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SIALIDASES AND CANCER:

HUMAN SIALIDASE NEU3 ENHANCES EGFR ACTIVATION IN COLORECTAL CANCER

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List of abbreviations

AKT, Protein Kinase B (PKB)
4MU-NANA, 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid
β-ACT, β-actin
BBF, Bromophenol blue
BRAF, v-raf murine sarcoma viral oncogene homolog B
BSA, bovine serum albumin
CRC, colorectal cancer
DMEM, Dulbecco's Modified Eagle's Medium
DTT, dithiothreitol
ECL, enhanced chemioluminescence
EDTA, ethylenediaminetetraacetic acid
EGF, epidermal growth factor
EGFR, epidermal growth factor receptor
EGTA, ethylene glycol tetraacetic acid
EMEM, Eagle's minimal essential medium
Erk1/2, extracellular regulated kinase 1 and 2
FBS, fetal bovine serum
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase
GFP, green fluorescent protein
GPI, glycosylphosphatidylinositol
GTP, Guanosine-5'-triphosphate
HRP, horseradish peroxidase
IP, immunoprecipitation
KRAS, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK, mitogen-activated protein kinase
MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
N3, sialidase NEU3
NP40, nonidet P-40
PBS, phosphate buffered saline
PI3K, phosphatidylinositol 3-kinase
PMSF, phenylmethylsulfonyl fluoride
POL2, RNA polymerase I

PTEN, Phosphatase and tensin homolog
Q-PCR, quantitative-polymerase chain reaction
RIPA, radio immunoprecipitation assay
RPMI, Roswell Park Memorial Institute Medium
RT-PCR, reverse transcription-polymerase chain reaction
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNA, Sambucus nigra agglutinin
ST6-Gal1, Beta-galactoside alpha-2,6 sialyltransferase 1
TBS, tris buffered saline
TEMED, N,N,N´,N´-tetramethylethylenediamine

Abstract

Abnormal glycosylation is known to be associated with cancer malignancy. In the cell, this process is finely regulated by many enzymes, including sialidases or neuraminidases; these are glycohydrolases widely distributed in nature that remove sialic acid residues from glycoproteins and glycolipids. In mammals, four sialidases with different subcellular localizations and biochemical features have been described: a lysosomal sialidase (NEU1), a cytosolic sialidase (NEU2), a plasma membrane-associated sialidase (NEU3) and a mitochondrial sialidase (NEU4).

Studies performed over the last decade have focused on the involvement of sialylation in the progression of cancer and moreover on the association of sialidase deregulation to the tumorigenic transformation.

In particular, recent studies on the Japanese population have shown that sialidase NEU3 is often deregulated in colorectal cancer and also that it co-immunoprecipitates with the epidermal growth factor receptor (EGFR), the molecular target of the most recent therapies based on monoclonal antibodies.

In collaboration with the Istituto Nazionale dei Tumori of Milan (Italy) (IRCCS) and with the Istituto Cantonale di Patologia of Locarno (Switzerland) we recruited a cohort of 85 Caucasian patients resected for a colorectal cancer. By real-time PCR experiments we observed a deregulation of the mitochondrial sialidase transcripts and, on the contrary, an up regulation of NEU3 mRNA in tumor tissues compared to paired normal mucosa.

Moreover, by comparing NEU3 and EGFR mRNA levels, we observed a statistically significant correlation, suggesting that the increase in EGFR expression could be associated with NEU3 increment. Vice versa, no correlation was observed between the overexpression of NEU3 sialidase and mutations in KRAS, BRAF, PIK3CA and PTEN diagnostic markers.

Experiments performed on colorectal cancer cell lines have demonstrated that overexpression of *wild type NEU3* enhanced EGFR activation, compared to colon normal mucosa CCD841 cell line, irrespectively of mRNA and protein levels, mutational and gene status of the receptor in all the cell

lines tested. The only exception was represented by SW620 cells, that are commonly used as EGFR negative control.

Moreover, Western Blots of *wild type* NEU3 overexpressing cells revealed increased EGFR and ERK1/2 phosphorylation in SW480 colorectal cell line and also in DIFI cells, which represents the best cellular model to study the EGFR pathway, while mRNA and total EGFR protein contents remained constant. On the contrary, we could not detect any EGFR activation in cells overexpressing a totally inactive mutant of NEU3.

In addition we performed MTT based test in transfected cells. Western blot analyses showed a significant increase of cell viability, only upon overexpression of *wild type* NEU3, the inactive mutant being completely ineffective in this respect.

Having demonstrated that the human NEU3 sialidase is more strongly anchored to the membrane its murine counterpart, we proved, not only by the lectin binding assay but also by mass spectrometry, that this sialidase directly modified the sialylation level of EGFR extracellular domain.

Moreover we also showed that NEU3 overexpression modulated the response to the pharmacological treatment with Cetuximab. The overexpression of the active form of NEU3 sialidase lead to a significant increase in cell viability in all tested cell lines, also under pharmacological treatment with Cetuximab, with the exception of SW48 cells that presented a hyperactivating mutation in the tyrosine kinase domain of EGFR, causing the receptor to act independently from the dimerization.

Our data suggest that, in the cell lines in which EGFR acts correctly as a dimer, sialidase overexpression caused an increment of viability even in those presenting hyperactivating mutations in the downstream pathways, that influence the efficacy of the therapy.

We decided to extend the analysis of the deregulation of human sialidases to other types of cancer, in order to identify and deepen the knowledge of common variations of these enzymes also in the Western population.

On the whole, by demonstrating the role of sialidase NEU3 in CRC, our work strongly suggests that this enzyme might be taken into consideration as a new effective molecular marker for CRC diagnosis and treatment.

Furthermore, these data confirm the need of further studies concerning the role played by sialidases as a defining factor in cancer progression, opening up potential applications in diagnosis and therapy.

1. Introduction

1.1 *Sialic acids*

Sialic acids are 9-carbon amino-sugar molecules largely distributed in animal cells, from the echinoderms, such as starfish,to humans (Reuter G. and Gabius HJ., 1996; Corfield AP. *et al*, 1982) .
Their existence in lower animals of the protostomate lineage or in plants is not yet proved, with the exception of buckwheat (Bourbouze R. *et al*, 1982). The only known exception is the occurrence of polysialic acid in the larvae of the insect *Drosophila* (Roth J. *et al,* 1992). Sialic acids are present also in some protozoa, viruses and bacteria (Schauer R. *et al*, 1997; Barry GT., 1959; Yamasaki R. *et al*, 1993). Thus, several strains of *Escherichia coli* contain long saccharide stretches consisting of up to 200 sialic acid molecules, the so-called colominic acid.
Sialic acids are present on cell surfaces as well as in intracellular membranes (e.g., of the Golgi apparatus). In higher animals they are also important components of the serum and of mucous substances.

1.1.1 *Sialic acid structure*

Sialic acids are a family of about 40 derivatives of the nine-carbon sugar neuraminic acid (Schauer R. *et al*, 1997; Varky A., 1992). The amino group at position 5 and the carboxyl group at position 1 confers a negative charge to the molecule under physiological conditions and characterizes it as a strong organic acid (pK 2.2)(Figure 1.1).
The unsubstituted form, neuraminic acid, does not exist in nature.
The amino group is usually acetylated, leading to *N*-acetylneuraminic acid (Neu5Ac), the most widespread form of sialic acid (Figure 1.1).

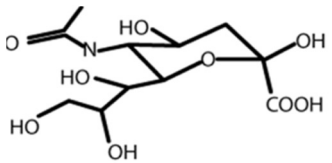
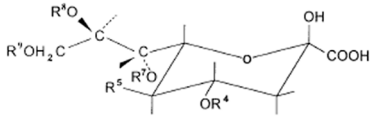


Figure 1.1 ***N-acetylneuraminic acid structure (Neu5Ac).***

Substituting one of the hydrogen atoms in the methyl moiety of the acetyl group by a hydroxyl group results in *N*-glycolylneuraminic acid (Neu5Gc), which is common in many animal species but has been found in humans only in the case of particular cancers. Sialic acid molecules can be substituted in more than one position, for example in 7,8,9-tri-*O*-acetyl-*N*-acetyl- or *N*-glycolylneuraminic acid. Modifications to hydroxyl groups include methylation, acylation, phosphorylation, and sulfation, or unsaturation at C-2/C-3 (Traving C. and Schauer R., 1998) (Figure1.2).



R^5	$R^{4,7,8,9}$	
$-NH-C(=O)-CH_3$	$-H$	(4,7,8,9)
$-NH-C(=O)-CH_2-OH$	$-C(=O)-CH_3$	(4,7,8,9)
$-NH-C(=O)-CH_2-O-C(=O)-CH_3$	$-C(=O)-CH(OH)-CH_3$	(9)
	$-CH_3$	(8)
$-OH$	$-SO_3H$	(8)
	$-PO_3H_2$	(9)

Figure 1.2 ***Chemical structure of the sialic acid molecule and a list of natural substituents*** (Traving C. and Schauer R., 1998).

These molecules usually represent the terminal, non-reducing sugar moiety in glycoproteins or glycolipids linked to galactose (α2-3 or α2-6), *N*-acetylgalactosamine (GalNAc), or *N*-acetylglucosamine (α2-6) (GlcNAc). Sialic acids that occur as side chains are linked via C2 to position 3 or 6 of the penultimate sugar, while sialic acids that are internal to oligosaccharides, polysaccharides of glycoproteins, or glycolipids are linked to position 8 of another sialic acid molecule, as well as in bacterial capsules and gangliosides of higher animals (Schauer R., 1982). Co-polymers of sialic

acid via α2-8, α 2-9, or α 2-8/α2-9 linkages result in polysialic acids, common in embryonic tissue glycoproteins, and could play a role in cell adhesion and organogenesis (Roth J. *et al*, 1992).
A specific pattern of sialic acid derivatives characterizes the tissue and the developmental stage of each individual species (Varky A., 1992). The variability of sialic acids is further extended by their location on cells and molecules. In humans, normally only small amounts of sialic acids are present as free molecules in plasma or in cellular environment.

1.1.2 *Sialic acid function*

Sialic acids play important roles in various biological processes by influencing the conformation of glycoproteins, recognizing and masking the biological sites of the molecules and their binding sites to the cells (Miyagi T. *et al*, 2008).The structural diversity of sialic acid is reflected in the variety of its functions including blood protein half-life regulation, toxin neutralization, cellular adhesion and glycoprotein lytic protection (Schauer R., 1982; Kelm S. and Schauer R., 1997; Schauer R., 1985).
Due to their negative charge, sialic acids are involved in binding and transport of positively charged molecules (e.g. Ca^{2+}) as well as in attraction and repulsion phenomena related to cellular and molecular recognition. Since they are exposed in carbohydrate chains of glyco-conjugates, sialic acids canfunction as a protective shield both for biological macromolecules preventing, for example, protease degradation, and for cells, as demonstrated for the mucous layer of the respiratory epithelium. Indeed, in infectious processes the colonization of bacteria is prevented by the sialic acid coat covering the host cell surface.
Another phenomenon is the spreading effect that is exerted on sialic acid-containing molecules due to the repulsive forces acting between their negative charges (Muller HE., 1974). This stabilizes the correct conformation of enzymes or cell membrane glycoproteins, and is important for the slimy character and the resulting protective function of mucous substances, such as those found on the eye surface and mucous epithelium.

Sialic acids also contribute in the masking process of cells and molecules. It has been demonstrated that erythrocytes are covered by a dense layer of sialic acid molecules. During the normal life span of red blood cells, sialic acids are removed stepwise from the cell surface by the action of serum sialidase and by spontaneous chemical hydrolysis. The exposure of the penultimate galactose residues represents a degradation and the unmasked erythrocytes are bound to macrophages and phagocytized (Figure 1.3) (Bratosin D. *et al*, 1995). Thus, sialic acids prevent erythrocytes from being degraded, because they mask the subterminal galactose residues. The same mechanism works on other blood cells (thrombocytes and leucocytes) and on various serum glycoproteins, which are bound by hepatocytes after exposure of subterminal galactose residues.

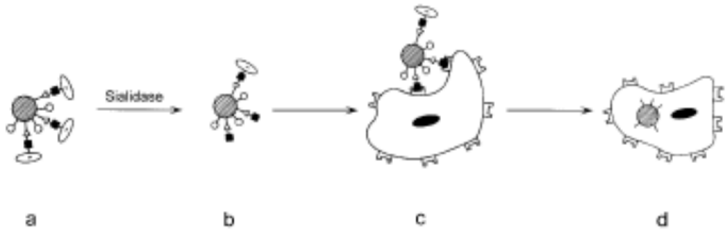


Figure 1.3 ***The masking function of sialic acids: mechanism of binding*** *(b)* ***and phagocytosis*** *(c)* ***of sialidase-treated erythrocytes*** *(a)* ***by macrophages.***
(Traving C. and Schauer R., 1998)

In addition sialic acids are also well suited as ligands for mediating selective recognition processes between cells and molecules (Schauer R. *et al*, 1997; Kelm S. and Schauer R., 1997). Thus, the immune system can distinguish between *self* and *non-self* structures according to their sialic acid pattern. Sialic acid represents not only a blood group antigenic determinant but is also a necessary component of receptors for many endogenous substances such as hormones and cytokines. Moreover, many pathogenic agents such as toxins (e.g. cholera toxin), viruses (e.g.influenza), bacteria (e.g. *Escherichia coli*, *Helicobacter pylori*) and protozoa (e.g. *Trypanosomacruzi*) also bind to host cells via sialic acid-containing receptors (Schauer R. *et al*, 1997).

Another group of sialic acid-recognizing molecules belongs to the so-called lectins,oligomeric glycoproteins from plants, animals and invertebrates that bind to specific sugar residues. Very common sialic acid-binding lectins are wheat germ agglutinin (WGA), *Limulus polyphemus* agglutinin (LPA), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), which are specific for α2-6-linked and α2-3-linked sialic acids. In plants, that do not have sialic acids, lectins might be helpful in the defense against sialic acid-containing microorganisms or plant-eating mammals. In mammals, there exist counterparts of lectins, including selectins as well as members of the sialoadhesin family ('Siglecs') (Crocker PR. *et al*, 1998) such as sialoadhesin (Sn), CD22, CD33, myelin-associated glycoprotein (MAG) and Schwann cell myelin protein (SMP) (Kelm S. *et al*, 1996). Selectin and Siglec molecules consist of several domains, one of which is responsible for sialic acid binding. Selectins play an important function in the initial stage of adhesion of white blood cells to endothelia, the so-called rolling of the cells, which may be followed by extravasation of leukocytes from the circulation into tissue sites. They are located on endothelial cells and recognize sialic acids in the sialyl LewisX $(Le)^x$ and sialyl LewisA $(Le)^a$ structure on leucocytes surface. Because these molecules are also present on tumor cells, selectins might be involved in tumor metastasis. CD22, a member of the immunoglobulin superfamily, is a type I membrane glycoprotein expressed in B cells. It mediates binding of B cells to other B or T cells, neutrophiles, monocytes or erythrocytes. The ligand of CD22 is sialic acid bound in α2-6 linkage to branched *N*-linked oligosaccharides. Sialoadhesin was found on macrophages and is thought to be important for the development of myeloid cells in the bone marrow and trafficking of leucocytes in lymphatic tissues. Binding between these receptors and their ligands depends on the presence of only a few functional groups of the oligosaccharide. Thus, these receptors bind also to low-affinity ligands that are present in high density on the cell surface and to a low number of high-affinity ligands. Thus, a better fine tuning of cell-cell

interactions is possible, as required for high specific recognition processes (Crocker PR. *et al*, 2007; Varky A. and Angata T., 2006).

1.1.3 *Pathobiological significance of sialic acid diversity*

One of the primary pathobiological implication of sialic acids is related to pathogen binding process as recognized for influenza A and B viruses (Suzuki Y., *et al*, 2000; Shinya K. *et al*, 2006). Most influenza viruses that infect and spread among wild and domesticated birds preferentially recognize sialic acids that are α2-3-linked to the underlying glycan chains. One of the most recent example is the so called H5N1 'bird flu' virus.
Humans are at least partly resistant to infection by such viruses,because they display α2-6-linked sialic acids on the epithelium of the upper airways. Thus, in order for avian influenza viruses to become human pathogens, certain specific mutations must occur in the sialic acid-binding pocket of the virus hemagglutinin. This is thought to occur in intermediate hosts. However, there has been at least one instance, the 1918 pandemic influenza, where in the virus adjusted to bind both types of linkages (Russell CJ. and Webster RG., 2005; Stevens J. *et al*, 2006) and 'jumped' directly from birds to humans. Recent infections of a few humans with the bird flu have fortunately been traced not to such a change in virus-binding specificity, but likely to exposure to a very large dose of virus. Another example of a pathology in which sialic acids are important in the pathogen binding process is represented by malaria. The invasion of red blood cells by the merozoite stage of *Plasmodium falciparum* is often dependent on the presence of sialic acids. Indeed, different proteins on the *Plasmodium* merozoites govern the binding to red blood cells although some sialic acid-independent binding mechanisms have emerged in this virulent pathogen (Figure 1.4) (Martin MJ. *et al*, 2005).
Sialic acids are also involved in the progression and spread of human malignancies.
As described in previous chapter, sialic acids are responsible for masking of endogenous structures. This can also have a detrimental effect, as can be seen in the case of some tumors that are sialylated to a much higher degree than the corresponding normal tissues. Consequently, the

transformed cells become 'invisible' to the immune defense system. The immunosuppressive effect of the higher degree of sialylation in tumors is due to an increased activity of sialyl-transferases. Thus, terminal galactose residues, that would otherwise inhibit further cell growth and spreading, are masked. This might be a reason for the loss of contact inhibition of cancer cells (Wieser RJ. *et al*, 1995). The masking effect of sialic acids also helps to hide antigenic sites on parasite cells from the host immune system. This is the case for microbial species like certain *E. coli* strains and gonococci as *Neisseria gonorrhoeae* (Jarvis GA., 1995).

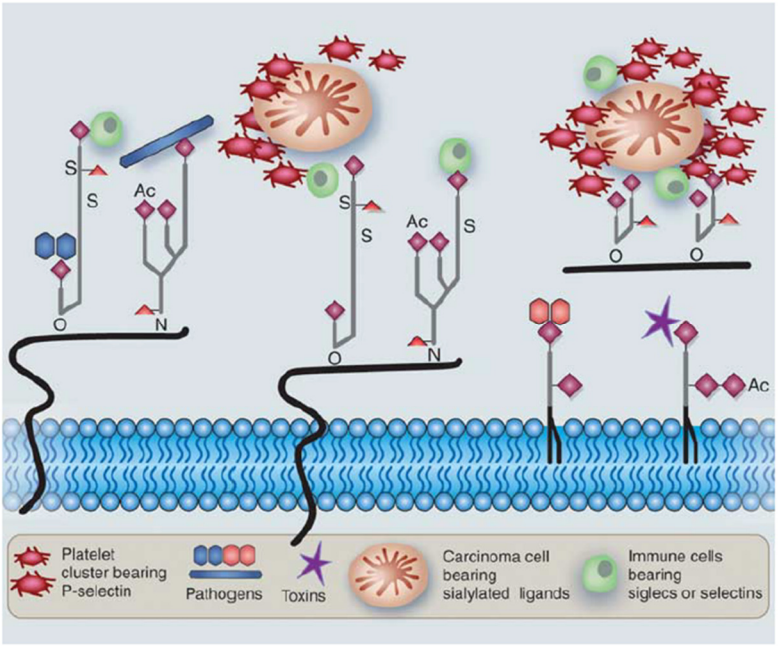


Figure 1.4 ***Examples of pathobiological interactions involving sialic acids***
(Varki N. M. and Varki A., 2007)

Moreover, an enhanced expression of terminal α2-6-linked Sias on cell surface N-linked glycans and of Sialyl-Lewis X on O-linked glycans often correlates with poor prognosis of many human malignancies. This may relate to the effects of α2-6-linked sialic acids on integrin function (Seales

EC. *et al*, 2005). In the case of Sialyl-Lewis X expression, it appears that tumor cells use this selectin ligand to facilitate interactions with the selectins. Tumor emboli and microthrombi result when carcinoma selectin ligands on carcinoma cells interact with selectins on platelets, innate immune cells and endothelium, serving to propagate metastases (Varky NM. and Varky A., 2002; Laubli H. *et al*, 2006). The serendipitous finding that some clinically approved heparins can block many of these selectin-mediated processes at clinically acceptable levels might explain why heparins reduce the incidence of metastasis, when utilized during the 'window of therapeutic opportunity' (Varky NM. and Varky A., 2002; Laubli H. *et al*, 2006; Stevenson JL. *et al*, 2005).

Gangliosides are glycolipids carrying sialic acids, which are found in all tissues and cell types, but are particularly enriched incells of neuroectodermal origin. Malignant melanomas express very high levels of the disialoganglioside GD3 as well as modified 9-O-acetylated and 5-N-deacetylated forms of this ganglioside (Cheresh DA. *et al*, 1984; Kohla G. *et al*, 2002; Chammas R. *et al*, 1999). There is evidence that whereas GD3 enhances apoptosis, 9-O-acetyl-GD3 has the opposite effect (Malisan F. *et al*, 2002) (Figure 1.4).

1.1.4 *Sialic acid biosynthesis and degradation*

In view of the different structures of sialic acids and especially of the various biological roles attributed to these sugars, enzymes involved in the metabolism of sialic acids assume great significance.

Several of the sialic acid-metabolizing enzymes are listed in Table 1.1.

Table 1.1 ***Sialic acid-metabolizing enzymes.***
(Achyuthan KE. and Achyuthan AM., 2001)

Enzyme	Catalytic activity/function
Neu5Ac aldolase	Sialic acid synthesis from pyruvic acid and *N*-acetyl-D-mannosamine
Sialyl transferase	Transfers sialic acid to polysaccharides, glycoproteins or glycolipids. Also transfer of nucleotide-linked sialic acid to glycoconjugates
Esterase	Hydrolyzes *O*-acetyl groups in sialic acid
Endosialidase	Hydrolyzes sialic acid linkages internal to molecules
Trans-sialidase	Transfers sialic acid from polysaccharides to water or with greater preference to glycan chains
Sialidase L	Releases 2,7-anhydro-Neu5Ac from Neu5Ac-α2−3-galactose linkages
KDNase	Releases KDN residues from sialoglyco-conjugates; some can also hydrolyze α-ketosides of Neu5Ac
Bi-functional enzyme	Hemagglutinin-sialidase of paramyxoviruses; capable of hemagglutinin and sialidase activity
Exo-α-sialidase	Hydrolyzes terminal sialic acid in glycoconjugates

The reactions of sialic acid synthesis and degradation are distributed among different cell compartments (Figure 1.5). The *N*-acetylneuraminic acid (Neu5Ac) is synthesized from *N*-acetylmannosamine-6-phosphate and phospoenolpyruvate in the cytosol by the catalytic activity of Neu5Ac aldolase. After dephosphorylation of the reaction product, Neu5Ac-9-phosphate, the molecule is activated in the nucleus by the transfer of a cytidine monophosphate (CMP) residue from cytidine triphosphate (CTP) through CMP-Neu5Ac synthase. This sugar nucleotide is the only natural case of a *β*-linkage between sialic acid and another compound, because in glycoconjugates an α linkage is always present. CMP-Neu5Ac is then translocated either into the Golgi apparatus or the endoplasmic reticulum (Schauer R., 1991; Kelm S. and Schauer R., 1997). There, the transfer of the activated sialic acid (nucleotide-linked sialic acid) to the oligosaccharide chain of a nascent glycoconjugate is accomplished by sialyltransferases.

Then sialic acid can be modified by *O*-acetylation or *O*-methylation before transport of the mature glycoconjugate to the cell surface, whereas the only modification taking place before the transfer on the glycoconjugate is the hydroxylation of the *N*-acetyl group of CMP-Neu5Ac, which leads to CMP-Neu5Gc in the cytosol (Traving C. and Schauer R., 1998).

The key enzymes of sialic acid catabolism are sialidases, exoglycosidase that hydrolyze the glycosidic linkage between sialic acid molecules and the penultimate sugar of the carbohydrate chains of oligosaccharides and glycoconjugates. Sialic acid residues can be removed from cell surfaces or serum sialoglycoconjugates by membrane-bound sialidases.

Usually, the glycoconjugates that are prone to degradation are taken up by receptor-mediated endocytosis in higher animals. After fusion of the endosome with a lysosome, the terminal sialic acid residues are removed by lysosomal sialidases. In contrast, the corresponding microbial enzymes are mostly secreted to get into contact with their substrates in the environment. Esterases hydrolyze the *O*-acetyl groups in sialic acids (Klein A. and Roussel P., 1998). A prerequisite for the effective action of sialidases is the removal of *O*-acetyl groups by the hydrolytic action of *O*-acetyl esterases (Schauer R. *et al*, 1997), whereas bound Neu5Gc is a fairly good substrate. Free sialic acid molecules (Neu5Ac or Neu5Gc) are transported through the lysosomal membrane into the cytosol, from where they can be recycled by activation and transfer onto another nascent glycoconjugate molecule in the Golgi. Alternatively, they can be retransferred onto glycoconjugates by sialyltransferases after activation to CMP-Neu5Ac or further degraded to acylmannosamine and pyruvate by acylneuraminate lyases.

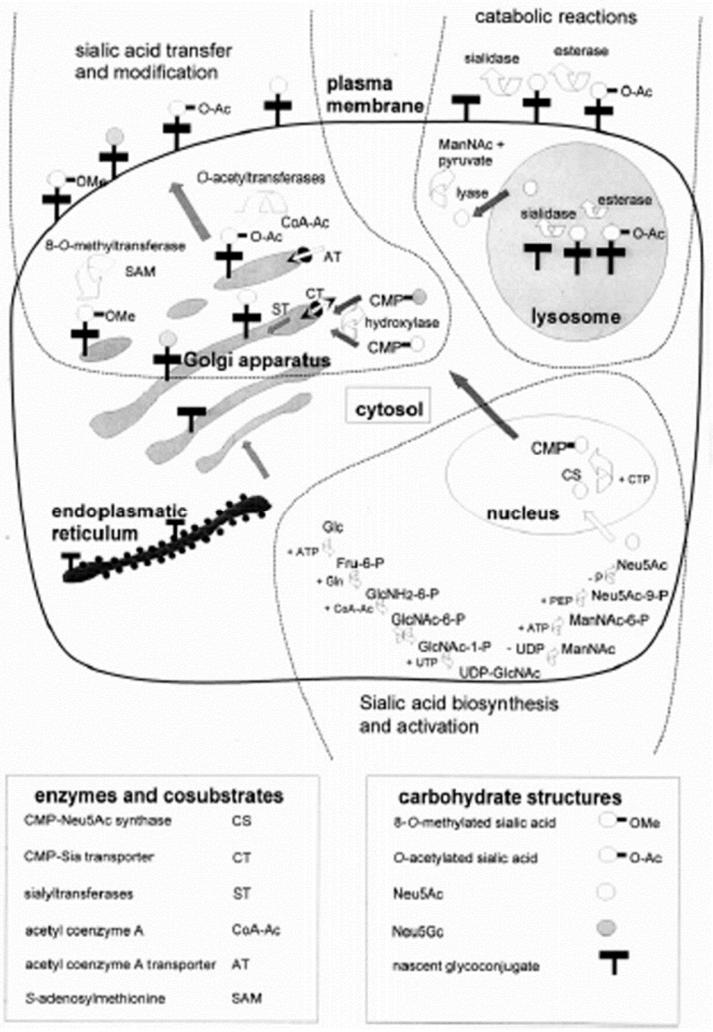


Figure 1.5 ***Metabolism of sialic acids.***
(Traving C. and Schauer R., 1998)

1.2 Sialidases (EC 3.2.1.18)

Hirst (1941) first discovered sialidase activity while investigating red cell agglutination in the presence of influenza virus. Sialidases have been called 'neuraminidases' or RDEs (receptor-destroying enzymes), because they were first described in viruses (Burnet FM. *et al*, 1946). The terms 'sialidase' and 'neuraminidase' were first proposed, respectively, by Heimer, Meyer and Gottschalk (Heimer R. and Meyer K., 1956; Gottschalk A., 1957). Currently, 'sialidase' is deemed appropriate to describe eukaryotic enzymes, whereas 'neuraminidase' the prokaryotic enzymes (Cabezas JA. *et al*, 1983).

Sialidases or neuraminidases [EC 3.2.1.18] are glycosidases that catalyze the removal of a glycosidically linked sialic acid residues from carbohydrate groups of glycoproteins and glycolipids.

They are widely distributed in metazoan animals and also in viruses and microorganisms including fungi, protozoa and bacteria. Perhaps the most widely studied sialidase is the enzyme from influenza virus, which is involved in viral replication and released from infected cells. The studies on influenza virus neuraminidases led to the development of specific inhibitors approved for therapeutical use in the treatment of human influenza (Gubareva LV. *et al*, 2000).

1.2.1 Sialidase gene evolution

Sialidases have been extensively investigated, especially in microbial species. Primary structure data enable to hypothesize the evolution of these enzymes. Close contact between the partners exchanging genetic information would be necessary, as is the case in host-parasite interactions for gene transfert. Possible vehicles for this process are viruses. It is also possible that free DNA molecules can be taken up by several naturally transformable bacterial species by the process of transfection. For some strains of *Salmonella typhimurium* it has been shown that sialidase genes are spread between strains of this species by horizontal gene transfer (Hoyer LL. *et al*, 1992).

The alignment of sialidase primary structures (Roggentin P. *et al*, 1993) reveals that, apart from some conserved regions, the overall homology between the sequences is low. The conserved areas point to a single evolutionary origin of sialidases. The percentage of identical amino acids between each pair of sequences can be calculated and the values used to construct a dendrogram of sialidase relationships. For example, it can be seen that clostridial sialidases form a group of closely related enzymes. The highest degree of similarity was found between the 'small' sialidase isoenzyme from *C. perfringens* (Roggentin P. *et al*, 1988) and the enzyme from *C. sordellii* (Rothe B. *et al*, 1989), whereas the close relationship between the 'large' isoenzyme from *C. perfringens* (Traving C. *et al*, 1984) and the one from *C. septicum* (Rothe B. *et al*, 1991), is expected, as suggested by 16S ribosomal RNA (rRNA) analysis.

It has been hypothesized (Traving C. *et al*, 1997) that the genetic information for sialidases was spread by horizontal gene transfer at two time points in evolution. Genes that were acquired by microbial species at an earlier time are well adapted to their environment, as demonstrated by their broad substrate specificity and their secretion into the medium to make contact with their substrates (e.g. the 'large' isoenzyme from *C. perfringens*). On the other hand, sialidase genes that have been transferred during a more recent event reveal a limited substrate specificity and have not been fully adapted to their new 'host' because they still lack a signal for excretion of the enzyme proteins (e.g. the 'small' sialidase isoenzyme from *C. perfringens*).

1.2.2 Sequence and three-dimensional structure of sialidases

The alignment of sialidase primary structures shows the existence of conserved regions. The most important conserved sequence and structure motif is the so-called Asp-box, which is a short repeat motif with consensus sequence distinguishing bacterial from influenza neuraminidases (**S**-X-**D**-X-**G**-X-**T**-**W,** where X indicates any amino acid) (Roggentin P. *et al*, 1993; Roggentin P. *et al*, 1989). The name is due to the presence of an aspartic acid residue. The five different residues are conserved to different degrees. This motif is found four to five times throughout all microbial sequences,

(Crennell SJ. *et al*, 1993) three to four times in mammalian sequences, whereas in viral sialidases it is only found once or twice or is even absent. The third Asp box is more strongly conserved than Asp boxes 2 and 4. Interestingly, the space between two sequential Asp boxes is also conserved between different primary structures.

Asp boxes have been detected in a number of protein families from bacterial ribonucleases to reelin, netrins, sulfite oxidases, and some lipoprotein receptors, as well as in a series of GHs (Copley RR. and Russell RB., 2001). The Asp boxes do not participate in catalytic action. Increasing insight into the three-dimensional structure of sialidases confirm a simple structural role (Crennell SJ. *et al*, 1993).

In the N-terminal part of the amino acid sequences there is the 'FRIP' motif (X-**R**-X-**P**). In this motif, the arginine and the proline residues are absolutely conserved. In particular, the arginine is directly involved in catalysis by binding of the substrate molecule (Chong AK. *et al*, 1992). Also important for the catalytic reaction of sialidases is a glutamic acid-rich region, which is located between Asp boxes 3 and 4, as well as two further arginine residues.

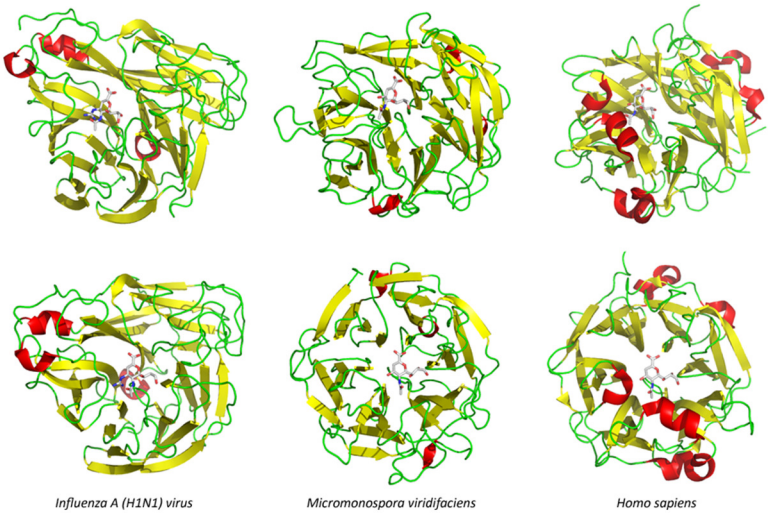


Figure 1.6 ***Comparison between crystallographic structure of viral, bacterial and mammalian sialidases.***
Ribbon diagrams of *Influenza A (H1N1) virus*, *Micromonospora viridifaciens* and human NEU2 sialidases in complex to inhibitors mimicking sialic acid (upper panel: lateral view; pannel below: top view).

The enzyme is mainly β-sheet with two small α-helical segments, with a shallow active site crevice. In particular, the secondary structure consists of six four-stranded antiparallel β-sheets arranged as the blades of a propeller around an axis passing through the active site, forming a β-barrel structure (Crennell SJ. *et al*, 1993)(Figure 1.7).

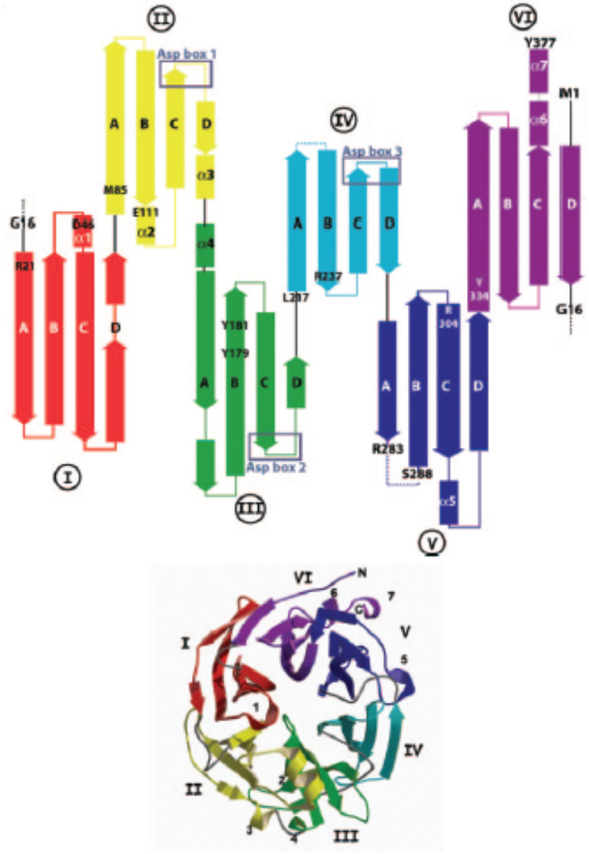


Figure 1.7 ***Structure of human sialidase NEU2.***

In the upper panel, topology diagram representing the arrangement of the Neu2 secondary structure elements. Arrows representβ-strands and rectangles α-helices.The six blades of the β -propeller are coloredin the same way as in a. Residues of the active site are labeled in bold black letters. Asp boxes are highlighted by blue rectangles. In the panel below, ribbon diagram of Neu2, viewed into the active site. Individual six blades (I–VI) of the β-propeller are colored differently. Loops between the blades are shown in gray (Chavas LMG. *et al*, 2005).

In human NEU2 the three Asp boxes are grouped far away from the active site, closer to the acidic cleft opening on the opposite part of the β-propeller (Chavas LM. *et al*, 2005) (Figure1.8).

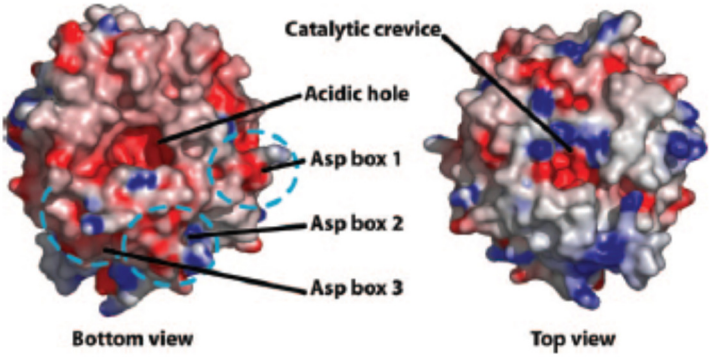


Figure 1.8 ***Electrostatic Surface of Neu2.***
Molecular surface representation of Neu2 colored according to the electrostatic potential, the bottom (left), and catalytic side (right). Positive and negative potential are indicated by blue and red surfaces, respectively, over the range from 10 to -10 kBT. Asp boxes are marked by blue dotted ovals (Chavas LMG. *et al*, 2005).

The Asp boxes are located at equivalent positions on the surface of the protein monomers from the different species, namely at the turn between the third and fourth strands of the first four sheets. Their aromatic residues form a hydrophobic core stabilizing the turn, whereas the aspartic acid residues point to the solvent. Thus, they might represent contact sites between subunits of the protein. On the other hand, there are only one or two Asp boxes in tetrameric influenza virus sialidases. In agreement with the location of these motifs on the surface of the enzyme, another possible role for these regions might be involvement in the process of enzyme secretion. The carboxylate group of the substrate is stabilized by three arginine residues in the catalytic crevice (Crennell SJ. *et al*, 1996). One of them is part of the FRIP region.

1.2.3 The active site and the catalytic mechanism of sialidases

The elucidation of the catalytic mechanism of sialidases is very important because these enzymes are involved in the pathogenesis of several infectious diseases and the development of inhibitors is of great importance in medicine and pharmaceutical industry for example as potential drugs against influenza infections (von Itzstein M. *et al*, 1993).
Some insight into the catalytic mechanism of these enzymes can be gained from the analysis of the corresponding primary and three-dimensional structures. These data, together with mutagenesis experiments (Roggentin T. *et al*, 1992), provide enough information to develop a model of the catalytic process (Crennell SJ. *et al*, 1993; Chong AK. *et al*, 1992).
Observing the crystal structure of human sialidase NEU2 solved in complex to the DANA (2-deoxy-2,3-dehydro-N-acetylneuraminic acid) inhibitor, a molecule mimicking the sialic acid in the catalytic crevice, a dynamic nature of substrate recognition has been inferred (Figure 1.9). The two loops of human NEU2, that are disordered in the apo-form, become ordered into two short α-helices, covering the inhibitor upon binding of DANA (Chavas LM. *et al*, 2005).

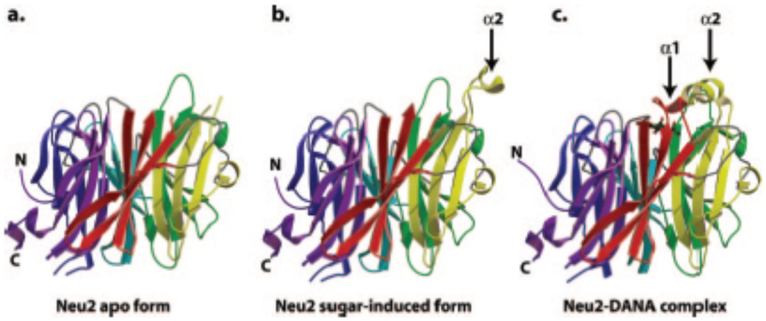


Figure 1.9 ***Structural changes of NEU2 upon maltose and DANA binding.***
(a) Ribbon diagram of NUE2 apo form, viewed from the side. The active site is located on the top part of the protein. (b) NEU2 sugar-induced form. The arrow indicates the loop that becomes ordered and forms helix α2. (c) NEU2-DANA complex. DANA is represented as a ball-and-stick model. The arrows indicate two helices (α1 and α2) that are formed upon inhibitor binding. (Chavas LMG. *et al,* 2005)

The overall structure of the catalytic site is preserved. There is a strictly conserved arginine triad, that aids in the positioning of the carboxylate group of DANA within the active site region, as well as threeother residues directly involved in catalysis: a Tyr/Glu nucleophile pair and an aspartic acid acting as the acid/base catalyst. Whereas the latter can be most likely identified with D46, located on the a1 mobile loop that partially covers the transition state analogue in NEU2/DANA structure, only one member of the nucleophile pair, namely Y334, could be identified in the inner part of the active site, close to C2 of DANA molecule. A superimposition of NEU2 active site with those of *Micromonospora viridifaciens* and *Influenza A virus 1918/H1N1* allows the identification of a conserved aspartic acid residue (E218), which could represent the second member of the nucleophile pair located below the DANA ring in exosialidases (Mozzi A. *et al*, 2012) (Figure 1.10).

The N-acetyl and glycerol moieties of DANA inhibitor are recognized by NEU2 residues not shared by bacterial sialidases and viral neuraminidases, which can be regarded as a key structural difference for potential drug design against bacterial, influenza and other viruses.

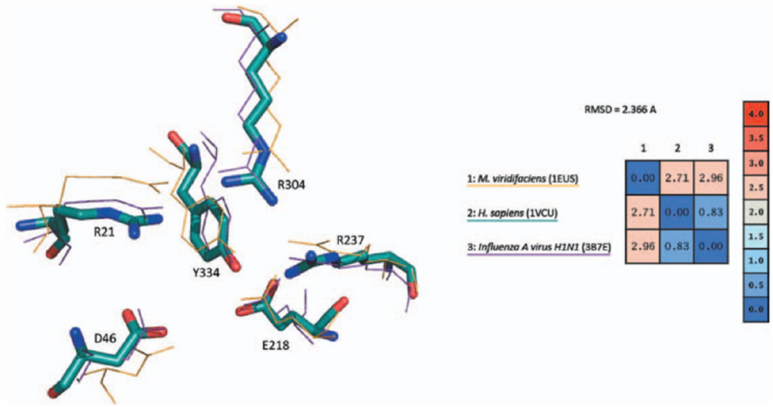


Figure 1.10 ***Superimposition of the active site of sialidases.***
Human sialidase NEU2 (stick model, cyan), *M. viridifaciens* sialidase (lines, orange) and Influenza A virus H1N1 sialidase (lines, purple) superimposition was calculated by overlapping the ligand coordinates of the three complexes. RMSD calculation was performed by MOE software (Mozzi A. *et al*, 2012).

While much of the information regarding the catalytic mechanism has come from studies of non-mammalian sialidases, some general features of the reaction are common to all exo-α-sialidases (Smith LE. and Eichinger D., 1997).

The recognition portion of the substrate (sialic acid) probably binds initially to the enzyme active site in a boat configuration. Depending on the source of the sialidase, the reactive substrate configuration of the sugar following binding could vary from boat, twist-boat, flattened-boat, chair, flattened-chair, or half-chair. Sugar distortion appears to be a common feature for all sialidase catalysis studied thus far. The enzyme catalysis process has four steps. The first involves the distortion of the α-sialoside from a 2C5 chair conformer to a boat conformer when the sialoside binds to the sialidase. In the second step the glycosidic oxygen is protonated, leading to a partial positive charge at carbon atom 2 of the sialosylcation (oxocarbonium ion intermediate), and a tetrahedral transition complex is thus formed. A water molecule, or an activated hydroxonium ion (H_3O^+), serves as the nucleophilic proton donor to the positive charge at C-2, necessary for the hydrolysis reaction. The final two steps of the enzyme mechanism are the formation and release of Neu5Ac from the active site of the sialidase. Neu5Ac is initially released as the α-anomer. It is conceivable that expulsion of product from the active site is favored by the mutarotation of the α-anomer to the thermodynamically more stable β-anomer for Neu5Ac in solution (Taylor NR. and von Itzstein M., 1994).

1.2.4 Sialidase enzymatic features

Exo-α-sialidases cleave the terminal, non-reducing sialic acid residue from glycoproteins, oligosaccharides, polysaccharides, glycolipids, gangliosides, and synthetic glycosides. The ketosidic bond linking sialic acid to various sialidase substrates has the α-D configuration and is equatorial to the pyranose ring. Only α-ketosides are susceptible to sialidase attack as demonstrated by Meindl and Tuppy (Achyuthan KE. and Achyuthan AM., 2001). Perhaps the only naturally occurring linkage of ketosidically bound Neu5Ac that is not hydrolyzed by sialidase is cytidine-5'-monophosphate-

Neu5Ac. Optical studies showed that the anomeric carbon in this compound was in the β-configuration (Comb DG. *et al*, 1966). When released by sialidase catalysis, the α-D-sialic acid anomer rapidly undergoes mutarotation in solution to produce β-D-sialic acid. A small portion of sialic acid in solution exists in the thermodynamically unfavorable open-chain form. The susceptibility of a sialic acid residue to sialidase attack is greatly influenced by adjacent residues, usually a sugar such as galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, another sialic acid, or an aglycone 'reporter' group in the case of synthetic substrates. While α2-3 and α2-6 linkages of sialic acid are preferentially cleaved by sialidase, other types of linkages are known, including α2-4, α2-9 (Huang RT. and Orlich M., 1972) and α2-8 in polysialic acids (Roth J. *et al*, 1992). Internal sialic acids are generally resistant to the action of *exo*-α-sialidase, presumably due to steric hindrance. In general, the rates of human exo-α-sialidase cleavage are as follows: α2-3 > α2-6 > α2-8. As with the chemical structure of the substrate, the susceptibility of a glycosidic linkage to hydrolysis by sialidases varies with enzyme source.

The presence of a free carboxyl group in sialic acid is essential for sialidase catalysis. Methyl esters of sialic acid are resistant to sialidase attack (Yu RK. and Ledeen R., 1969). Modification of the hydroxyl groups of sialic acid by various *O*-substitutions affects reactivity. For example, 4-*O*-acetylation of sialic acid renders it resistant to bacterial and mammalian (including human) sialidases, but not to viral sialidases. This may be because the C-4 binding pockets in bacterial and mammalian sialidases active sites are considerably smaller compared to viral sialidases (Holzer CT. *et al*, 1993). As stated above, substitution of an -OH residue at C-5, instead of the usual *N*-acetyl or *N*-glycolyl groups, resulted in KDN and KDN-glycosides that are resistant to the action of *exo*-α-sialidases. Changes in the 'natural' nitrogen group substituent of sialic acid, from *N*-acetyl or -glycolyl to *N*-formyl, -succinyl, or -propionyl, also reduced the susceptibility to sialidase hydrolysis (Brossmer R. and Nebelin E., 1996). Exo-α-sialidases generally hydrolyze the Neu5Ac linkage at a faster rate than the Neu5Gc linkage.

Various modifications to the glycerol side chains have been reported to be associated with resistance to sialidase hydrolysis. Shortening of the C7-C9 carbon chain resulted in resistance to sialidase activity. Inhibitors targeting

influenza virus sialidase, but not human or bacterial sialidases, were designed through such modifications (Taylor G., 1996; Holzer CT. *et al*, 1993; von Itzstein M. and Colman P., 1996; Wade RC., 1997). In addition, the nature of the aglycone has a profound influence on the susceptibility to, as well as the rates of, sialidase catalysis. Changes to the pH optimum and/or kinetic parameters (K_m, V_{max} , and k_{cat}) of sialidases are common, depending on the chemical nature of the aglycone.

The divalent cation requirements for sialidases vary with source, and Ca^{2+} is considered as either essential for sialidase activity, or at least capable of stimulating it. Several membrane-bound sialidases were reported to be stimulated by the presence of detergents, or a physiologic activator (Fingerhut R. *et al*, 1992). Multi-protein complexation is essential for the stable expression of placental sialidase. Chelators (ethylen-ediaminetetraacetic acid, EDTA) and metals (Cu^{2+} and/or Zn^{2+}) were reported to inhibit sialidases from certain sources, but not others. Free sialic acid is only a weak inhibitor of sialidases (IC_{50} = 1-2 mM). Transition state analogs of sialic acid have been modeled to generate potent inhibitors of influenza virus sialidases.

1.2.5 Sialidases in diseases

Viral and bacterial sialidasesare implicated as virulence factors in a range of diseases.

The influenza type A and B viruses have two surface glycoproteins: hemagglutinin (HA), which recognizes sialic acid for attachment but is also involved in the fusion of viral and cell membranes, and neuraminidase. The role of the neuraminidase is to process progeny virus particles when they bud from an infected cell, removing viral sialic acids to halt self-agglutination of viruses. The neuraminidase forms tetramers on the viral surface, anchored by N-terminal transmembrane regions (Parks GD. and Pohlmann S., 1995).

Sialidases are produced by a wide range of bacteria, and are often one of several virulence factors secreted by bacteria involved in important diseases (Corfield T., 1992; Tang HB. *et al*, 1996; Dwarakanath AD. *et al*,

1995). Many pathogenic and nonpathogenic sialidase-producing bacteria can use sialic acid as a carbon and energy source, and possess both permeases to transport the sugar inside the cell, and the enzymes for its catabolism. In certain cases the enzyme also has a defined role in disease. For example, *Vibrio cholerae* sialidase removes sialic acid from higher order gangliosides to create GM1, the binding site for cholera toxin (Galen JE. *et al*, 1992).

Bacterial sialidases vary in size from 40kDa to 120kDa. Most exist as monomers, but higher oligomeric states have been reported. Most are secreted as soluble proteins, others are tethered to the bacterial surface as in *Streptococcus pneumoniae* (Camara M. *et al*, 1994), and some are not secreted as the small sialidase of *Clostridiurn perfringens* (Roggentin P. *et al*, 1995). Bacterial sialidases share little sequence identity, typically 30%, but contain two conserved sequence motifs: the first is the RIP/RLP motif (Arg-Ile/Leu-Pro), the second is the Asp-box motif (Ser/Thr-X-Asp-[X]-Gly-X-Thr-Trp/Phe; where X represents any amino acid), which can occur several times along the chain (Roggentin P. *et al*, 1989).

Several pathogenic bacteria and various tumour cells express poly-α2-8-linked sialic acid on their surfaces. One such bacterium is *Escherichia coli* K1, which can cause high mortality rates in cases of neonatal meningitis. An endosialidase that binds to and hydrolyzes such polysialic acid substrates has been isolated from bacteriophage K1E and K1F (Long GS. *et al*, 1995; Petter JG. and Vimr ER., 1993). The enzyme is a trimer of 74kDa monomers that contains two Asp boxes. This enzyme may find a use in the diagnosis and therapy of K1 meningitis.

Mammalian sialidases, unlike viral and bacterial ones, do not represent a virulent factor in deseases. Their pathological effect is evident in two genetic diseases due to the loss of sialidase activity: sialidosis and galactosialidosis.

Sialidosis is an inherited, autosomal, recessive lysosomal storage disease associated with lysosomal acid sialidase deficiency and subsequent lysosomal storage. There are two major clinical manifestations: sialidosis type I (non-dysmorphic, late, adult onset) and type II (early, infantile onset); the latter is the more severe condition and is manifested as coarse face, myoclonus, mental retardation, cherry-red spots, psychomotor

retardation, bone abnormalities, progressive neurological disorders, vacuolated lymphocytes and hepatosplenomegaly, with severe cases being fatal (Lowden JA. and O'Brien JS., 1979).
Galactosialidosis is an autosomal, recessively inherited, lysosomal storage disease, showing depressed levels of sialidase activity in affected patients (Okamura-Oho Y. *et al*, 1994; Suzuki Y., 1995; Rudenko G. *et al*, 1995). Like sialidosis, patients with galactosialidosis accumulate and excrete large amounts of a complex carbohydrate mixture of glycopeptide fragments rich in sialic acid. In galactosialidosis there is a combined deficiency of sialidase and β-galactosidase (Lowden JA. and O'Brien JS., 1979).
Only in the past few years many of scientific papers pointed out the potential involvement of mammalian sialidases in the occurrence of various kind of tumors. This role will be describe in depth in the following sections.

1.2.6 Mammalian sialidases

Whereas in microorganisms sialidases are likely to function for nutritional purposes and in the processes of adhesion to and invasion of host cells, in mammals they have been implicated not only in lysosomal catabolism but also in the modulation of functional molecules involved in many biological processes (Corfield T., 1992; Miyagi T. and Yamaguchi K., 2007; Miyagi T. *et al*, 2004). Four types have been identified and characterized to date, namely NEU1, NEU2, NEU3, and NEU4. They are encoded by different genes and differ in major subcellular localization and enzymatic properties, including substrate specificity (Monti E. *et al*, 2002). The first three are localized predominantly in the lysosomes, cytosol, and plasma membranes, respectively, and the fourth sialidase, NEU4, has been suggested to exist in lysosomes, or in mitochondria and endoplasmic reticulum (Bonten E. *et al*, 1996; Milner CM. *et al*, 1997; Pshezhetsky AV. *et al*, 1997; Bigi A. *et al*, 2010).
The charactheristics of these types of sialidase are listened in Table 1.2.
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.

Observation of their primary structure reported in Figure 1.11 shows that all isoforms contain several Asp boxes (-Ser-X-Asp-X-Gly-X-Thr-Trp-) and the Arg-Ileu-Pro conserved sequence, also found in microbial sialidases (Roggentin P. *et al*, 1989). NEU1 features a possible lysosomal C-terminal targeting motif (YGTL), NEU3 possesses long hydrophobic stretch as a putative transmembrane domain (pTM), and NEU4 consists of two isoforms differing in the presence of 12 N-terminal amino acid residues (Mt), which may act in mitochondrial targeting (Miyagi T. *et al*, 2008).

Table 1.2 ***Enzymatic features of mammalian sialidases.***
(Miyagi T., 2008)

	Neu1	Neu2	Neu3	Neu4
Major subcellular localization	Lysosomes	Cytosol	Plasma membrane	Lysosomes[41] Mitochondria[9] Intracellular membranes[9,42]
Good substrates	Oligosaccharides Glycopeptides	Oligosaccharides Glycoproteins Gangliosides	Gangliosides	Oligosaccharides Glycoproteins Gangliosides
Optimal pH	4.4–4.6	6.0–6.5	4.6–4.8	4.4–4.5
Total amino acids				
(human)	415	380	428	496 (484)
(mouse)	409	379	418	478
Chromosome location				
(human)	6p 21.3	2q 37	11q13.5	2q37.3
(mouse)	17	1	7	10
Possible function	Degradation in lysosomes	Myoblast differentiation	Neural differentiation	Apoptosis
	Immune function Elastic fiber assembly	Neural differentiation	Apoptosis Adhesion	

Regarding comparative expression levels of human sialidases, NEU1 generally shows the highest expression, 10–20 times higher than those of NEU3 and NEU4, while NEU2 expression is extremely low, only four- to tenthousandth of NEU1 level at most in a wide range of tissues, as assessed by quantitative real time RT-PCR using a standard curve for each cDNA, although these profiles differ among the human, rat and mouse (Yamaguchi K. *et al*, 2005; Miyagi T., 2008). Although many functional

aspects are not fully understood, recent progress in gene cloning has facilitated elucidation of important biological roles such as involvement in events contributing to cell differentiation, cell growth, and apoptosis.

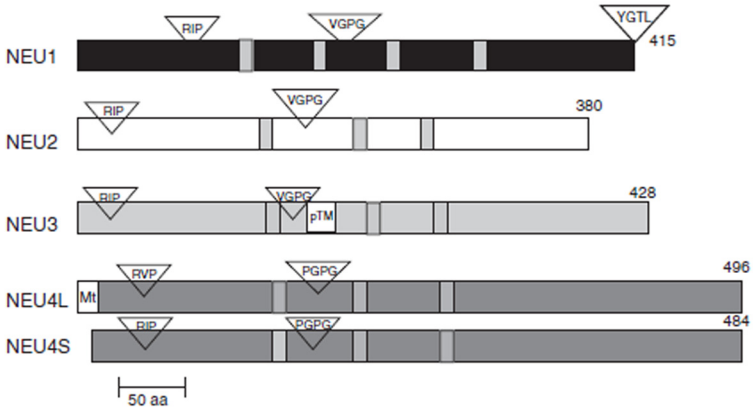


Figure 1.11 ***Schematic representation of primary structure of human sialidases.***
(Miyagi T. *et al*, 2008)

1.3 The plasma membrane-associated sialidase NEU3

1.3.1 General features

The membrane-associated sialidase NEU3 was first cloned from bovine brain in 1999, as a plasma membrane-associated sialidase specific for gangliosides (Miyagi T. *et al*, 1999).
The human homologue of NEU3 was identified by Monti *et al*. in 2000, starting from an expressed sequence tag (EST) clone. NEU3 gene maps to chromosome 11q13, and is organized in three exons, with the ATG codon located in the second one. The cDNA encodes a 428 aa protein, showing an high sequence identity (78%) with the membrane–associated sialidase recruited in *Bos Taurus*.

The primary structure of NEU3 human homologue reveals the presence of the canonical FRIP motif and three Asp-boxes, typical of sialidases and also of a Caveolin binding domain and a pYXNX motif, recognized by Grb-2 SH2 domain, as shown in Figure 1.12 (Miyagi T. *et al*, 2008).

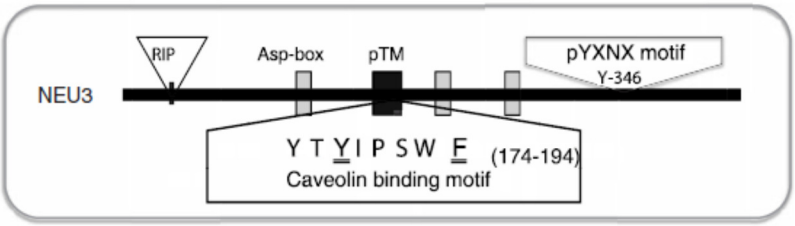


Figure 1.12 ***Schematic representation of binding motifs of NEU3.***
(Miyagi T. *et al*, 2008)

NEU3 was subsequently characterized at the molecular level from various animal species and was confirmed, at least by *in vitro* enzyme assays, to be highly specific for gangliosides (Table 1.3) (Monti E. *et al*, 2002; Oehler J. *et al*, 2002; Ha KT. *et al*, 2004). Unlike the bovine and the murine enzyme with only one activity optimum at a pH near 4.6, human NEU3 shows two optima at pH 4.5-4.8 and at 6.0-6.5 (Wada T. *et al*, 1999).
Indeed a detailed kinetic characterization of the enzyme was carried out by transient transfection studies, using cDNAs encoding highly homologous plasma membrane-associated sialidase from various mammalian species.
Sialidases recognize gangliosides other than GM1 as preferential substrates, GD3 being the best one, followed by GM3, GD1a, GD1b, and GT1b, and thus act on both sialyl linkages of α2-3 and α2-8.
Interestingly, whereas NEU3 from *B. taurus* and *H. sapiens* hardly recognized GM2, the corresponding enzymes from rodents showed a considerable activity on this ganglioside substrate.
Human homologue shows only 1/20th of the activity observed toward GD3.
All the mammalian enzymes hardly act on sialoglycoprotein fetuin. (Miyagi T. *et al*, 1999; Wada T. *et al*, 1999; Hasegawa T. *et al*, 2000; Hasegawa T. *et al*, 2001).

These differences in substrate specificities are related to very similar proteins in terms of amino acid sequences, with similarity values up to 89%, which show the same subcellular localization and probably have the same biological function within the cell. This suggests that the observed differences reflect variation of substrate levels *in vivo* and/or the involvement of the enzyme(s) in species-specific metabolic network(s).

Table 1.3 ***Sialidase Activities toward various substrates in the homogenate of COS cells transfected with NEU3cDNAs from different mammalian species.***
(Monti E. *et al*, 2002)

	Hydrolysis relative to GD3 (%)			
Substrates	Hs Neu3	Mm Neu3	Rn Neu3	Bt Neu3
GD3	100	100	100	100
GD1a	79	49	75	76
GD1b	52	NA	NA	64
GT1b	NA	NA	NA	57
GM3	84	86	85	97
GM2	2	14	20	ND
GM1	NA	NA	NA	ND
Fetuin	1	2	4	2
Sialyllactose α(2-3)	5	30	17	13
4MU-NeuAC	11	13	27	7

Note: The reaction mixture contained up to 50 nmol of substrate as bound sialic acid, 0.2 mg of bovine serum albumin, 10 mmol of sodium acetate buffer, pH 4.6, and 0.2 mg of Triton X-100 in a final volume of 200 ml. After incubation of 37°C for up to 30 min, released sialic acid was measured with the thiobarbituric acid method (Miyagi, T., and Tsuiki, S. 1985. Purification and characterization of cytosolic sialidase from rat liver. J. Biol. Chem. 260:6710–6716).
Sialidase activity toward 4-methylumbelliferyl-neuraminic acid (4MU-NauAc) was assayed by spectrofluorometrical measurement of 4-methylumbelliferone (4 MU) released.
ND, Not detectable.
NA, Not assessed

1.3.2 Subcellular localization and membrane anchoring mechanism

The puzzling mechanism of NEU3 anchorage to the membrane has been studied in COS7 and HeLa cells, overexpressing murine NEU3. In these cells the enzyme behaves as a peripherally associated membrane protein, present in both the plasma membrane and the membranous structures corresponding to the recycling endosomal compartment, from which it can be released by treatment with carbonate (Zanchetti G. *et al*, 2007).
Recently the association of NEU3 with lipid raft markers was demonstrated (Kalka D. *et al*, 2001). Lipid rafts are small (10–200 nm), heterogeneous, highly dynamic platforms or aggregates resulting from the preferential packing of some proteins, cholesterol, and sphingolipids (namely ceramide, sphingomyelin, and gangliosides) that float within the liquid disordered bilayer of cellular membranes (Schmitz G. and Grandl M., 2008). These structure are involved in many cellular processes such as membrane trafficking, cell polarization and signaling, as well as in pathogen invasion, lipid homeostasis, angiogenesis, and neurodegenerative diseases (Simons K. and Toomre D., 2000; Simons K. and Ehehalt R., 2002).
Further experiments confirmed the co-fractionation with caveolin-1 in low-density Triton-X-100-insoluble membrane fractions for both the endogenous and overexpressing enzyme, using HeLa and COS1 cell, respectively (Wang Y. *et al*, 2002).
NEU3 was found not only in membrane microdomains associated with caveolin but also in tetraspanin-enriched microdomains (TERMs)(Odintsova E. *et al*, 2006); these are membrane proteins involved in membrane compartmentalization and dynamics that provide, through self-interactions, platforms for recruiting other proteins such as integrins, tyrosine kinases, and G-protein-coupled receptors into TERMs (Yunta M. and Lazo PA., 2003; Levy S. and Shoham T., 2005).
Metabolic labeling of the sphingolipids with tritiated sphingosine demonstrated that NEU3 overexpression led to a decrease of about one third of GM3 and GD1a ganglioside cell content, with a parallel 35% increase in ganglioside GM1.
Moreover, a mixed culture of NEU3-overexpressing cells with sphingosine-labeled cells (the latter acting as substrate carriers) showed that the

enzyme present at the cell surface was able to remove sialic acid residues from the oligosaccharide moieties of ganglioside exposed on the membrane of neighboring cells. Overall these results demonstrated that the enzyme on the cell surface modifies the ganglioside pattern not only of the cells where it resides (cis-activity), but also of adjacent cells (trans-activity), indicating a possible involvement of the enzyme in cell-to-cell interactions.

These results are further supported by the enrichment of the NEU3 at the plasma membrane sites corresponding to cellular contact regions (Figure 1.13).

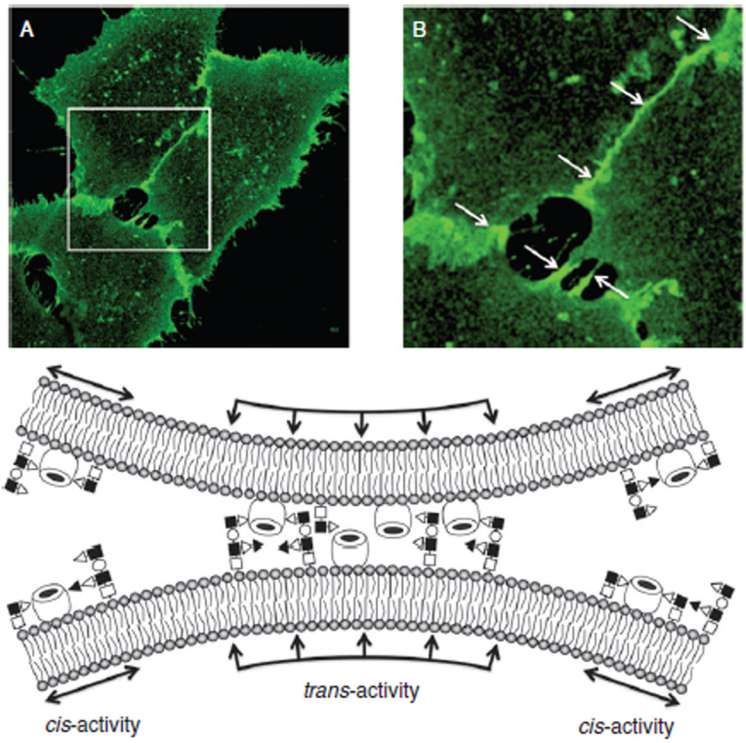


Figure 1.13 ***NEU3 localization at the membrane surface and in the cell-to-cell contact areas (***A-B***), and schematic view of cis/trans activity mechanism (***C***).***
(Monti E. et al, 2010)

1.3.3 Physiological roles

Considering its unique localization and substrate specificity, the biological action of sialidase NEU3 is exerted through its activity on gangliosides, wich are the main structural and functional components of the membrane microdomain. If NEU3 is regardered as the physiolocal regulator of the membrane structure, this enzyme can be thereby involved in all the pivotal cellulas processes involving the membrane, like trafficking, lipid

homeostasis, cell polarization, cell differentiation, pathogen recognition, angiogenesis, hormone sensing and apoptosis.

Murine NEU3 was demonstrate to regulate cell apoptosis in human fibroblasts by producing ceramide from GM3 in plasma membranes through further degradation of the sugar unit (Valaperta R. *et al*, 2006).

Biochemical evidences suggested an essential role of gangliosidesialidase in the processes of proliferation control and differentiation; NEU3 was indeed confirmed to participate positively in neurite formation in mice (Hasegawa T. *et al*, 2000) and in human neuroblastoma cells (Proshin S*. et al*, 2002), and in the regulation and regeneration of rat hippocampus neurons (Rodriguez GA. *et al*, 2001; Da Silva GS. *et al*, 2005).

NEU3 was confirmed to be also involved in skeletal muscle differentiation and apoptosis; in fact NEU3 knock-down in murine C2C12 cells inhibits myotube formation and sensitization to apoptotic stimuli through accumulation of GM3 subsequent to epidermal growth factor receptor blockage (Aureli M. *et al*, 2010). Moreover NEU3 was considered a physiological modulator of smooth muscle cell physiology because its expression led to reduced TNF-alpha-induced MMP-9 expression in vascular smooth muscle cells by decreasing MMP-9 promoter activity in response to TNF-alpha, contributing to plaque instability in atherosclerosis (Moon SK. *et al*, 2007).

In caveolae, NEU3 has been proposed to control PDGF-induced Src mitogenic signaling and DNA synthesis by modification of cell surface GM1 level (Veracini L. *et al*, 2008).

Moreover ganglioside GM3 depletion by NEU3 causes increased EGFR phosphorylation and inhibition of the EGF-induced tyrosine phosphorylation of caveolin-1, leading to activation of EGFR signaling by retention of caveolin-1 in caveolae (Wang Y. *et al*, 2002).

Human NEU3 has been found to contribute to the development of adaptive immune responses; it is up-regulated together with NEU1 during differentiation of monocytes into dendritic cells, and enhances LPS-induced production of cytokine including IL-6, IL-12p40, and TNF-alpha through alteration of the sialic acid content of specific cell surface glycoconjugates (Stamatos NM. *et al*, 2010).

Moreover the role of the sialidase NEU3 in the process of pathogen recognition and invasion has been confirmed.
Whereas the presence on the envelope of influenza viruses of the sialidase(s) or neuraminidase(s) is well-known, as well as the role in the virus penetration into cells, much less is known about the possible involvement in viral biology of the endogenous sialidase(s) associated to the surface of the mammalian cells.
Among the sialic acid-containing molecules acting as receptors, gangliosides have been reported to interact with Newcastle Disease Virus (NDV), a virus belonging to the *Paramyxoviridae* family, causative agent of a contagious bird disease that causes severe economic losses in industrial poultry breeding (Crennell S. *et al*, 2000). It has been demonstrated that an extensive modification of the cell-surface ganglioside pattern induced by the overexpression of NEU3 had no significant effect on NDV binding to the cells, whereas it caused a significant decrease of NVD infection, as well as viral propagation through cell-cell fusion (Anastasia L. *et al*, 2008). These results have shed light on the complex series of events governing virus-host cell interactions, and have suggested a role in this process of the endogenous enzymes on the host's plasma membrane, which are involved in the fine regulation of ganglioside levels.

1.4 Sialidase and cancer

Aberrant sialylation in cancer cells is thought to be a characteristic feature associated with malignant properties including invasiveness and metastatic potential. In fact, altered glycosylation of functionally important membrane glycoproteins may affect tumor cell adhesion or motility, resulting in invasion and metastasis (Yogeeswaran G. and Salk PL., 1981; Fogel M. *et al*, 1983; Passaniti A. *et al*, 1988).
In the last few years several observations on the alteration of endogenous sialidase activity in cancer cells suggested that these enzymes might be related totumorigenic transformation and tumor invasiveness.

In literature there are a lot of examples: Schengrund *et al.* (1973) described increased sialidase activity toward gangliosides in BHK transformed cells; Bosmann *et al.* (1974) observed elevated sialidase activity in human cancer tissues using fetuin as a substrate; Nojiri *et al.* (1982) reported that in the human promyelocytic leukemia cell line HL-60, stimulation of sialidase activity toward 4MU-NeuAc occurs during cell differentiation into granulocytes by retinoic acid or DMSO; again Yogeeswaran and Hakomori (1975) observed a loss of cell density-dependent suppression of membrane bound sialidase activity for gangliosides in 3T3-transformed cells. Since four types of mammalian sialidases exist differing in localization and biochemical properties, including substrate specificity,it was still uncertain whether the activities recorded were due to a single isoform, or to different sialidases. Advances in the molecular cloning of mammalian sialidases has facilitated elucidation of the molecular mechanisms and significance of these alterations. Using a differential assay procedure for each form, Miyagi *et al.* (1990) observed that, in rat hepatomas, intra-lysosomal and membrane-bound sialidase activities were elevated, whereas cytosolic sialidase activity was reduced, as compared with normal liver.

The four types ofsialidases identified to date (NEU1, NEU2, NEU3, and NEU4) were found to behave in different manners during carcinogenesis. Different sialidases have been observed to promote or oppose malignant phenotypes.

Sialidases are indeed closely related to malignancy and are thus potential targets for cancer diagnosis and therapy.

1.4.1 Sialidases NEU1 and NEU2 in cancer

A good inverse relationship between lisosomal sialidase NEU1 expression level and metastatic ability was found in mouse adenocarcinoma colon cells with different metastatic potentials (Miyagi T. *et al*, 1994; Sawada M. *et al*, 2002).

To investigate whether overexpression of NEU1 sialidase can reverse metastatic ability, rat lysosomal sialidase gene was introduced into Bl6-BL6

mouse melanoma variant subclone derived from B16 melanoma cells, known to be highly invasive and metastatic (Kato T. *et al*, 2001). As expected, sialidase overexpressing cells showed suppression of experimental pulmonary metastasis and tumor progression. NEU1 expressing transfectants exhibited reduced 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.

In the same cell lineit was also investigated how cytosolic sialidase NEU2 expression influences metastasis (Tokuyama S. *et al*, 1997). Intravenous injection of stable transfectants into syngenic mice resulted in a marked decrease of experimental pulmonary metastasis, invasiveness and cell motility but no change in cell growth or cell attachment to fibronectin, collagen type VI or laminin. Sialidase overexpression did not lead to any significant changes in cell surface or intracellular glycoproteins, while a decrease in ganglioside GM3 and an increase in lactosylceramide was assessed by thin layer chromatography.

When the sialidase gene was transfected into highly metastatic mouse colon 26 adenocarcinoma cells, changes in the sialyl Le^x level were observed, in addition to marked suppression of metastasis (Sawada M. *et al*, 2002). Stable transfection of NEU2 in NL17 cells showed marked inhibition of lung metastasis, invasion and cell motility with a concomitant decrease in sialyl Le^x and GM3 levels. The results together indicate that sialidase level is a determining factor affecting metastatic ability, irrespective of sialic acid contents. In addition, NEU2 may participate in cell apoptosis, as reported by Tringali *et al.* (2007): NEU2 gene introduction into leukemic K562 cells induced increased sensitivity to apoptotic stimuli by impairing Bcr-ABl/Src kinase signaling.

Expression levels of human ortholog NEU1 decreased in human colon cancer tissues as compared with that in the adjacent non-cancerous mucosa (Yamaguchi K. *et al*, 2005). Human NEU1 overexpression suppressed cell migration and invasion in human colon adenocarcinoma HT-29 cell, whereas its knock down resulted in the opposite effects. When NEU1-overexpressing cells were injected transsplenically into mice, the *in*

vivo liver metastatic potential was significantly reduced (Uemura T. *et al*, 2009).

1.4.2 Sialidase NEU4 in cancer

When NEU4 mRNA levels were compared between human colon cancer and adjacent non-cancerous tissues, a marked decrease in its expression was noted in the tumors (Yamanami H. *et al*, 2007). In these cultured cancer cells, the enzyme was upregulated in the early stage of apoptosis, induced by either the death ligand TRAIL, or serum-depletion. Transfection of NEU4 gene into DLD-1 and HT-15 colon adenocarcinoma cells resulted in acceleration of apoptosis and a decreased invasiveness and cellular motility. On the other hand, siRNA-mediated NEU4 targeting caused a significant inhibition of apoptosis and promotion of cellular invasiveness and motility. Lectin blot analyses revealed that the desialylated forms of approximately 100kDa glycoproteins were prominently increased in NEU4-transfectants, whereas only slight changes in glycolipids were found. These results suggested that NEU4 plays important roles in the maintenance of normal mucosa, mostly through desialylation of glycoproteins and that downregulation may contribute to invasive properties and protect against programmed cell death in colon cancer.

1.4.3 Sialidase NEU3 in cancer

Investigation of plasma membrane-associated sialidase accomplished by Kakugawa *et al*. (2002) revealed that NEU3 mRNA levels were increased up to 100-fold in human colon cancer tissues compared to adjacent non-tumor mucosa, and a significant elevation of sialidase activity in the tumors was also observed. Moreover, *in situ* hybridization analysis with antisense probes demonstrated positive signals to be localized in carcinoma cells, rather than in the surrounding stromal cells, with no clear signals when using the sense probe.

Experiments using NEU3 transgenic mice established the importance of NEU3 up-regulation for the promotion of colorectal carcinogenesis *in vivo* (Shiozaki K. *et al*, 2009).

NEU3 level was downregulated by sodium butyrate treatment while NEU1 was upregulated. Transfection of an NEU3 gene into cancer cells was found to inhibit sodium butyrate induced apoptosis, accompanied by increased Bcl-2 protein and decreased caspase expression. Colon cancer tissues exhibit marked accumulation of lactosylceramide, a possible NEU3 product, and addition of this glycolipid to cultures reduced apoptotic cells during sodium butyrate treatment.

Moreover, knock down of NEU3 gene with a short interfering RNA (siRNA) resulted in enhanced apoptosis, indicating that high expression of NEU3 in cancer cells leads to protection against programmed cell death.

In colon cancer cells, NEU3 was noted to differentially regulate cell proliferation through integrin-mediated signaling depending on the extracellular matrix (Kato K. *et al*, 2006), causing increased adhesion to laminins and consequent cell division, but rather decreased cell adhesion to fibronectin and collagens I and IV. Triggered by laminins, NEU3 clearly stimulates phosphorylation of focal adhesion kinase (FAK) and extracellular signal-related kinase (ERK), without any activation of fibronectin. NEU3 markedly enhances tyrosine phosphorylation of intergrin β4 only on laminin-5, with recruitment of Shc and Grb-2, and is coimmunoprecipitated by anti-integrin β4 antibody, suggesting that the association of NEU3 with integrin β4 might facilitate promotion of integrin-derived signaling on laminin 5.

NEU3 was also found to be overexpressed in renal cell carcinomas (RCCs) (Ueno S. *et al*, 2006), correlating with an increase of interleukin IL-6, a pleiotropic cytokine that has been implicated in immune responses and the pathogenesis of several cancers. NEU3 activation by IL-6 directs IL-6-mediated signaling via the PI3K/Akt cascade in a positive feedback manner and thus contributes to the malignant phenotype, including suppression of apoptosis and promotion of cell motility in RCCs.

As described also for colon tumors, gycolipid analysis showed a decrease in ganglioside GM3 and an increase in lactosylceramide after NEU3 transfection. In ovarian clear cell adenocarcinomas, a high level of NEU3

expression is significantly correlated with the T3 factor (T: tumor size) of the pTNM classification (cancer stage classification) (Nomura H. *et al*, 2006).

Upregulation of NEU3 was also detected in prostate cancer, showing a significant correlation with malignancy, as assessed by Gleason score (Kawamura S. *et al*, 2012). In androgen sensitive LNCaP cells, forced overexpression of NEU3 significantly induced expression of EGR-1, a progression-related transcription factor, as well as androgen receptors and PSA, both with and without androgen, the cells becoming hormonesensitive. This NEU3-mediated induction was abrogated by inhibitors of PI-3 kinase and MAPK, confirmed by increased phosphorylation of AKT and ERK1/2 in NEU3-overexpressing cells. NEU3 siRNA introduction resulted in reduced growth of androgen-independent PC-3 cells in culture and of transplanted tumors in nude mice. These data have suggested that NEU3 regulates tumor progression of prostate cancer through androgen receptor signaling.

To further define the molecular mechanisms of NEU3 effects and their possible targets, the encoding gene was silenced by siRNA or overexpressed in human cancer cells (Wada T. *et al*, 2007). NEU3 silencing caused apoptosis without specific stimuli, accompanied by decreasedBcl-XL and increased mda7 and GM3 synthase mRNA levels in HeLa cells, whereas overexpression resulted in the opposite. 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 WI-38 and NHDF fibroblasts and NHEK 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), and in fact, siRNA introduction reduced phosphorylation of EGFR while overexpression promoted its phosphorylation in response to EGF.

To summarize, NEU3 sialidase activates molecules including EGFR, FAK, ILK, Shc, integrin β4 and also Met, often upregulated in carcinogenesis, and may thus cause accelerated development of malignant phenotypes in cancer cells.

NEU3 expression causes cell-type specific effects on cell proliferation, apoptosis and motility. Taking into account all of the evidence, NEU3 is certainly involved in the regulation of transmembrane signaling at the cell surface, possibly through both enzymatic modulation of gangliosides and interaction with other signal molecules like caveolin-1, Rac-1, integrin b4, Grb-2, and EGFR (Miyagi T. *et al*, 2008).

NEU3 overexpression leads to decreased levels of certain sialylated glycolipids, such as GM3, and an increased level of the unsialylated glycolipid LacCer.

One substrate of NEU3, GM3, interacts directly with EGF receptor and reduces its ability to respond to EGF ligand, possibly by sequestering EGFR in specialized membrane microdomains (Yoon SG. *et al*, 2006). In the presence of NEU3, GM3 is hydrolyzed to LacCer, relieving the inhibition of EGF signaling. In this way, NEU3 activity leads to increased EGF signaling and cell proliferation (Anastasia L. *et al*, 2008). NEU3 also modulates integrin signaling pathways, leading to increased proliferation and motility (Kato K. *et al*, 2006). NEU3-mediated depletion of GM3 has been shown to block integrin-mediated adhesion to fibronectin, consistent with other work that showed functional and physical interactions between α5β1 integrin and GM3 (Toledo MS. *et al*, 2005; Gopalakrishna P. *et al*, 2004). NEU3 activity also promotes integrin-mediated adhesion to laminin (Kato K. *et al*, 2006). The effect of these changes in adhesive properties is to stimulate cell proliferation. Additional work will be needed to clarify the molecular details of GM3's and LacCer's roles in these processes.

1.5 The colorectal cancer (CRC)

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western countries and the first when smoking-related cancers are excluded (Boyle P. *et al*, 1985; Bingham SA. *et al*, 2003). Estimated 5-year survival ratesrange from more than 90% for patients with stage I disease to less than 10% for patients with metastatic CRC (mCRC) (Venook AB., 2005a). At molecular level, intensive screening for genetic alterations led to the identification of two major types of CRC, one is characterized by normal caryotype, normal DNA index (Houlston RS., 2001) and genetic instability at microsatellite loci and is called MSI-positive cancer (Ilyas*et* M. *et al*, 1999) and the other is characterized by alterations in APC, KRAS, TP53 genes and in the tumor suppressor genes on chromosome 18q (Laurent-Puig P. *et al*, 1999). In addition, several other markers have been found to be altered in CRC and a good estimation is that more than 10-20 alterations occur in a single CRC case. Current strategies in the management of CRC are focused on the identification of drugs specifically addressing these alterations and, if possible, on predicting the efficacy of these compounds (named "targeted therapies").
Advances in chemotherapeutic agents have improved the outcome for patients with mCRC. Chemotherapies, however, are limited by their lack of specificity and by frequent and potentially severe dose-limiting toxicities. Therefore, there is an urgent need for more effective, better-tolerated treatments that specifically target the process pivotal to tumorigenesis and metastasis.

1.5.1 Epidemiology

Colorectal cancer (CRC) is one of the most frequent malignancy in the world: everyyear there are approximately 950,000 new cases and about 500,000 deaths (McCracken M. *et al*, 2007). With an increasing trend of incidence, colorectal cancer (CRC) is the third malignancy after breast cancer in women, prostate cancer in men and lung cancer for both sexes. In Italy 40 new cases per 100,000 inhabitants are observed every year and the

risk is significantly higher in the North than in the South of the country; the incidence has increased from 16,000 cases in 1970 to over 37,000 in 2009, as result of the significant improvement of the accuracy of the diagnostic techniques and of increased life expectancy of individuals (Negri E. *et al*, 2002). In both men and women the incidence becomes very high after 50 years with peaks close to 80 years, indeedmore than 90% of CRC are identified at 50 years of age (Benson AB., 2007).

The incidence of CRC varies around the word: it is more common in developed than developing countries (Figure 1.14) (Merika E. *et al*, 2010). Globally incidences vary 10-fold with highest rates in the Australia, New Zealand, Europe and the US and lowest rates in Africa and South-Central Asia (Ferlay J. *et al*, 2010).

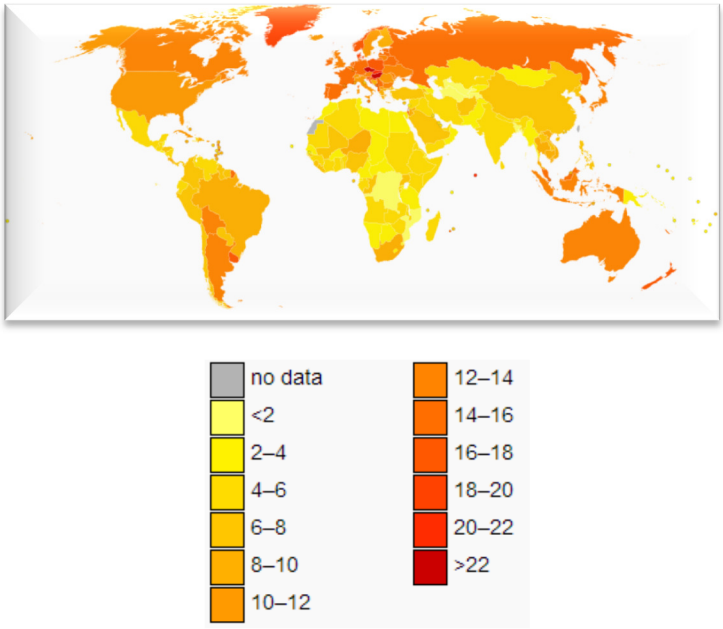


Figure 1.14 ***Colorectal cancer incidende in the world.***
In the legend is reported the death from colorectal cancer per 100,000 inhabitants.
(WHO Disease and injury estimates. World Health Organization. (2009))

The reasons for this variability may be related to the heterogeneous eating habits, lifestyle and opposed to the care taken in different screening and diagnosis of this pathology, according to the different socio-economic context. In fact immigrant populations from countries considered “low risk” into those classified “high risk” acquire, within a generation, the risk of the new country of residence.
Over the two past decades a gradual increase in the survival has been observed, especially due to the improved abilities to detect cancer in the early stages, thanks to mass screening campaign and to improving the therapies efficiency (Kuipers EJ. *et al*, 2013).
To this day the main cause of death for CRC is due to the dissemination of the tumor at a distance, which occurs mainly in the liver (in 20-70% of patients) and in the lung (10-20% of cases) (Penna C. and Nordlinger B., 2002).

1.5.2 Etiology and risk factors

In CRC onset a fundamental role is played by those generically are defined as “environmental factors”, that are established on a framework of susceptibility individual genetics and that are essential in the progression of carcinogenesis. Experimental and epidemiological studies have identified several factors that promoted the development of this tumor.
The diet seems toplay a major role in the development of CRC; potentially harmful dietary habits are represented by a low intake of fruits, vegetables and fiber. In addition the exposure to cigarette smoke and alcohol (especially beer and spirits) also contributes to CRC (Lin OS., 2009).
Diets rich in red meat are associated with an increased risk of developing dysplastic lesions of the colonic mucosa probably as a result of the high content of polyamines that, by exercising a key role in the process of carcinogenesis, are compared to markers of neoplastic proliferation (Linsalata M. and Russo F., 2008). Moreover an excessive fat intake increases the risk of colorectal cancer development.
A recent study of the European Prospective Investigation into Cancer and nutrition (EPIC) has shown a low incidence of colon cancer in populations

with diets rich in fiber. The mechanisms through fibers interfere with the development of cancer are various, but among these the most prominent are: the inhibition of polyamines formation, the increase of the feces water content with dilution of carcinogens that are in contact with the colonic mucosa, and the reduction of the transit time of the intestinal contents. The same conclusions was established by studying the protective role of fruit and vegetables.

A sedentary lifestyle, along with a Body Mass Index (Body Mass Index -BMI) higher than normal, is capable of influencing the development of the CRC. This seems to be due to the effect that being overweight has on production of insulin and inflammatory processes.

The insulin spikes after a meal can function as growth factor for cancer cells, while inflammation is the ideal process for the transformation and proliferation of neoplastic cells.

Epidemiological data collected in the last decade show that subjects with metabolic syndrome have an increased risk of colorectal cancer development. The mechanism is not completely understood but may be related to the insulin resistance shown by these patients (Giovannucci E., 2007). Other factors may induce or promote tumor progression: a fundamental role is played by chronic inflammatory deseases affecting the intestine; for example, patients affecting by ulcerative colitis, have a risk of developing CRC proportional to the duration and the extension of clinical disease.

Less significant is the association between Crohn's disease and CRC, even though the literature includes studies that establish a relationship proportional to the extension of intestinal tract affected, the clinical duration of disease, the age at diagnosis and the severity of histological inflammation (Zisman TL. and Rubin DT., 2008).

Other conditions predisposing to CRC include a family history of CRC, pelvic irradiation and the tendency of the intestine to form adenomatous polyps.

The latter are considered to be the substrate of tumor formation, the potential malignant transformation of which depends on the size, the degree of dysplasia, the presence of villosa component and the age of the lesion itself. Even after the surgical removal, the risk of forming new adenomas is about 30% higher than the unaffected population.

The 80% of CRC is considered sporadic and in most cases is developed from polyps. The remaining 20% of CRC is hereditary and includes a series of syndromes; the two predominant are the Familial Adenomatous Polyposis (FAP), characterized by mutations in the APC gene, and the Lynch syndrome or the Hereditary non-polyposis colorectal cancer (HNPCC) that is not associated with polyposis, characterized by mutations in the genes of mismatch-repair (MMR) system.

1.5.3 Prevention and Screening

The evolution of the CRC is relatively slow. An effective prevention plan must be intended both to correct the risk factors of the disease (primary prevention) and to the early detection of affected patients (secondary prevention).
Screening is an important tool for the early diagnosis and to organize a better therapeutic strategy.
According to the AIRC (American Institute for Cancer Research) more than 30% of tumors is imputable to a sedentary lifestyle, since it has been estimated that about 70% of cases of CRC could be prevented by a healty diet.
There are several tests for the early detection of this tumor, the main are:

- determination of the Faecal Occult Blood Test (FOBT);
- rectal examination;
- sigmoidoscopy;
- colonoscopy.

1.5.4 Anatomical and pathological features

CRC is a cancer that affects the last part of the intestine called large intestine, anatomically divided into six parts: caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum (Figure 1.15).

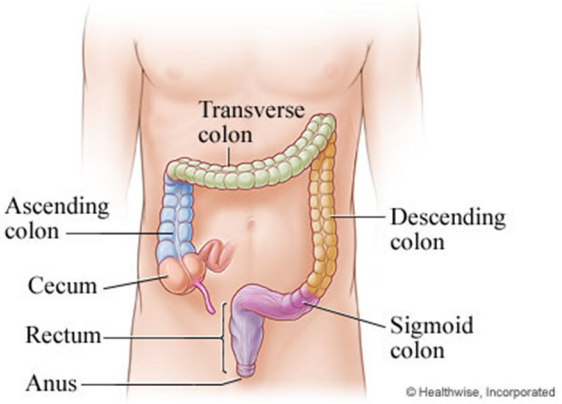


Figure 1.15 ***Schematic representation of the large intestine.***

CRC originates from epithelial cells lining the gastro-intestinal mucosa, which is characterized by a frequent cell turnover. More than 90% of intestinal cancer cases develops from polyps of the mucosa, the so-called adenomas, originated from the epithelial cells of the intestinal wall (Figure 1.16). The sizes vary from a few mm to not more than 10 cm in diameter.

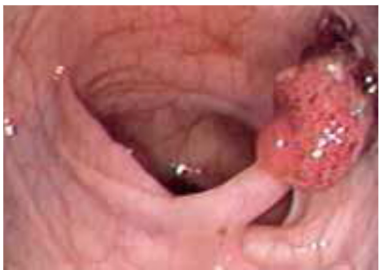


Figure 1.16 ***Endoscopy: a polyp of sigmoidal colon.***

Rare cases of CRC may develop from the cells that secrete hormones, immune cells or by cells of the underlying connective tissue.
CRC is manifested by a different distribution in the various segments of the large intestine: 16% in caecum, 8% in ascending colon, 6% in transverse and descending colon, 20% in sigmoid colon and 50% in rectum (Papadopoulos VN. *et al*, 2004) (Figure 1.15).
The tumors in the proximal portion (caecum, ascending colon and transverse colon) have a different aspect from those arising in the distal portions of the intestine (Descending colon, sigmoid colon and rectum): while the former tend more frequently to have a defined aspect and a growth within the intestinal lumen, those arising in the distal portions occur most often in the form of infiltrating carcinomas following the circumferential spread.
Each intestinal segment has its own clinical, therapeutic and prognostic specificity.

1.5.5 Adenoma-carcinoma sequence

CRC arises as an abnormal proliferation of cells of the intestinal mucosa and progresses from a state of adenoma to carcinoma.
There are two main mechanisms determining CRC onset. The first type of carcinogenesis (tumors in the context of familial syndromes HNPCC and a small subgroup of sporadic tumors) is characterized by normal karyotype, normal mitotic indexand, at the gene level, by microsatellite instability and loss of protein expression of MMR (Ilyas M. *et al*, 1999; Houlston RS., 2001). In this type of carcinogenesis there is a correlation between morphological alterations and gene alterations.
The second type of carcinogenesis is due to the progressive acquisition of molecular alterations that affect oncogenes (promoting cellular proliferation) and tumor suppressor genes (promoting cell differentiation and inhibiting proliferation) to which different morphological aspects of the lesion are associated. This process, which occurs in 90% of adenocarcinomas of sporadic origin and in those patients with the

hereditary syndrome FAP, is represented from a model originally proposed by Vogelstein and is known as adenoma-carcinoma sequence (Fearon ER. and Vogelstein B., 1990). This model, that is shown in Figure 1.17, involves mutations in the APC gene, or rather in the pathway APC-β-catenin, as initial event which determines proliferation and then formation of theprimary hyperplastic adenoma. The late stage of adenoma is achieved by hyperactivating mutations in K -Ras gene. The formation of carcinoma in situ depends on the loss of chromosome 18q oncosuppressor genes, among which the most important is the DCC gene.

As a result of alterations in the TP53 gene (typically inactivating mutations) the tumor gains the capability to metastasize (Laurent-Puig*et al.*, 1999). Despite the adenoma-carcinoma sequence has not yet been directly proved, a series of epidemiological, clinical, histopathological and genetic data indirectly support this model (Boyle *et al*, 1985; Chung DC., 2000; Lesley A. *et al*, 2002). The analysis of all these markers on the same patients simultaneously has allowed to observe how these mutations are able to combine each other through two preferential mechanisms: APC/K-Ras/DDC/TP53 and APC/DCC/TP53.

Extensive research on this disease have shown evidence that other genes may be involved in colorectal carcinogenesis (Frattini *et al.*, 2004; Sanford K and McPherson RA., 2009). Several markers were identified including growth factors, tyrosine kinase receptors (eg. EGFR) and transcription factors.

The carcinogenesis of the colon-rectum is more complicated with respect of that proposed by previous models, in agreement with other works published in literature (Smith G. *et al*, 2002).

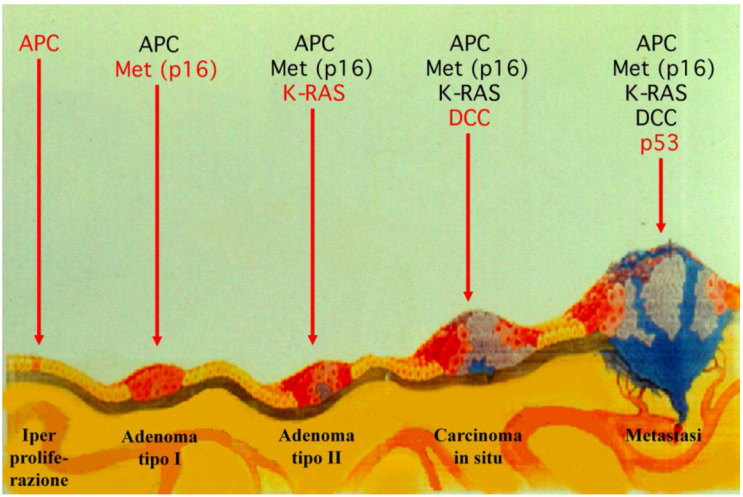


Figure 1.17. ***Schematic model of colorectal carcinogenesis proposed by Vogelstein.***
(Fearon ER. and Vogelstein B., 1990)

1.5.6 Treatment of CRC

Surgery is the best therapeutic option in patients with CRC and involves the removal of the entire tumor mass, lymph nodes and the satellites resectable metastases. The tumors in stage I and II are surgically curable, those in stage III are curable, up to 70% of cases, with surgery and adjuvant chemotherapy, and stage IV tumors are unfortunately incurable in most cases (Markowitz SD. and Bertagnolli MM., 2009). The surgical treatment however is not always healing: in fact about 50% of the patients die within five years of diagnosis of the disease, while 80% develop relapses in the two years subsequent diagnosis. The type of treatment depends on a plurality of CRC factors, mainly on the size, location and extent of thetumor, as well as the general condition of the patient. In addition to the surgery, various types of treatment are applicable, like radiation therapy, immunotherapy, or chemotherapy. The latter is only used to control the

cancer growth or to relieve symptoms of the disease, even after surgical treatment to eliminate residual cancer cells.
With the progression of the disease, a combination of different treatments becomes essential. Adjuvant chemotherapy includes the cytotoxic compound 5-Fluorouracil (5-FU) or its oral prodrug, capecitabine, which has reduced the risk of death by 30%. This drug is included in the two chemotherapy regimens most used for the CRC treatment: FOLFIRI (5- FU, folinic acid and Irinotecan) and FOLFOX (5- FU, folinic acid and oxaliplatin) (Ross JS., 2010). The addition of oxaliplatin to the treatment with 5-FU has increased disease-free survival by three years compared with only 5-FU, on the contrary the use of Irinotecan is not recommended in combination with 5-FU due to the high toxicity and lack of evidence of efficacy (Midgley R. and Kerr DJ., 2005).
Radiotherapy is only useful in the treatment of tumors of the cecum, which have frequent loco-regional relapse, and those of the rectum.
New therapies have further expanded the possibilities to control the disease even in the case of metastatic neoplasia; the targeted drugs (targeted therapies) that can selectively block functions essential to the survival and growth of the neoplastic cell led to great benefits for patients. In this field monoclonal antibodies directed against the extracellular domain of the epidermal growth factor receptor (EGFR) have particular relevance.

1.6 The epidermal growth factor receptor (EGFR) and related targeted therapies

1.6.1 EGFR activation mechanism

The epidermal growth factor receptor (EGFR) is a cell-surface receptor for members of the epidermal growth factor family (EGF-family) of extracellular protein ligands (Herbst RS., 2004).

The EGFR gene is located on chromosome 7 and encodes for a transmembrane receptor of 1201 amino acids, with a molecular mass of 134 kDa.

The epidermal growth factor receptor is a member of the ErbB family of receptors, a subfamily of four closely related tyrosine kinases receptors: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4).

EGFR (epidermal growth factor receptor) exists on the cell surface and is activated by binding of its specific ligands, including epidermal growth factor and also transforming growth factor α (TGFα).

It transduces cell signaling through ligand (EGF)-induced receptor dimerization, which then initiates its tyrosine kinase activity (Lemmon MA., 2009).

The extracellular domains of the EGFR family members are divided into four subdomains, with domains I and III participating in ligand binding and domains II and IV for dimerization (Lax I. *et al*, 1989; Ogiso H. *et al*, 2002). In the state without ligand stimulation, an intramolecular interaction between domains II and IV maintains EGFR in a tethered conformation to inhibit dimerization, and this conformation is opened up by EGF binding to expose the dimerization interface for receptor activation (Figure 1.18) (Cho HS. and Leahy DJ.,2002; Dawson JP. *et al*, 2005; Ferguson KM. *et al*, 2003).

EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity. As a result, autophosphorylation of several tyrosine (Y) residues in the C-terminal domain of EGFR occurs. These include Y992, Y1045, Y1068, Y1148 and Y1173 (Downward J. *et al*, 1984). This autophosphorylation elicits downstream activation and signaling by several other proteins associating with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains, that are involved in transmitting the mitogenic signalling through two main downstream pathways: the Ras/Raf/mitogen-activated protein kinase (MAPK) axis, mainly involved in cell proliferation, and the phosphoinositide-3-kinase (PI3K)/PTEN/AKT one, implicated in cell survival and motility (Figure 1.19) (Jorissen RN. *et al*, 2003).

EGFR is involved in cell proliferation, differentiation, metastasis, angiogenesis, and programmed cell-death (Carpenter G. and Cohen S., 1990).

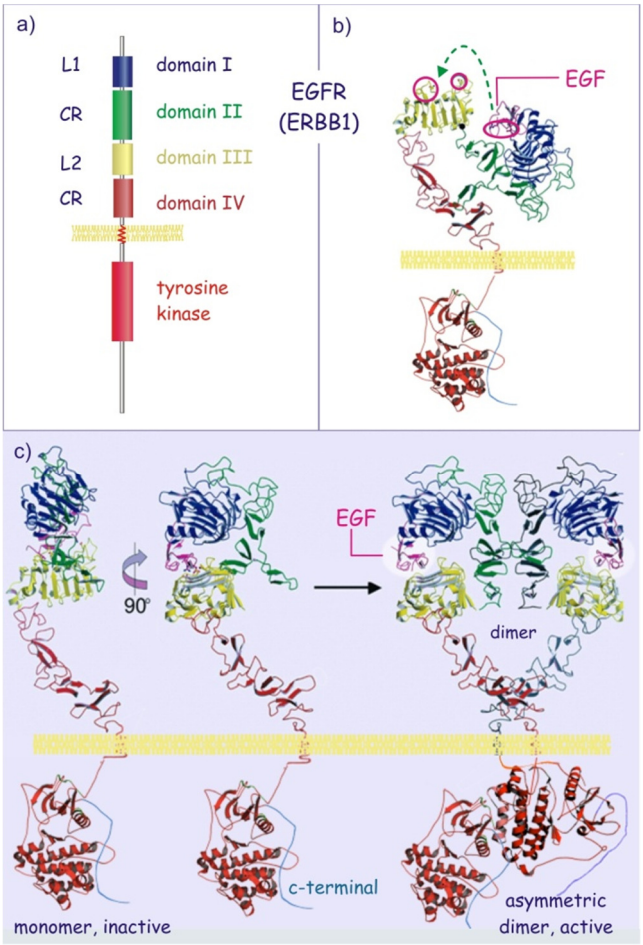


Figure 1.18 ***Modelled structures of the dimerization process of the extracellular domain of the EGFR.***

(a) Domain architecture of the EGF receptor. (b) Interaction between domain II and IV keeps the EGF receptor in a tethered conformation. EGF is bound to domain I but, because of (experimental) acidic conditions, cannot make contact with domain III. (c) EGF binding leads to the adaptation of an extended configuration and liberates the dimerization finger in domain II. This now interacts with another domain II and forms an EGFR dimer. (Textbook of Receptor Pharmacology (3rd edition))

1.6.2 EGFR downstream protein involved in mitogenic signal transduction

As previously described in the previous paragraph, after EGFR dimerization and activation, the transmission of the mitogenic signaling is spread and amplified through two main pathways; the first is the MAP kinase pathway, mainly involved in cell proliferation and including sequential activation of KRAS, BRAF, MEK and ERK, and the second is the PI3K-PTEN-AKT pathway, mainly involved in cell survival and leading to the activation of mTOR. Akt and MAP kinases in turn transduce the mitogenic signalling into the nucleus by regulating several transcription factors which control the expression of genes relevant for cell proliferation and survival (Figure 1.19) (Woodburn JR., 1999; Talapatra S. and Thompson CB., 2001; Venook AB., 2005b).

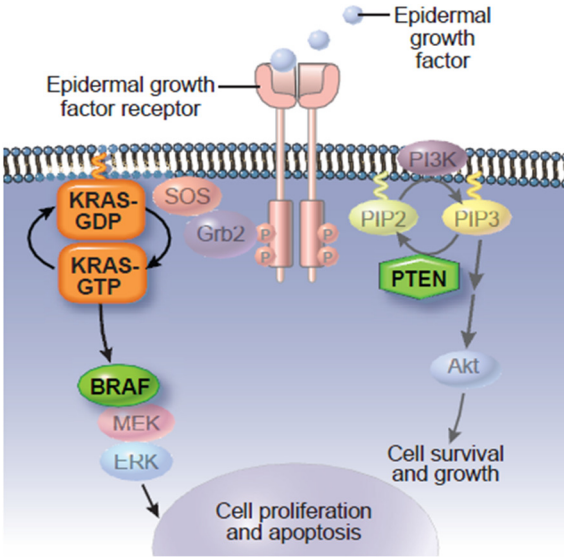


Figure 1.19 ***Schematic representation of EGFR downstream pathways.***
(Asghar U. *et al*, 2010)

The main EGFR downstream protein are briefly described and listen below:

K -Ras

The Ras family, encoded by the homonymous gene, includes GTPase enzymes involved in the intracellular signals transduction that regulates cell growth, differentiation and apoptosis. There are three different proteins with similar activity (H -Ras , K -Ras , N- Ras) that are specific for different cell types. Ras proteins normally alternate between the active conformation bound to GTP and an inactive conformation bound to GDP. This alternation is promoted by guanine nucleotide exchange factors (GEF), recruited from protein complexes present in the intracellular domain of the activated receptor. The signal ends when the complex Ras-GTP is hydrolyzed to Ras-GDP from activating GTPase activity protein (GAP) (Van Krieken H. and Tol J., 2008; Raaijmakers JH. and Hoff PM., 2009). After being activated by binding GTP, Ras recruits the protein encoded by the homonymous RAF oncogene that phosphorylates Mitogen-Activated Protein Kinase Kinase-1 (MAP2K) and subsequently MAP2K-2, thus activating the MAPK signal transduction pathway that leads to the expression of proteins important for cell growth, differentiation and survival.

Mutations in the K-Ras gene (located on chromosome 12) are among the most common alterations of human tumors, the protein being mutated in more than 30% ofCRC (Edkins S. *et al*, 2006). These mutations produce a constitutively active protein due to a defect inGTPaseactivity.

Contrary to normal Ras proteins that are inactivated in a short time, the aberrant Ras protein is able to costitutively activate the signal transduction in the absence of any external stimulus of the EGFR receptor.

More than 90% of K-Rasmutations involves codons 12 and 13: all mutations are missense and lead to an amino acid substitution (Kosaka T. *et al*, 2004). Mutations in other positions, as in the codon 61 and 146, have been identified but are less frequent (less 10% of the alterations mentioned) (Edkins S. *et al*, 2006).

BRAF

The BRAF gene, located on chromosome 7, codes for aRaseffector belonging to the family of serine-threonine RAFkinases. The BRAF protein is recruited to the plasma membrane upon binding Ras-GTP complex and represents a crucial step in signal transduction in the MAP kinases pathway. Mutations of BRAF gene have been identified in about 50 % of melanomas, in a smaller percentage of thyroid, colon and ovary carcinomas, and in some sarcomas (Michaloglou C. *et al*, 2008). All are missense point mutations within exons 11 and 15.

The most frequent mutation (in more than 90 % of cases) is a transversion of a thymine with an adenine in position 1799 of the nucleotidic sequence that leads to the substitution of the valine residue with a Glutamic acid in position 600 (V600E) in the amino acids sequence within the kinase domain. This change constitutively activates BRAF protein leading to MAPK activation and significantly contributing to the development of neoplastic cells (Davies H. *et al*, 2002; Michaloglou C. *et al*, 2008).

PI3K

The phosphatidylinositol 3-phosphate kinase (PI3K) is a protein belonging to the lipid kinases family that regulates signal transduction (Vivanco I. and Sawyers CL., 2002). These proteins consist of a catalytic and an adaptor/regulator subunit encoded by different genes and act directly or through adaptor proteins downstream of different receptor tyrosine kinases such as EGFR , HER2, IGF1R, cKIT, PDGFR and MET. The PI3K activation leads to the production of the second messenger phosphatidylinositol -3,4,5-triphosphate (PIP3) from phosphatidylinositol -4,5- bisphosphate (PIP2). The PIP3, through the activation of Akt kinase, controls different pathways involved in the regulation of multiple cellular mechanisms such as growth, transformation, adhesion, apoptosis, survival and motility (Yuan TL. and Cantley LC., 2008). The activation and constitutive overexpression of PI3K leads to an increase of the signal in this way, a phenomenon closely linked to cellular transformation and the onset of a neoplasia.

Only PI3K that contain the p110α catalytic subunit and the associated p85 regulatory subunitare involved in the development of cancer. The p110α

subunit is encoded by PIK3CA, a gene located on chromosome 3q26.3, composed of 20 exons. PIK3CA protein hyperactivating mutations have been identified in different tumors, such as breast, endometrium, urinary tract, ovary and brain.
In CRC are observed in 10-30 % of cases (Yuan TL. and Cantley LC., 2008; Samuels Y. *et al*, 2004). The majority of the mutations falls into two regions: exon 9 (E542K, E545K) and exon 20 (H1047R); these missense mutations confer constitutive kinase activity to the protein.

PTEN
PTEN is a tumor suppressor gene that encodes a phosphatase that dephosphorylates PIP3 to PIP2 and the latter to phosphoinositide-4-phosphate. In this way PTEN prevents the phosphorylation of Akt keeping it in its inactive form, thereby inhibiting cell cycle progression, modulating stop signals and stimulating angiogenesis (Sansal I. and Sellers WR., 2004).
The genetic alterations of PTEN vary from point mutations (missense and nonsense) to large chromosomal deletions (heterozygous / homozygous frameshifs, deletions in frames).
Most missense mutations are located in exons 5, 7 and 8; many of these determine the premature formation of stop codons inactivating the protein (Dicuonzo G. *et al*, 2001) . The loss of protein expression in CRC is observed in approximately 30% of cases, the possible correlation between PIK3CA mutations and hyperactivating loss of PTEN expression is still debated (Frattini M. *et al*, 2005; Saal LH. *et al*, 2005)

1.6.3 EGFR regulation in cancer and related targeted therapies

EGFR is overexpressed in a large proportion of carcinomas and is associated with disease progression and poor prognosis in CRC (Resnick MB. *et al*, 2004). The principal mechanism of deregulation of EGFR in CRC is represented by protein overexpression, demonstrated by immunohistochemistry in about 50% of patients (Mc Kay JA. *et al*, 2002). At odds with lung carcinoma, point mutations in the tyrosine kinase domain of EGFR rarely occur in CRC (Barber TD. *et al,* 2004). Therefore, the inhibition

of EGFR cascade in CRC can be performed by blocking the extracellular domain of the receptor through monoclonal antibodies rather than by small molecules against the EGFR tyrosine kinase domain.

Further advances in the understanding of molecular biology have led to the development of targeted-specific agents. In the treatment of metastatic disease Cetuximab and Panitumumab, targeted therapies against the Epidermal Growth Factor Receptor (EGFR), have been recently introduced, with satisfactory results.

Cetuximab and Panitumumab are two monoclonal antibodies (MoAbs) that, by their binding to the extracellular domain of EGFR, are able to block its dimerization and, therefore, its activation followed by the transduction cascade of mitogen signals. At clinical level, two phase II trials demonstrated that patients with advanced CRC have a response rate of 11% when Cetuximab is administered as single agent therapy, and 23% when combined with Irinotecan (Saltz LB. *et al,* 2004; Cunningham D. *et al*, 2004). As a consequence of these clinical studies, Cetuximab is currently indicated for treatment of patients with Irinotecan-resistant metastatic CRC (Venook AP., 2005a). These data suggest that Cetuximab is effective in a subgroup of patients with metastatic CRC. The same is true also for Panitumumab. Taking into consideration the importance of a timely identification of these patients, EGFR expression has been initially investigated by immunohistochemical analysis, the most widely method applied in routine diagnostic by laboratories of molecular pathology. However, it has been demonstrated that such a methodology does not represent the best way to evaluate EGFR alterations. Indeed, it has been shown that the type of fixative used, the storage time of unstained tissue sections (Atkins D. *et al*, 2004), the type of primary antibody used (Kersting C. *et al,* 2006) and the methods of immunohistochemical analyses and/or evaluation (Langner C. *et al*, 2004) might generate conflicting data. Moreover, it has also been demonstrated that EGFR-negative patients, as determined by immunohistochemistry, may respond to Cetuximab-based therapies (Chung KY. *et al*, 2005).

Therefore, researchers shifted the focus on EGFR gene status, using fluorescent *in situ* hybridization (FISH) and, although early data seemed to be very promising (Moroni M. *et al*, 2005; Sartore-Bianchi A. *et al*, 2007;

Frattini M. *et al*, 2007; Cappuzzo F. *et al*, 2007), it has recently been shown that FISH results for EGFR evaluation vary largely also among experienced international pathology centres, with fluctuations covering the whole range of cut-offs proposed by the literature (Sartore-Bianchi A. *et al*, 2012). Moreover, there is absence of correlation between EGFR protein expression by immunohistochemistry and EGFR gene status by FISH (Martin V. *et al*, 2009). Therefore, EGFR analysis, conducted either by immunohistochemistry or FISH, does not seem to be effective, at the moment, as a predictor of EGFR-targeted therapies efficacy. This finding can also be due to different mechanisms of EGFR regulation, which lead to uncoupling the gene status and the protein expression. In addition, other works have recently pointed out that the real efficacy of the two drugs depends on the alterations occurring in the EGFR downstream pathways, independently from EGFR status. In fact, it has been widely demonstrated that the presence of KRAS mutations leads to resistance to EGFR-targeted therapies in mCRC (Siena S. *et al*, 2009). Based on these results, the two international agencies Food and Drugs Administration (FDA) and European Medicine Agency (EMA) have approved the use of Cetuximab and Panitumumab only for mCRC patients whose tumors display a KRAS *wild type* sequence. Recent data have also shown that a similar role is played by NRAS (Douillard JY. *et al*, 2013), and therefore FDA and EMA have approved the NRAS mutational testing before the administration of Panitumumab. Concerning the NRAS predictive role, no data are currently available for Cetuximab.

Overall, at the moment, before the administration of EGFR-targeted therapies in CRC, diagnostic laboratories must investigate KRAS and NRAS mutational status, while there are no data concerning the precise mechanism of EGFR activation in CRC and, consequently, it is doubtful how EGFR deregulation could be linked to the efficacy of monoclonal antibodies against EGFR (Figure 1.20).

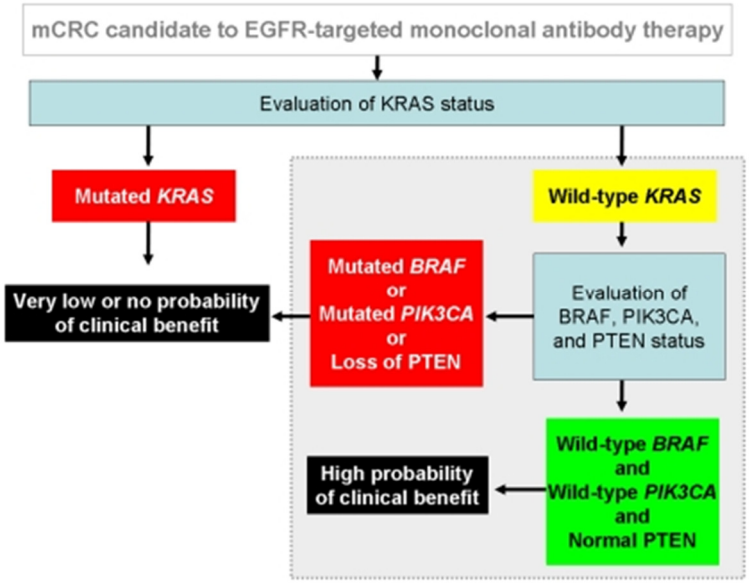


Figure 1.20 ***Algorithm of molecular diagnostics based on data discussed in this study for patients with mCRC candidates to Cetuximab- or Panitumumab-based therapies*** (Sartore-Bianchi *et al,* 2009).

1.6.4 Role of glycosilation of EGFR

A relevant role in the process of EGFR activation is played by the level of glycosylation, in particular by the presence of sialic acid residues.
Asparagine (N)-linked glycosylation is a highly regulated process that produces a large and diverse repertoire of cellular glycans that are mostly attached to proteins (Schwarz F. and Aebi M., 2011). Abnormal glycosylation is known to be associated with cancer malignancy. Among the sugars found on the cell surface there are sialic acids, which exist as terminal monosaccharide residues attached to cell surface glycan chains. The variety of sialicacid decorations on the cell surface governs many biological processes, including cell recognition, cell adhesion, receptor

activation and signal transduction. Studies performed over the last decade have focused on the involvement of sialylation in the progression of cancer (Ohtsubo K. and Marth JD., 2006; Varki NM. and Varki A., 2007), but the actual function of sialylation in tumorigenesis has received much less research attention (Schwarz F. and Aebi M., 2011). Recently, EGFR was identified as one of the sialylated glycoproteins in human lung cancer, since it has been demonstrated that sialylation is capable of regulating EGFR activity (Liu YC. *et al*, 2011). Thus, understanding the regulation of EGFR glycosylation may provide novel insights into cancer biology and suggest possible therapeutic strategies. Moreover, it has been demonstrated that the sialyltransferase ST6Gal-I induces cell adhesion and migration, and promotes radioresistance and protection from apoptosis in colon cancer cells (Lee M. *et al*, 2008; Lee M. *et al*, 2010a; Lee M. *et al*, 2010b). Furthermore ST6Gal-I overexpression significantly affects EGF-mediated cell growth and induces chemoresistance to gefitinib (a tyrosine kinase inhibitor against EGFR) in colon cancer cells (Park JJ. *et al*, 2012).

These data therefore suggest that the absence of sialic acid could play a relevant role in the activation of EGFR and, as a consequence, in the efficacy of EGFR-targeted therapies. The main family of enzymes able to regulate the level of sialic acid is represented by sialidases, that can directly activate EGFR (Soderquist AM. and Carpenter G., 1984).

2. Aim of the Thesis

Abnormal glycosylation is known to be associated with cancer malignancy. Among the sugars found on the cell surface there are sialic acids, which exist as terminal monosaccharide residues attached to cell surface glycan chains.

The studies performed over the last decade have focused on the involvement of sialylation in the progression of cancer (Ohtsubo K. and Marth JD., 2006; Varki NM. and Varki A., 2007), but the actual function of sialylation in tumorigenesis has received much less attention (Schwarz F. and Aebi M.,2011).

In this thesis we proposed to investigate in more depth the role of sialidases in human carcinogenesis and in particular the role of NEU3 in colorectal cancer.

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western countries and the first when smoking-related cancers are excluded. Newer therapeutic options for treating advanced CRC include targeted biologic therapies. Among these new drugs, the epidermal growth factor receptor (EGFR) antagonists represent a very promising class of targeted compounds that have been introduced into clinical management. Cetuximab and Panitumumab are two monoclonal antibodies (MoAbs) that, by binding EGFR, are able to block downstream pathways. However, these MoAbs have shown efficacy in about 10-20% metastatic colorectal cancer (mCRC) patients. It is now emerging that genetic alterations of EGFR and its downstream signaling effectors may predict the efficacy of EGFR-targeted drugs. However, the real deregulation of EGFR is currently unknown, also because of the lack of correlation between EGFR gene status and EGFR protein expression as detected by immunohistochemistry. Recent data pointed out that the inhibition of EGFR-tyrosine phosphorylation is highest in the presence of NEU3 deregulation, raising the possibility that NEU3 may largely stimulate EGFR phosphorylation.

In this thesis we proposed to investigate the role of NEU3, a sialidase that is able to directly activate EGFR, since its role in colorectal carcinogenesis is largely unknown.

We proposed the following specific aims:

AIM1: in-depth analysis of the membrane anchoring mechanism of human sialidase NEU3.

AIM2: analysis of sialidases deregulation in colorectal cancer.

AIM3: analysis of NEU3 deregulation with respect to alterations occurring in EGFR pathways, such as:

1) a study of the correlation between EGFR and NEU3 deregulation;

2) a study of the correlation of NEU3 expression with alterations involved in the classical model of colorectal cancer development occurring in EGFR downstream pathways.

AIM 4: investigation of NEU3 action on EGFR.

AIM 5: investigation of the role of NEU3 overexpression in the prediction of efficacy of EGFR-targeted therapies in colon cancer cell lines.

AIM 6: collection of data regarding the deregulation of the human sialidase in several types of cancer in the Western population.

3. Materials and Methods

3.1 CRC study subjects and tissue samples

We investigated tissue specimens from 88 patients surgically resected for a CRC at the Istituto Nazionale dei Tumori (Milano, Italy) or at the Istituto Cantonale di Patologia (Locarno, Switzerland) from 2008 to 2011. Fresh tissues from both primary tumors and paired normal mucosa were immediately frozen in liquid nitrogen and subsequently stored at -80°C until the analysis.
For all patients, tissues from formalin-fixed paraffin embedded sections are histologically evaluated and are available in the archives of these institutes. Our cohort was represented by 51 men and 37 women with a mean age at diagnosis of 66 years. Tumor site was variable: colon 59% of cases and rectum 41% of cases. Histopathological staging was detected in 87 patients because 1 patient was not evaluable: 9 patients (10%) were classified as pT1; 19 cases (22%) as pT2; 48 cases (55%) as pT3 and 11 cases (13%) as pT4. thirty-seven tumors (42.5%) were classified as N0, 50 tumors (57.5%) showed lymph node metastases (pN+) and 8 tumors (9%) 5 patients showed distant metastases (M1).

3.2 Cell cultures

SW48 (ATCC® CCL-231™), SW403(ATCC® CCL-230™), SW480 (ATCC® CCL-228™), SW620 (ATCC® CCL-227™), SW1116 (ATCC® CCL-233™), SW1463 (ATCC® CCL-234™), CO115, E705, MICOL24 and MICOL29 (kindly provided by Istituto Nazionale dei Tumori, Milano) cells were grown in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and maintained at 37°C in a humidified 5% CO_2 incubator.
Caco-2 (ATCC® HTB-37™) and CCD841 (ATCC® CRL-1790™) cells were grown in EMEM medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-Essential Amino acids (NEAA), 100 U/ml penicillin, 100 µg/mL streptomycin, and maintained at 37°C in a humidified 5% CO_2 incubator.

HT-29 (ATCC® HTB-38™) and COS-7(ATCC® CRL1651™) cells were grown in DMEM medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and maintained at 37°C in a humidified 5% CO_2 incubator.
T84 (ATCC® CCL-248™) cells were grown in Ham's F12/DMEM (1:1) medium supplemented with heat-inactivated 5% fetal bovine serum (FBS), 2 mM L-glutamine,100 U/mL penicillin, 100 µg/mL streptomycin, and maintained at 37°C in a humidified 5% CO_2 incubator.
DIFI cells, kindly provided by Dr. Josep Tabernero (Vall d'Hebron Institute of Oncology, Vall d'Hebron University Hospital, Universidad Autònoma de Barcelona, Spain), were grown in Ham's F12 medium supplemented with heat-inactivated 5% fetal bovine serum (FBS), 2 mM L-glutamine,100 U/ml penicillin, 100 µg/ml streptomycin, and maintained at 37°C in a humidified 5% CO_2 incubator.
All the reagents for cell culture were supplied by Lonza (Basilea, Switzerland).

3.3 Cancer tissue samples

We investigated cancer tissue specimens from surgically resected cancer patients at the Istituto Cantonale di Patologia (Locarno, Switzerland). Paraffin embedded tissues from both primary tumors and paired normal mucosa were collected from patients affected by gastric, gastrointestinal, ovarian, kidney, lung, prostatic and thyroid cancer.
For all patients, tissues from formalin-fixed paraffin embedded sections are histologically evaluated and are available in the archives of this institute.

3.4 RNA isolation

3.4.1 RNA extraction from frozen tissue samples and cells

Total RNA was isolated from frozen tissue samples and from cells using RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), according to

manufacturer's instructions. Biological samples were first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer which inactivates RNAse, ensuring the purification of intact RNA. We performed a DNase digestion step to eliminate genomic DNA contamination. Ethanol was added to ensure appropriated binding condition of RNA to the silica-based membrane and to wash away contaminants. High-quality RNA was eluted in 30 µL of RNAse-free water.

3.4.2 RNA extraction from formaline-fixed paraffin embedded tissues

Total RNA was isolated from formaline-fixed paraffin embedded tissues using RNeasy FFPE Kit (Qiagen, Chatsworth, CA, USA), according to manufacturer's instructions. 10-micrometer tissue sections obtained from formalin-fixed paraffin embedded specimens were initially treated with a Deparaffinization Solution. Next, samples were incubated in an optimized lysis buffer, which contains proteinase K, to release RNA from the sections. A short incubation at ahigher temperature partially reversed formalin crosslinking of the released nucleic acids, improving RNA yield and quality. We subsequently performed a DNase digestion step to eliminate all genomic DNA contamination including very small fragments that are often present in FFPE samples after prolonged formalin fixation and/or long storage times. Ethanol was added to improve RNA binding to the silica membrane and RNA was eluted in 20µl of RNase-free water.

3.5 Q-PCR

RNA (1 µg) was reverse-transcribed using SuperScript® II RT (Invitrogen, Paisley, UK) and random primers, according to the manufacturer's protocol. For each real-time PCR (Q-PCR), 50 ng cDNA were amplified using the SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) and specific primers of interest (100 nM), using the thermic profile below:

50°C	2 min	
95°C	10 min	
95°C	15 sec	
59°C	1 min	40 cycles
95°C	15 sec	
60°C	1 min	
95°C	15 sec	
60°C	15 sec	

Each sample was analyzed for NEU3 and EGFR expression and normalized for total RNA content using β-actin as housekeeping gene for tissue samples derived from patients CRC affected, since it showed high stability, and Pol2 as housekeeping gene for cells. The samples derived from formaline-fixed paraffin embedded tissues were analyzed for NEU1, NEU3 and NEU4 sialidases expression and normalized for total RNA content using as housekeeping gene β-actin.

The relative expression level was calculated with the $2^{[-\Delta\Delta C(T)]}$ method and was expressed as a fold change ± standard deviation. The accuracy was monitored by the analysis of melting curves (Livak KJ. and Schmittgen TD., 2001). The primers used for Q-PCR are listed in Table 3.1.

Table 3.1 ***List of primers used for Q-PCR***

Gene	Forward Primer	Reverse Primer
EGFR	5'-GGTGTGTGCAGATCGCAAAG-3'	5'-GACATGCTGCGGTGTTTTCAC-3'
NEU1	5'-CCTGGATATTGGCACTGAA-3'	5'-CATCGCTGAGGAGACAGAAG-3'
NEU3	5'-TGAGGATTGGGCAGTTGG-3'	5'-CCCGCACACAGATGAAGAA-3
NEU4	5'-ACCGCCGAGAGTGTTTTGG-3'	5'-CGTGGTCATCGCTGTAGAAGG-3'
Pol2	5'-AGGAGCAAAGCCTGGTGTT-3'	5'-ACCCAAAGCTGCCAGAAGT-3'
β-actin	5'-CGACAGGATGCAGAAGGAG-3'	5'- ACATCTGCTGGAAGGTGGA-3'

3.6 Genomic DNA extraction and mutational status analysis

Cells grown in monolayer on the plate were detached by scraping and centrifuged for 5 min at 300g. Genomic DNA was isolated from the cell pellet using QIAamp® DNA Micro Kit according to the procedure recommended by the supplier.
We investigated the mutational status of EGFR, KRAS, BRAF, PIK3CA, APC and TP53 genes. We searched for point mutations first of all in EGFR, in exon 18-21 corresponding to the tyrosine kinase domain, where iperactivating point mutations usually occur. Then we analyzed KRAS exon 2 (including codons 12 and 13), as already reported (Frattini *et al*, 2007). We investigated BRAF point mutations in exon 15 (including codon 600) and PIK3CA point mutations in exon 9 (including codons 542 and 545) and exon 20 (codon 1047) as previously described (Moroni M. *et al*, 2005; Frattini M. *et al*, 2007), because more than 95% of activating mutations occur in these regions in each gene.
First of all we amplified genomic DNA by PCR.

The program used was:

	50°C	2 min
	96°C	10 min
Denaturation	96°C	30 sec
Annealing	XX°C	30 sec
Extension	72°C	30 sec
	72°C	10 min

30 cycles

The primers and the annealing temperature used for each target are listened in the Table 3.2.
PCR products were sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analysed with appropriate software (SeqScape Software Version 2.5, Applied Biosystems). Each sequence reaction was performed at least twice, starting from independent PCR reactions.

3.7 Immunohistochemical analysis of PTEN

PTEN expression was analyzed in 3-µm formalin-fixed paraffin-embedded tissue sections using a mouse monoclonal primary antibody anti-PTEN (MMAC1 Ab-4, Neomarkers, Fremont, CA, USA) 1:50 in Antibody Diluent Reagent (Ventana, Tucson, AZ, USA) as previously reported (Frattini M. *et al*, 2007). Loss of PTEN was defined when a decrease of at least 50% of the signal was detected in more than 50% of the cells. Normal endometrium was used as external positive control.

Table 3.2 ***List of primers used for amplified genomic DNA***

Gene	Exon	Primers	T Annealing
EGFR	**18**	Fw: 5'-TCCAGCATGGTGAGGGCTGAG-3' Rv: 5'-GGCTCCCCACCAGACCATG-3'	58°C
	19	Fw: 5'-TGGGCAGCATGTGGCACCATC-3' Rv: 5'-AGGTGGGCCTGAGGTTCAG-3'	58°C
	20	Fw: 5'-CCTCCTTCTGGCCACCATGCG-3' Rv: 5'-CATGTGAGGATCCTGGCTCC-3'	58°C
	21	Fw: 5'-CCTCACAGCAGGGTCTTCTC-3' Rv: 5'-CCTGGTGTCAGGAAAATGCT-3'	58°C
K-Ras	**2**	Fw: 5'-TGGTGGAGTATTTGATAGTGTA-3' Rv: 5'-CATGAAAATGGTCAGAGAA-3'	55°C
BRAF	**15**	Fw: 5'-TCATAATGCTTGCTCTGATAGGA-3' Rv: 5'-GGCCAAAAATTTAATCAGTGGA-3'	52°C
PIK3CA	**9**	Fw: 5'-GGGAAAAATATGACAAAGAAAGC-3' Rv: 5'-CTGAGATCAGCCAAATTCAGTT-3'	56°C
	20	Fw: 5'-CTCAATGATGCTTGGCTCTG-3' Rv: 5'-TGGAATCCAGAGTGAGCTTTC-3'	55°C

3.8 FISH analysis

Fluorescent *in situ* hybridization was performed on cell lines previously described and on tissue samples.
Each cell line was incubated overnight at 37°C with Demecolcine Solution 10µg/mL (Sigma-Aldrich, St. Louis, MO, USA), then treated with 2ml Trypsin-EDTA (Lonza, Basilea, Switzerland) from 3 to 10 minutes at 37°C and later centrifuged at 400g for 5 minutes.
The obtained pellet was serially treated with 10mL of KCl (0,56% in distilled water) at 37°C for 7 min, 5mL of aqueous acetic acid (5%), 5mL of methanol and 5 mL of fixative (3:1 ethanol-acetic acid), the latest repeated twice. After each step the pellet was centrifuged at 400g for 8 minutes. Finally cells were firmly attached on glasses.
EGFR gene status evaluation on tissues was performed on 3-micrometer tissue sections obtained from neutral buffered formalin-fixed paraffin embedded specimens. Tissue sections were treated using Paraffin Pretreatment kit II (Vysis, Downer's Grove, IL, USA).
FISH EGFR assay was performed using the dual color probe LSI EGFR/CEP7 (Vysis, Downer's Grove, IL, USA) (Martin V. *et al*, 2009). Fluorescent signals were evaluated through an automated microscope (Zeiss Axioplan 2 Imaging, Oberkochen, Germany) equipped with single and triple band pass filters. Images were captured using an Axiocam camera (Zeiss AxiocamMRm) and processed with the AxioVysion Software (Zeiss).
In order to define objective and consistent criteria we classified cells in 4 groups (disomic, low or high polisomic and amplified) using descriptive features previously reported in literature. In particular, samples were classified using descriptive criteria (ICP criteria) as follows: cases showing 2 chromosomes 7 in more than 60% of cells were defined as disomic; samples with 3 or 4 chromosomes 7 in >40% of cells were defined as low polisomic; tumour samples with an aberrant number of chromosome 7, defined as >4 copies in >40% of cells, were classified as high polysomic; specimens with a ratio >2 between EGFR gene and chromosome 7 centromere signals in >10% of cells were defined as carrying EGFR gene amplification. According to the literature (Colorado score), cell lines carrying either a high polysomic profile or gene amplification were

classified as FISH+; samples carrying either a low polysomic profile or disomic profile were classified as FISH- (Martin V. *et al*, 2009; Varella-Garcia M. *et al*, 2009).

3.9 Vectors

The cDNA coding for human sialidase NEU3 had been previously subcloned in our laboratory, either in frame with C-terminal haemagglutinin (HA) epitope and in frame with C-terminal green fluorescent protein (GFP) into plasmid pcDNA3I (Invitrogen, Paisley, UK), according to Monti E. *et al*. (2000).

3.10 Transfection

The pcDNA3I vector containing *wild type* or mutated NEU3 cDNAs was used for transfection, carried out in a 2% serum medium using X-treme gene 9 DNA transfection reagent with 3:1 ratio (Roche, Basel, Switzerland), according to the manufacturer's instructions. After 6 hours the medium was replaced with the complete medium.

3.11 Confocal fluorescence microscopy

Cells were transfected using pcDNA3I vector containing *wild type* NEU3 cDNA in frame with GFP coding sequence. GFP fluorescence was detected by laser scanning confocal microscopy using the Bio-Rad MRC-600 microscope (Bio-Rad, Hemel Hempstead, UK) coupled with an upright epifluorescence microscope Nikon Optiphot-2 (Nikon, Tokyo, Japan) and equipped with a 20x Nikon Planapochromat dry objective. Fluorescence was excited at 395 nm using a 25mW argon laser and the emission was detected above 509 nm. The accumulation of 50–100 frames enable to collect images with a reduced noise level, even from low fluorescent signals.

3.12 Solubilization experiments

3.12.1 Ultracentrifugation

Membrane isolation and solubilisation was performed also by ultracentrifugation. Briefly, 24 h after transfection, cells were washed twice with cold PBS, harvested by scraping and collected at 800 g for 10 min at 4°C. Pellet was resuspended in 500 µL of cold PBS supplemented with protease inhibitors. Cells were lysed by two cycles of 10 sec bland sonication on ice and then centrifuged at 800 g for 10 min at 4°C. The resulting crude extract was ultracentrifuged at 200,000 g for 20 min at 4°C. After centrifugation, the membrane fraction was resuspended in 200 µL cold PBS. Aliquots of the starting sample and membrane fraction were subjected to western blotting for detection of NEU3.

3.12.2 RIPA buffer

Membrane solubilization was performed using RIPA buffer. Briefly, 24 h after transfection, cells were washed twice with cold PBS and harvested by scraping in 50 µL of RIPA buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% NonidetP-40, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with protease inhibitors) per plate. Cells were then lysed by 4 cycles of 10 sec probe sonication on ice and collected at 800 g for 10 min at 4°C. The resulting crude extract was centrifuged at 13,000 g for 30 min at 4°C to collect cell membranes. The pellet was resuspended in RIPA buffer. Aliquots of the starting sample and membrane fraction were subjected to western blotting for detection of NEU3.

3.12.3 Sodium carbonate extraction

Sodium carbonate extractions were performed as described by Zanchetti *et al.* (2007). Briefly, 36 h after transfection, cells were washed twice with cold PBS, harvested by scraping and then collected by centrifugation at 800

g for 10 min at 4°C. Cells were suspended in ice-cold 10 mM Tris-HCl, pH 7.5, containing protease inhibitors, and sonicated at the minimum setting for 5 sec. After centrifugation at 800 *g* for 10 min at 4°C, the supernatant (crude extract) was centrifuged at 100,000 *g* for 1 h at 4°C to collect total cell membranes. The pellet was resuspended in lysis buffer and then split into identical aliquots. To obtain peripheral protein extraction, membrane samples were then treated with an equal volume either of ice-cold 0.2 M Na_2CO_3, pH 12.0 or 10 mM Tris-HCl, pH 7.5, 3 M NaCl or lysis buffer alone, as a control, and incubated for 30 min on ice. After centrifugation at 100,000 *g*, pellets were resuspended in the appropriate buffer to yield the membrane fractions, while the supernatants represented the soluble fractions. Samples containing sodium carbonate were quickly brought to pH 7.5 by the addition of acetic acid. Finally, soluble and membrane fractions were adjusted to the same final volume and then subjected to western blotting for detection of NEU3.

3.12.4 Triton X-114 extraction

Membrane solubilization with Triton X-114 was performed as described by Bordier (1981). Briefly, 24 h after transfection, cells were washed twice with cold PBS, harvested by scraping and collected at 800 g for 10 min at 4°C. Cells were lysed by 5 sec probe sonication (Bandelin Sonoplus 2070 sonicator) in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, supplemented with protease inhibitors, and then centrifuged at 800g for 10 min at 4°C. The resulting crude extract was diluted in 100 µl of the same buffer to yield a final protein concentration of 1.0 mg/ml. Protein extraction was performed by addition to the sample of a corresponding volume of 2% (v/v) precondensed Triton X-114 in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, followed by incubation for 1 h on ice. Detergent-extracted samples (200 µl) were then layered onto a cushion of 6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.06% Triton X-114 (300 µl), incubated 3 min at 30°C and centrifuged at 300 g for 3 min at room temperature. After centrifugation, the upper aqueous phase was removed and treated again with 1% fresh Triton X-114. A second phase separation was then performed

as above using the same sucrose cushion. Finally, the detergent and aqueous phases were adjusted to the same final volume with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl. Aliquots of the starting sample and separated phases were subjected to western blotting for detection of NEU3 .

3.13 Homology modeling

HsNEU2 (Q9Y3R4) and HsNEU3 (Q9UQ49) amino acidic sequence were retrieved from Uniprot (Universal Protein Resource)database and were aligned in FASTA format using ClustalW (Thompson JD. *et al,* 1994) with a Blosum scoring matrix, an opening and end gap penalty of 10 and an extending and separation gap penalty of 0,05.
Considering the high sequence identity between the two sialidases and the availability of NEU2 crystal structure of in PDB, we performed a homology modeling of NEU3 (auto mode) in the Swiss Model workspace using NEU2 structure (PDB ID: 2F25) as a template (Arnold K. *et al,* 2006; Schwede T. *et al,* 2003 ; Guex N. and Peitsch MC*.,* 1997). The model was visualized using PyMOL.

3.14 Site-directed mutagenesis

Sialidase mutant was obtained by PCR using QuikChange Site directed Mutagenesis Kit (Stratagene La Jolla, CA, USA), according to the procedures recommended by the supplier. PCR program used was:

95°C	30sec	
95°C	30sec	
60°C	1 min	16 cycles
68°C	16 min	
68°C	16 min	

Primers used to introduce D50A and Y370F mutations are listed in Table 3.3.

All constructs were hosted and amplified in *E. coli* strain DH5α; the presence of the mutations was subsequently verified by automated sequencing (Bio-Fab Research, Pomezia, Italy), using commercially available vector oligonucleotide primers.

Table 3.3 ***List of primers used for mutagenesis PCR***

Mutation	Primer
D50A	Fw: 5'-CGTTCTACGAGGAGAGCTGAGGATGCTCTCCAC-3'
	Rv: 5'-GTGGAGAGCATCCTCAGCTCTCCTCGTAGAACG-3'
Y370F	Fw: 5'-GTGGGCCCTGTGGCAACTCTGATCTGGCTGC-3'
	Rv: 5'-GCAGCCAGATCAGAGTTGCCACAGGGCCCAC-3'

3.15 Sialidase activity assay

24h after transfection COS-7 cells were harvested by scraping, washed in PBS and resuspended in the same buffer containing 1 mM EDTA, 1 µg/mL pepstatin A, 10 µg/mL aprotinin and 10 µg/mL leupeptin. Crude extracts, obtained by gentle sonication, were centrifuged at 800 g for 10 min to eliminate unbroken cells and nuclear components. Supernatants were subsequently centrifuged at 200,000 g for 15 min to obtain a cytosolic fraction and a membrane fraction. The activity was then evaluated on the pellets resuspended in PBS. Protein concentration was determined by Bradford assay (Coomassie Protein Assay Reagent, Pierce)(Bradford MM., 1976).

NEU3 sialidase activity was determined towards two different substrates according to Monti *et al.* (2000). All reaction mixtures were set up in triplicate with 30 µg of total protein in a final volume of 100 µL in the presence of 12.5 mM sodium citrate/phosphate buffer, pH 3.8. In all cases, one unit of sialidase activity was defined as the liberation of 1 µmol of NeuAc/min at 37°C.

Using 0.12 mM 4MU-NANA (4-methylumbelliferyl α-NANA) as an artificial substrate, the amount of sialic acid hydrolyzed was evaluated by spectrofluorimetric measurement of the 4-methylumbelliferone released after an incubation at 37°C up to 30 min stopping the reaction with 1.5 mL 0.2 M Glycine/NaOH, pH 10.8.

The activity was also measured towards G_{D1a} ganglioside by a radiochemical method. The mixture containing 60 nmol G_{D1a}+[3H]G_{D1a} and TRITON X-100 0.1% was incubated at 37°C for 1 h and then 400 µL of tetrahydrofuran were added.

The mixture was centrifuged at 10,000 g for 5 min and 10 µL of resulting supernatant were subjected to high-performance TLC on silica-gel plate with chloroform/methanol/0,2% $CaCl_2$ (50:42:11 vol) as a solvent system to separate the reaction products from the substrate (Chigorno V. *et al*, 1986). Glycolipids separated were quantified by radiochromatoscanning (Beta Imager 2000; Biospace Mesures Paris, France).

3.16 SDS-PAGE and Western blotting

With the purpose to analyse EGFR pathway activation, 36 h after transfection cells were washed with ice-cold PBS and lysed in RIPA buffer, containing both protease and phosphatase inhibitors and 1 mM PMSF. After lysis on ice, homogenates were obtained by passing 5 times through a blunt 20-gauge needle fitted to a syringe and then centrifuged at 15,000 g for 30 min. Supernatants were analyzed for protein content. Protein concentration was determined by the BCA protein assay (Smith PK. *et al*, 1985) using Pierce® BCA Protein Assay Kit (Thermo Scientific).

SDS-PAGE and Western blotting were carried out by standard procedures. Equal amounts of protein (60 µg) were separated on 10% acrylamide/bis-acrylamide SDS-PAGE, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) and probed with the appropriated antibodies. Membranes were blocked with 5% dried milk in PBS, 0.1% Tween20 (PBS-T), and incubated with appropriated dilution of primary antibody in blocking solution overnight at 4°C. After washes in PBS-T and incubation with HRP-conjugated IgG antibody, detection was performed

using ECL detection system (Millipore, Billerica, MA, USA). Protein levels were quantified by densitometry of immunoblots using ScionImage software (Scion Corp., Frederick, MD, USA). Primary antibodies tested were anti EGFR, phospho-EGFR (Tyr1068), p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Akt (pan), phospho-Akt (Ser473), PTEN, GAPDH mAbs from Cell Signaling Technology, Danvers, MA, USA. Anti-mouse and anti-rabbit IgG HRP-conjugated antibodies were also from Cell Signaling Technology, Danvers, MA, USA.

3.17 Viability assay

Cell viability was investigated using an *in vitro* toxicology assay kit MTT based (Sigma, St. Louis, MO, USA), in accordance with manufacturer's protocols.

Cells were seeded in 96-well micro titer plates at a density of 1×10^4 cells/well and cultured in complete medium without phenol red. After an incubation at 37°C for 36 h post transient transfection, 10 µL of MTT solution (5 mg/mL) were added to each well. After a further 4 h incubation time, absorbance upon solubilization was measured at 570 nm using a micro plate reader to assay the effect of overexpression of *wild type* or inactive form of NEU3.

The results were expressed as mean values ± standard deviation of three determinations.

Determination of half maximal inhibitory concentration (EC50) was performed on cells seeded in 96-well micro titer plates and cultured in complete medium without phenol red in the presence of Cetuximab at different concentration, from 0.01 µg/mL up to 200 µg/mL. After incubating cells for 36 h at 37°C, reconstituted MTT was added to each well and after a 4 h incubation time, absorbance upon solubilization was measured at 570 nm using a micro plate reader. EC50 was defined as the drug concentration yielding a fraction of cells affected equal to 0.5, compared to control.

The effect of overexpression either of *wild type* or mutated form of NEU3 was assayed at the dose corresponding to the estimated EC50 for each cell

line tested. Cells, after transient transfection, were cultured in a completed medium containing Cetuximab. After 36h of treatment the same procedure described above was performed and the optical density at 570 nm was measured.
The results were expressed as mean values ± standard deviation of three determinations.

3.18 EGFR Immunoprecipitation

Briefly, 36h after transfection, DIFI and SW480 cells were washed in PBS and harvested by scraping in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% NP-40 containing phosphatase inhibitor, 1 µg/mL pepstatin A, 10 µg/mL aprotinin and 10 µg/mL leupeptin as protease inhibitors.
Crude extracts, obtained by gentle sonication, were then centrifuged at 15,000 g for 15 min to clarify the lysate. The protein content of supernatant was assayed by the Bradford method.
The volume corresponding to 1 mg of total protein extract was incubated overnight at 4°C with 2 µL of EGFR antibody with gentle rocking. Samples were then incubated for 4 h at 4°C with 20 mg of protein A-Sepharose previously re-hydrated (Amersham Pharmacia Biotech, Uppsala, Sweden). After washes, immunoprecipitates were collected by centrifugation, boiled in 2x SDS-sample buffer without β-mercaptoethanol and subjected to electrophoresis and Western blot analysis.

3.19 Lectin affinity assay

EGFR immunoprecipitates were separated on 10% acrylamide/bis-acrylamide SDS-PAGE, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) and probed with a biotinylated form of the lectin *Sambucus Nigra* agglutinin (SNA) (Vector Laboratories, Burlingame, CA, USA). Membrane was blocked with Carbo-free blocking solution, 0.1% Tween-20 (TBS-T) overnight at 4°C, and incubated with biotinylated SNA 4

μg/mL in TBS 0.05% Tween-20 1 hour at room temperature. After washes in TBS-T, membrane was incubated using VECTASTAIN® elite ABC reagent 30 minutes at room temperature. After washes in TBS-T, α2,6-sialylated EGFR was detected using ECL (Millipore, Billerica, MA, USA). Protein levels were quantified by densitometry of immunoblots using ScionImage software (Scion Corp., Frederick, MD, USA).

3.20 Acid silver stain

In order to analyze sialylation levels by mass spectrometry, immunoprecipitated EGFR was loaded onto a SDS-PAGE and the gel was fixed for 1 h in 40% ethanol, 10% acetic acid and overnight in 5% ethanol, 5% acetic acid.

The gel was subsequently washed in 30% ethanol and incubated for 1 min in 0.8 M sodium thiosulfate. Then it was incubated in 12 mM silver nitrate containing 0.02% formaldehyde for 20 min. After washing with water, development was carried out with 556 mM sodium carbonate containing 0.02% formaldehyde and 0.02 mM sodium thiosulfate. Reaction was stopped with 50% ethanol, 12% acetic acid. After washing with water, the gel was conserved at 4°C in 1% acetic acid.

3.21 Mass spectrometry analysis

The gel bands corresponding to EGFR protein were excised, cut into smaller pieces, and dried in a Speed Vac. Dried gels were reduced by 10 mM dithiothreitol at 56 °C for 1 h. Following cysteine derivatisation by iodoacetamide at 25 °C the gels were digested overnight with trypsin sequencing grade (ratio 1:10=protease:protein) at 37 °C. The in-gel tryptic digest was extracted with 50% acetonitrile in 0.1% trifluoroacetic acid and the peptide mixture was desalted using C18 ZipTipe (Millipore) before mass spectrometry analysis. The tryptic digest was used for MALDI-TOF and ESI analysis.

3.21.1 MALDI-TOF analysis

The extract was subjected to matrix-assisted laser desorption ionization/time-of-flight mass spectrometric analysis by using a AutoflexIII (Bruker Daltonics) instrument equipped with a nitrogen laser (337 nm) and operated in reflector mode with a matrix of α-ciano-4-hydroxycinnamic cinnamic acid. External standards were used for calibration (Bruker peptide calibration standard. In order to assess the different level of sialylation, the fingerprint spectra of mock, *wild type* NEU3 and double mutant NEU3 samples were compared focusing on peaks not present in all samples.

3.21.2 ESI analysis

Samples were separated by liquid chromatography using an UltiMate 3000 HPLC (Dionex, now Thermo Fisher Scientific). Buffer A was 0.1% v/v formic acid, 2% acetonitrile; buffer B was 0.1% formic acid in acetonitrile. Chromatography was performed using a PepMap C18 column (15 cm, 180 lm ID, 3 lm resin, Dionex). The gradient was as follows: 5% buffer B (10 min), 5-40% B (60 min), 40-50% B (10 min), 95% B (5 min) at a flow rate of 0.3 lL/min.
Mass spectrometry was performed using a LTQ-Orbitrap Velos (Thermo Fisher Scientific) equipped with a nanospray source (Proxeon Biosystems, now Thermo Fisher Scientific). Eluted peptides were directly electro-sprayed into the mass spectrometer through a standard non-coated silica tip (New Objective, Woburn, MA, USA) using a spray voltage of 2.8 kV. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350–2000) in the Orbitrap and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan. Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific). Data Base searching was performed using the Sequest search engine contained in the Proteome Discoverer 1.1 software (Thermo Fisher Scientific). The following parameters were used: 10 ppm for MS and 0.5 Da for MS/MS tolerance,

carbamidomethylation of Cys as fixed modification, Lys ubiquitination as variable modifications, trypsin (2 misses) as protease.

3.22 Statistical analysis

All values are presented as means ± standard deviation (SD).
To define the statistical significance of the gene expression correlation rate in tissues and cell lines we used Fisher's exact test, a test useful in the analysis of contingency tables in which the significance of the deviation from a null hypothesis can be calculated exactly.
Statistical analyses were usually performed using Student's t-test comparing treated cells data with mock cells data. Sometimes comparisons among different samples were performed. Significance was defined as *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

4. Results

4.1 Study of the anchoring mechanism of the human sialidase NEU3 to the plasma membrane

Zanchetti *et al.* (2007) have shown that the murine form of sialidase NEU3 is associated with the outer leaflet to the plasma membrane. This evidence is well in accordance with the ability of NEU3 to degrade gangliosides inserted into the plasma membrane of adjacent cells. Moreover, the mechanism of the protein association with the lipid bilayer is an indirect one, involving a polar interaction with some membrane proteins, as shown by the fact that murine NEU3 can be solubilized by carbonate extraction and not by treatment with Triton X-114.

Starting from these data we performed the same experiments on the human isoform of NEU3.

First of all, we transfected COS7 cells with pCDNA3I-Hs-NEU3-HA and after 36h we harvested cells in PBS buffer and performed a cellular fractionation by ultracentrifugation.

As shown in Figure 4.1A, after Western blot we recovered NEU3 in the membrane fraction. Unexpectedly the protein was detected in SDS electrophoresis at the beginning of the running gel suggesting protein aggregation.

We therefore tried to solubilize NEU3 with detergents using RIPA buffer. The protein was again recruited in the insoluble fraction showing the same electrophoretic pattern.

These experiments were repeated GFP-tagged sialidase in order to assess whether the protein solubility was conditioned by the tag. As reported in Figure 4.1B, we confirmed NEU3 localization in the membrane fraction and also its unsuccessful solubilization upon detergent treatment, the only difference being in a partial migration in SDS-PAGE.

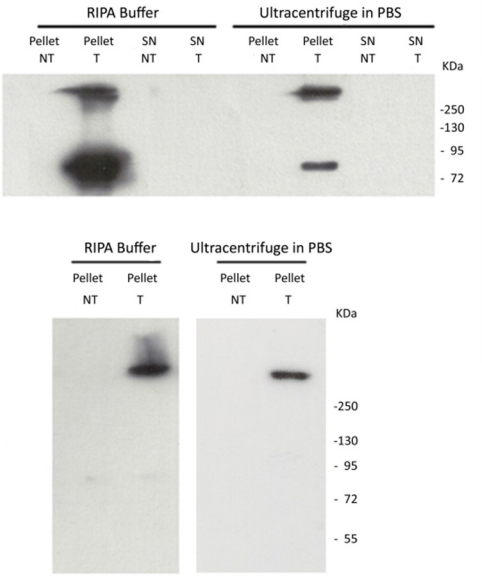


Figure 4.1 ***Western blot analysis of human sialidase NEU3 after ultracentrifugation in PBS and RIPA buffer treatment.***
A. COS-7 cells transfected with the construct pcDNA3I-Hs-NEU3-HA were lysed in PBS and fractionated by ultracentrifugation or treated with RIPA buffer. Equal amounts of protein were subjected to SDS-PAGE and western blotting. NEU3 was detected using an anti-HA antibody. B. The same experiment performed on COS-7 cells transfected with the pcDNA3I-Hs-NEU3-GFP construct. NEU3 was detected using an anti-GFP antibody. Blots are representative of three independent experiments. (NT: not transfected, T: transfected, SN: supernatant)

We subsequently tested if, as reported for murine NEU3, carbonate extraction could lead to a complete solubilization of the human isoform. Noteworthily, under alkaline conditions the lipid structure is normally preserved, without affecting the association of integral proteins with the lipid bilayer. As reported in Figure 4.2, human sialidase NEU3 was again

detected in the insoluble fraction, although after this treatment we observed a correct migration in SDS-PAGE.

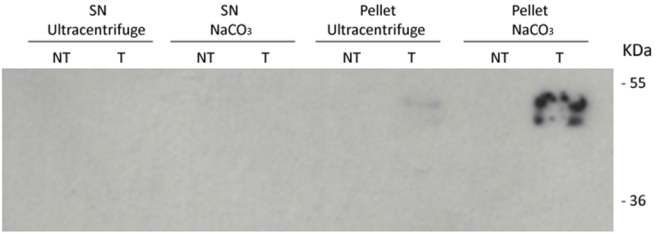


Figure 4.2 ***Western blot analysis of human sialidase NEU3 after carbonate treatment.***
COS-7 cells transfected with the pcDNA3I-Hs-NEU3-HA construct were treated with carbonate and fractionated by ultracentrifugation. Equal amounts of protein of each fraction were subjected to SDS-PAGE and western blotting. NEU3 was detected using an anti-HA antibody.
Blot is representative of three independent experiments.
NT: not transfected, T: transfected, SN: supernatant.

Conversely from the data reported in literature for murine NEU3, human sialidase is apparently more strongly anchored to membrane.
Using as alternative solubilization method, the temperature-induced phase separation in Triton X-114, we observed that the human NEU3 was still detected in the detergent-insoluble pellet as shown in Figure 4.3.
Usually with this treatment ectoenzymes anchored by a single membrane spanning polypeptide are recovered in the detergent-rich phase, while ectoenzymes with a covalently attached glycosyl-phosphatidylinositol (G-PI) membrane anchor are predominantly recovered in the insoluble fraction.

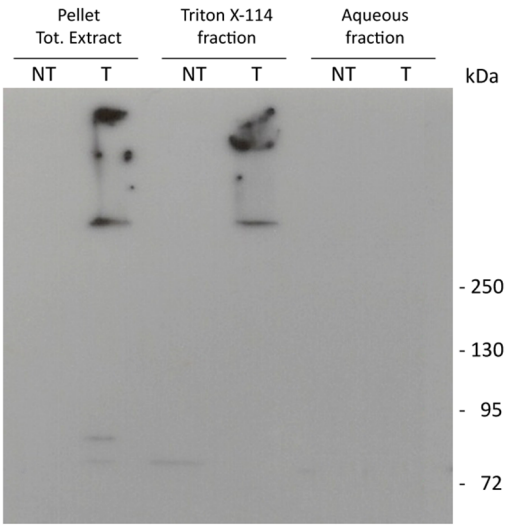


Figure 4.3: ***Western blot analysis of human sialidase NEU3 after temperature-induced phase sepharation in Triton X-114.***
COS-7 cells transfected with pcDNA3I-Hs-NEU3-HA construct were treated with Triton x-114. Equal amounts of protein of each fraction were subjected to SDS-PAGE and western blotting. NEU3 was detected using an anti-HA antibody.
Blotis representative of three independent experiments.
NT: not transfected, T: transfected.

We therefore compared the amino acids sequence of human sialidase NEU3 to the murine homologue in order to find differences that could explain these data.
A bioinformatic analysis using the ExpPASy platform was carried out: in particular we used the TMpred program that makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins.
We confirmed the results performing the same analysis by TMHMM tool, a membrane protein topology prediction method based on a hidden Markov model (HMM), that predicts transmembrane helices in proteins.

The sequence alignment, reported in Figure 4.4, shows that the two sequences are strongly conserved, differing mainly in the C-terminus and in two brief portions in the inner region of the sequence (from 190 to 196 and from 305 to 317 amino acid position on human sequence).
Observing the hydrophobicity plots obtained through TMPred, the general trend of the two sequences is conserved. In both cases an inner portion of about 20 amino acids was found, predicted to be a transmembrane helix, from the 178 to the 203 in *Mus musculus* NEU3 and from 175 to 194 in human NEU3, respectively.
In the plot of the human sialidase NEU3 a sequence of 21 amino acids was also identified, from position 92 to 112, that was predicted to be a transmembrane helix with an outside-to-inside orientation (Figure 4.5).

```
CLUSTAL W (1.83) multiple sequence alignment

MmNEU3    MEEVPPYSLSSTLFQQEEQSGVTYRIPALLYLPPTHTFLAFAEKRTSVRDEDAACLVLRR   60
HsNEU3    MEEVTTCSFNSPLFRQEDDRGITYRIPALLYIPPTHTFLAFAEKRSTRRDEDALHLVLRR   60
          ****.. *:.*.**:**:: *:**********:**************:: *****  *****

MmNEU3    GLMKGRSVQWGPQRLLMEATLPGHRTMNPCPVWEKNTGRVYLFFICVRGHVTERCQIVWG   120
HsNEU3    GLRIGQLVQWGPLKPLMEATLPGHRTMNPCPVWEQKSGCVFLFFICVRGHVTERQQIVSG   120
          **  *: ***** : **********************:::* *:************** *** *

MmNEU3    KNAARLCFLCSEDAGCSWGEVKDLTEEVIGSEVKRWATFAVGPGHGIQLHSGRLIIPAYA   180
HsNEU3    RNAARLCFIYSQDAGCSWSEVRDLTEEVIGSELKHWATFAVGPGHGIQLQSGRLVIPAYT   180
          :*******: *:*******.**:***********:*:***************:****:****:

MmNEU3    YYVSRWFLCFACSVKP--HSLMIYSDDFGVTWHHGKFIEPQVTGECQVAEVAGTAGNPVL   238
HsNEU3    YYIPSWFFCFQLPCKTRPHSLMIYSDDLGVTWHHGRLIRPMVTVECEVAEVTGRAGHPVL   240
          **:. **:**  . *.  *********:*******::*.* ** **:****:* **:***

MmNEU3    YCSARTPSRFRAEAFSTDSGGCFQKPTLNPQLHEPRTGCQGSVVSFRPLKMPNTYQDSIG   298
HsNEU3    YCSARTPNRCRAEALSTDHGEGFQRLALSRQLCEPPHGCQGSVVSFRPLEIPHRCQDSSS   300
          *******.* ****:*** *  **: :*. ** **  ************::*:  *** .

MmNEU3    KGAPATQKCPLLDSPLEVEKGAETPSATWLLYSHPTSKRKRINLGIYYNRNPLEVNCWSR   358
HsNEU3    KDAPTIQQS-SPGSSLRLEEEAGTPSESWLLYSHPTSRKQRVDLGIYLNQTPLEAACWSR   359
          *.**: *:.   .*.*.:*: * *** :*********:::*::**** *:.***. ****

MmNEU3    PWILNRGPSGYSDLAVVEEQDLVACLFECGEKNEYERIDFCLFSDHEVLSCEDCTSPSSD   418
HsNEU3    PWILHCGPCGYSDLAALEEEGLFGCLFECGTKQECEQIAFRLFTHREILSHLQGDCTSPG   419
          ****: **.******.:**:.*..****** *:* *:* * **:.:*:**  :  ..*..

MmNEU3    ---------                                                      -
HsNEU3    RNPSQFKSN                                                      428
```

Figure 4.4 ***Sequence alignment of the human and the murine isoforms of NEU3 sialidase.***
The two sequences in FASTA format were recruited from the UniProt database. The aligment was performed using CLUSTAL W tool, from the ExPASy bioinformatic platform.

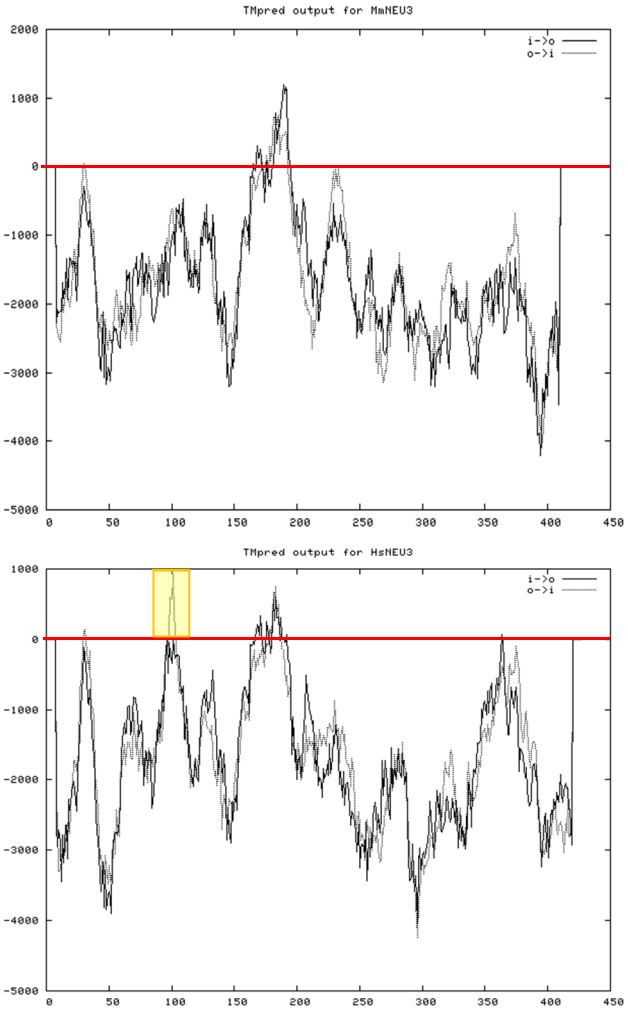


Figure 4.4 ***Hydrophobicity prediction plot of the human and the murine isoforms of NEU3 sialidase.*** The two sequences in FASTA format were recruited from the UniProt database. The plot was obtained using TMPred tool, from the ExPASy bioinformatic platform. In the yellow box the idrofobic portion identified only in the human sequence is highlighted.

4.2 Human sialidases NEU3 and NEU4 transcripts deregulation in a Caucasian cohort of CRC patients

We investigated 85 Caucasian patients surgically resected for a CRC. Fresh tissues from both primary tumors and paired normal mucosa were immediately frozen in liquid nitrogen and subsequently stored at -80°C until the analysis.

Total RNA was isolated from primary tumors or from adjacent normal mucosa by the RNeasy kit (QIAGEN), as recommended by the manufacturer. First strand cDNAs were synthesized by reverse transcription and used as templates for real-time PCR experiments (SYBER-green assay). B-actin gene was used as housekeeping gene. The fold increase/decrease in tumor was calculated through the $2^{-\Delta\Delta Ct}$ method using paired normal tissue as calibrator. We considered gene overexpressing tumors those showing a ≥ 3-fold expression level with respect to paired normal mucosa.

As shown in Figure 4.5, the transcription rate is generally up-regulated and in 27 out of 85 evaluable cases (32%) NEU3 mRNA overexpression was detected in tumors.

In 74 of 85 cases the transcription level of NEU4 sialidase was also evaluated, a human isoform previously demonstrated to localize in the mitochondria membrane. As shown in Figure 4.6, the transcription rate was mostly downregulated and in 31 out of 74 cases (42%) a <3 fold decrease of NEU4 mRNA level was detected in tumors.

By comparing NEU3 and NEU4 mRNA expression levels in the cohort of samples analyzed, we observed that both transcripts were overexpressed only in 4 cases (5%) (Figure 4.7).

In most cases we observed an opposite trend of transcription of the two sialidases, with an increase of NEU3 mRNA levels and a decrease of NEU4 mRNA levels.

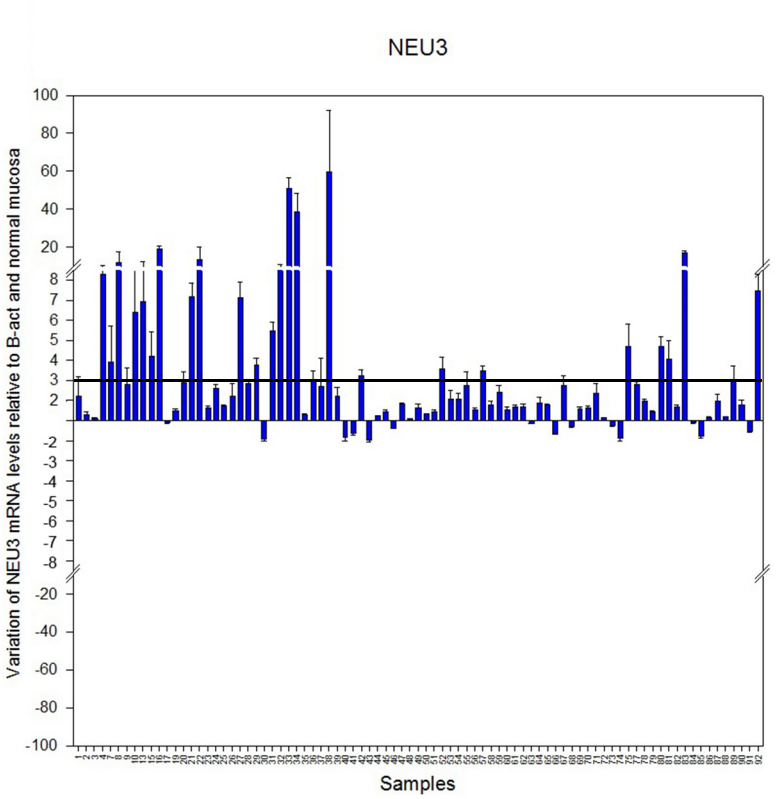


Figure 4.5 ***NEU3 mRNA expression in 85 patient affected by CRC.***
Real-time PCR analysis of NEU3 mRNA levels in tumor tissues of 85 Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed in triplicate.

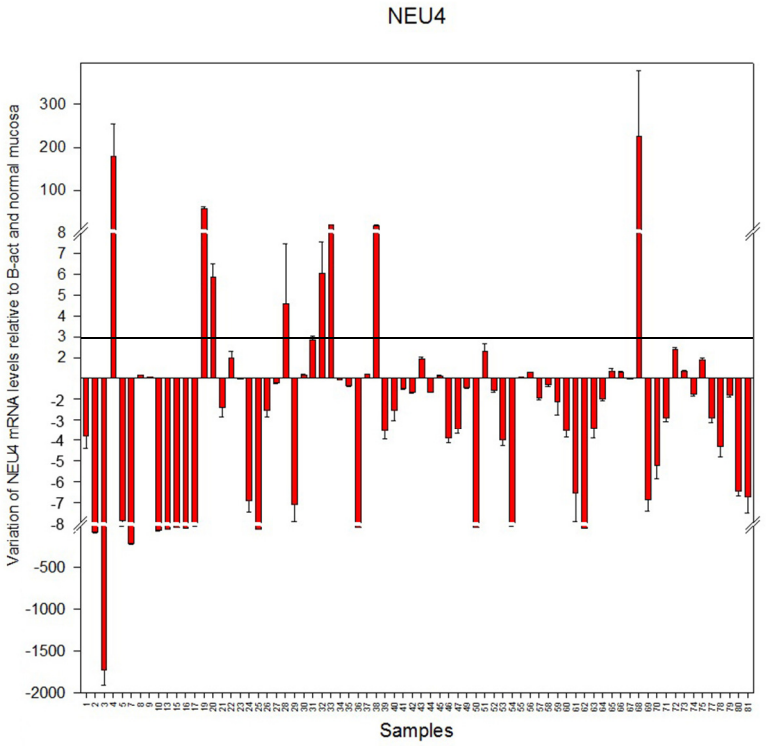


Figure 4.6 ***NEU4 mRNA expression in 74 patient affected by CRC*.**
Real-time PCR analysis of NEU3 mRNA levels in tumor tissues of 74 Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed in triplicate.

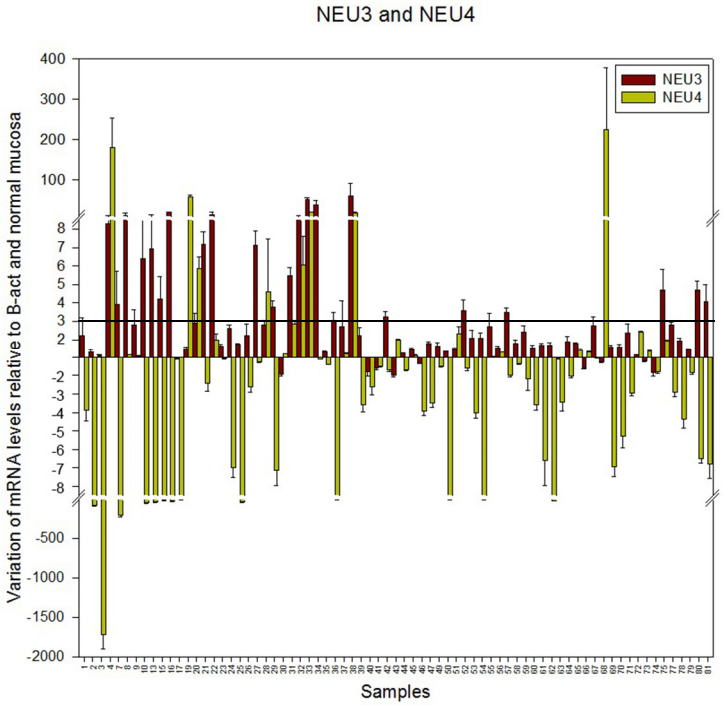


Figure 4.7 ***NEU3 and NEU4 mRNA expression in 73 patient affected by CRC.*** Real-time PCR analysis of NEU3 mRNA levels in tumor tissues of 73 Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed in triplicate.

4.3 EGFR transcripts deregulation in a Caucasian cohort of CRC patients

We also evaluated, in the same cohort of patients resected for CRC, the variation of the mRNA levels of EGFR in order to record data in Western population. As described in the paragraph above, we analyzed EGFR transcription rate by Real-time PCR using B-actin gene as housekeeping and the paired normal tissue as calibrator. The data collected are reported in the Figure 4.8. We observed EGFR mRNA overexpression in only 10 out of 85 evaluable cases (12%).

By comparing NEU3 and EGFR mRNA expression levels, we observed that NEU3 was overexpressed in 20 out of 75 (27%) EGFR negative cases and in 7 out of 10 (70%) EGFR overexpressing cases (Figure 4.9). This difference is statistically significant (p=0.010, two-tailed Fisher's Exact Test). These data suggest therefore that a strict correlation between NEU3 and EGFR mRNA expression exists (Table 4.1).

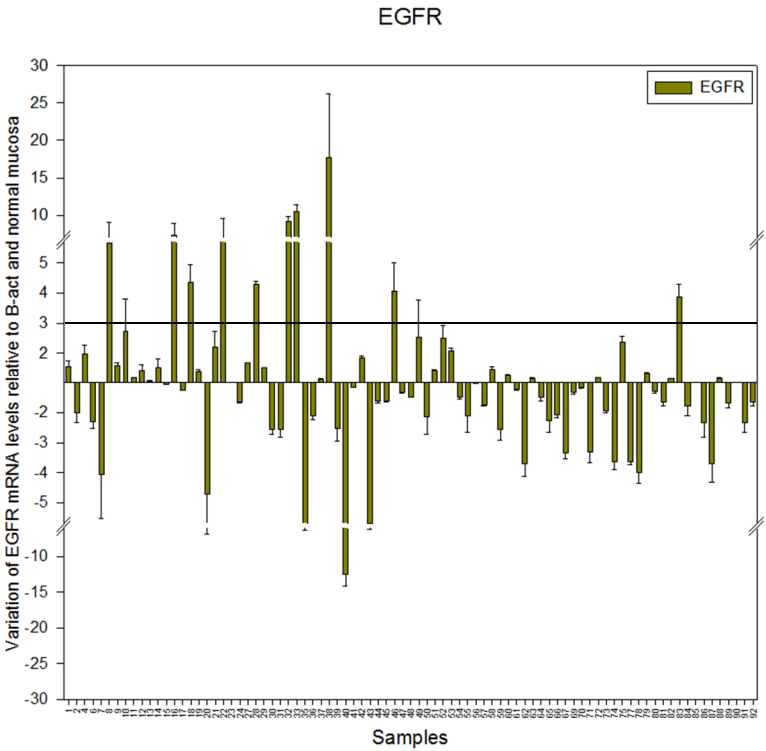


Figure 4.8 ***EGFR mRNA variation in 85 patient affected by CRC.***
Real-time PCR analysis of NEU3 mRNA levels in tumor tissues of 85 Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed at least in triplicate.

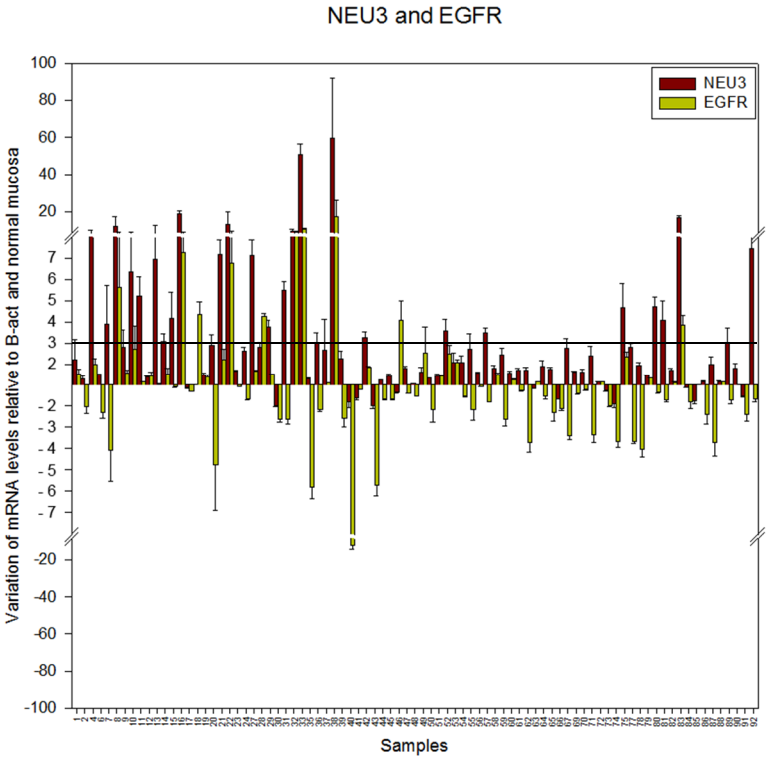


Figure 4.9 ***EGFR and NEU3 mRNA variation in 85 patient affected by CRC.*** Real-time PCR analysis of NEU3 mRNA levels in tumor tissues of 85 Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed at least in triplicate

Table 4.1 ***Correlation analysis between NEU3 and EGFR expression levels in 85 CRC patients by two-tailed Fisher Exact's Test***.
(+: overexpressing cases; -: cases with normal expression)

		NEU3		
		+	**-**	**Tot**
EGFR	**+**	7	3	10
	-	20	55	75
	Tot	27	58	85

p=0,010

To evaluate the activation of EGFR downstream pathways, we investigated the mutational status of KRAS, BRAF and PIK3CA genes by direct sequencing and PTEN protein expression by immunohistochemistry (Table 4.2).

We identified KRAS mutations in 33 out of 88 evaluable cases (37.5%), of which 29 at codon 12 and 4 at codon 13. The most frequent mutations identified were G12V and G12D (each detected in 10 patients) changes; the other mutations observed were: G12C in 3 cases, G12A, G12R and G12S in 2 cases. All mutations occurring at codon 13 were represented by the classical G13D change, one of which was detected in a patient showing the concomitant mutation D33E.

We only found 2 mutated cases out of 87 evaluable patients (2%) for BRAF gene; the mutation was the classical change V600E.

We observed 11 mutations in 83 (12.5%) evaluable patients for PIK3CA gene (exons 9 and 20); in particular, 8 patients showed an alteration in exon 9 (1 E542K change, 1 Q546K change and 6 E545K changes) and 3 in exon 20 (all represented by the classical change H1047R). The frequency and the type of mutations perfectly match with those reported in the literature.

As for PTEN protein expression on FFPE tissue, we found that 49 out of 71 evaluable patients (69%) showed a positive staining, demonstrating normal expression of the protein, whereas 22 out of 71 evaluable patients (31%) showed a negative staining, demonstrating loss of expression of the protein in at least 50% of tumoral cells.

Table 4.2 ***KRAS, BRAF, PIK3CA mutational analyses and PTEN immunohistochemical analysis of 88 FFPE evaluated patients***.
Neg: negative staining; NV: not evaluable; Pos: positive staining; WT: *wild type*.
In red: cases with alteration (gene mutation or absence of protein expression).

Sample	KRAS	BRAF	PIK3CA 9/20	PTEN
1	WT	WT	WT	Neg
2	WT	WT	WT	Neg
4	G12D	WT	WT	Pos
5	WT	WT	WT	Neg
6	WT	WT	WT	Neg
7	G12D	WT	WT	Neg
8	WT	WT	WT	Pos
9	G12D	WT	E545K	NV
10	WT	WT	WT	Pos
11	WT	WT	WT	Neg
12	WT	WT	WT	Pos
13	WT	V600E	WT	Pos
14	G13D	WT	WT	NV
15	G12V	WT	E545K	Neg
16	WT	WT	WT	Pos
17	WT	WT	WT	Pos
18	WT	WT	WT	Pos
19	G13D	WT	WT	Neg
20	WT	WT	WT	Pos
21	WT	WT	WT	Neg
22	G12R	WT	WT	NV
23	WT	V600E	WT	Pos
24	G13D	WT	WT	NV
27	WT	WT	WT	Pos

28	WT	WT	WT	Neg
29	G12V	WT	WT	NV
30	WT	WT	WT	NV
31	G12V	WT	WT	Neg
32	G12V	WT	WT	Pos
33	WT	WT	WT	Neg
35	WT	WT	WT	NV
36	G12D	WT	WT	Pos
37	WT	WT	WT	Neg
38	G12C	WT	WT	Neg
39	WT	WT	WT	Pos
40	G12D	WT	WT	NV
41	G12D	WT	E542K	Pos
42	WT	WT	WT	NV
43	WT	WT	WT	NV
44	WT	WT	WT	NV
45	G12C	WT	E545K	Pos
46	WT	WT	WT	NV
47	G12C	WT	E545K	Pos
48	G12D	WT	WT	Pos
49	WT	WT	WT	Pos
50	WT	WT	H1047R	Pos
51	WT	WT	WT	Pos
52	G12A	WT	WT	Pos
53	G12D+D33E	WT	WT	Pos
54	G12V	WT	WT	Pos
55	G12R	WT	WT	Pos
56	WT	WT	WT	Pos
57	WT	WT	H1047R	Pos
58	WT	WT	WT	Pos
59	WT	WT	WT	Pos
60	G12A	WT	WT	Pos
61	WT	WT	WT	Pos
62	WT	WT	WT	Pos
63	G12V	WT	E545K	NV
64	WT	WT	WT	Pos

65	G12S	WT	WT	Pos
66	WT	WT	WT	Pos
67	WT	WT	WT	Pos
69	G12S	WT	WT	Neg
70	G12D	WT	WT	Pos
71	WT	WT	WT	Pos
72	G12D	WT	WT	Neg
73	WT	WT	WT	Pos
74	G12V	WT	WT	Pos
75	WT	WT	WT	Neg
77	WT	WT	H1047R	Pos
78	WT	WT	WT	Pos
79	WT	WT	WT	Pos
80	WT	WT	NV	Pos
81	WT	WT	WT	Pos
82	G12V	WT	Q546K	Neg
83	WT	WT	WT	NV
84	WT	WT	WT	Neg
85	G12D	WT	E545K	NV
86	G12V	WT	WT	Pos
87	WT	WT	WT	Neg
88	WT	WT	WT	Neg
89	WT	WT	WT	Pos
90	WT	WT	WT	NV
91	G12V	WT	WT	NV
92	WT	WT	WT	Pos
93	WT	WT	WT	Pos
94	WT	WT	WT	Neg

Using the two-tailed Fisher's Exact Test, we evaluated possible correlations existing between these molecular markers (KRAS, BRAF, PIK3CA and PTEN) and NEU3 and EGFR mRNA expression levels. No correlations were observed among these markers.

4.4 Analysis of deregulation of NEU3 and EGFR in human colon cancer cell lines

We evaluated the correlation between NEU3 and EGFR expression and activation in 13 commercial human colon cancer cell lines (SW48, CO115, SW403, SW1116, SW480, SW1463, E705, MICOL29, MICOL24, HT-29, SW620, T84 and CACO2), as well as in DIFI cells, a fibroblast cell line characterized by high levels of EGFR gene amplification.

We initially evaluated NEU3 and EGFR gene expression levels through Q-PCR by comparing mRNA expression levels in colon cancer and fibroblast cells with those observed in CCD841 normal intestinal healthy mucosa cells, using as cut-off value a ≥3-fold increase.

As shown in Figure 4.10A, the level of NEU3 mRNA was increased by 3- to 30-fold in all cell lines tested, compared to the normal mucosa cell line, with MICOL24 and CACO2 cells showing the highest levels.

As for EGFR, we observed mRNA EGFR overexpression in 7 out of 14 (50%) analyzed cell lines, namely SW48, SW1116, SW480, MICOL29, MICOL24, CACO2 and DIFI (Figure 4.10B). Three out of 7 cell lines without EGFR overexpression were characterized by EGFR downregulation, with SW620 cells showing the strongest EGFR transcript decrease (Figure 4.10B). No significant correlation was observed between NEU3 and EGFR mRNA levels.

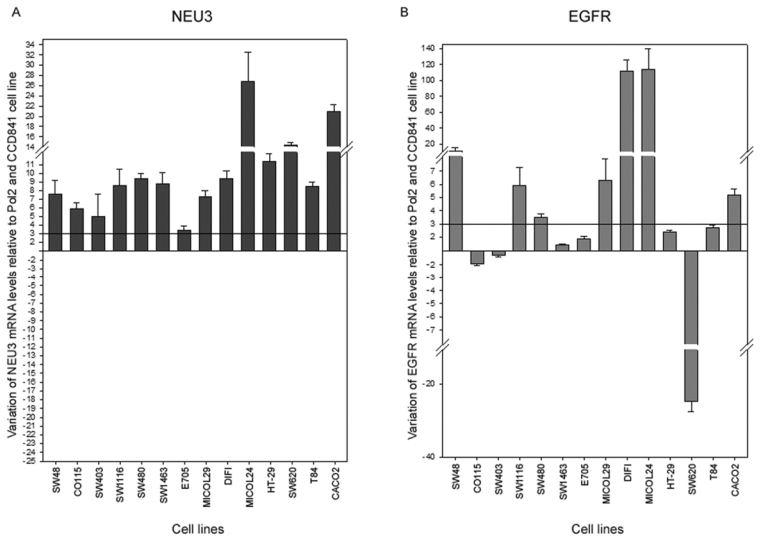


Figure 4.10 ***Relative quantification of NEU3 and EGFR mRNA levels by Real-time quantitative PCR in colon and fibroblast cell lines.***

The relative expression levels of colon cancer and DIFI fibroblast cell lines were calculated with the Livak method (2[-ΔΔC(T)]) and were expressed as a fold change ± standard deviation, using Pol2 gene as internal reference control and the CCD841 normal healthy mucosa as calibrator. mRNA overexpression was defined using a cut-off value of ≥3-fold increase. Values are presented as means ± standard deviation (SD).

Concerning EGFR gene sequence, except one, all cell lines were characterized by *wild type* sequences of exons 18-21 (encoding the tyrosine kinase domain). The exception is represented by SW48 cell line showing a G719S substitution, located in exon 18 and able to constitutively activate the EGFR protein.

Table 4.3 ***EGFR mutational status by direct sequencing in CRC cell lines.***
WT: *wild type*. In red: mutated cell lines.

Cell line	EGFR
SW48	**G719S**
CO115	WT
SW403	WT
SW1116	WT
SW480	WT
SW1463	WT
E705	WT
MICOL29	WT
DIFI	WT
MICOL24	WT
HT-29	WT
SW620	WT
T84	WT
CACO2	WT
CCD841	WT

Subsequently, we investigated EGFR gene status by FISH experiments to characterize the chromosomal status (Table 4.4).
Two colon cancer cell lines (SW48 and E705) and the CCD841 normal intestinal mucosa cell line were classified as FISH-, according to the Colorado score. In particular, 2 of these cell lines (E705 and CCD841) showed disomy, whereas the SW48 cell line showed low polysomy.
Of the remaining 12 cell lines, all classified as FISH+, 2 were characterized by EGFR gene amplification (the MICOL24 colon cancer cell line and, as expected, the DIFI fibroblast transformed cell line), 1 by concomitant high

polysomy and low gene amplification (CACO2) and the remaining 9 by high polysomy (Table 4.4).

Table 4.4 ***EGFR gene status by FISH in CRC cell lines.***
Legend: 2N: disomy, A: gene amplification; FISH -: FISH negative; FISH +: FISH positive; HP: high polysomy; LowA: low gene amplification; LP: low polysomy.

Cell line	FISH EGFR	
	ICP score	Colorado score
SW48	LP	FISH -
CO115	HP	FISH +
SW403	HP	FISH +
SW1116	HP	FISH +
SW480	HP	FISH +
SW1463	HP	FISH +
E705	2N	FISH -
MICOL29	HP	FISH +
DIFI	A	FISH +
MICOL24	A	FISH +
HT29	HP	FISH +
SW620	HP	FISH +
T84	HP	FISH +
CACO2	HP+LowA	FISH +
CCD841	2N	FISH -

We subsequently evaluated the levels of total EGFR expression through Western blotting experiments. The results are reported in Figure 4.11A, showing the corresponding intensity ratios with respect to the relative loading control in Figure 4.11B. Five cell lines, namely SW48, SW1116,

SW480 and MICOL24 colon cancer cells, as well as the fibroblast transformed DIFI cell line, displayed high levels of EGFR expression. Among cell lines not overexpressing EGFR, SW620 cells were characterized by the lowest level: the level of EGFR protein was substantially undetectable.

In addition, we evaluated the level of EGFR activation in Western blotting experiments, using monoclonal antibodies recognizing the receptor's phosphorylated form. The results are reported in Figure 4.11A, with the corresponding intensity ratios, expressed by comparing P-EGFR/total EGFR ratio of each cell with P-EGFR/total EGFR ratio of CCD841 normal mucosa cell line in Figure 4.11C. We observed EGFR hyperphosphorylation in all the investigated cell lines, except SW620 cells and CCD841 normal intestinal cell line.

By comparing all the experiments concerning EGFR investigation (mRNA expression, gene sequence, gene/chromosomal status, protein expression and phosphorylation status), we observed that:

1) The CCD841 normal intestinal mucosa is characterized by no EGFR overexpression and by absence of EGFR phosphorylation (as expected);
2) the SW48 cell line, characterized by no copy number gain (FISH-) but by the presence of a hyperactivating point mutation, displayed mRNA and protein overexpression, and also EGFR activation;
3) both cell lines with a strong EGFR gene amplification, namely DIFI and MICOL24, showed EGFR mRNA and protein overexpression, as well as EGFR hyperactivation;
4) the E705 cancer cell line, characterized by a normal EGFR gene status, showed normal levels of EGFR mRNA and protein expression but EGFR protein hyperactivation;
5) The SW620 colon cancer cell line, although characterized by high polysomy, did not show any EGFR protein expression and, as a consequence, any EGFR phosphorylation. This finding is not unexpected because this cell line is commonly used as a negative control for EGFR (Park JJ. *et al*, 2012).
6) out of 9 remaining FISH positive cell lines, 4 showed EGFR mRNA overexpression, namely SW1116, SW480, MICOL29 and CACO2, and among these only 2 (SW1116 and SW480) were also characterized by

EGFR protein overexpression. The 5 remaining FISH positive cell lines without mRNA overexpression, namely CO115, SW403, SW1463, HT-29 and T84, showed no EGFR overexpression. However, irrespective of mRNA and protein expression levels, all these FISH+ cell lines showed EGFR hyperactivation.

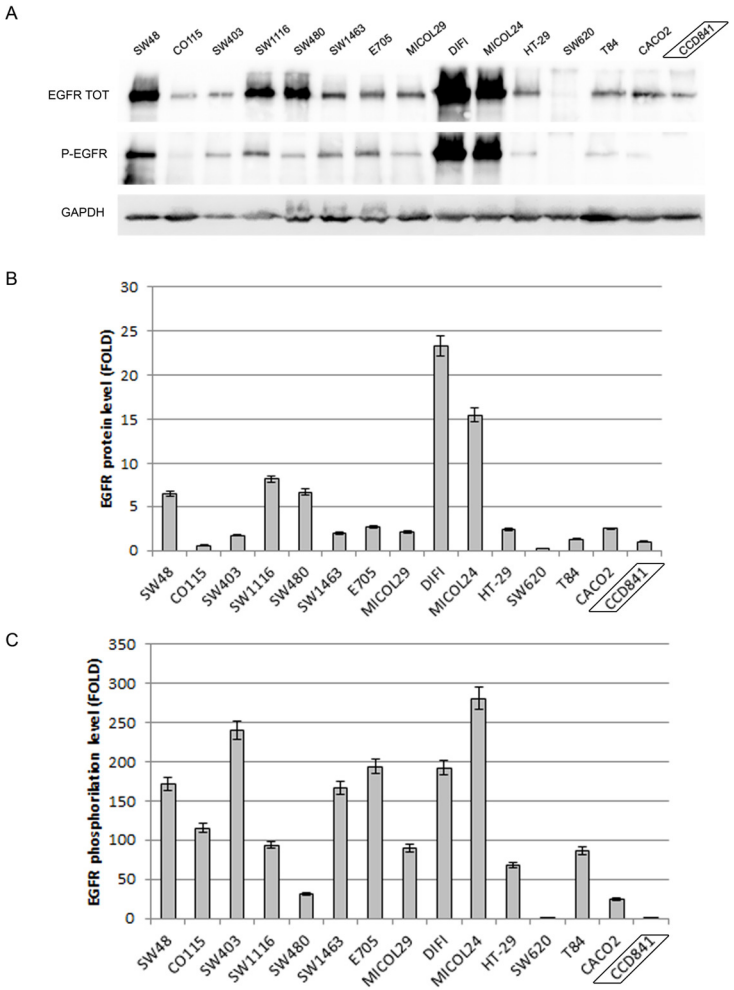


Figure 4.11 ***Western blot analysis of EGFR expression and activation in colon cancer, fibroblast and normal mucosa cell lines.***

Analysis of total protein content and phosphorylation level (A) of EGFR performed by western blot techniques. GAPDH was used as a loading control. (B) Densitometric analysis of total protein content. Data are expressed as folds compared to loading control. (C) Determination of phosphorylation rate by densitometric analysis. Data are expressed by comparing P-EGFR and total

EGFR ratio of each cell to P-EGFR and total EGFR ratio of CCD841 normal mucosa cell line. Values are presented as means ± standard deviation (SD).

In conclusion, if we exclude SW620 cells, characterized by a complete absence of EGFR protein, all colon cancer cell lines as well as DIFI cells were characterized by overexpression of the phosphorylated form of EGFR, independently from mRNA and protein expression, or gene status. Having in mind that all the cell lines were characterized by NEU3 mRNA overexpression, it is conceivable that NEU3 may influence EGFR activation.

4.5 Rational design of an inactive form of NEU3 sialidase

In accordance with the work of Albohy and colleagues (2010), an investigation of human NEU3 structure was carried out using molecular modeling, in order to predict residues involved in the hydrolysis of sialic acid from glycolipid substrates, based on the assumption of a high conservation level of catalytic residues within the active site.
Starting from human NEU2 crystal structure, which shows 42% sequence identity with NEU3, we obtained a homology model for NEU3in which D50 and Y370 were identified as the acidic and the nucleophile tyrosine residues, respectively, both essential for NEU3 catalytic activity (Taylor G., 1996; Chavas MG. *et al*, 2005) (Figure 4.12 and 4.13).
To confirm the role played by these amino acids, site-directed mutagenesis experiments were carried out. A plasmid containing the double-site mutant NEU3 encoding cDNA (D50A Y370F) was generated and transfected in COS-7 cells in order to evaluate sialidase enzymatic activity on the artificial substrate 4MU-NANA, as well as on the natural substrate GD1a ganglioside, in comparison with *wild type* NEU3 activity. The double mutant exhibited no activity on either substrate, as demonstrated by the measured values that are superimposable to those observed in the control (mock), represented by untransfected cells, and corresponding to endogenous sialidase activity. On the contrary, as expected, the *wild type* form of NEU3 showed significant activity on both substrates (Table 4.5).

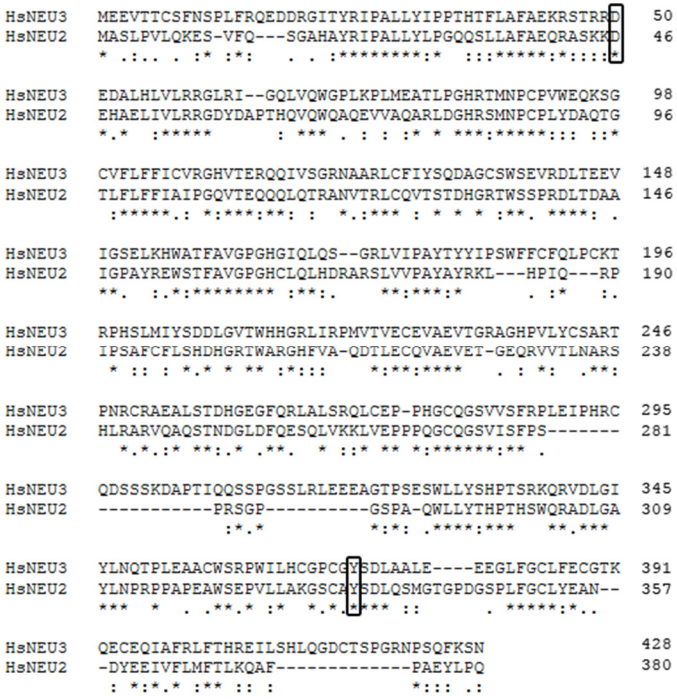


Figure 4.12 Sequence alignment of human NEU3 and NEU2 sialidases.
The two sequences in FASTA format were recruited from the UniProt database. The aligment was performed using CLUSTAL W tool, from the ExPASy bioinformatic platform. Catalytic residues studied by mutagenesis are indicated by black bold boxes.

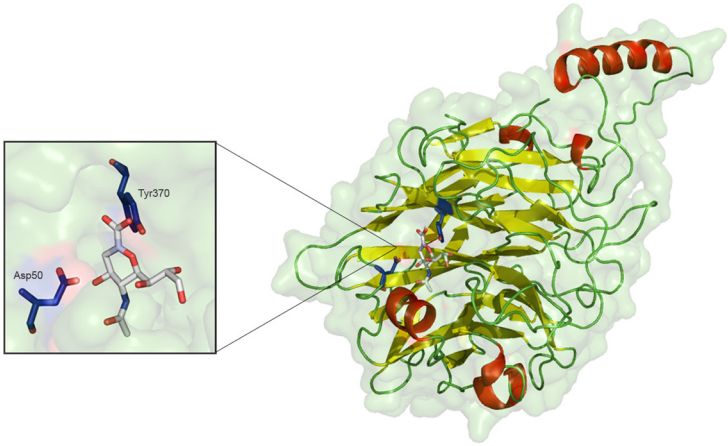


Figure 4.13 ***Three-dimensional model of NEU3 human sialidase.***
Molecular surface representation of NEU3 docked with 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA), a sialic acid mimetic, represented as a stick model. β-sheets are colored in yellow, α-helix portions in red and loops in green. Residues involved in catalysis are shown as blue sticks in the enlargement aside.

Table 4.5 ***Evaluation of sialidase activity in membrane cells extracts***

	Specific Activity vs 4MU-NANA (nmoli/h mg protein)	**Specific Activity vs GD1a (nmoli/h mg protein)**
Mock	23,3 ± 1,2	1,8 ± 0,1
NEU3 wt	46,8 ± 2,3**	110,6 ± 5,1***
NEU3 D50A Y370F	23,0 ± 1,1	2,2 ± 0,2

Values are presented as means ± standard deviation (SD).
Legend: 4MU-NANA: 4-methylumbelliferyl α-N-acetylneuraminic acid; mock: cells transfected with empty vector; wt: *wild type*. **p<0.01 and ***p<0.001 (Student's t-test).

4.6 Regulation of EGFR pathway by NEU3 sialidase activity

To evaluate the possible role played by NEU3 in the complex series of events triggered by EGFR activation in colon cancer, specific cell lines were transfected with either the active or the inactive form (i.e.: corresponding to the double mutant) of the enzyme. For this purpose, two different cell lines were chosen: the fibroblast DIFI cells, which represents one of the most suitable cellular system to study the EGFR pathway (Dolf G. *et al*, 1991), and the SW480 colon cancer cells, which are widely used to study colon cancer (Trainer DL. *et al*, 1988). Both these cell lines showed NEU3 and EGFR mRNA upregulation, as well as EGFR overexpression and hyperactivation (Figures 4.11).

We determined a transfection efficiency of 30% and 25% in DIFI and SW480 cells respectively, visualizing the fluorescence of cell expressing GFP in fusion to NEU3 by confocal microscopy as described in paragraph 3.11.

Upon transfection, using a plasmid containing either the *wild type* or the double mutant NEU3, Q-PCR experiments revealed a 20-fold and 100-fold NEU3 mRNA increase in DIFI and SW480 cells, respectively, in comparison with the control (Figure4.14A). No variations of EGFR mRNA levels were reported independently of the status of transfected NEU3 form with respect to mock (Figure 4.14A). As regard EGFR protein expression, Western blot analysis demonstrated that the total EGFR content did not vary upon transfection with either plasmids, thus showing that NEU3 overexpression does not modify the transcription rate and the overall protein content of the receptor.

By contrast, when the *wild type* sequence of NEU3 was transfected, the resulting NEU3 overexpression led to a marked increase of EGFR phosphorylation and in turn to ERK1/2 hyperactivation in both cell lines (Figure 4.14B-C). The activation was further increased upon stimulation with EGF (data not shown). Conversely, upon transfection of the double mutant inactive form of NEU3, there was no evidence of EGFR activation, thus highly suggesting that NEU3 sialidase activity regulates EGFR activation (Figure 4.14B-C).

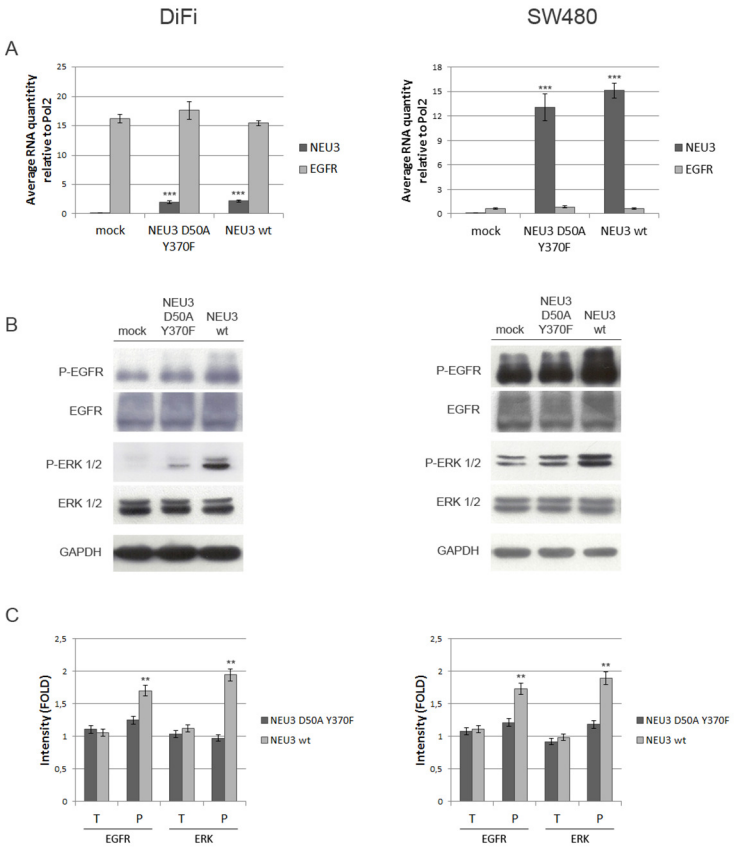


Figure 4.14 ***Analysis of EGFR pathway activation after sialidase NEU3 overexpression.***

A. Relative quantification of NEU3 and EGFR mRNA levels by real-time quantitative PCR on DiFi and SW480 cell lines transfected with empty vector (mock), pcDNA3I-HsNEU3 D50A Y370F (double mutant) and pcDNA3I-Hs NEU3 (*wild type*) B. Representative Western blot analyses performed on crude extracts, using anti-EGFR, anti-P-EGFR, anti-ERK1/2, anti-P-ERK1/2 antibodies. The experiments were performed in triplicate. C. Densitometric analyses were performed with Scion Image Software. Values are expressed by comparing the data obtained after transfection with either *wild type* or the double mutant

form of NEU3 with those obtained after transfection with the empty vector (mock). Statistical analyses were performed using Student's t-test. Values are presented as means ± standard deviation (SD). *p<0.05, **p<0.01, and ***p<0.001 (Student's t-test).

The effect of NEU3 transfection on cell viability, assayed using the MTT procedure, was studied on the same cell lines. Results showed a significant increase of cell viability (p<0.01) upon transfection with the *wild type* enzyme, whereas in the case of cells overexpressing the double mutant, no difference in cell viability was detected between transfected and control (mock) cells (Figure 4.15).

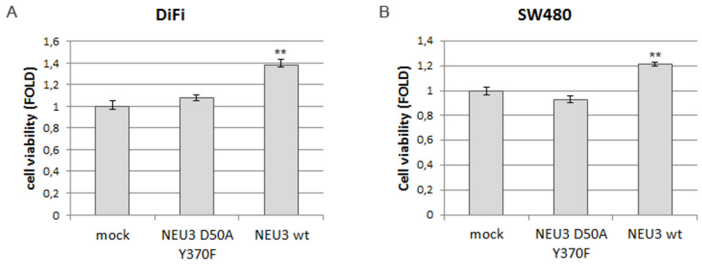


Figure 4.15 ***Evaluation of cell viability*** .
MTT tests were performed on DiFi (A) and SW480 (B) cell lines transfected with empty vector (mock), pcDNA3I-HsNEU3 D50A Y370F (double mutant) and pcDNA3I-Hs NEU3 (*wild type*). Data were normalized on absorbance measured at 570 nm upon solubilization of cell transfected with the empty vector. Statistical analyses were performed using the Student's t-test. Values are presented as means ± standard deviation (SD).**p<0.01 .

These data strongly suggest not only that NEU3 activity regulates EGFR downstream pathway but also that its overexpression enhances cell viability.

4.7 EGFR is a substrate of human sialidase NEU3

In order to understand whether NEU3 acts directly on EGFR, causing a variation of receptor sialylation and its activation, we carried out EGFR immunoprecipitation experiments in DIFI cell lines transfected with either active or the inactive form of NEU3. The investigation of sialylation level was carried out by lecting binding assay. The lysate, obtained after DIFI cell line transfection with the empty vector, or with the plasmid containing cDNA coding either for *wild type* NEU3 or mutant NEU3, was incubated with anti-EGFR antibodies. The samples were subsequently incubated with protein A-Sepharose (previously re-hydrated). After washing, immunoprecipitates was collected by centrifugation, boiled in 2x SDS-sample buffer without β-mercaptoethanol and subjected to electrophoresis followed by Western blotting.

In order to investigate sialylation, immunoprecipitated EGFR was loaded onto a SDS-PAGE.

The sialylation was confirmed in DIFI cells by lecting blotting using a biotinilated SNA (*Sambucus nigra* agglutinin) and an avidin-horseradich peroxidase. As reported in Figure 4.16, the level of α2,6-sialylation of EGFR was found to be reduced in cells overexpressing the active form of NEU3 sialidase. Conversely, upon transfection of the double mutant inactive form of NEU3, no reduction of EGFR sialylation was detected, thus strongly suggesting that EGFR sialylation is regulated by NEU3.

To confirm these data, we performed a mass spectrometry analysis , in collaboration with Prof. Gabriella Tedeschi from the Università degli Studi di Milano. Immunoprecipitated samples were loaded onto a SDS-PAGE and then stained with acid silver.

In particular after a MALDI-TOF mass spectrometry analysis three EGFR sialylated peptides were identified in the samples obtained from mock cells and from cells overexpressing the inactive form of NEU3 sialidase, that were absent in the sample obtained from cells overexpressing *wild type* NEU3 (Table 4.6). The modification was also confirmed by ESI analysis.

As expected, the two peptides identified are located in the extracellular domain of the EGFR receptor, from 25 to 28 and from 253 to 261 amino acid position on human sequence. The third peptide was not identified,

because the basal peak (1097.38), present in all samples, is absent in the theoretical fingerprint of EGFR. Therefore it is assumed that this peptide can contain further post-translational modified residues (eg, phosphorylation, acetylation).

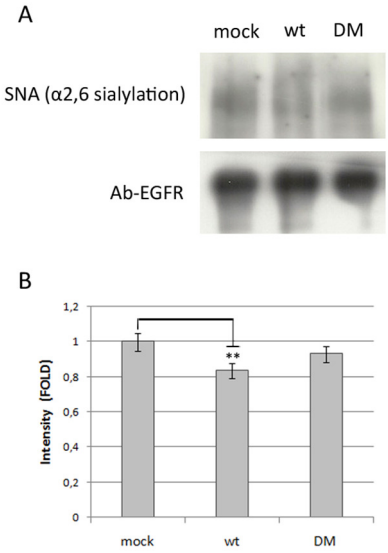


Figure 4.16 ***Analysis of EGFR sialylation after sialidase NEU3 overexpression.*** A. Representative Western blot analyses and lectin affinity assay performed on EGFR immunoprecipitated samples from DiFi cell lines transfected with empty vector (mock), pcDNA3I-Hs NEU3 (*wild type*) and pcDNA3I-HsNEU3 D50A Y370F (double mutant), using anti-EGFR antibody. The experiments were performed in triplicate. B. Densitometric analyses were performed with Scion Image Software. Values were obtained by comparing the data obtained after transfection with either the *wild type* or the double mutant form of NEU3 with those obtained after transfection with the empty vector (mock). Statistical analyses were performed using Student's t-test. Values are presented as means ± standard deviation (SD). **p<0.01 (Student's t-test).

Table 4.6 ***Evaluation of EGFR sialylation by mass spectrometry analysis.***
In brackets the number of sialic acid molecules identified

Peptide	Predicted peak (m/z)	Identified peak (m/z)		
		mock	***wild type***	**DM**
^{25}LEEK^{28}	519.96	519.96	519.96	519.96
		1757.04 (+4)	1757.04 (+4)	1757.04 (+4)
		2066.31 (+5)	NN	2066.31 (+5)
^{253}KFRDEATCK^{261}	1152.13	1152.13	1152.13	1152.13
		1771.7 (+2)	NN	1771.7 (+2)
		2080.97 (+3)	NN	2080.97 (+3)
Not Identified		1097.38	1097.38	1097.38
		1406.65 (+1)	NN	1406.65 (+1)

4.8 NEU3 overexpression influences pharmacological response to Cetuximab treatment

Before evaluating the effect of NEU3 overexpression on pharmacological treatment of CRC with Cetuximab, the monoclonal antibody targeted to the EGFR extracellular domain, we investigated the mutational status of markers able to predict the efficacy of this targeted therapy.
In Table 4.7 the mutational profiles of KRas, BRAF and PIK3 genes are reported.

Table 4.7 ***KRAS, BRAF, PIK3CA mutational analyses and PTEN immunohistochemical analysis of CRC cell lines.***
WT: *wild type*. Colored bold: gene mutation.

Cell lines	KRAS 2	BRAF	PIK3CA 9	PIK3CA 20
SW48	WT	WT	WT	WT
CO115	WT	**V600E**	WT	WT
SW403	**G12V (50%)**	WT	WT	WT
SW1116	**G12A (omo)**	WT	WT	WT
SW480	**G12V (omo)**	WT	WT	WT
SW1463	**G12C (omo)**	WT	WT	WT
E705	WT	WT	WT	**H1047H**
MICOL29	**G12D (omo)**	WT	WT	WT
DIFI	WT	WT	WT	WT
MICOL24	WT	WT	WT	WT
HT29	WT	**V600E**	WT	WT
SW620	**G12V (omo)**	WT	WT	WT
CACO2	WT	WT	WT	WT
CCD841	WT	WT	WT	WT

Cell line	IC50 (µg/mL)
SW48	>200
CO115	>200
SW480	>200
SW620	>200
DIFI	0,044 ± 0,005
E705	0,142 ± 0,06
HT-29	>200
CACO2	>200
CCD841	>200

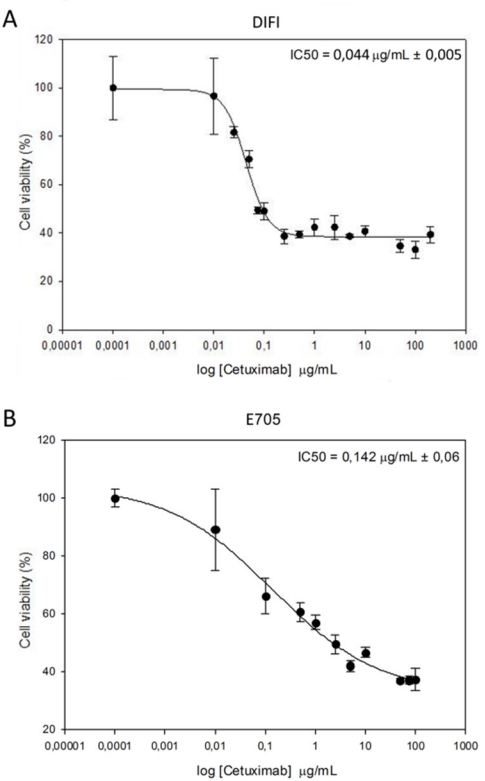


Figure 4.17 ***Evaluation of Cetuximab EC50 by MTT test.***
In the table are reported the Cetuximab EC50 values determined in the cell lines.
A. Dose-response curve of Cetuximab in the human fibroblast DIFI cell line. B. Dose-response curve of Cetuximab in the human E705 colorectal cancer cell line.

All the cell lines were transfected with the following constructs: pcDNA3I, pcDNA3I-HsNEU3, pcDNA3I-HsNEU3 D50A Y370F. Five hours after transfection, the cell lines were subjected to Cetuximab subministration at a defined concentration.

5 cell lines were taken into consideration: DIFI cells that posses a normal mutational status of EGFR and the downstream pathway; SW48 cells, presenting the G719S iperactivating point mutation of EGFR; SW480 and CO115 cells that show mutations in the MAP kinases pathway, in KRAS and BRAF genes respectively; and E705 showing an activating mutation in the PIK3 gene.

In all cell lines considered, with the exception of SW48, after transfection with *wild type* (WT) NEU3, we observed a significant increase in the cell viability compared to that observed after transfection with the empty vector (mock, negative control). This effect was not observed in the same cells after transfection with the vector containing the cDNA coding for the inactive sialidase (double mutant) (Figure 4.18).

Moreover, after Cetuximab treatment, we observed an enhanced cell viability in the presence of NEU3 overexpression in all the cell lines tested, irrespective of their mutational status.

The only exception is represented by SW48 cells, which did not show any significant variation of viability after overexpression of either the active or the inactive form of the sialidase, even in the absence of the drug (Figure 4.18).

We also analyzed EGFR pathway by western blot on DIFI cell lines, in order to understand through which mechanism NEU3 enhances resistance to the pharmacological treatment with Cetuximab.

As regard EGFR protein expression, Western blot analysis demonstrated that total EGFR content vary upon Cetuximab treatment.

When the *wild type* sequence of NEU3 was transfected, the resulting active NEU3 overexpression led to an increase of EGFR phosphorylation and in turn to ERK1/2 hyperactivation (Figure 4.19).

Conversely, upon Cetuximab addition to the culture media there was no evidence of ERK1/2 activation (Figure 4.19), while a strong activation of the AKT kinase was observed, compared to that observed when overexpressing *wild type* NEU3 in the absence of the drug.

No evident variation we detected for the PTEN phosphatase level in all the condition tested.

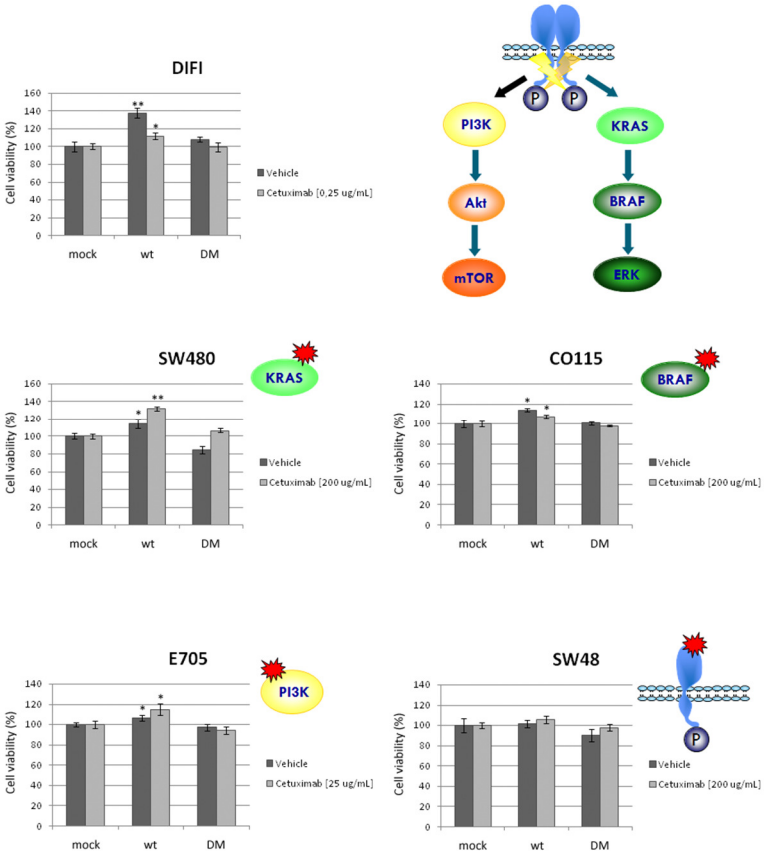


Figure 4.18 ***Evaluation of cell viability by MTT test after NEU3 overexpression and Cetuximab treatment.***

MTT tests were performed on DiFi, SW480 (KRAS mutated), CO115 (BRAF mutated), E705 (PI3K mutated), and SW48 (EGFR mutated) cell lines transfected with empty vector (mock), pcDNA3I-Hs NEU3 (*wild type*) and pcDNAI-HsNEU3 D50A Y370F (double mutant). Data were normalized on absorbance measured at 570 nm upon solubilization of cell transfected with the empty vector, either in absence and in presence of Cetuximab. Statistical analyses were performed using the Student's t-test. Values are presented as means ± standard deviation (SD); *p<0.05,**p<0.01 .

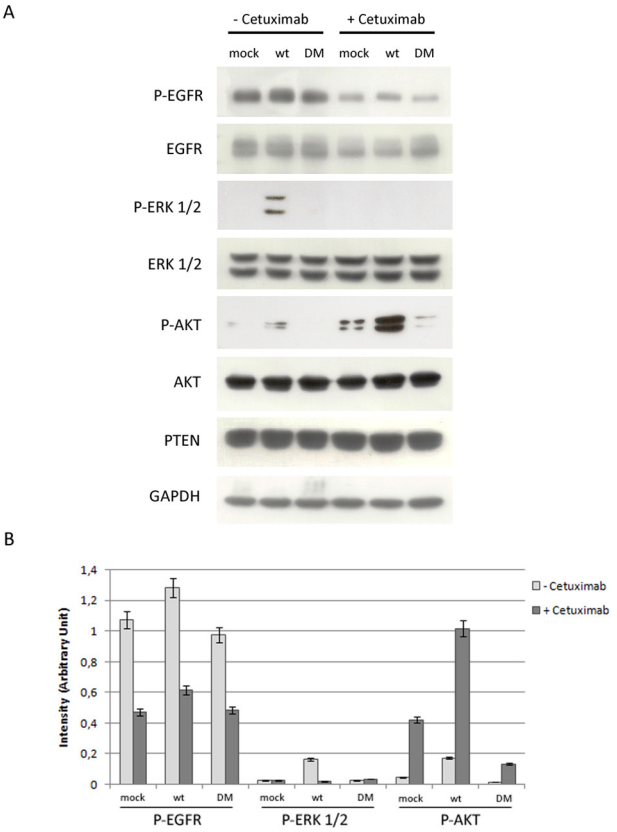


Figure 4.19 ***Analysis of EGFR pathway activation after sialidase NEU3 overexpression and Cetuximab treatment.***

A. Representative Western blot analyses performed on crude extracts on DiFi cell lines transfected with empty vector (mock), pcDNA3I-Hs NEU3 (*wild type*) and pcDNA3I-HsNEU3 D50A Y370F (double mutant), using anti-EGFR, anti-P-EGFR, anti-ERK1/2, anti-P-ERK1/2, anti-AKT, anti-P-AKT, anti-PTEN and anti-GAPDH antibodies. The experiments were performed in triplicate. B. Determination of phosphorylation rate by densitometric analysis. Data are expressed by comparing P-protein and total protein ratio.

4.9 Evaluation of sialidases deregulation in cancer

Finally we performed a relative quantification of human sialidases NEU1, NEU3 and NEU4 mRNA levels in different types of cancer tissues by Real-time PCR, and we determined the variation of transcription rate in the tumor transformation.

All paraffin embedded tissues from both primary tumors and paired normal mucosa were collected from a cohort of Caucasian patients affected by gastric, gastrointestinal, ovarian, kidney, lung, prostatic and thyroid cancer in collaboration with the Istituto Cantonale di Patologia of Locarno, Switzerland. We initially evaluated the relative expression of the lysosomal sialidase NEU1, the membrane-bound sialidase NEU3 and the mitochondrial sialidase NEU4, by comparing the mRNA level to B-actin gene.

The cytosolic sialidase NEU2 was not considered in our analysis because of the very low levels of basal expression which are often below detection.

In Figure 4.20 the relative expression on human sialidase NEU1 in normal tissue is reported; as shown in the chart, the highest transcription levels in kidney and gastrointestinal tissue. However, sialidase NEU1 transcript levels were generally lower transcript levels than those of the other two isoforms considered.

High levels of NEU3 mRNA were observed in the stomach, in the gastrointestinal tissue and in the kidney as well as in the prostate and the thyroid tissues (Figure 4.21).

Regarding the mitochondrial sialidase NEU4 we observed high mRNA levels in all tissues analyzed. The only exception was the lung, tissue in which very low transcription levels were observed also for the other two isoforms (Figure 4.22).

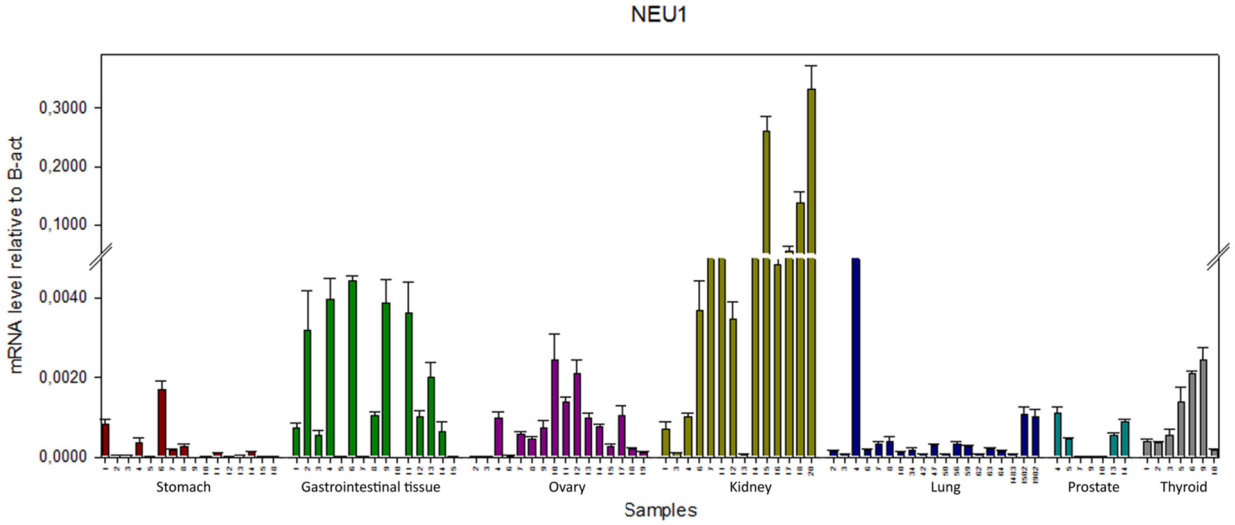


Figure 4.20 ***Evaluation of NEU1 mRNA level in different human tissues.***
Real-time PCR analysis of NEU1 mRNA levels in various tissues of Caucasian patients. mRNA expression levels were normalized to B-act mRNA. The experiments were performed at least in triplicate. In red: mRNA level observed in gastric tissue; in green: mRNA level in gastrointestinal stromal mucosa; in purple: mRNA level in ovarian tissue; in yellow: mRNA level in kidney; in blue: mRNA level in lung; in cyan: mRNA level in prostate; in grey: mRNA level in thyroid tissue.

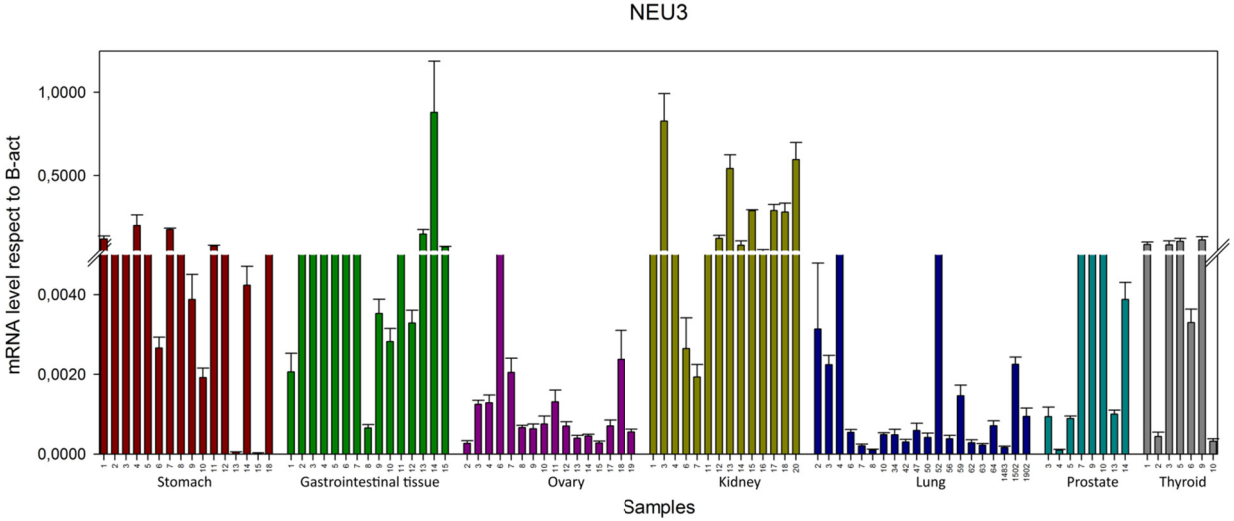


Figure 4.21 ***Evaluation of NEU3 mRNA level in different human tissues.***
Real-time PCR analysis of NEU3 mRNA levels in various tissues of Caucasian patients. mRNA expression levels were normalized to B-act mRNA. The experiments were performed at least in triplicate. In red: mRNA level observed in gastric tissue; in green: mRNA level in gastrointestinal stromal mucosa; in purple: mRNA level in ovarian tissue; in yellow: mRNA level in kidney; in blue: mRNA level in lung; in cyan: mRNA level in prostate; in grey: mRNA level in thyroid tissue.

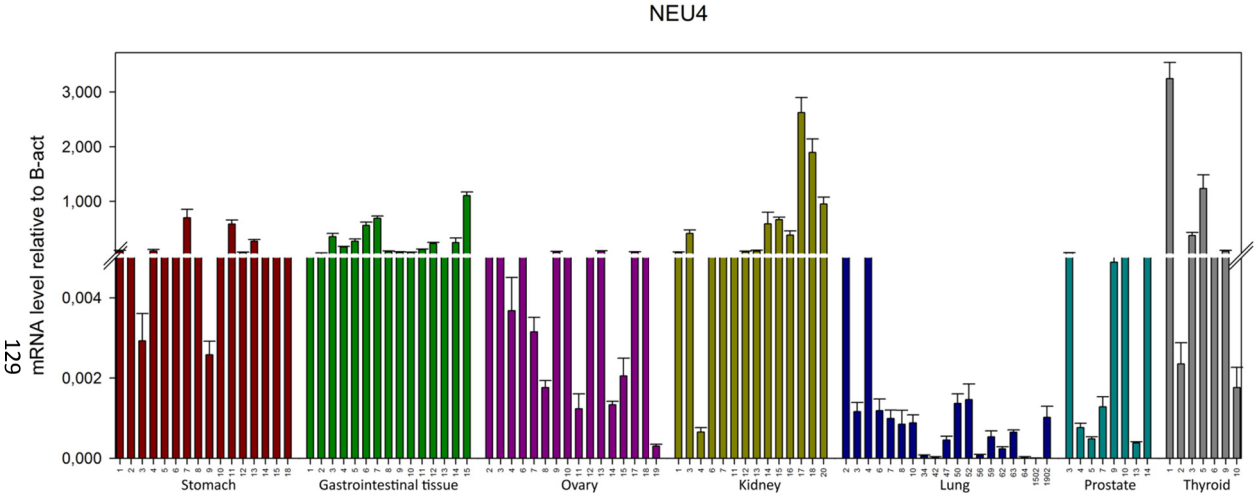


Figure 4.22 ***Evaluation of NEU4 mRNA level in different human tissues.***
Real-time PCR analysis of NEU4 mRNA levels in various tissues of Caucasian patients. mRNA expression levels were normalized to B-act mRNA. The experiments were performed at least in triplicate. In red: mRNA level observed in gastric tissue; in green: mRNA level in gastrointestinal stromal mucosa; in purple: mRNA level in ovarian tissue; in yellow: mRNA level in kidney; in blue: mRNA level in lung; in cyan: mRNA level in prostate; in grey: mRNA level in thyroid tissue.

We subsequently evaluated the relative variation of sialidases transcripts in tumors. B-actin gene was used as a housekeeping gene. The fold increase/decrease in tumor was calculated through the $2^{-\Delta\Delta Ct}$ method using paired normal tissue as calibrator. As previously reported we considered gene overexpressing tumors those showing a ≥ 3-fold expression level with respect to paired normal mucosa.

It was noted that while for the sialidase NEU1 and NEU3 the upregulation of mRNA levels was observed as a general trend, sialidase NEU4 was mostly downregulated (Figure 4.23-4.25).

The lysosomal sialidase NEU1 appears to be transcribed at high levels particularly in ovarian and prostatic cancers, although the number of patients analyzed so far is small. No great evidence of deregulation was detected in the gastrointestinal stromal and thyroid cancers (Figure 4.23).

Like NEU1, the membrane sialidase NEU3 was transcribed at high levels in ovarian cancer (Figure 4.24). We observed a 3-fold increase of NEU3 transcripts in the 25% of the samples in gastric cancer, as well as in the lung and in thyroid tumors. In the gastrointestinal stromal tumor, the transcript of sialidase NEU3 was up-regulated in the 67% of the samples, with an increment of 3-fold increase in the 33% of the samples.

Regarding the transcription levels of mitochondrial sialidase NEU4, a decrease was observed in all tumor tissues analyzed, although less evidently in ovarian and prostatic cancer (Figure 4.25).

To date we have analyzed only a small number of patients. A more detailed survey on Western population should be performed enlarging the cohort of patients.

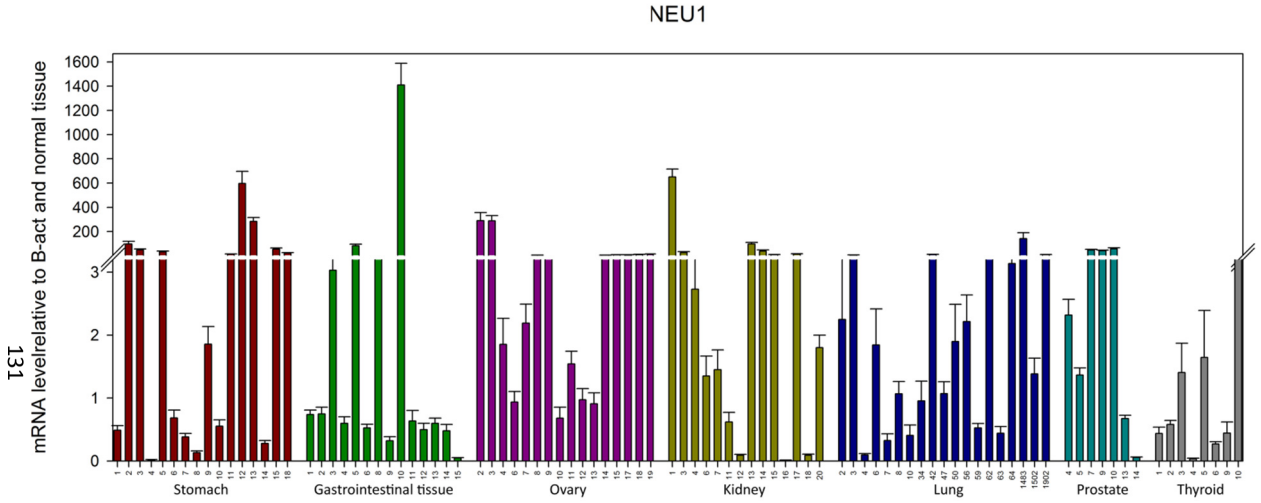


Figure 4.23 ***NEU1 mRNA variation in different tumor tissues.***
Real-time PCR analysis of NEU1 mRNA levels in tumor tissues of Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed at least in triplicate. In red: mRNA level observed in gastric tissue; in green: mRNA level in gastrointestinal stromal mucosa; in purple: mRNA level in ovarian tissue; in yellow: mRNA level in kidney; in blue: mRNA level in lung; in cyan: mRNA level in prostate; in grey: mRNA level in thyroid tissue.

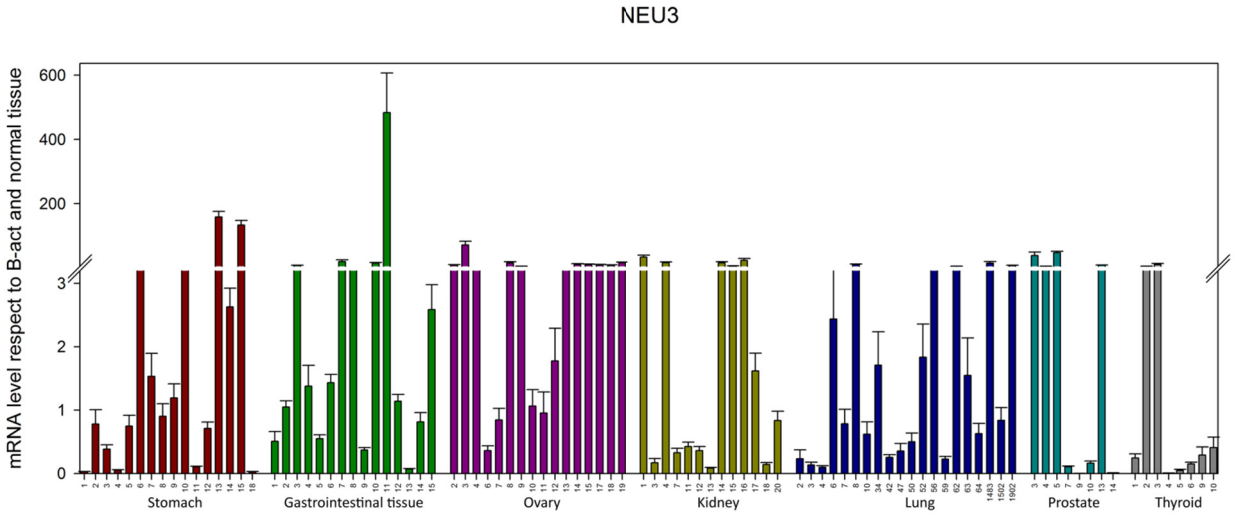


Figure 4.24 ***NEU3 mRNA variation in different tumor tissues.***
Real-time PCR analysis of NEU3 mRNA levels in tumor tissues of Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed at least in triplicate. In red: mRNA level observed in gastric tissue; in green: mRNA level in gastrointestinal stromal mucosa; in purple: mRNA level in ovarian tissue; in yellow: mRNA level in kidney; in blue: mRNA level in lung; in cyan: mRNA level in prostate; in grey: mRNA level in thyroid tissue.

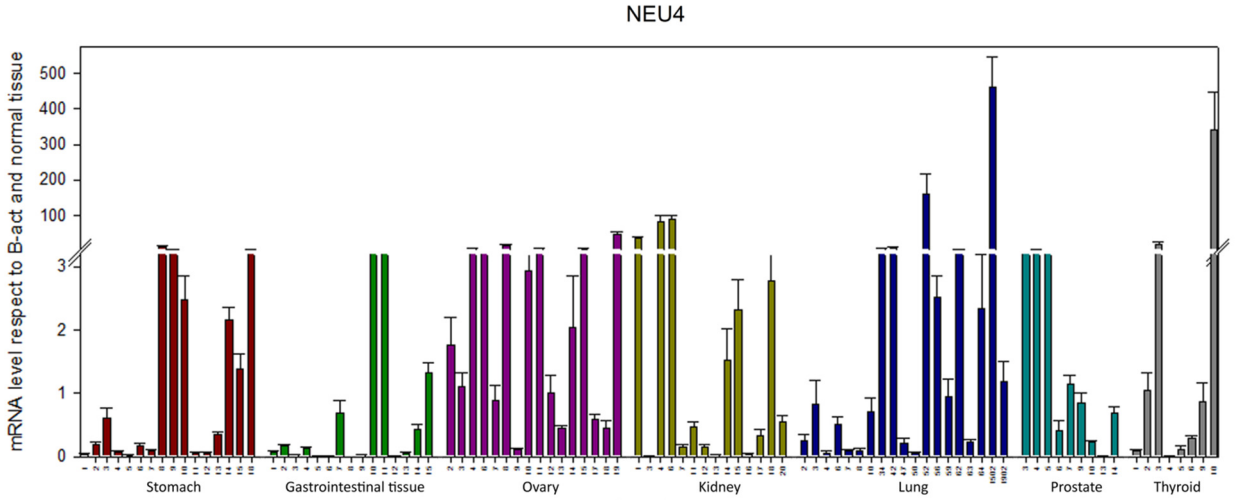


Figure 4.25 ***NEU4 mRNA variation in different tumor tissues.***

Real-time PCR analysis of NEU4 mRNA levels in tumor tissues of Caucasian patients. mRNA expression levels were normalized to B-act mRNA and to paired normal mucosa. A gene was considered overexpressed when its fold is above 3. The experiments were performed at least in triplicate. In red: mRNA level observed in gastric tissue; in green: mRNA level in gastrointestinal stromal mucosa; in purple: mRNA level in ovarian tissue; in yellow: mRNA level in kidney; in blue: mRNA level in lung; in cyan: mRNA level in prostate; in grey: mRNA level in thyroid tissue.

5. Discussion

5.1 Human sialidase NEU3 is a peripheral membrane protein

The puzzling mechanism of sialidaseNEU3 anchorage to the membrane had been previously studied in COS7 and HeLa cells, overexpressing murine NEU3. This enzyme was demonstrated to be a peripherally associated membrane protein, present in both the plasma membrane and the membranous structures corresponding to the recycling endosomal compartment, from which it can be released by treatment with carbonate (Zanchetti G. *et al*, 2007).

We have confirmed a localization in the membrane fraction also for the human isoform of NEU3 sialidase, after ultracentrifuge separation; however we have observed some differences in the mechanism of anchorage to the membrane.

A bioinformatics analysis failed to detect any palmitoylation or myristilation site in human NEU3 amino acids sequence. Besides, no G-PI anchor motif was found, as expected considering the high percentage of identity between human and murine sequences.

Nevertheless, submitting the human enzyme sequence to the TMPred tool, we identified two putative sequences predicted to be transmembrane helices. In particular we found a brief portion of 21 amino acids that is not found in murine NEU3, corresponding to amino acids 92-112 of the human enzyme.

However, in our experiments human NEU3 could not be released from the cellular membrane after Triton X-114 extraction, thus ruling out the possibility of it being a transmembrane protein, as predicted by the bioinformatic tool. This was also confirmed by the homology model of NEU3, obtained using human cytosolic sialidase NEU2 crystallographic structure as a template. Indeed in this model the two brief sequences predicted to be transmembrane helices are structured as β-sheets of the conserved β-propeller of sialidases, as reported in Figure 6.1. Since this overall 3D structure is conserved in all sialidases characterized so far, from viruses and bacteria enzymes, to mammalian ones, it seems very unlikely that only human NEU3 could possess a different structure.

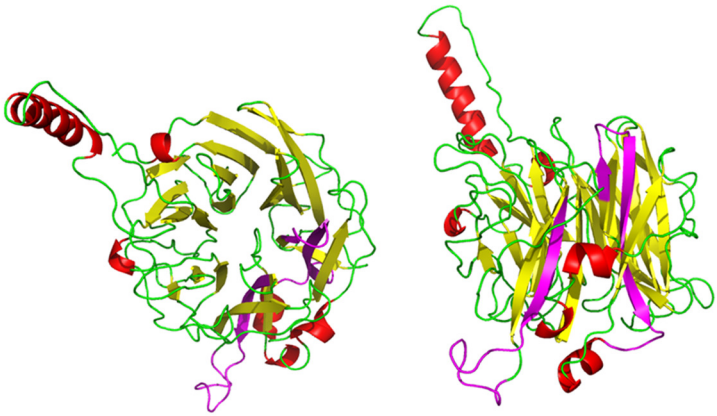


Figure 6.1 Homology model *of human sialidase NEU3.*
Ribbon representation of human sialidase NEU3 (left: top view; right: lateral view). The α-helices are colored in red, β-sheet in yellow, and loops in green. The sequences predicted to be transmembrane portion by TMPRED tool are colored in purple. The figure was drawn using PyMOL.

Our experiments also showed that, unlike murine sialidase, the human enzyme was not recruited into the soluble fraction after treatment with carbonate.

Taking into consideration the high similarity between the primary structures of the two enzymes, we can suppose that the human sialidase is also anchored by electrostatic interactions to other membrane proteins; however the different behavior shown upon carbonate extraction strongly suggests that this is not the only mechanism. According to the hydrophobicity prediction we cannot exclude additional hydrophobic interactions with other membrane proteins or lipid components, that have not been observed for murine sialidase.

This might explain the difficulties encountered in the purification of recombinant human sialidase NEU3 from *E. coli*, yeast and also mammalian cellular systems. Several attempts to obtain recombinant human NEU3 in a purified form were unsuccessful, yielding an aggregated unsoluble protein.

NEU3 structure was therefore confirmed through studies of site-directed mutagenesis of conserved sites with respect to the cytosolic sialidase NEU2.
On the whole, our data show that the human isoform is more firmly attached to the cell membrane with respect to the murine enzyme.

5.2 Deregulation of sialidases NEU3 and NEU4 in colorectal cancer in Caucasian population

We characterized NEU3 mRNA expression in 85 pathological specimens from Caucasian patients affected by CRC. In addition we extended our analysis evaluating, in 74 out of 85 previously considered cases, the transcription level of NEU4 sialidase, a human isoform demonstrated to localize in the mitochondrial membrane (Bigi A. *et al*, 2010).
On the whole, we observed that expression of both NEU3 and NEU4 sialidase (referring gene expression to the internal control β-actin) vary markedly from patient to patient both in normal mucosa and in tumors.
In the only work reported in literature investigating NEU3 expression at mRNA level, Miyagi and colleagues showed in a Japanese cohort of CRC patients that NEU3 expression was increased by 3 to 100-fold in all cases (Miyagi T. *et al*, 2008). We found that only 32% of our cohort (including Western population only) showed NEU3 overexpression. However the transcription rate was generally up-regulated.
We found that, opposite to NEU3, the transcription rate was mostly downregulated and in 31 out of 74 cases (42%) a <3 fold decrease of NEU4 mRNA level was detected in tumors.
By comparing NEU3 and NEU4 mRNA expression levels in the cohort of samples analyzed, we observed an opposite trend of transcription of the two sialidases, with an increase of NEU3 mRNA levels and a decrease of NEU4 mRNA levels. Both transcripts were overexpressed only in 4 cases (5%). NEU4 expression at mRNA level had previously been investigated in a Japanese cohort of 41 CRC patients (Yamanami H. *et al*, 2007). They

observed a marked decrease ofNEU4 mRNA levels in tumors, with an average reduction of 2.8 fold compared to normal mucosa.
In our clinical records the average reduction was 23.7 fold, showing a deeply marked downregulation of NEU4 sialidase transcripts.
These discrepancies can be explained by ethnical differences, since Western and Japanese population are characterized by significant different lifestyles.
However we confirm that sialidases transcription rate was strongly deregulated in cancer, suggesting an important role of these enzymes in malignant transformation.

5.3 Effects of sialidase NEU3 deregulation on EGFR expression and activation in CRC

In the same 85 pathological specimens from patients affected by CRC previously analyzed for evaluating sialidases transcriprion, we also characterized EGFR mRNA expression. As it had previously been demonstrated that NEU3 co-immunoprecipitates with EGFR (Wada T. *et al*, 2007), we tested whether a correlation existed between NEU3 and EGFR mRNA expression levels and we found a strict correlation.
We observed EGFR mRNA overexpression in only 10 out of 85 evaluable cases (12%).
By comparing NEU3 and EGFR mRNA levels, we observed a statistically significant correlation (p=0.010, two-tailed Fisher's Exact Test). In particular, we found NEU3 overexpression in 20 out of 75 evaluable cases with normal EGFR expression (27%) and in 7 out of 10 EGFR overexpressing cases (70%). This correlation suggests that the increase in EGFR expression could be associated with NEU3 increment, whereas we could not assert the opposite. Therefore it seems that NEU3 can affect EGFR activity only when the latter is deregulated, that is only in an EGFR-dependent tumor.
As KRAS, BRAF, PIK3CA and PTEN may be deregulated in a consistent number of CRC and as it seems that NEU3 and EGFR expression are correlated, we proposed to investigate whether NEU3 expression levels

could be related also to deregulations in EGFR downstream pathways. No correlation was observed among markers.

Based on these results on tissue samples, we proposed to evaluate if the correlation existing between NEU3 and EGFR could affect the sensitivity to EGFR-targeted therapies, using CRC cell lines as an experimental model.

First of all, we studied the expression levels of NEU3 in a number of cell lines widely used for studies on colorectal carcinogenesis. Results showed the up-regulation of NEU3 mRNA levels in all the cellular systems with respect to the CCD841 normal mucosa cell line, pointing out the relevance of this sialidase in CRC.

Our findings are in agreement with the observations carried out, so far, only on CRC Asiatic patients (Kakugawa Y. *et al*, 2002) and further support NEU3 as a biomarker with a relevant role for colorectal carcinogenesis.

Since co-immunoprecipitation assays have demonstrated the ability of NEU3 to interact with EGFR, whose signaling pathway plays a major role in CRC (Wada T. *et al*, 2007) we also analyzed EGFR expression levels in the same cell lines. EGFR transcript was found to be overexpressed in only 50% of tested cell lines, confirming data reported in literature for CRC patients by McKay and colleagues (McKay JA. *et al*, 2002).

Furthermore, we evaluated EGFR gene status by FISH analysis, as well as the receptor total protein content and its phosphorylation rate by Western blotting. Our data show that EGFR gene status corresponds to mRNA and protein levels in disomic and amplified cell lines, with the exception of SW48 cell line which has a disomic chromosomal asset but carries an hyperactivating mutation leading to EGFR constitutive activation. On the contrary, we could not find any correlation between the amount of EGFR transcript and the protein content in cell lines classified as highly polysomic.

Moreover, we observed EGFR activation, compared to the colon normal mucosa CCD841 cell line, irrespectively of mRNA or protein levels in all but one cell lines. The exception is represented by SW620 cells, which, although characterized by an abnormally high number of chromosomes 7 (and of EGFR gene, as a consequence), do not express EGFR protein at all and, therefore, show no EGFR phosphorylation. On the other hand the

result of SW620 was not unexpected, since this cell line is routinely used as a negative control of EGFR (Park JJ. *et al*, 2012).

Overall, these findings, together with those showing NEU3 mRNA up-regulation in all cell lines, led us to assume that NEU3 might activate EGFR, possibly by modulating EGFR activation directly through receptor modification, without effects on its mRNA or protein content. Indeed, it is commonly known that modifications of the sialylation pattern of molecules exposed on the cell surface or released in the extracellular milieu represent a hallmark of malignant differentiation (Schauer R., 2009; Varki A., 2008). Although the biochemical mechanisms underlying sialylation and cancer are only poorly understood, several findings about a direct role played by the enzymes involved in the biosynthesis, degradation and fine tuning of the sialic acid cell content, namely sialyltransferases (STs) and sialidases (NEUs), have been produced (Hakomori S., 2002; Bos PD. *et al*, 2009; Miyagi T. *et al*, 2008). Among these, in colon cancer cells NEU3 may regulate cell proliferation by markedly enhancing tyrosine phosphorylation of integrin β4, with recruitment of Shc and Grb-2, in order to stimulate phosphorylation of focal adhesion kinase and ERK 1/2 (Kato K. *et al*, 2006). As a result of the increased ganglioside(s) catabolism triggered by NEU3 overexpression, an accumulation of lactosylceramide has been detected in different colon cancer tissue specimens (Chung KY. *et al*, 2005). Moreover, it has been demonstrated that NEU3 directly interacts with EGFR (Wada T. *et al*, 2007).

Based on these findings, it is therefore possible to postulate that human membrane sialidase NEU3 plays a relevant role in the regulation of transmembrane signaling directly at the cell surface either through the modulation of gangliosides content or by a direct action/interaction on specific signal proteins.

To test this hypothesis, we have examined the effect of NEU3 overexpression on EGFR expression and activation, as well as on cell viability, with *in vitro* transfection experiments using as a negative control a double mutant form of the enzyme specifically designed for this study and completely devoid of catalytic activity, as demonstrated by enzymatic assay both on the artificial substrate 4MU-NANA and the natural substrate GD1a ganglioside.

We selected two specific cell lines from the previously characterized panel: DIFI cells, representing the best cellular model to study the EGFR pathway, and SW480 cells, the most studied model of CRC development (Dolf G. *et al*, 1991; Trainer DL. *et al*, 1988).

After transient transfection with NEU3 active *wild type* form we detected an increase in NEU3 mRNA expression level by 20- to 100-fold in DIFI and SW480 cell lines, respectively. No variation of EGFR mRNA was detected, independently of the status of NEU3 transfected form. Moreover, Western Blot of will typeNEU3 overexpressing cells revealed increased EGFR and ERK1/2 phosphorylation in both cell lines. On the contrary we could not detect any EGFR activation in cells overexpressing the double mutant inactive NEU3. Therefore, we confirmed our hypothesis that NEU3 overexpression leads to a strong activation of EGFR and EGFR downstream pathways, also in the presence of a high basal level of EGFR downstream pathways activation. The activation of EGFR and EGFR downstream cascades in DIFI cells is noteworthy, because this cell line is characterized, at a basal level, by a high content of the receptor due to the presence of EGFR gene amplification.

We also found that, while mRNA and total EGFR protein contents remained constant, the overexpression of the active *wild type* NEU3 markedly enhanced EGFR phosphorylation, suggesting the possibility of a direct modification (i.e.: activation) of the receptor. Moreover, we also found an enhanced activation of MAP kinases pathway, in particular of ERK1/2 proteins. On the contrary, upon overexpression of the totally inactive NEU3 mutant, we did not observe any activation of the EGFR pathway in any tested cell line.

In addition, in order to evaluate if, after hyperactivation of EGFR, NEU3 active form is also able to increase cell viability, we performed MTT based test in transfected cells. According to Western blot analyses, we reported a significant increase of cell viability, only upon overexpression of *wild type* NEU3, the inactive mutant being completely ineffective.

Overall these results confirm our hypothesis that NEU3 overexpression leads to a strong activation of EGFR and of its downstream pathways (especially the MAP kinase pathway), even in the presence of a high basal level of EGFR, as demonstrated in the case of DIFI cell line. Although the

actual mechanism by which NEU3 exerts its effect on EGFR remains to be elucidated, it is highly probable that protein/protein interaction(s) or modification of the receptor's sialylation rate play a relevant role, since NEU3 physically interacts with EGFR (Wada T. *et al*, 2007).

In order to elucidate the mechanism by which NEU3 exerts its effect on EGFR, by protein/protein interaction(s) or modification of the receptor's sialylation rate, since NEU3 physically interacts with EGFR (Wada T. *et al*, 2007) we performed EGFR immunoprecipitation experiments on DIFI cells.

We have demonstrated, by SNA lectin binding assay, that the overexpression of the active form of the membrane sialidase NEU3, leads to a reduction of sialylation rate of the receptor. In fact there are many evidences that the receptor is glycosilated (Liu YC. *et al*, 2011).

To confirm that the sialidase NEU3 directly modified the receptor and in particular to identified the sites of these modifications on the EGFR sequence, we perfomed a mass spectrometry analysis on the same immunoprecipitated samples.

Our data shows two sites on the extracellular domain of the receptor, that are sialylated in the control (mock) and also in cells overexpressing high levels of NEU3 inactive mutant. On the other hand, these sites were not found to be sialylated in cells overexpressing the *wild type* form of the sialidase.

We can suppose that the decrease of sialylation of the receptor can facilitate the activation process, favouring the recognition of the epidermal growth factor or the dimerization process even in the absence of ligand.

On the other hand, our data showing that an altered level of sialylation affects EGFR activation are supported by the recent contribution by Park and colleagues, who investigated β-Galactoside α2-6-sialyltransferase (ST6Gal-I), an enzyme exerting the opposite function of NEU3 (Park JJ. *et al*, 2012). The authors demonstrated that a reduced activity of ST6Gal-I is able to activate EGFR, thus also affecting the efficacy of tyrosine kinase inhibitors against EGFR.

A corollary of our analyses is represented by the evaluation of the relationship among the level of mRNA and protein content, the phosphorylation status as well as the gene status of EGFR. Initial studies demonstrated that EGFR must be overexpressed in a patient in order to

obtain a clinical benefit from the administration of EGFR-targeted therapies. However, this requisite was abrogated after the publication of the work of Chung and colleagues, who reported the efficacy of EGFR-targeted therapies even in the absence of EGFR overexpression (Chung KY. *et al*, 2005). Later on, starting from the hypothesis (not supported by any data) that a copy number gain of EGFR gene is associated with EGFR activation, it appeared that EGFR gene status evaluation by FISH might represent a methodology able to predict the clinical efficacy of EGFR-targeted therapies, but recently other works did not confirm these preliminary findings (Frattini M. *et al*, 2007; Cappuzzo F. *et al*, 2008; Sartore-Bianchi A. *et al*, 2012; Custodio A. and Feliu J., 2013). Our data, indicating EGFR activation also in cells without EGFR protein overexpression, as well as in FISH negative cases, may therefore explain the failure of EGFR evaluation by protein expression or gene status as a methodology able to early identify patients who can be treated with EGFR-targeted drugs.

In conclusion, alteration of sialidase expression may be proposed as a defining factor in cancer progression, opening up potential applications in diagnosis and therapy. In this field, NEU3 may play a dual role in colorectal carcinogenesis:

1) its expression may alter the efficacy of monoclonal antibodies against EGFR, and further studies must be drawn to investigate this issue;

2) most importantly, NEU3 can be considered an ideal, and new, druggable marker for CRC, since it is overexpressed in the totality of cases.

These data confirm the need of further studies concerning the role played by NEU3 in CRC cancer progression, suggesting to consider this sialidase as a new effective molecular marker for CRC treatment. Therefore, designing NEU3-targeted therapies may significantly help in fighting CRC.

5.4 Role of NEU3 overexpression in the prediction of efficacy of EGFR-targeted therapies in colon cancer cell lines

Cetuximab, a monoclonal antibody against EGFR, has entered now into the routine management of colorectal cancer patients. This new drug (like all drugs) has been approved for clinical trials and patient management even before large and significant studies on the involved targeted molecules became available. However, to avoid inefficacious treatments, it will be mandatory to carefully select patients who can possibly benefit from these treatments. Current selection criteria are limited to the identification of KRAS and NRAS mutations which, however, only account for 30-40% of non responsive patients. The identification of additional genetic determinants of primary resistance to EGFR-targeted therapies is clearly a priority. Very interestingly, the target of these therapies does not seem to play any role in the identification of patients who can benefit from Cetuximab.

Having shown that sialidase NEU3 directly activates EGFR, our aim was to evaluate whether NEU3 overexpression may also affect the response to EGFR-targeted therapies, by examining its effect after Cetuximab treatment in cellular models.

We initially determined the mutational status of the markers able to predict the efficacy of this targeted therapy.

After determining Cetuximab EC50 value for each cell line considered, by MTT viability test, on the basis of the mutational status of EGFR, KRAS, BRAF and PIK3 genes, we selected 5 cell lines characterized by different mutations in EGFR pathway (Figure 6.2). These were transfected with the *wild type* or the inactive form of NEU3 both in the absence and in the presence of the drug.

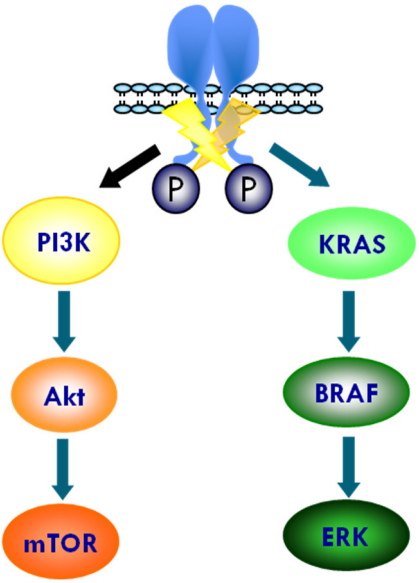


Figure 6.2 ***Schematic representation of EGFR downstream pathways.***

The overexpression of the active form of NEU3 sialidase lead to a significant increase in cell viability in all cell lines tested, also under pharmacological treatment with Cetuximab, with the exception of SW48 cell line. In these cells the observed increase in viability following overexpression of NEU3 could not be considered statistically significant, a behavior probably associated to the presence of a hyperactivating mutation in the tyrosine kinase domain of EGFR that causes the receptor to act independently from the dimerization: EGFR mutation makes it able to activate the pathway acting as a monomer and not as a dimer.

These data suggest that in the cell lines in which EGFR acts correctly as a dimer we observed an increment of viability even in those presenting hyperactivating mutations in the downstream pathways that influence the efficacy of the therapy.

Moreover, a recent contribution investigating the effect of ST6-Gall on EGFR agonists (using, however, tyrosine kinase inhibitors instead of

monoclonal antibodies) demonstrated that absence of sialic acid residues on EGFR protein is able to revert, at least partially, the negative effect played by KRAS mutations on Gefitinib efficacy (Park JJ. *et al*, 2012).
In particular we have investigate the EGFR downstream pathway activation by western blot techniques in response to pharmacological treatment with Cetuximab and the overexpression of the active form of the NEU3 sialidase in DIFI cell line. In these condition we have reported a strong activation of the Akt kinase, leading to the cell survival.
Our data provide important information on the mechanisms regulating EGFR activation and can be useful for improving the prediction of tailored chemotherapeutic regimens, to avoid inefficacious treatments, and to maintain the costs related to the clinical use of these novel drugs under control. In addition to the possible effect on EGFR-targeted therapies, our data suggest NEU3 as a potentially targetable marker for new specific therapies, using inhibitors or antibodies to prevent cancer progression.

5.5 Deregulation of Human Sialidases in Cancer

Aberrant glycosylation is thought to be a characteristic feature of cancer cells. In particular, altered sialylation is associated with malignant properties including invasiveness and metastasis. Sialidase expression levels was confirmed to change in response to various cellular phenomena and especially during carcinogenesis.
All the data presented in the literature concerning the changes in mRNA levels of genes coding for the human sialidases were collected only in the Japanese population. Miyagi and coworkers (2012) reported that whereas NEU1 and NEU4 showed downregulation in colon cancer, the plasma membrane-associated sialidase NEU3 was upregulated in various human cancers including colon, renal, ovarian and prostate cancers, except for the downregulation in acute lymphoblastic leukemia in relation to disease progression (Mandal C. *et al*, 2010). They showed that the aberrant expression of each sialidase exerted a influence on cancer progression.

We partially confirm their data on the Western population. Has been observed considerable variability among the samples considered regarding the data collected both on the normal and on cancer tissues.

NEU1 was reported to have an inverse relationship between NEU1 expression level and metastatic ability (Sawada M. *et al*, 2002). We confirm a general trend of deregulation in tumor tissues with some exceptions in ovarian and thyroid cancer.

We also confirm the upregulation of plasma-membrane associated NEU3 in colorectal, in ovarian and in prostate cancer but not in the renal cancer.

We also confirm the general trend of down-regulation for the sialidase NEU4 in all the tumor tissues analyzed. These data are in accordance to the data reported in literature that correlated the decrease of NEU4 sialidase to the inhibition of the apoptosis and to the promotion of cellular invasiveness and motility, processes that characterize the tumor progression (Shiozaki K. *et al*, 2011).

Noteworthy, in our cohort of patients, was the increase of transcription rate of all three sialidases in ovarian cancer, suggesting peculiar features of this tumor.

To date we have analyzed only a small number of patients. A more detailed survey on Western population will perform enlarging the cohort of patients to confirm these interesting preliminary data.

Investigation of mammalian sialidases in cancer has uncovered a great deal of information regarding the molecular bases of aberrant sialylation related to malignancy.

The altered expression of three human sialidases, NEU1, NEU3, and NEU4, may definitely influence the malignant properties of cancer cells, including cell survival, motility, invasion, and metastasis, through modification of various glycoconjugates as substrates.

Alteration in the sialidase expression may be a defining factor in cancer progression, because close links between promotion of malignancy and aberrant sialylation can now be explained, at least partly, as the results of altered expression of sialidases.

Sialidase alterations open up potential applications in cancer cure and diagnosis.

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- Looking at human cytosolic sialidase NEU2 structural features with an interdisciplinary approach. Monti E, Benaglia G, Gangemi F, Castiglioni E, **Mozzi A**, Fusi P, Fornili SL, Longhi G, Abbate S. *(Submitted)*

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