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Sialidase significance for cancer progression

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Abstract Aberrant glycosylation is a characteristic feature of cancer cells. In particular, altered sialylation is closely associated with malignant properties, including invasiveness and metastatic potential. To elucidate the molecular mechanisms underlying the aberrancy, our studies have focused on mammalian sialidase, which catalyzes the removal of sialic acid residues from glycoproteins and glycolipids. The four types of mammalian sialidase identified to date show altered expression and behave in different manners during carcinogenesis. The present review briefly summarizes results on altered expression of sialidases and their possible roles in cancer progression. These enzymes are indeed factors defining cancer malignancy and thus potential targets for cancer diagnosis and therapy.

Keywords Sialidase · Sialic acid · Cancer · Invasion · Metastasis · Glycosylation · Sialylation · Glycoprotein · Ganglioside · Transmembrane signaling

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Abbreviations

DANA	2-deoxy-2,3dehydro-*N*-acetylneuraminic acid
4MU-NANA	4-methylumbelliferyl sialic acid
NeuAc	*N*-acetyl neuraminic acid
EGFR	Epidermal growth factor receptor
siRNA	Small interfering RNA
RT-PCR	Reverse transcription polymerase chain reaction

Altered sialylation in cancer progression

Sialic acids are generally found in the non-reducing termini of glycoproteins and glycolipids, primarily localized in outer membranes of cells. This outer location makes them readily accessible to other cells and external signaling molecules, and facilitates rapid responses to environmental change. The subject of cell surface sialic acids on cancer cells received much attention in the 1960's and early 1970's and a 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 resulted in decreased surface charge followed by suppression of malignancy. However, no definite conclusions could be drawn because of controversial experimental results regarding sialic acid contents. Investigations into biochemical properties of the cell surface were then pursued extensively, and characteristic features in cancer cells were identified [1, 2].

Carbohydrate portions of glycoproteins and glycolipids undergo neoplasia-associated alterations, including increase in branching of asparagine-linked glycans, increase in the number and/or length of polylactosaminnoglycan chains, and increase in sialylation [3]. In fact, acquision of an

invasive potential on expression of ras oncogene in NIH 3T3 fibroblasts is concomitant with such changes of cell surface N-linked oligosaccharides. Sialylation of N-glycans is required for maintenance of a transformed phenotype with oncogene expression. Early structural studies were further supported by observation of altered expression of glycosyltransferases involved in the formation of glycans, with increased sialic acid density and increased tri- or tetraantennary glycopeptides from surface glycoproteins after introduction of an N-ras proto-oncogene [4]. Altered sialylation of glycolipids is also observed as a ubiquitous phenotype, leading to the appearance of tumor-associated antigens, aberrant adhesion, and blocking of transmembrane signaling [5]. Similar to the case of N-glycans, ganglioside expression seems to affect and also to be affected by oncogenic phenotypes. For example, v-Jun induces transformed cell clones with reduced levels of GM3 and GM3 synthase in C3H 10T1/2 and DF1 cells, showing enhanced anchorage- independent growth and increased growth rates. Re-expression of GM3 synthase restored control cell growth characteristics, and reduced the ability of the cells to form colonies in agar [6]. Since cellular sialic acid contents are mainly controlled metabolically by sialyltransferase and sialidase, studies on these enzymes should facilitate understanding of the molecular mechanisms underlying altered sialylation in cancer. To understand causes and consequences of such aberrant sialylation, our studies have focused on mammalian sialidases, which regulate the cellular sialic acid contents and function of glycoconjugates by desialylation. Alteration of sialidase expression, which occurs in cancer, is closely related with the malignant phenotype.

Altered expression of sialidase and its significance for cancer progression

Sialidases (EC 3.2.1.18, also called neuraminidases) are glycosidases catalyzing the removal of α−glycosidically linked sialic acid residues from carbohydrate groups of glycoproteins and glycolipids, which is an initial step in the degradation of these glycoconjugates. Sialidase reactions exert a great influence on biological processes through changing the conformation of glycoproteins and creating or masking of binding sites on functional molecules. Sialidases exist in common in vertebrates and also in microorganisms including viruses, bacteria, fungi, mycoplasma, and protozoa, even in organisms mostly lacking sialic acids. Since sialidase activity in higher organisms was first detected in preparations of Cohn Fraction VI, numerous papers have demonstrated its presence in a wide variety of mammalian cells and tissues. Sialidases of mammalian origin have been found to differ from those of microbial origin in the overall primary sequences and enzymatic properties, although they contain Asp boxes (−Ser-X-Asp-X-Gly-X-Thr-Trp-) and the RIP (−Phe (Tyr)-Arg-Ileu-Pro-) sequence in the primary sequences, and possess the canonical six-blade β-propeller in the tertiary structure, conserved in viral and bacterial sialidases. Endogenous mammalian sialidases generally are unstable with low expression compared to microbial sialidases. They are involved in many biological processes as well as in lysosomal catabolism, whereas the same enzymes of microorganisms appear to play roles limited to nutrition and pathogenesis [7]. Four types of mammalian sialidase have been identified and characterized to date, designated as NEU1, NEU2, NEU3 and NEU4. Their overall properties and altered expression in cancer are summarized in Table 1. NEU1, NEU2 and NEU3 are now known to be localized mainly in the lysosomes, cytosol and plasma membranes, respectively, whereas NEU4 may be found in lysosomes, or in mitochondria and endoplastic reticulum. However, recent observations have revealed that the subcellular localization can vary with particular cell stimuli. More detailed information about the four sialidases is available in the recent literature [8, 9]. Focusing on sialidases in cancer, the four types appear to behave in different manners during carcinogenesis, but their alterations mostly influence or lead to a malignant phenotype including uncontrolled growth, invasion and metastasis.

Roles of sialidase NEU1 in cell signaling as a modulator of cell receptors as well as in lysosomal catabolism In 1996–1998, the genes encoding NEU1were identified in humans [10–12] and mice [13, 15] as major histocompatibility complex (MHC)– related sialidase genes on chromosome 6 and 17, respectively. NEU1 is associated with a protective protein/cathepsin A (PPCA) and a β-galactosidase as a complex in lysosomes, dissociation of the complex leading to sialidase inactivation [16, 17] . NEU1 has been extensively investigated as a target gene for neurodegenerative lysosomal storage disorders, sialidosis and galactosialidosis. The former features a sialidase deficiency caused by mutations in the genomic DNA including frameshift insertions and missense mutations, and the latter is defined as a combined deficiency of NEU1 and β−galactosidase due to the absence of a functional PPCA, leading to defective or deficient enzyme activity [18]. Human NEU1 is located in plasma membranes as well as within lysosomes under various cellular conditions [19–21], apparently connected to novel physiological functions. NEU1 possesses narrow substrate specificity, with oligosaccharides and glycopeptides serving as good substrates in *in vitro* sialidase activity assays [22], but recent observations have shown that the sialidase can react on glycoproteins at the cellular level by making complexes with other proteins on cell surfaces. NEU1 negatively regulates lysosomal exocytosis, a cellular process for recruitment of lysosomes to the plasma membrane. In the cells from NEU1 deficient mice, hypersialylated lysosomal-associated

Table 1 Properties of four mammalian sialidases and their neoplastic alterations

	NEU1	NEU2	NEU3	NEU4
Major subcellular localization	Lysosomes	Cytosol	Plasma membrane	Lysosomes[1)] Mitochondria[1)] and ER
Good substrates in activity assays	Oligosaccharides Glycopeptides	Oligosaccharides Glycoproteins Gangliosides	Gangliosides	Oligosaccharides Glycoproteins including mucins Gangliosides
Endogenous substrates identified in cellular level	LAMP-1 IR, IGF-1R PDGF-R Integrinβ4	Sialyl- Le^x GM3	GM3 GD3, GD1a	Sialyl- Le^x Sialyl- Le^a PolySia-NCAM GD1a
Proposed functions	Lysosomal catabolism Exocytosis Phagocytosis Immune function Elastogenesis Muscle differentiation	Myoblast differentiation Neural differentiation	Neuronal differentiation Apoptosis Adhesion	Neuronal differentiation Apoptosis Adhesion
Neoplastic alterations:				
Frequent changes in expression	tendency of decrease	tendency of decrease	increase	decrease (in colon cancer)
Apoptosis	increased sensitivity	increased sensitivity	suppression	acceleration
Motility and Invasion	suppression	suppression	acceleration	suppression
Metastasis (*in vivo*)	suppression	suppression	tendency of increase	tendency of decrease

[1] For the subcellular localization of human NEU4, two different descriptions have been reported on the basis of gene transfection studies

membrane protein (LAMP)-1 accumulates at the cell surfaces, concomitantly with enhanced secretion of lysosomal proteases and glycosidases. The increase in hypersialylated LAMP-1 is due to abnormal processing of its sialic acid residues and decreased turnover rate in the absence of NEU1, indicating LAMP-1 to be a natural substrate of the enzyme [23]. A direct involvement of LAMP-1 in the exocytosis is evidenced by silencing of LAMP-1 in the cells leading to reduced exocytosis. NEU1 is also involved in cellular signaling for immune responses and inflammation through its transportation to plasma membranes [24]. For example, during PMA-induced monocyte differentiation, NEU1 is up-regulated and targeted together with PPCA to MHC II-positive vesicles that subsequently merge with the plasma membranes [21]. Another example is that NEU1 contributes to regulation of phagocytosis in macrophages and dendritic cells through desialylation of surface receptors [25]. In this case, cell surface NEU1 activates phagocytosis in immune cells probably by affecting sialylation and phosphorylation of multiple receptors involved in phagocytosis, including Fc receptors for immunoglobulin G. Furthermore, there is evidence that NEU1 is an important regulator of cell proliferation. Facilitation of elastic fiber assembly occurs probably through desialylation of microfibrillar glycoproteins and other adjacent matrix glycoconjugates, contributing to the maintenance of cellular quiescence. Under this condition, elastin binding protein, which is identical to the spliced variant of β-galactosidase, forms a complex with PPCA and NEU1 at the cell surface [26]. The carboxypeptidase activity of PPCA is required for elastin-binding protein complex formation, participating in regulation of blood pressure as an endothelin-1 inactivating enzyme [27]. NEU1, as a subunit

of the elastin receptor, inhibits proliferation of aortic smooth muscle cells and fibroblasts by desialylation of both platelet derived growth factor (PDGF) and insulin-like growth factor (IGF)-1 receptors, followed by reduction of PDGF-BB- and IGF-2-induced mitogenic responses [28]. NEU1 also desialylates insulin and IGF-1 receptors in skeletal myoblasts and regulates proliferative responses of the cells to insulin in a different manner, depending on the concentration of insulin [29]. These observations indicate that, in addition to lysosomal catabolism, NEU1 regulates various important cellular phenomena by modulating cell signaling through desialylation of various cell receptors.

Crucial roles of sialidase NEU1 in determination of metastatic potential of cancer cells In line with the observations above on augmentation of cell proliferation with decreased expression of NEU1, cancer cells and tissues tend to show lower NEU1 expression than adjacent noncancerous counterparts. Interestingly, a good inverse relationship between the NEU1expression level and metastatic ability has been noted in various cell types. After transfection of oncogenes, several cell lines have demonstrated reduced endogenous NEU1 expression and acquisition of metastatic ability. In rat 3Y1 fibroblasts, NEU1 decreased after src-transformation, and v-fos transfer to these transformed cells induced more severe decrease in the sialidase activity associated with higher metastatic potential [30]. In mouse adenocarcinoma colon 26 cells, highly metastatic NL17 and NL 22 cells exhibit lower expression of NEU1 [31]. As expected, introduction of murine *Neu1* gene into Bl6-BL6 murine melanoma cells, a highly invasive and metastatic line, resulted in suppression of experimental pulmonary metastasis and tumor growth in syngeneic mice, with reduction of anchorage- independent growth and increased sensitivity to apoptosis [32], although no significant alteration was observed in invasiveness, cell motility and cell attachment. The natural substrate molecule associated with the NEU1-mediated effects was not identified, but desialylation of a cell surface glycoprotein of 83 kD was prominent in the *Neu1* transfectants, when changes in glycoproteins were determined by galactose oxidase labeling. When expression levels of human NEU1 were evaluated in colon cancer by quantitative RT-PCR and by activity assays using a synthetic substrate, 4MU-NeuAc, the cancer tissues showed a tendency towards decrease in the mRNA and activity levels as compared with the adjacent non-cancerous mucosa. Interestingly, the activity level in the cancer tissues seemed to be inversely correlated with the extent of invasion and poor differentiation [33]. To investigate how human NEU1 expression affects malignant behavior, the gene was introduced with a protective protein into colon adenocarcinoma HT-29 cells. Overexpression of NEU1 brought about alterations of the human cancer cells [34] similar to those observed in the murine cells, with additional suppression of cell migration and invasion. *NEU1* knockdown resulted in the opposite effects. Furthermore, injection of NEU1-overexpressing cells trans-splenically into nude mice caused a significant decrease in *in vivo* liver metastasis. One of the target molecules for NEU1 was found to be integrinβ4, overexpression of which is associated with cancer metastasis. The integrin in the cells underwent desialylation and decreased phosphorylation followed by attenuation of FAK and ERK1/2 pathway and down-regulation of matrix metalloproteinase-7. Treatment of the cells with GalNAc-α-*O*-benzyl, an inhibitor of *O*-glycosylation, increased PNA-positive integrin β4 with its decreased phosphorylation, indicating that sialic acid removal from integrin *O*-glycans may result in decreased phosphorylation. Biotinylation and immunofluorescence staining showed the presence of some NEU1 molecules at the cell surface accessible to the integrin. These observations indicate that NEU1 plays an essential role in regulation of integrin β4-mediated signaling, leading to suppression of metastasis.

Roles of sialidase NEU2 in muscle and neuronal differentiation and alterations in cancer NEU2 was the first example of a mammalian sialidase for which cDNA cloning was achieved from rat skeletal muscle [35]. Homologues were subsequently cloned from cDNA libraries of CHO [36], mouse brain [37, 38], and mouse thymus [39] and from a genomic library of human skeletal muscle [40], all showing high amino acid identity (70–98 %) to the rat gene. NEU2 is able to hydrolyze a wide range of glycoproteins, oligosaccharides and gangliosides at an optimum pH of about 6.0–6.5. Determination of the three-dimensional structure of human NEU2 by X-ray crystallography [41] provided evidence for a canonical six-blade beta-propeller, observed in viral and bacterial sialidases, with the active site in a shallow crevice. The enzyme participates in muscle cell and neuronal differentiation, and the rat *Neu2* gene is highly expressed in skeletal muscle. The rat gene contains two E-box pairs, known to be consensus binding sites for muscle-specific transcription factors, in the 5′-flanking enhancer/promotor region. This region exhibits transcriptional activity in rat L6 [42] and murine C2C12 [43] myogenic cells, and activity as well as the mRNA level is increased during myotube formation, in line with the enzyme playing a critical role in muscle cell differentiation. In this context, it is interesting that the SJL mouse, a model for human dysferlinopathy, shows down-regulation of NEU2, this probably contributing to impaired muscle regeneration [44]. In PC12 cells, NEU2 may participate in neuronal differentiation through nerve-growth factor-induced transcriptional activation [45]. It should be noted here that the endogenous level of human NEU2 is extremely low at only four- to ten- thousandths of the value for NEU1, which shows the strongest expression (10–20 times greater than those of NEU3 and NEU4) in

tissues, as assessed by quantitative real time RT-PCR [46]. In rat and mouse tissues, different situations are observed.

Introduction of the rat *Neu2* gene into a B16-BL6 mouse melanoma cell line caused a marked decrease in pulmonary metastasis, with a decrease in the ganglioside GM3 and an increase in lactosylceramide [47]. In confirmation of the substrate specificity *in vitro*, the results indicate that NEU1 and NEU2 desialylate natural molecules different from each other, even in the same cells. As described earlier, NEU1 activity results in marked desialylation of a glycoprotein but hardly any ganglioside changes. When the rat *Neu2* gene was transfected into highly metastatic mouse colon 26 adenocarcinoma cells [31], 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. 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. It is interesting that sublines of cells featuring low spontaneous metastasis possessed a relatively high level of endogenous 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. On the other hand, highly metastatic cells exhibit rather decreased sialic acid contents, both total and cell surface, as compared to low metastasis examples. The results together indicate that the sialidase level may be a determining factor for metastatic ability independent of the cell type and the sialic acid content. Although the occurrence of glycoconjugates in the cytosol may be considered as an unusual event, there have been reports of cytosolic presence of glycoproteins, oligosaccharides and gangliosides, and also of glycosidases and lectins involved in metabolism and recognition of the glycan molecules [48], implying that NEU2 may play physiologically and pathologically significant roles in the cytosol.

Another line of the experiments with NEU2 in human cancer cells reported by other investigators exhibited significant effects on cell growth and apoptosis. For example, transfection of the Chinese hamster ovary *Neu2* gene into the A431 human epidermoid carcinoma cell line reduced GM3 levels and enhanced cell growth and tyrosine autophosphorylation of EGF receptors at low EGF concentration [49]. Introduction of the human *NEU2* gene into leukemic K562 cells caused a marked decrease in anti-apoptotic factors Bcl-XL and Bcl-2, resulting in increased sensitivity to apoptotic stimuli [50]. NEU2 overexpression in the cells reduced gene expression and activity of Bcr-Abl, together with a decrease in Bcr-Abl-dependent Src and Lyn kinase activity probably brought about by desialylation of some cytosolic glycoproteins. However, it is uncertain at present 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 marked decrease of NEU2 expression in human as opposed to other species remains to be elucidated.

Roles of sialidase NEU3 in transmembrane signaling The plasma membrane- associated sialidase NEU3 was first cloned from a bovine brain library [51], based on peptide sequence information from the purified enzyme protein, and later from a human skeletal muscle cDNA library [52] and from the human genome data base [53]. Primary sequences covering the entire coding region of the corresponding human, mouse [38], and rat [54] genes display approximately 80 % overall identity with the bovine gene. In *in vitro* assays, the bovine and human enzymes specifically hydrolyze gangliosides other than GM1 and GM2, and the murine enzyme acts on oligosaccharides, 4MU-NeuAc and glycoproteins to a certain extent. Gangliosides GD3, GM3, GD1a and even GD1b are good substrates. With administration of the radiolabeled ganglioside GD1a to murine *NEU3*-transfected cells, NEU3 was shown to hydrolyze ganglioside substrates in intact living cells at a neutral pH, with probable involvement in cell-cell interactions through hydrolysis of gangliosides at the surfaces of neighboring cells [55]. Unlike the bovine and mouse NEU3, human NEU3 is not always detected on the cell surface and also may exist in other intra-membranous components. In response to growth stimuli such as EGF treatment, NEU3 mobilizes to membrane ruffles together with Rac-1, a small G protein participating in actin reorganization and cell motility, and enhances cell movement [56]. On the basis of an analysis of membrane topology, Zanchetti *et al*. [57] has suggested that the sialidase might be localized partially on the cell surface as a peripheral membrane protein and also in endosomal structures. NEU3 participates in neurite formation [38, 58] and in regulation and regeneration of rat hippocampus neurons [59, 60]. It is located in rafts of neuroblastoma cells [61] and in caveolae of HeLa cells [62], closely associated with caveolin-1. Our recent observations have further provided evidence that NEU3 regulates transmembrane signaling by interacting with signaling molecules including caveolin-1, Rac-1, interin β4, Grb-2 and EGFR as well as by modulation of gangliosides as an enzyme [63].

Crucial roles of NEU3 in acceleration of cancer progression Up-regulation of NEU3 has been observed in various neoplasms including colon, renal, ovarian and prostate cancers, the one obvious exception being down-regulation in acute lymphoblastic leukemia in relation to disease progression [64]. In human colon cancers, *NEU3* mRNA levels were found to be increased 3- to 100-fold as

compared to adjacent non-tumor mucosa [65]. *In situ* hybridization analysis with an antisense probe demonstrated positive signals to be localized to carcinoma cells, rather than surrounding stromal cells. During sodium butyrate-induced apoptosis, human colon cancer cells showed down-regulation of NEU3 expression and in contrast, up-regulation of NEU1. Transfection of a *NEU3* gene into cancer cells was found to inhibit this sodium butyrate-induced apoptosis, accompanied by increase in Bcl-2 protein and decreased caspase expression. Human colon cancer specimens have been found to exhibit marked accumulation of lactosylceramide, a possible NEU3 product, compared with adjacent non-cancerous mucosa. In line with these results, addition of the glycolipid to cultures reduced apoptotic cells during sodium butyrate treatment. In colon cancer cells, NEU3 may differentially regulate cell proliferation through integrin-mediated signaling depending on the extracellular matrix [66], causing increased adhesion to laminins and consequent cell-division, but rather decrease in cell adhesion to fibronectin, collagen I and IV. Triggered by laminins, NEU3 markedly enhanced tyrosine phosphorylation of intergrin β4, with recruitment of Shc and Grb-2, stimulating phosphorylation of focal adhesion kinase and ERK1/2. Significant increase of *NEU3* mRNA levels has been noted in renal cell carcinomas [67], correlating with elevation of interleukin (IL)-6, a pleiotropic cytokine. NEU3 activated by IL-6 directs IL-6-mediated signaling via the PI3K/Akt cascade in a positive feedback manner and thus contributes to a malignant phenotype, including suppression of apoptosis and promotion of cell motility in renal cell carcinoma ACHN cells. Up-regulation of NEU3 has also been detected in prostate cancer,with a significant correlation to malignancy as assessed by the Gleason score [68]. In androgen-sensitive LNCaP cells, forced overexpression of *NEU3* significantly induced expression of the progression-related transcription factor EGR-1, the androgen receptor and PSA both with and without androgen, the cells becoming sensitized to androgen. NEU3-mediated induction was abrogated by inhibitors of PI-3 kinase and MAPK, in line with increased phosphorylation of AKT and ERK1/2 in *NEU3*-overexpressing cells. NEU3 siRNA introduction caused reduction of cell growth of androgen-independent PC-3 cells in culture and of transplanted tumors in nude mice. There is further evidence of involvement in expression of malignant properties, in that NEU3 knockdown contributed to decreased cell motility, invasion and *in vivo* bone metastasis with decreased expression of the matrix metalloproteinases MMP-2 and MMP-9, in PC-3 cells [69]. The data suggest that NEU3 regulates tumor progression of prostate cancer through androgen receptor signaling, and is likely to be involved in bone metastasis.

To define further the molecular mechanisms of NEU3 influence and its possible targets, the encoding gene has been silenced by siRNA or overexpressed in various human cancer cells [70]. Silencing caused apoptosis without specific stimuli, accompanied by decreased Bcl-xL and increased mda7 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, appeared to be similarly affected by treatment with the NEU3 siRNA, but interestingly non-cancerous human fibroblasts and keratinocytes showed no significant changes. NEU3 siRNA was found to inhibit Ras activation and NEU3 overexpression to stimulate it with consequent influence on ERK and Akt. Ras activation by NEU3 was largely abrogated by PP2 (a src inhibitor) or AG1478 (an EGFR inhibitor). In fact, siRNA introduction reduced phosphorylation of EGFR while overexpression promoted its phosphorylation in response to EGF. NEU3 co-immunoprecipitated with EGFR, and EGF-stimulation yielded a higher amount of immunoprecipitable NEU3. These results indicate that NEU3 suppresses apoptosis of cancer cells by promoting EGFR phosphorylation, probably through its association with EGF receptors and consequent activation of Ras cascades, especially via the Ras/ERK pathway, as illustrated in Fig. 1. Up-regulation of NEU3 has also been established to have importance for the promotion stage of colorectal carcinogenesis *in vivo*, from experiments using NEU3 transgenic mice [71]. Thus NEU3 was found to increase azoxymethane-induced aberrant crypt foci formation in colon mucosa by suppression of apoptosis, possibly via activation of EGF signaling. To summarize, the sialidase activates the molecules including FAK, ILK, Shc, integrin β4 and also Met, often upregulated in

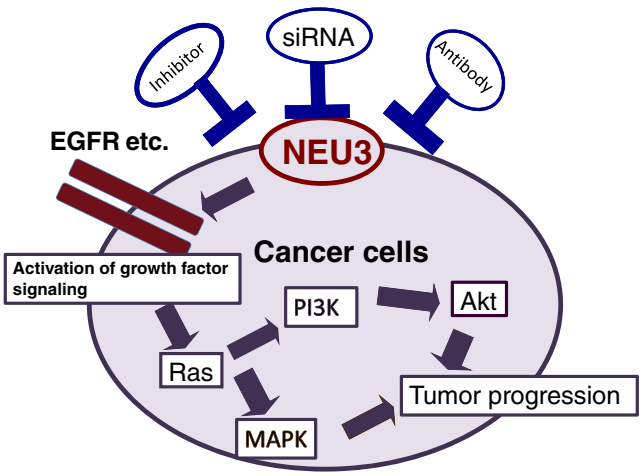

Fig. 1 Proposed effects of down-regulation of NEU3 as a potential target for cancer therapy. Down-regulation of NEU3 expression by treatment with a specific siRNA, antibody or inhibitor may lead to prevention of cancer progression. In particular, taking advantage of its limited effects on normal cells, NEU3 siRNAs causing apoptosis in cancer cells could offer a useful tool

carcinogenesis,which may cause acceleration of progression to a more malignant phenotype in cancer cells [72]. In this context, sialidase could be a useful target for cancer diagnosis and therapy.

Roles of sialidase NEU4 in sialyl- Lewis antigen expression and in polysialylation and its relation to the cancer phenotype The mouse and human genes for NEU4 were identified based on cDNA sequences in public databases [73–76]. With regard to subcellular localization of human NEU4, two different descriptions have been reported on the basis of gene transfection studies: one featuring targeting to the lysosomal lumen [75], and the other to mitochondria and endoplasmic reticulum [76, 77]. The sialidase exists as two iso-forms, which differ in their possession of 12 N-terminal amino acid residues for mitochondrial targeting. The isoforms, NEU4L and NEU4S, are also differentially expressed in a tissue-specific manner, brain, muscle and kidney containing both, and the liver and colon possessing predominantly the short form [76], as assessed by RT-PCR. There are similarly two isoforms of mouse NEU4 generated by alternating splicing, designated NEU4a and NEU4b [78]. Mouse NEU4a has an additional 23 amino acid stretch at the N-terminus and exhibits lower enzymatic activity than NEU4b. The mouse *Neu4* gene is expressed dominantly in brain [73] and is only found at very low levels in other tissues. NEU4 possesses broad substrate specificity like NEU2, but it seems to be only a sialidase hydrolyzing mucins. Seyrantepe *et al*. [79] have reported that despite the lack of gross morphological abnormalities, NEU4 deficient mice show ganglioside patterns featuring increased GD1a and decreased GM1 in the brain, suggesting a regulatory role of NEU4 in ganglioside catabolism in nervous tissue. As our recent observations have shown that NEU4 expression is predominant in the mouse brain and the level is significantly higher than NEU3 [80], it is possible that this form may be a major sialidase for ganglioside catabolism in this site.

Human NEU4 may be involved in neuronal cell apoptosis, based on the observation that NEU4L including the mitochondrial targeting signal exists predominantly in brain and probably impacts on the level of GD3 in neuroblastoma cells [81], in line with the proposal that GD3 can act as an apoptosis-inducing ganglioside [82]. The expression level of the mouse gene increases 3 to 14 days after birth [78], further suggesting a role in brain development. In contrast to NEU3, the murine form of NEU4 sialidase appears to negatively regulate neurite formation, being down-regulated in Neuro2a cells during retinoic acid-induced differentiation, although definite target molecules have yet to be identified. Furthermore, recent observations revealed that mouse NEU4 catalytically degrades polysialic acids (polySia), a homopolymer of α-2-8-linked sialic acid residues, on neural cell adhesion molecules (NCAMs) [80]. PolySia have been implicated in control of synaptic plasticity, neuronal differentiation and cell migration [83, 84]. Levels are high during embryonic development, whereas its expression in the adult is restricted to brain regions featuring persistent neural plasticity or neural generation, such as the hippocampus [85]. For the biosynthesis of polySia, two polysialyltransferases, ST8SiaII and ST8SiaIV, have been identified [86], but physiological turnover remains to be clarified. Although a bacteriophage-derived endosialidase essential for bacterial infection is well known, NEU4 was found to be the major degradation enzyme for polySia in vertebrates, thus negatively regulating neurite outgrowth of hippocampal neurons [80].

When *NEU4* mRNA levels were compared between human colon cancer and adjacent non-cancerous tissues, marked decrease in expression was apparent in the tumors [87], in clear contrast to the NEU3 case. Levels were not significantly correlated with the histological differentiation or the pathological stage, but rather with degree of venous invasion. In cultured human colon cancer cells, the enzyme was up-regulated in the early stage of apoptosis induced by either the death ligand TRAIL, or serum-depletion. Indeed, 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 and promotion of cellular invasiveness and motility. To elucidate the significance of NEU4 down-regulation and its close relation with venous invasion in colon cancer, sialyl-Lewis antigens, sialyl-Le^a and sialyl-Le^x, as endogenous substrates for the sialidase, were investigated [88], 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 the 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. In addition, 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 here that the sialidase did not change the level of a normal glycan, disialyl-Le^a, generally expressed in non-malignant 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 in various cells.

It is feasible that desialylation by NEU4 may occur specifically with cancer related sialyl-Lewis antigens and thus maintainance of the normal glycan level can be achieved in colon mucosa highly expressing NEU4. In addition to nervous tissue, polysia-NCAM has been reported to be expressed in malignant tumors, including gliomas, lung and colon cancers [89, 90], the presence being correlated to tumor development, invasion and poor prognosis. Considering regulation of polySia by NEU4, it is likely that down-regulation of NEU4 might be involved in the presence in cancers, at least in the colon. On the other hand, Tringali *et al*. [91] reported that experimental overexpression of NEU4L in neuroblastoma cells influenced the differentiation/proliferation behavior of the cells by activation of the Wnt/β-catenin signaling pathway. In the cells enhanced proliferation and reduced differentiation occurred together with increased expression of pluripotency genes, such as MYC, NANOG, OCT-4, CD133 and NES (nestin), probably due to modification of sialylation level of cellular glycoproteins. Because no information is available on the actual level of NEU4 in human neuroblastomas, the role of the enzyme remains uncertain, but functions may vary with the target molecules.

Conclusion

Investigation of mammalian sialidases in cancer has uncovered a great deal of information regarding the molecular bases of aberrant sialylation related to malignancy. The results overall show that altered expression of three human sialidases, NEU1, NEU3, and NEU4, in particular may definitely influence the malignant properties of cancer cells, including cell survival, motility, invasion, and metastasis, through modification of various glycoconjugates as substrates. In general, down-regulation of NEU1 and NEU4 now seems to facilitate metastasis, while NEU3 up-regulation is essential for the cell survival and also possibly even for metastasis. Alteration in the sialidase expression thus may be a defining factor in cancer progression, because close links between promotion of malignancy and aberrant sialylation can now be explained, if not completely, at least partly as the results of altered expression of sialidases. Sialidase alterations, therefore, open up potential applications in cancer cure and diagnosis. In particular, as illustrated in Fig. 1, down-regulation of NEU3 expression by treatment with specific siRNAs, antibodies or inhibitors may lead to prevention of cancer progression.

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