NEU3 activity enhances EGFR activation without affecting EGFR expression and acts on its sialylation levels

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Abstract

Several studies performed over the last decade have focused on the role of sialylation in the progression of cancer and, in particular, on the association between deregulation of sialidases and tumorigenic transformation. The plasma membrane-associated sialidase NEU3 is often deregulated in colorectal cancer (CRC), and it was shown that this enzyme co-immunoprecipitates in HeLa cells with epidermal growth factor receptor (EGFR), the molecular target of most recent monoclonal antibody-based therapies against CRC. To investigate the role of NEU3 sialidase on EGFR deregulation in CRC, we first collected data on NEU3 gene expression levels from a library of commercial colon cell lines, demonstrating that NEU3 transcription is upregulated in these cell lines. We also found EGFR to be hyperphosphorylated in all cell lines, with the exception of SW620 cells and the CCD841 normal intestinal cell line. By comparing the effects induced by overexpression of either the wild-type or the inactive mutant form of NEU3 on EGFR, we demonstrated that the active form of NEU3 enhanced receptor activation without affecting EGFR mRNA or protein expression. Moreover, through western blots and mass spectrometry analysis, we found that EGFR immunoprecipitated from cells overexpressing active NEU3, unlike the receptor from mock cells and cells overexpressing inactive NEU3, is desialylated. On the whole, our data demonstrate that, besides the already reported indirect EGFR activation through GM3, sialidase NEU3 could also play a role on EGFR activation through its desialylation.

Key words: cell lines, colorectal cancer, EGFR, NEU3, sialylation

Introduction

Defects in glycosylation are known to play a role in cancer malignancy (Ohtsubo and Marth 2006). In particular, altered levels of sialylation have been associated with invasiveness and metastatic potential in cancer cells, although how this process is involved in the regulation of tumorigenesis is poorly understood (Miyagi et al. 2012). Sialidases (EC 3.2.1.18) are glycohydrolases that catalyze the removal of terminal α-glycosidic-linked sialic acid residues from the carbohydrate moieties of glycoproteins and glycolipids. To date, four types of sialidases have been identified and characterized in humans—NEU1, NEU2, NEU3 and NEU4—which differ in their subcellular localizations and enzymatic properties, including substrate specificity (Monti and Miyagi 2012). In particular, NEU3 localizes in plasma membranes (Zanchetti et al. 2007), where it acts not only on gangliosides within its own membrane but also on gangliosides belonging to the plasma membranes of neighboring cells (Papini et al. 2004). Therefore, NEU3 is an important protein for many phenomena that occur at the cell surface, particularly those involved in the regulation of trans-membrane signaling (Miyagi et al. 2008;Monti and Miyagi 2012).

Several studies have shown that NEU3 is upregulated in various neoplastic diseases, including melanoma, colon, renal, ovarian and prostate cancers (Monti and Miyagi 2012). Furthermore, it was observed that NEU3 mRNA levels were increased from 3- to 100-fold in human colon cancer tissues compared with adjacent non-tumor mucosal tissues (Kakugawa et al. 2002). Finally, it was demonstrated that NEU3 could promote colorectal carcinogenesis in transgenic mice (Shiozaki et al. 2009).

Recent evidence has uncovered a link between NEU3 and activation of the epidermal growth factor receptor (EGFR) pathway (Odintsova et al. 2006; Tringali et al. 2012). In particular, it was shown inHeLa cells that human sialidaseNEU3 co-immunoprecipitates with EGFR (Wada et al. 2007). EGFR is a sialoglycoprotein with intrinsic tyrosine kinase activity, and it possesses 12 N-glycosylation sites in its extracellular domain (Zhen et al. 2003; Liu et al. 2011). Following ligand binding, EGFR is able to dimerize, and through a process known as cross-autophosphorylation, it can activate a number of downstream pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) axis, which is primarily involved in cell proliferation, and the phosphoinositide-3-kinase (PI3K)/PTEN/AKT axis, which is involved in cell survival and motility (Jorissen et al. 2003). EGFR is deregulated in colorectal cancer (CRC) essentially following gene copy number gain (due to gene amplification or polysomy), rarely by point mutations.

An alternative mechanism of EGFR constitutive activation is represented by ligands (e.g., AREG and EREG) overexpression (Kuramochi et al. 2012).

Although many studies have been published concerning the role of EGFR in CRC and EGFR-targeted therapies have been successfully introduced for the treatment of CRC patients, all the detailed mechanism(s) that control EGFR activation are still unclear. In particular, it isunknown how sialylation levels could affect receptor activation. Elucidating these mechanisms should provide novel insights into cancer biology and may lead to the development of innovative therapeutic approaches, as well as to a further demonstration of the role played by glycosylation for cell survival. Therefore, the aim of this study was to evaluate the effects of NEU3 deregulation on EGFR expression and activation in CRC cell lines.

Materials and methods

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 Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) colon cancer 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% CO2 incubator. CACO-2 (ATCC® HTB-37™) colon cancer cell line and CCD841 (ATCC® CRL-1790™) healthy mucosa cell linewere grown inEMEM medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 µg/ml streptomycin and maintained at 37°C in a humidified 5% CO2 incubator. HT-29 (ATCC® HTB-38™) colon cancer cell line and COS-7 (ATCC® CRL1651™) cells were grown in DMEM medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 100 U/ mL penicillin, 100 µg/mL streptomycin and maintained at 37°C in a humidified 5% CO2 incubator. T84 (ATCC® CCL-248™) colon cancer cells were grown in Ham's F12/DMEM (1:1) medium supplemented with heat-inactivated 5% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C in a humidified 5% CO2 incubator. DIFI human CRC cells, kindly provided by Dr Josep Tabernero (Vall d'Hebron Institute of Oncology, Vall d'Hebron University Hospital, Universitat Autònoma de Barcelona, Spain), were grown in Ham's F12 medium supplemented with heat-inactivated 5% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C in a humidified 5% CO2 incubator. ATCC validated cell lines by short tandem repeat profiles that are generated by simultaneous amplification of multiple short tandem repeat loci and amelogenin (for gender identification). All the reagents for cell culture were supplied by Lonza (Lonza Group, Basel, Switzerland).

RNA isolation and Q-PCR

Total RNA was isolated from cells using RNeasy Mini Kits (Qiagen, Chatsworth, CA), according to manufacturer's instructions. RNA was reverse-transcribed using SuperScript® II RT (Invitrogen, Carlsbad, CA), oligo dT and random primers, according to the manufacturer's protocol. For quantitative real-time PCR (Q-PCR), SYBR Green methodwas used for NEU3 and EGFR expression, TaqMan assay for AREG, EREG and ST6Gal-I expression. Briefly, 50 ng cDNA was amplifiedwith SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) or TaqMan Gene Expression MasterMix (Applied Biosystems) and specific primers (100 nM) or FAM-MGBprobes, using an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 59°C annealing for 1 min. Each sample was analyzed for NEU3, EGFR, AREG and EREG expression and normalized for total RNA content using Pol2 gene as an internal reference control. The relative expression level was calculated with the Livak method (2[−ΔΔC(T)]) (Livak and Schmittgen 2001) and was expressed as a fold change ± SD. The accuracy was monitored by the analysis of melting curves. The following primers were used: NEU3 Fw 5′-TGAGGATTGGGCAGTTGG-3′ and Rv 5′-CCCGCACACAGATGAAGAA-3′; EGFR Fw 5′-GGTGTGTGCAGATCGCAAAG-3′ and Rv5′-GACATGCTGCGGTGTTTTCAC-3′; Pol2 Fw 5′-AGGAGCAAAGCCTGGTGTT-3′ and Rv 5′-ACCCAAAGCTGCCAGAAGT-3′. Assay IDs were Hs00155832_m1_for AREG, Hs00914313_m1 for EREG, Hs00949382_m1 for ST6Gal-I and Hs00172187_m1 for Pol2 (Applied Biosystems).

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) was performed on cell lines previously described. Each cell line was incubated overnight at 37°C with Demecolcine Solution 10 μg/mL (Sigma-Aldrich, St. Louis, MO), treated with 2 mL trypsin–EDTA (Lonza) for 3–10 min at 37°C and centrifuged at 400 × g for 5 min.

The pellet was treated with 10 mL of KCl (0.56% in distilled water) at 37°C for 7 min, 5 mL of 5% acetic acid, 5 mL of methanol and 5 mL of fixative (3:1 ethanol-acetic acid), the latest repeated twice. After each step, the pellet was centrifuged at 400 × g for 8 min. Finally, cells were firmly attached on glasses.

FISH EGFR assay was performed using the dual color probe LSIEGFR/CEP7 (Vysis, Downer's Grove, IL) (Martin et al. 2009). Fluorescent signalswere evaluated through an automated microscope (Zeiss Axioplan 2 Imaging, Oberkochen, Germany) equipped with singleand triple band pass filters. Images were captured using an Axiocam camera (Zeiss Axiocam MRm) and processed with the AxioVysion Software (Carl Zeiss GmbH, Germany). We classified cell lines in four groups (disomic, low or high polysomic and with gene amplification) using descriptive features (Martin et al. 2009; Varella-Garcia et al. 2009).

EGFR mutational status

Total DNAwas isolated fromcells and analyzed by direct sequencing. We investigated exons 18–21 corresponding to the tyrosine kinase domain, where activating point mutations may occur. Sequencing of the PCR products was done using a 3130 Genetic Analyzer (Applied Biosystems) and analyzed with appropriate software (SeqScape Software Version 2.5, Applied Biosystems). Each sequence reaction was performed twice.

Homology modeling

HsNEU2 and HsNEU3 amino acidic sequences were aligned using ClustalW program (Thompson et al. 1994). Considering the high sequence identity between the two sialidases and the availability of the NEU2 crystal structure in the Protein Data Bank (PDB, www.pdb.org), we performed homology modeling of NEU3 (auto mode) in the Swiss Model workspace using NEU2 (PDB ID: 2F25) as a template (Guex and Peitsch 1997; Schwede et al. 2003; Arnold et al. 2006). The model was visualized using PyMOL software (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC, www.pymol.org).

Vector

The cDNA coding for human sialidase NEU3 was previously subcloned into plasmid pcDNAI (Invitrogen), in frame with C-terminal haemagglutinin epitope (Monti et al. 2000).

Site-directed mutagenesis

Sialidase double mutant was obtained by PCR using QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to recommended procedures. The following primers were used to introduce the D50A and the Y370F mutations:

D50A Fw 5′-CGTTCTACGAGGAGAGCTGAGGATGCTCTCCAC-3′,

D50A Rv: 5′-GTGGAGAGCATCCTCAGCTCTCCTCGTAGAACG-3′;

Y370F Fw 5′-GTGGGCCCTGTGGCAACTCTGATCTGGCTGC-3′,

Y370F Rv: 5′-GCAGCCAGATCAGAGTTGCCACAGGGCCCAC-3′.

All constructs were hosted and amplified in E. coli strain DH5α; the presence of the mutations was subsequently verified by automated sequencing, using commercially available vector oligonucleotide primers.

Transfection

Cells were seeded at 1 × 106 cells/100 mm dish and transiently transfected with pcDNA3I vector containing wild-type or double-mutant NEU3 cDNAs in a 2% serum medium using X-treme Gene 9 DNA Transfection Reagent (Roche, Basel, Switzerland), according to the manufacturer's instructions. After transfection, cells were grown for 36–48 h in a complete medium without EGF treatment.

Sialidase activity assay

COS-7 cells were harvested 48 h after transfection 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 amembrane fraction. The activity was evaluated on PBS resuspended pellets. Protein concentration was determinedby Bradford Assay (Coomassie Protein Assay Reagent, Bio-Rad,Hercules, CA) (Bradford 1976). NEU3 sialidase activitywas determined towards two different substrates (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, 1 U 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-N-acetylneuraminic acid) as 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 0.2M alycine/NaOH, pH 10.8.

The activity was also measured using GD1a ganglioside by a radiochemical method. The mixture containing 60 nmol GD1a+[3H]GD1a and Triton X-100 0.1% was incubated at 37°C for 1 h and then400 μL of tetrahydrofuran was added (Monti et al. 2000). The mixture was centrifuged at 10,000 × g for 5 min and 3 μL of resulting supernatant (1000 dpm/sample) were subjected to high-performance TLC on silica-gel plate with chloroform/methanol/0.2% $CaCl_2$ (60:40:9 vol) as solvent system to separate the reaction products from the substrate (Chigorno et al. 1986). Glycolipids separated were quantified by radiochromatoscanning (Beta Imager 2000; Biospace Mesures, Paris, France).

SDS–PAGE and western blot

Thirty-six hours after transfection, SW480, DIFI, SW620 and CCD841 cells were washed with ice-cold PBS and lysed in RIPA buffer, containing protease and phosphatase inhibitors and 1 mMPMSF. After lysis on ice, homogenates were obtained by passing five 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 by the BCA protein assay (Smith et al. 1985). SDS–PAGE and western blot were carried out by standard procedures. Sixty micrograms of proteins were separated on 10% acrylamide/ bis-acrylamide SDS–PAGE, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA), probed with the appropriated antibodies and visualized using ECL detection system (Millipore). Protein levels were quantified by densitometry of immunoblots using

ScionImage software (Scion Corp., Frederick, MD). We used the following primary antibodies (all purchased by Cell Signaling Technology, Danvers, MA): anti-EGFR (dilution 1:1000), phospho-EGFR (Tyr1068; dilution 1:1000), p44/42 MAPK (Erk1/2; dilution 1:1000), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; dilution 1:1000), Akt (dilution 1:1000), phospho-Akt (Ser473; dilution 1:1000), PTEN (dilution 1:1000) and GAPDH (dilution 1:10,000). IgG HRP-conjugated secondary antibodies (purchased by Cell SignalingTechnology) were diluted 1:10,000.

EGFR immunoprecipitation

DIFI cells, 36 h after transfection, 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 1 μg/mL pepstatin A, 10 μg/mL aprotinin and 10 μg/mL leupeptin as protease inhibitors. Crude extracts, obtained by gentle sonication, were centrifuged at 15,000 Å~ g for 15 min to clarify the lysate. The protein content of supernatant was assayed by the Bradford method (Bradford 1976). The volume corresponding to 1 mg of total protein extract was incubated 1 h at 4°C with 20 mg of protein A-Sepharose previously re-hydrated (Amersham Pharmacia Biotech, Uppsala, Sweden) to clear the lysates and centrifuged at 15,000 Å~ g for 10 min. Supernatants were incubated overnight at 4°C with 2 μL of EGFR antibody with gentle rocking and incubated for 4 h at 4°C with 20 mg of protein A-Sepharose previously re-hydrated (Amersham Pharmacia Biotech). After washes, immunoprecipitated were collected by centrifugation, boiled in 2Å~ SDS-sample buffer without β-mercaptoethanol.

Lectin affinity assay

EGFR immunoprecipitated samples were separated on 6% acrylamide/ bis-acrylamide SDS–PAGE, transferred onto a nitrocellulose membrane (Millipore) and probed with a biotinylated form of the lectin SNA (Vector Laboratories, Burlingame, CA). The 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 h at room temperature. After washes in TBS-T, the membrane was incubated using VECTASTAIN® elite ABC reagent 30 min at room temperature. After washes in TBS-T, α2,6-sialylated EGFR was detected using ECL (Millipore). Proteinlevels were quantified by densitometry of immunoblots using Scion Image software (Scion Corp.).

Acid silver stain

EGFR immunoprecipitated samples were loaded on 6% acrylamide/ bis-acrylamide 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 incubatedfor 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 mMsodium thiosulfate. Reactionwas stopped with 50% ethanol and 12% acetic acid. After washing with water, the gel was conserved at 4°C in 1% acetic acid.

Mass spectrometry analysis

Upon SDS–PAGE, the bands corresponding to EGFR protein were excised, cut into smaller pieces and dried in a Speed Vac. Dried gels were reduced by 10 mMdithiothreitol (Sigma-Aldrich) at 56°C for 1 h. Following

cysteine derivatization by iodoacetamide (Sigma-Aldrich) at room temperature, gels were digested overnight with trypsin sequencing grade (Sigma-Aldrich) (ratio 1:10 = protease:protein) at 37°C. The in-gel tryptic digest was extracted with 50% acetonitrile (Sigma-Aldrich) in 0.1% trifluoroacetic acid (Sigma-Aldrich) and the peptide mixture was desalted using C18ZipTip (Sigma-Aldrich). Matrix-assisted laser desorption ionization/time-of-flight mass spectrometric analysis was performed by using a AutoflexIII (Bruker Daltonics, Bremen, Germany) instrument equipped with a nitrogen laser (337 nm) and operated in reflector mode with a matrix of α-ciano-4-hydroxycinnamic cinnamic acid (Bruker Daltonics). External standards were used for calibration (Bruker peptide calibration standard).

Viability assay

Cell viability was investigated using in vitro toxicology assay kit MTT based (Sigma), according to 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) was 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.Results were expressed as mean values ± SD of three determinations.

Statistical analysis

Correlation analyses were performed by Fisher's exact test or by Student's t-test. The significance was defined as $p < 0.05$.

Results

Analysis of NEU3, EGFR, AREG and EREG deregulation in human colon cancer cell lines

We evaluated the correlation between NEU3, AREG and EREG expression and EGFR activation in 14 commercial human colon cancer cell lines, including DIFI cells, a CRC cell line used as a paradigm of EGFR activation in this particular cancer due to high levels of EGFR gene amplification. We initially analyzed NEU3, EGFR, AREG and EREG gene expression levels using Q-PCR by comparing mRNA levels in CRC cells with those observed in healthy CCD841 intestinal mucosa cells, using a ≥3-fold increase as the cut-off value. As shown in Figure 1A, compared with the normal mucosa cell line, NEU3 mRNA levels were increased by 3- to 30-fold in all cell lines tested, with MICOL24 and CACO2 cells showing the highest levels.With respect to EGFR, we observed overexpression of EGFR mRNA in 7 of the 14 (50%) tested cell lines, namely SW48, SW1116, SW480, MICOL29, MICOL24, CACO2 and DIFI (Figure 1B). In contrast, of the seven cell lines that did not show EGFR overexpression, three showed down-regulation of EGFR, with SW620 cells showing the strongest decrease in EGFR transcript levels (Figure 1B). AREG and EREG were upregulated in all cell lines, except CO115 and SW480, with a wide range of overexpression (ranging from 14- to 5000-fold for AREG; from 175- to 30,000-fold for EREG). The highest AREG overexpression was found in E705, SW1463, CACO2 and T84 cell lines (all >1000-fold), while EREG was found overexpressed at highest levels in E705, SW403, HT29 and SW1463 cells (all >10,000-fold) (Figure 1C and D).

Comparing mRNA expression levels of the four markers, a significant correlation was observed only between AREG and EREG: the two ligands were upregulated in the same cell lines (P = 0.01, two tailed Fisher's exact test). In contrast, NEU3, AREG and EREG mRNA levels showed no correlation to EGFR gene expression. All tested cell lines contained wild-type sequences for EGFR exons 18–21, encoding the tyrosine kinase domain, with the exception of SW48 cell line, which carries a G719S substitution in exon 18, leading to a constitutively activation of EGFR. Subsequently, we investigated the chromosomal status of the EGFR gene in the same cell lines, through FISH experiments. Two colon cancer cell lines (SW48 and E705), as well as the CCD841 normal intestinalmucosa cell line, were classified as FISH negative, according to the Colorado score; in particular, E705 and CCD841 showed disomy, whereas SW48 showed low polysomy. The remaining 12 cell lines (CO115, SW403, SW1116, SW480, SW1463, MICOL29, DIFI, MICOL24, HT29, SW620, T84 and CACO2) were classified as FISH positive, two of them (MICOL24 and DIFI cell lines) being characterized by EGFR gene amplification, one by concomitant high polysomy and low gene amplification (CACO2 cells) and the remaining nine by high polysomy. Next, we evaluated total EGFR expression levels through western blot experiments. The results are reported in Figure 2A, and the corresponding intensity ratios with respect to the relative loading control are shown in Figure 2B. Five cell lines, namely SW48, SW1116, SW480, DIFI and MICOL24 cells, displayed high levels of EGFR expression. Among the cell lines that did not show EGFR overexpression, SW620 cells showed the lowest levels of EGFR, which was nearly undetectable in these cells. In addition, we evaluated EGFR activation level using western blot experiments with monoclonal antibodies against the phosphorylated form of the receptor (Figure 2A). Finally, the results are expressed as the P-EGFR/total EGFR ratios and normalized to the corresponding value observed in the CCD841 normal mucosa cell line (Figure 2C). We observed EGFR hyperphosphorylation in all tested cell lines, with the exception of the SW620 colon cancer cells and the CCD841 normal intestinal cells. Considering the results obtained from all of the EGFR characterization experiments (mRNA expression, gene sequencing, gene/ chromosomal status, protein expression and phosphorylation status), we concluded the following:

(i) the CCD841 normal intestinal mucosa line did not show EGFR overexpression and lacked observable EGFR phosphorylation, as expected;

(ii) the SW48 cell line—which did not show a copy number gain (FISH negative) but contained a hyperactivating point mutation —displayed EGFR mRNA and protein overexpression as well as EGFR activation;

(iii) the two cell lines with strong EGFR gene amplification, DIFI and MICOL24, showed EGFR mRNA and protein overexpression as well as EGFR hyperactivation;

(iv) the E705 cell line, which had a normal EGFR gene status, showed normal levels of EGFR mRNA and protein expression but displayed hyperactivation of the EGFR protein;

(v) the SW620 colon cancer cell line, although characterized by high polysomy, did not show EGFR protein expression, and as a consequence, lacked EGFR phosphorylation. Indeed, this finding was not unexpected, as this cell line is commonly used as a negative control for EGFR expression (Park et al. 2012);

(vi) of the nine remaining FISH-positive cell lines, four were characterized by overexpression of the EGFR mRNA, namely SW1116, SW480, MICOL29 and CACO2, although among these, only two (SW1116 and SW480) were characterized by overexpression of the EGFR protein. The five remaining FISH-positive cell lines that lacked EGFR mRNA overexpression, namely CO115, SW403, SW1463, HT-29 and T84, showed no EGFR overexpression.

However, irrespective ofmRNA and protein expression levels, all these FISH-positive cell lines showed EGFR hyperactivation.

In conclusion, excluding SW620, which showed a complete lack of EGFR expression, all tested colon cancer cell lineswere characterized by increased levels of the phosphorylated form of the receptor, independent of mRNA/ protein expression or gene status. Sincewe did not observe any correlation between AREG and EREG expression and EGFR activation, and considering that all these cell lines also show overexpression of the NEU3 mRNA, it is possible that NEU3 may influence EGFR activation.

Rational design of an inactive form of the NEU3 sialidase

Based on the work of Albohy and colleagues (Albohy et al. 2010), we carried out a structural investigation of human NEU3 using molecular modeling to predict residues that might be involved in the hydrolysis of sialic acid from glycolipid substrates, assuming catalytic residues within the active site would be highly conserved (Taylor 1996; Buschiazzo and Alzari 2008). Starting from the human NEU2 crystal structure (Chavas et al. 2005), which shows 42% sequence identity with NEU3, we created a NEU3 homology model and identified residues D50 and Y370 as the acidic and nucleophilic tyrosine residues, respectively, both of which are essential for NEU3 catalytic activity (Taylor 1996; Buschiazzo and Alzari. 2008) (Figure 3A). To confirm the role played by these amino acids, site-directed mutagenesis experiments were carried out. A plasmid containing a cDNA encoding a double-site (D50A Y370F) mutant version of NEU3 was generated and transfected into COS7 cells to evaluate, in the corresponding cell extracts, sialidase enzymatic activity of the mutant protein compared with wild-type NEU3, using the artificial substrate 4 MU-NANA, as well as the natural substrate GD1a ganglioside. The double mutant exhibited no activity against either substrate, demonstrated by the fact that the measured values were comparable with those observed in the untransfected cells (mock), representing endogenous sialidase activity (Figure 3B). Incontrast, as expected, upon transfection with wild-type NEU3, cell extracts showed significant enzymatic activity for both substrates (Figure 3B).

Regulation of the EGFR pathway by NEU3 sialidase activity

To evaluate a possible role for 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 (double mutant) form of the enzyme. Three different CRC cell lines were chosen for this experiment: DIFI cells, which are one of the most investigated cellular systems for studying the EGFR pathway (Dolf et al. 1991), SW480 cells, which are widely used to study CRC (Trainer et al. 1988) and SW620, a CRC cell line not expressing EGFR (Park et al. 2012). Furthermore, CCD841 cells were used as healthy control cells. Both SW480 and DIFI cells showed NEU3 and EGFR mRNA upregulation, compared with CCD841 cells, as well as EGFR overexpression and hyperactivation; on the other hand, SW620 cells showed NEU3 overexpression but no EGFR expression, confirming previous data (Figures 1 and 2). Focusing the attention on DIFI and SW480 cells, following transfection with plasmids containing either wild-type or double-mutant inactive NEU3, Q-PCR experiments revealed an increase in NEU3 mRNA levels of 20- and 100-fold, respectively, compared with controls (Figure 4A). ST6Gal-I mRNA expression was also evaluated through Q-PCR and was found unaffected by wild-type active NEU3 overexpression (data not shown). Overexpression of either active or inactive NEU3 did not affect endogenous EGFR mRNA levels (Figure 4A), neither total EGFR protein expression, as shown in western blot experiments (Figure 4B and C). Taken together, these findings indicate that NEU3 overexpression does not

alterEGFR gene transcription rate nor protein expression. However, overexpression of wild-type NEU3 led to a marked increase in EGFR phosphorylation as well as to a significant activation of EGFR downstream pathways ERK1/2 and AKT in DIFI and SW480 cells, ERK1/2 pathway being hyperactivated at the same level (2-fold) in both cell lines, whereas AKT pathway showed a stronger activation in DIFI cells (~3-fold) than in SW480 cells (~1.35-fold) (Figure 4B and C). PTEN expression levels were unaffected by overexpression of either wild-type or double-mutant NEU3 (Figure 4B and C). AREG and EREG mRNA expression levels were unaffected as well (data not shown). Conversely, no evidence of EGFR activation and of its downstream pathways was observed upon transfection with the inactive double-mutant form of NEU3, strongly suggesting that EGFR activation is mediated by NEU3 sialidase activity (Figure 4B and C). EGFR pathway activationwas evaluated also on SW620 CRC cells and on CCD841 healthy intestinal cells. As expected, upon transfection with either active or inactive NEU3, neither EGFR expression nor activation of downstream pathways was observed in SW620 (Figure 5). In CCD481 healthy cells, following transfection with either active or inactive NEU3, EGFR was expressed at the same level of mock cells; however, neither receptor phosphorylation nor activation of ERK1/2 and AKT pathways were detected (Figure 5).

Analysis of EGFR sialylation following sialidase NEU3 overexpression

To determine whether NEU3 can directly act on EGFR, altering receptor sialylation level to affect its activation, EGFR immunoprecipitation experiments were carried out in DIFI cells transfected with either the active or the inactive form of NEU3. To assess sialylation levels, we used a lectin-binding assay based on biotinylated Sambucus nigra agglutinin (SNA) and avidin-conjugated horseradish peroxidase. Asshown in Figure 6, the level of EGFR α2,6-sialylation was reduced in cells overexpressing the active form of the NEU3 sialidase. Conversely, following transfection with the inactive double-mutant form of NEU3, no reduction in EGFR sialylationwas detected, strongly suggestingthat EGFR sialylation is regulated by NEU3.

These data were further confirmed by mass spectrometry: immunoprecipitated EGFR from DIFI cells overexpressing either wild-type or double-mutant NEU3, as well as from mock cells, was subjected to trypsin digestion. Analysis in MALDI-TOF mass spectrometry led to the identification of interesting peptides; in particular, a peptide with a molecular mass of 2080.97 Da was found in cells overexpressing inactive NEU3 and in mock cells, but not in cells overexpressing the wild-type NEU3. This peptide was identified, using GlycoMod software (Cooper et al. 2001), as the N-glycosilated (on N528) NVSR peptide, spanning residues 528–531, with the following predicted glycan structure: (Hex)1 (HexNAc)2 (NeuAc)1 (NeuGc)2 (Pent)1 (Table I). On the other hand, cells overexpressing wild-type NEU3 showed the presence of an ion with a molecular mass of 1953.78 Da (not detected in mock cells and in cells overexpressing inactive NEU3); GlycoMod software allowed its identification as the DCVSCRNVSR peptide, spanning residues 522-531, originated from a missed trypsin cut at position 527 and carrying the same glycosidic chain, shortened by 2 NeuGc and 1 NeuAc residues (Table I).

Overexpression of human NEU3 sialidase enhances cell viability

Finally, we studied the effects of NEU3 transfection on cell viability in the above cell lines using MTT assay. Results showed a significant increase in cell viability ($P < 0.01$) following transfection with the wildtype NEU3

enzyme, whereas no difference in cell viability was detected between transfected and control (mock) cells for the inactive double-mutant enzyme (Figure 7).

Discussion

In the present study, we analyzed the effects of NEU3 sialidase deregulation on EGFR expression and activation in CRC cell lines. We initially determined NEU3 expression levels in a number of cell lines commonly used in colorectal carcinogenesis studies, showing that NEU3 mRNA levels were upregulated in all cell lines, compared with CCD841 normal mucosa cells. These results suggest a role for this membrane-associated sialidase in CRC and confirm previous data obtained in Japanese CRC patients (Kakugawa et al. 2002).

Since the ability of NEU3 to interact with EGFR, a receptor playing a major signaling role in CRC (Wada et al. 2007) had been previously demonstrated through co-immunoprecipitation assays in HeLa cells, EGFR expression levels were also analyzed. EGFR transcripts were found to be overexpressed in 50%of the tested cell lines, which is consistent with the body of literature for CRC patients (McKay et al. 2002).

Furthermore, we evaluated EGFR gene status in these cells using FISH analysis and we also analyzed EGFR total protein content and phosphorylation rate using western blot techniques. Our data indicate that EGFR gene status correlates with mRNA and protein levels in all cell lines with either chromosome 7 disomy or EGFR gene amplification, with the exception of the SW48 cells, which are characterized by a disomic chromosomal asset but also carry a hyperactivating mutation leading to constitutive EGFR activation. In contrast,we could not establish any correlation between EGFR transcription and protein levels in cell lines classified as highly polysomic.Moreover, using CCD841 normal colon mucosa cells as a control, we observed EGFR activation in all cell lines, irrespective of mRNA or protein levels, with the exception of SW620 cells. In this cell line, although an abnormally high chromosome 7 copy number is present, EGFR is not expressed, SW620 cells being therefore routinely used as a negative control for EGFR expression (Park et al. 2012).

We also investigated the two most important EGFR ligand (AREG and EREG), for which a putative role in the prediction of efficacy of EGFR-targeted therapies has been proposed (Saridaki et al. 2011; Jonker et al. 2014). Although AREG and EREG expression levels were strictly correlated, in particular both ligands are overexpressed in the same cell lines and of the two EREG is consistently more overexpressed than AREG, no correlation was observed between their expression and EGFR mRNA, protein or activation level. Considering these findings together with the fact that NEU3 mRNA was found to be upregulated in all tested cell lines and that NEU3 had previously been shown to interact with EGFR (Wada et al. 2007), we suggest that NEU3 might activate EGFR through a direct modification of the receptor, without affecting overall EGFR mRNA or protein levels. It is well known that modifications of sialylation patterns on molecules exposed at the cell surface or released into the extracellular milieu are a hallmark of malignant differentiation (Varki 2008; Schauer 2009). Although the biochemical mechanisms linking sialylation and cancer are only poorly understood, several studies have been published indicating a direct role for sialyltransferases (STs) and sialidases (NEUs) in the biosynthesis, degradation and fine-tuning of cell sialic acid content (Hakomori 2002; Wada et al. 2007; Bos et al. 2009). For example, in colon cancer cells, NEU3 may regulate cell proliferation by enhancing tyrosine phosphorylation on integrin β4 through the recruitment of Shc and Grb-2, which in turn stimulates phosphorylation of focal adhesion kinase and ERK 1/2 (Kato et al. 2006). Furthermore, as a result of increased ganglioside catabolism triggered by NEU3 overexpression, accumulation of lactosyl-ceramide

has been detected in a number of colon cancer tissue specimens (Chung et al. 2005). Many reports have established the involvement of gangliosides, in particular GM3 on EGFR activation (Yang et al. 2011; Huang et al. 2013; Hakomori and Handa 2015). In particular, it has been well demonstrated that sialidase NEU3 can decrease GM3 content, thus removing the inhibitory effect exerted by this ganglioside on the receptor activation (Papini et al. 2004, 2012; Scaringi et al. 2013). In addition, to test whether sialidase NEU3 might play a direct role in the regulation of EGFR signaling at the cell surface, through direct actions and/or interactions with specific signaling proteins, we examined the effects of NEU3 overexpression on EGFR expression/activation and cell viability through in vitro transfection experiments using a completely inactive double-mutant form of NEU3, as a control. For this purpose, three specific CRC cell lines from the previously characterized panel were chosen: DIFI cells (Dolf et al. 1991), as the optimal cellular model for studying the EGFR pathway, SW480 cells, as the most studied model for CRC development (Trainer et al. 1988), and SW620, a CRC cell line not expressing EGFR; finally, CCD841 were used as control healthy cells.

Although EGFR mRNA and total protein levels, as well as AREG and EREG expression, were unaffected, overexpression of active wildtype NEU3 markedly enhanced EGFR phosphorylation, indicating the potential for direct modification (i.e., activation) of the receptor by this enzyme. Moreover, we also observed enhanced activation of both EGFR downstream pathways, the MAP kinase pathway (evaluated as ERK1/2 proteins activation) and the PI3K–mTOR pathway (evaluated as AKT activation). In contrast, following overexpression of the inactive double-mutant NEU3, we did not observe any activation of the EGFR downstream pathways. Finally, a significant increase in cell viability was only observed following overexpression of wildtype NEU3, whereas the inactive double-mutant form had no effect on viability.

Overall, these results confirm that NEU3 overexpression leads to a strong activation of EGFR and its downstream pathways, even in the context of cells with high basal levels of EGFR pathway activation, such as DIFI cells. Moreover, our data show that sialidase activity is required for EGFR activation and that NEU3 is involved in the regulation of EGFR sialylation level, confirming previously published data (Odintsova et al. 2006; Wada et al. 2007; Tringali et al. 2012).

However, sialidase activity could in principle be involved either directly, on EGFR desialylation, or indirectly, through gangliosides pattern modulation. While indirect NEU3 action promoting tumorigenesis is well established (Wada et al. 2007), our data show for thefirst time that direct EGFR desialylation by the membrane-associated sialidase could also be involved, at least in CRC cell lines used in this study, in its activation. In particular, mass spectrometry results confirm glycosylation of N528, previously observed by other authors (Zhen et al. 2003; Wu et al. 2005; Liu et al. 2011). These studies were performed on purified commercial EGFR, aswell as on the receptor of different cell lines, such as A431 human epidermoid carcinoma cells and CL1-0 and CL1-5 human lung cancer cells, and clearly showed the presence on N528 of a paucimannose-type biantennary sialylated glycan, whose precise composition varies in different cell lines. In this perspective, the presence of negatively charged sialylated glycan(s) on the extracellular domain of EGFR could influence both recognition of EGF ligand, a small acidic protein, and its dimerization. Our results should also be considered in the context of recent findings by Park and colleagues, showing that β-galactoside α2-6-sialyltransferase (ST6Gal-I) affects EGFR activation by altering its sialylation level (Park et al. 2012). In particular, these authors demonstrated that reduced ST6Gal-I activity can activate EGFR and can also affect the efficacy of tyrosine kinase inhibitors against EGFR. However, in our experiments, ST6Gal-I mRNA

expression was found unchanged in both DIFI and SW480 cells, showing that, at least in our cell systems, NEU3 overexpression does not affect its regulation and the activation effect on EGFR could be obtained by decreasing the its overall content of sialic acid through the action of an hydrolytic enzyme, NEU3, instead of acting on the biosynthetic side. Intriguingly, recent data demonstrated that EGF induced phospholipase D1 (PLD1) activation and the resulting release of phosphatidic acid mediates activation and traslocation of NEU3 to the cell surface (Shiozaki et al. 2015). In this perspective, EGFR stimulation could useNEU3 sialic acid removal as a positive feedback in a regulatory loop of the receptor activity. In conclusion, we propose that altered sialidase expression may be a defining factor in cancer progression, which suggests several potential diagnostic and therapeutic applications for this enzyme. In the field of CRC, NEU3 will likely have two immediate effects: (i) its expression may alter the efficacy of monoclonal antibodies against EGFR, which should be further investigated; and most importantly, (ii) NEU3 appears to be a promising, novel, druggable marker for this particular kind of cancer, due to its apparent overexpression in nearly all cases of CRC. Therefore, according to flexible and multipurpose strategies, designing NEU3-targeted therapies could represent a significant tool to fight CRC.

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Conflict of interest statement

None declared.

Abbreviations

CRC, colorectal cancer; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FISH, fluorescent in situ hybridization; MAPK, mitogen-activated protein kinase; PDB, Protein Data Bank; PLD1, phospholipase D1; SNA, Sambucus nigra agglutinin; STs,sialyltransferases.

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