysosomal sialidase affects malignant properties including the metastatic ability of cancer cells, in a manner different from that of cytosolic sialidase.

Next, we introduced a plasma membrane-associated sialidase into B16-Bl6 melanoma cells, and no significant changes in metastatic potential were observed before, versus after, transfection (Sawada *et al*. unpublished data). However, it has been suggested that gangliosides and sphingolipids modulate transmembrane signaling essential for tumor cell growth, invasion, and metastasis, and in fact, sialidase activity was found to be related to malignant transformation of murine cells in previous reports by Schengrund *et al.* [76] and by ours [80,82]. We, therefore, investigated the sialidase expression in matched tumor and adjacent non-tumorous mucosa from 50 colon cancer patients [92]. The mRNA levels were increased by 3- to 100-fold in colon cancer tissues compared to adjacent non-tumor mucosa associated with significant sialidase activity elevation in the tumors. *In situ* hybridization showed sialidase expression in epithelial elements of adenocarcinomas. To understand the significance of the increased expression, cultured human colon cancer cells were treated with sodium butyrate, and changes in expression during differentiation and apoptosis were observed. The sialidase level was down-regulated by the treatment, while lysosomal sialidase was up-regulated. Transfection of the ganglioside specific membrane sialidase gene into cancer cells inhibited apoptosis accompanied by increased Bcl-2 and decreased caspase expression. Colon cancer tissues exhibited a marked accumulation of lactosylceramide (Lac-cer), a possible NEU3 product, and addition of the glycolipid to the culture reduced apoptotic cells during sodium butyrate treatment (Figure 3). These results indicate that high expression of NEU3 in cancer cells leads to protection against programmed cell death, probably via modulation of gangliosides.

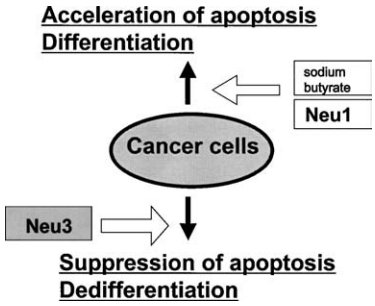

Figure 3. A possible role of increased Neu3 in human colon cancer.

Outlook

Recent progress in molecular cloning of mammalian sialidases has provided evidence of the multiple nature of sialidases encoded by different genes and has confirmed the enzymatic properties as well as intracellular localizations of these enzymes to be distinct from each other. Remodeling of sialic acid residues in glycoconjugates by introduction of these sialidase genes will provide important clues to controlling the degree of sialylation of functional molecules in cells. In fact, the use of these genes as tools has made it possible to elucidate some of their functions and the significance of their altered expressions in cancer, as listed in Table 2. In summary, the observations described herein indicated that the expression level of lysosomal sialidase (Neu1) may be a critical and defining factor in malignancy, and increased expression of plasma membrane-associated sialidase (Neu3) may be essential for the survival of various cancer cells. Whatever the mechanism and biological significance of altered expression, sialidase could be a useful target for cancer diagnosis and therapy. In particular, discovery of a specific inhibitor for Neu3 would throw light on the development in a cure for cancer. It is also of great importance to investigate the detailed mechanism of Neu1 involved in immune response which possibly leads to elucidation of autoimmune disease. Further investigation of mammalian sialidases would clarify the molecular basis of numbers of pathological phenomena as a result of aberrant sialylation.

Table 2. Cellular changes induced by transfection of mammalian sialidase

	Gene origin	*Transfected cells*	*Phenotype (possible target molecule)*	*References*
Neu1	Rat	B16-Bl6 cells	Metastasis↓ Anchorage-dependent growth↓ Apoptosis↑	[91]
Neu2	Rat	B16-Bl6 cells	(GM3)↓	[86]
		Colon adenocarcinoma	(GM3↓ , sialylLe^x ↓) Metastasis↓ Cell motility↓ Cell invasion↓	[85]
	Hamster	A431	Cell growth ↑ EGF-R phosphorylation↑	[90]
Neu3	Mouse	Neuro2a	Neurite growth ↑	[38]
		Hippocampal neuron	Neurite growth ↑ Neurite regeneration↑	[72]
	Human	NB-1	Neurite growth ↑	[71]
	Human	Colon cancer cells	Apoptosis↓	[92]
	Human	SSC	Apoptosis ↓	[75]

Arrowheads indicate direction of changes in sialidase expression.

References

1 Abercrombie M, Ambrose EJ, The surface properties of cancer cells, *Cancer Res* **22**, 525–48 (1962).
2 Weiss L, Neuraminidase, sialic acids, and cell interactions, *J Natl Cancer Inst* **50**, 3–19 (1973).
3 Warren L, Buck CA, Tuszynski GP, Glycopeptide changes and malignant transformation, a possible role for carbohydrate in malignant behaviour, *Biochim Biophys Acta* **516**, 97–127 (1978).
4 Dennis JW, Laferte S, Tumor cell surface carbohydrate and the metastatic phenotype, *Cancer Metastasis Rev* **5**, 185–204 (1987).
5 Kobata A, Altered glycosylation of surface glycoproteins in tumor cells and its clinical application, *Pigment Cell Res* **2**, 304–8 (1989).
6 Bhavanandan VP, Cancer-associated mucins and mucin-type glycoproteins, *Glycobiology* **1**, 493–503 (1991).
7 Hakomori S, Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism, *Cancer Res* **56**, 5309–18 (1996).
8 Hakomori S, Glycosylation defining cancer malignancy: New wine in an old bottle, *Proc Natl Acad Sci USA* **99**, 10231–3 (2002).
9 Yogeeswaran G, Salk PL, Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines, *Science* **212**, 1514–6 (1981).
10 Fogel M, Altevogt P, Schirrmacher V, Metastatic potential severely altered by changes in tumor cell adhesiveness and cell-surface sialylation, *J Exp Med* **157**, 371–6 (1983).
11 Collard JG, Schijven JF, Bikker A, La Riviere G, Bolscher JG, Roos E, Cell surface sialic acid and the invasive and metastatic potential of T-cell hybridomas, *Cancer Res* **46**, 3521–7 (1986).
12 Dennis JW, Lafarté S, Waghorne C, Breitman ML, Kerbel RS, β1-6 Branching of Asn-linked oligosaccharides is directly associated with metastasis, *Science* **236**, 582–5 (1987).
13 Passaniti A, Hart GW, Cell surface sialylation and tumor metastasis. Metastatic potential of B16 melanoma variants correlates with their relative numbers of specific penultimate oligosaccharide structures, *J Biol Chem* **263**, 7591–603 (1988).
14 Basu S, Basu M, Basu SS, Biological specificity of sialyltransferases. In *Biology of the sialic acids*, edited by Rosenberg A (Plenum Press, New York, 1995), pp. 69–94.
15 Basu S, Basu M, Dastgheib S, Hawes JW, Biosynthesis and regulation of glycoshingolipids. In *Comprehensive natural products chemistry*, edited by Barton D, Nakanishi K, Meth-Cohen O, vol. 3 (Pinto BM ed.). (Pergamon Press, New York, 1999), pp. 107–28.
16 Pilatte Y, Bignon J, Lambré CR, Bacterial sialidases-roles in pathogenicity and nutrition, *Glycobiology* **3**, 201–17 (1993).
17 Saito M, Yu RK, Biochemistry and function of sialidases. In *Biology of the sialic acids*, edited by Rosenberg A (Plenum Press, New York, 1995), pp. 261–313.
18 Miyagi T, Wada T, Yamaguchi K, Multiple forms of mammalian sialidase and their altered expression in physiological and pathological conditions. In *Sialobiology and other novel forms of glycosylation*, edited by Inoue Y, Lee YC, Troy II FA (Gakushin Publishing Co., Osaka, 1999), pp. 197–205.
19 Monti E, Preti A, Venerando B, Borsani G, Recent development in mammalian sialidase molecular biology, *Neurochem Res* **27**, 649–63 (2002).
20 Warren L, Spearing CW, Mammalian sialidase(neuraminidase), *Biochem Biophys Res Commun* **3**, 489–92 (1960).
21 Carubelli R, Trucco RE, Caputto R, Neuraminidase activity in mammalian organs, *Biochim Biophys Acta* **60**, 196–7 (1962).
22 Mahadevan S, Nduaguba JC, Tappel AL, Sialidase of rat liver and kidney, *J Biol Chem* **242**, 4409–13 (1967).
23 Taha BH, Carubelli R, Mammalian neuraminidase: Intracellular distribution and changes of enzyme activity during lactation, *Arch Biochem Biophys* **119**, 55–61 (1967).

24 Horvat A, Touster O, On the lysosomal occurance and the properties of the neuraminidase of rat liver and of Ehrlich ascites tumor cells, *J Biol Chem* **243**, 4380–90 (1968).

25 Schengrund C-L, Rosenberg A, Intracellular location and properties of bovine brain sialidase, *J Biol Chem* **245**, 6196–6200 (1970).

26 Schengrund C-L, Jensen DS, Rosenberg A, Localization of sialidase in the plasma membrane of rat liver cells, *J Biol Chem* **247**, 2742–6 (1972).

27 Tettamanti G, Preti A, Lombardo A, Bonali F, Zambotti V, Parallelism of subcellular location of major particulate neuraminidase and gangliosides in rabbit brain cortex, *Biochim Biophys Acta* **306**, 466–77 (1973).

28 Visser AEP, Studies on plasma membranes. XX sialidase in hepatic plasma membranes, *J Membrane Biol* **14**, 73–84 (1973).

29 Kishore GS, Tulsiani DRP, Bhavanandan VP, Carubelli R, Membrane-bound neuraminidases of rat liver. Neuraminidase activity in Golgi apparatus, *J Biol Chem* **250**, 2655–9 (1975).

30 Saito M, Fronda CL, Yu RK, Sialidase activity in nuclear membranes of rat brain, *J Neurochem* **66**, 2205–8 (1996).

31 Miyagi T, Tsuiki S, Rat-liver lysosomal sialidase. Solubilization, substrate specificity and comparison with the cytosolic sialidase, *Eur J Biochem* **141**, 75–81 (1984).

32 Miyagi T, Tsuiki S, Purification and characterization of cytosolic sialidase from rat liver, *J Biol Chem* **260**, 6710–16 (1985).

33 Miyagi T, Sagawa J, Konno K, Handa S, Tsuiki S, Biochemical and immunological studies on two distinct ganglioside-hydrolyzing sialidases from the particulate fraction of rat brain, *J Biochem (Tokyo)* **107**, 787–93 (1990).

34 Miyagi T, Konno K, Emori Y, Kawasaki H, Suzuki K, Yasui A, Tsuiki S, Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase, *J Biol Chem* **268**, 26435–40 (1993).

35 Ferrari J, Harris R, Warner TG, Cloning and expression of a soluble sialidase from Chinese hamster ovary cells: Sequence alignment similarities to bacterial sialidases, *Glycobiology* **4**, 367–73 (1993).

36 Monti E, Rossi PA, Ballabio E, Borsani A, Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases, *Genomics* **57**, 137–43 (1999).

37 Fronda CL, Zeng G, Gao L, Yu RK, Molecular cloning and expression of mouse brain sialidase, *Biochem Biophys Res Commun* **258**, 727–31 (1999).

38 Hasegawa T, Yamaguchi K, Wada T, Takeda A, Itoyama Y, Miyagi T, Molecular cloning of mouse ganglioside sialidase and its increased expression in Neuro2a cell differentiation, *J Biol Chem* **275**, 8007–15 (2000).

39 Kotani K, Kuroiwa A, Saito T, Matsuda Y, Koda T, K-Ochiai S, Cloning, chromosomal mapping, and characteristic 5′-UTR sequence of murine cytosolic sialidase, *Biochem Biophys Res Commun* **286**, 250–8 (2001).

40 d'Azzo A, Hoogeveen A, Reuser AJ, Robinson D, Galjaard H, Molecular defect in combined beta-galactosidase and neuraminidase deficiency in man, *Proc Natl Acad Sci USA* **79**, 4535–9 (1982).

41 van der Horst GT, Galjart NJ, d'Azzo A, Galjaard H, Verheijen FW, Identification and *in vitro* reconstitution of lysosomal neuraminidase from human placenta, *J Biol Chem* **264**, 1317–22 (1989).

42 Bonten E, van der Spoel A, Fornerod M, Grosveld G, d'Azzo A, Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis, *Genes Dev* **10**, 3156–69 (1996).

43 Milner CM, Smith SV, Carrillo MB, Taylor GL, Hollinshead M, Campbell RD, Identification of a sialidase encoded in the human major histocompatibility complex, *J Biol Chem* **272**, 4549–58 (1997).

44 Pshezhetsky AV, Richard C, Michaud L, Igdoura S, Wang S, Elsliger MA, Qu J, Leclerc D, Gravel R, Dallaire L, Potier M, Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis, *Nat Genet* **15**, 316–20 (1997).

45 Carrillo MB, Milner CM, Ball ST, Snoek M, Campbell RD, Cloning and characterization of a sialidase from the murine histocompatibility-2 complex: Low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele, *Glycobiology* **7**, 975–86 (1997).

46 Igdoura SA, Gafuik C, Mertineit C, Saberi F, Pshezhetsky AV, Potier M, Trasler JM, Gravel RA, Cloning of the cDNA and gene encoding mouse lysosomal sialidase and correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts, *Hum Mol Genet* **7**, 115–21 (1998).

47 Rottier RJ, Bonten E, d'Azzo A, Hum Mol Genet. A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse, **7**, 313–21 (1998).

48 van der Spoel A, Bonten E, d'Azoo A, Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A, *EMBO J* **17**, 1588–97 (1998).

49 Lukong KE, Seyrantepe V, Landry K, Trudel S, Ahmad A, Gahl WA, Lefrancois S, Morales CR, Pshezhetsky AV, Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail, *J Biol Chem* **276**, 46172–81 (2001).

50 Miyagi T, Wada T, Iwamatsu A, Hata K, Yoshikawa Y, Tokuyama S, Sawada M, Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides, *J Biol Chem* **274**, 5004–11 (1999).

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

52 Wada T, Yoshikawa Y, Tokuyama S, Kuwabara M, Akita H, Miyagi T, Cloning, expression, and chromosomal mapping of a human ganglioside sialidase, *Biochem Biophys Res Commun* **261**, 21–7 (1999).

53 Monti E, Bassi MT, Papini N, Riboni M, Manzoni M, Venerando B, Croci G, Preti A, Ballabio A, Tettamanti G, Borsani G, Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane, *Biochem J* **349**, 343–51 (2000).

54 Hasegawa T, Feijoo Carnero C, Wada T, Itoyama Y, Miyagi T, Differential expression of three sialidase genes in rat development, *Biochem Biophys Res Commun* **280**, 726–32 (2001).

55 Roggentin P, Rothe B, Lottspeich F, Schauer R, Cloning and sequencing of a Clostridium perfringens sialidase gene, *FEBS Lett* **238**, 31–4 (1988).

56 Hoyer LL, Hamilton AC, Steenbergen SM, Vimr ER, Cloning, sequencing and distribution of the Salmonella typhimurium LT2

sialidase gene, nanH, provides evidence for interspecies gene transfer, *Mol Microbiol* **6**, 873–84 (1992).
57 Crennell SJ, Garman EF, Laver WG, Vimir ER, Taylor GL, Crystal structure of a bacterial sialidase (from Salmonella typhimurium LT2) shows the same fold as an influenza virus neuraminidase, *Proc Natl Acad Sci USA* **90**, 9852–6 (1992).
58 Wang Y, Yamaguchi K, Shimada Y, Zhao X, Miyagi T, Site-directed mutagenesis of human membrane-associated ganglioside sialidase: Identification of amino-acid residues contributing to substrate specificity, *Eur J Biochem* **268**, 2201–8 (2001).
59 Sato K, Miyagi T, Genomic organization and the 5′-upstream sequence of the rat cytosolic sialidase gene, *Glycobiology* **5**, 511–6 (1995).
60 Sato K, Miyagi T, Involvement of an endogenous sialidase in skeletal muscle cell differentiation, *Biochem Biophys Res Commun* **221**, 826–30 (1996).
61 Landolfi NF, Leone J, Womack JE, Cook RG, Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase, *Immunogenetics* **22**, 159–67 (1985).
62 Chen XP, Enioutina EY, Daynes RA, The control of IL-4 gene expression in activated murine T lymphocytes: A novel role for neu-1 sialidase, *J Immunol* **158**, 3070–80 (1997).
63 Chen XP, Ding X, Daynes RA, Ganglioside control over IL-4 priming and cytokine production in activated T cells, *Cytokine* **12**, 972–85 (2000).
64 Yamamoto N, Kumashiro R, Conversion of vitamin D3 binding protein (group-specific component) to a macrophage activating factor by the stepwise action of beta-galactosidase of B cells and sialidase of T cells, *J Immunol* **151**, 2794–802 (1993).
65 Yamamoto N, Naraparaju VR, Role of vitamin D3-binding protein in activation of mouse macrophages, *J Immunol* **157**, 1744–9 (1996).
66 Usuki S, Hoops P, Sweeley CC, Growth control of human foreskin fibroblasts and inhibition of extracellular sialidase activity by 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, *J Biol Chem* **263**, 10595–9 (1988).
67 Kopitz J, von Reitzenstein C, Muhl C, Cantz M, Role of plasma membrane ganglioside sialidase of human neuroblastoma cells in growth control and differentiation, *Biochem Biophys Res Commun* **199**, 1188–93 (1994).
68 Kopitz J, Muhl C, Ehemann V, Lehmann C, Cantz M, Effects of cell surface ganglioside sialidase inhibition on growth control and differentiation of human neuroblastoma cells, *Eur J Cell Biol* **73**, 1–9 (1997).
69 Kopitz J, von Reitzenstein C, Burchert M, Cantz M, Gabius HJ, Galectin-1 is a major receptor for ganglioside GM1, a product of the growth-controlling activity of a cell surface ganglioside sialidase, on human neuroblastoma cells in culture, *J Biol Chem* **273**, 11205–11 (1998).
70 Kopitz J, von Reitzenstein C, Andre S, Kaltner H, Uhl J, Ehemann V, Cantz M, Gabius HJ, Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3, *J Biol Chem* **276**, 35917–23 (2001).
71 Proshin S, Yamaguchi K, Wada T, Miyagi T, Modulation of neuritogenesis by ganglioside-specific sialidase (Neu 3) in human neuroblastoma NB-1 cells, *Neurochem Res* **27**, 841–6 (2002).
72 Rodriguez JA, Piddini E, Hasegawa T, Miyagi T, Dotti CG, Plasma membrane ganglioside sialidase regulates axonal growth and regeneration in hippocampal neurons in culture, *J Neurosci* **21**, 8387–95 (2001).
73 Kalka D, von Reitzenstein C, Kopitz J, Cantz M, The plasma membrane ganglioside sialidase cofractionates with markers of lipid rafts, *Biochem Biophys Res Commun* **283**, 989–93 (2001).
74 Wang Y, Yamaguchi K, Wada T, Hata K, Zhao X, Fujimoto T, Miyagi T, A close association of the ganglioside-specific sialidase Neu3 with caveolin in membrane microdomains, *J Biol Chem* **277**, 26252–9 (2002).
75 Sun P, Wang XQ, Lopatka K, Bangash S, Paller AS, Ganglioside loss promotes survival primarily by activating integrin-linked kinase/Akt without phosphoinositide 3-OH kinase signaling, *J Invest Dermatol* **119**, 107–17 (2002).
76 Schengrund CL, Lausch RN, Rosenberg A, Sialidase activity in transformed cells, *J Biol Chem* **248**, 4424–8 (1973).
77 Bosmann HB, Hall TC, Enzyme activity in invasive tumors of human breast and colon, *Proc Natl Acad Sci USA* **71**, 1833–7 (1974).
78 Yogeeswaran G, Hakomori S, Cell contact-dependent ganglioside changes in mouse 3T3 fibroblasts and a suppressed sialidase activity on cell contact, *Biochemistry* **14**, 2151–6 (1975).
79 Nojiri N, Takaku F, Tetsuka T, Saito M, Stimulation of sialidase activity during cell differentiation of human promyelocytic leukemia cell line HL-60, *Biochem Biophys Res Commun* **104**, 1239–46 (1982).
80 Miyagi T, Goto T, Tsuiki S, Sialidase of rat hepatomas: Qualitative and quantitative comparison with rat liver sialidase, *Gann* **75**, 1076–82 (1984).
81 Miyagi T, Konno K, Sagawa J, Tsuiki S, Neoplastic alteration of a membrane-associated sialidase of rat liver, *Jpn J Cancer Res* **81**, 915–9 (1990).
82 Miyagi T, Sagawa J, Kuroki T, Matsuya Y, Tsuiki S, Tumor-promoting phorbol ester induces alterations of sialidase and sialyltransferase activities of JB6 cells, *Jpn J Cancer Res* **81**, 1286–92 (1990).
83 Miyagi T, Sato K, Hata K, Taniguchi S, Metastatic potential of transformed rat 3Y1 cell lines is inversely correlated with lysosomal-type sialidase activity, *FEBS Lett* **349**, 255–9 (1994).
84 Martinez-Zorzano VS, Feijoo C, Paez de la Cadena M, Butron M, Fernandez-Briera A, Rodriguez-Berrocal FJ, Human colon sialidase: Characterization and activity levels in normal mucosa and colonic adenocarcinoma, *Enzyme Protein* **48**, 282–90 (1994).
85 Sawada M, Moriya S, Saito S, Shineha R, Satomi S, Yamori T, Tsuruo T, Kannagi R, Miyagi T, 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**, 180–85 (2002).
86 Tokuyama S, Moriya S, Taniguchi S, Yasui A, Miyazaki J, Orikasa S, Miyagi T, Suppression of pulmonary metastasis in murine B16 melanoma cells by transfection of a sialidase cDNA, *Int J Cancer* **73**, 410–5 (1997).
87 Gillard BK, Thurmon LT, Marcus DM, Variable subcellular localization of glycosphingolipids, *Glycobiology* **3**, 57–67 (1993).

88 Sakakibara K, Momoi T, Uchida T, Nagai Y, Evidence for association of glycosphingolipid with a colchicine-sensitive microtubule-like cytoskeletal structure of cultured cells, *Nature* **293**, 76–8 (1981).
89 Gillard BK, Thurmon LT, Marcus DM, Association of glycosphingolipids with intermediate filaments of mesenchymal, epithelial, glial, and muscle cells, *Cell Motil Cytoskeleton* **21**, 255–71 (1992).
90 Meuillet EJ, Kroes R, Yamamoto H, Warner TG, Ferrari J, Mania-Farnell B, George D, Rebbaa A, Moskal JR, Bremer EG, Sialidase gene transfection enhances epidermal growth factor receptor activity in an epidermoid carcinoma cell line, A431, *Cancer Res* **59**, 234–40 (1999).
91 Kato T, Wang Y, Yamaguchi K, Milner CM, Shineha R, Satomi S, Miyagi T, 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 (2001).
92 Kakugawa Y, Wada T, Yamaguchi K, Yamanami H, Ouchi K, Sato I, Miyagi T, 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**, 10718–23 (2002).