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α2,3-Sialylation regulates the stability of stem cell marker CD133

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CD133 is widely used as a marker for the isolation and characterization of normal and cancer stem cells. The dynamic alternation of CD133 glycosylation contributes to the isolation of normal and cancer stem cells, and is supposed to be associated with cell differentiation. Although CD133 has been identified as a *N*-glycosylated protein, the specific glycosylation status of CD133 remain unclear. Here, we found that CD133 could be sialylated in neural stem cells and glioma-initiating cells, and the sialyl residues attach to CD133 *N*-glycan terminal via α2,3-linkage. Furthermore, desialylation of CD133 by neuraminidase specifically accelerates its degradation in lysosomes-dependent pathway. Taken together, our results characterized CD133 as an α2,3-sialylated glycoprotein and revealed that the sialylation modification contributes to the stability of CD133 protein, providing clues to understanding the function of CD133 molecular and to understanding the utility of glycosylated CD133 epitopes in defining neural stem cells and tumour-initiating cells.

Keywords: CD133/sialylation/stability/tumour-initiating cells.

Abbreviations: CHX, cycloheximide; CSCs, cancer stem cells; GalNAc, N-acetylgalactosamine; GICs, glioma-initiating cells; MAA, *Maackia amurensis* agglutinin; NSCs, neural stem cells; PNGase F, peptide-N-glycosidase F; PSA, polysialic acid; SAs, sialic acids; SNA, *Sambucus nigra* agglutinin; TICs, tumour-initiating cells; NHE1, Na^+/H^+ exchanger isoform 1.

CD133, also known as Prominin 1, is a founding member of pentaspan transmembrane glycoproteins that are thought to act as the organizers of plasma-membrane protrusions (*1*, *2*). The general interest in CD133 has grown rapidly, since it appears to be an important cell surface marker widely used to identify and isolate normal and cancer stem cells (CSCs) from various tissues including brain (*3*–*5*). CD133-positive human brain tumour cell fraction (as opposed to CD133-negative) contains cells that initiate tumour information in immunodeficient mice (*6*–*10*), raising the exciting possibility that CD133 becomes a molecular target for effective cancer therapies.

However, it remains unclear at this point how CD133 contributes to CSCs characteristics such as resistance to therapy, self-renewal and differentiation. CD133 protein is predicted to contain eight N-linked glycosylation sites, all within the putative extracellular domains (*4*, *11*, *12*). Because the anti-CD133 antibodies typically used, that are AC133 and AC141 mAbs, recognize undefined glycosylated epitopes (*4*, *12*), it is hypothesized that the glycosylation status of CD133, rather than the expression of CD133 protein itself, can act as an indirect marker of the stem cells (*13*). This idea is supported by the findings in K. Kemper's publication, where it is reported that CD133 mRNA and protein are not decreased when CSCs differentiate and lose their 'stemness', but that the epitope for AC133 is lost (maybe due to a change in glycosylation and a resulting distinct overall tertiary structure) (*14*), suggesting that the dynamic glycosylation of CD133 might play a critical role in its biological functions. Thus, identification of CD133 glycosylation status, which might be different in cells at different stages of differentiation and in different tissues, means a crucial step in defining the potential role of CD133 in normal and CSCs.

Glycosylation is one of the most frequently occurring co- or post-translational modifications made to proteins (*15*). The polysaccharide chains attached to the target proteins are primarily localized on the plasma-membrane surface of cells and participate in many key biological processes including cell adhesion, molecular trafficking, receptor activation, signal transduction and endocytosis (*15*–*21*). Normal and CSCs may be distinguished by the expression of the carbohydrate antigens (*22*). The carbohydrate antigens carried by oligosaccharides are mainly localized on the plasma membrane surface of the cells and they serve as excellent biomarkers for various stages of cellular differentiation (*22*, *23*). Furthermore, oligosaccharides have also been suggested to have a wide range of receptor and signaling functions in NSCs (*22*, *23*). Thus, understandings the glycosylation modification of proteins regulating the characteristics of stem cells provide us the clues for understating stem cell biology.

Here, we found that CD133 could be terminally sialylated in both neural stem cells (NSCs) and glioma-initiating cells (GICs). These sialyl residues

attached to the *N*-glycan of CD133 via α2,3-linkage. Furthermore, desialylation reduced the stability of CD133 which was degraded by the lysosome-dependent pathway rather than by the protease-dependent pathway. Our results identified the characteristics of terminal sialic acid (SA) of CD133 as a α2,3-linkage, enhancing our understanding of the utility of glycosylated CD133 epitopes in defining NSCs and tumour-initiating cells (TICs).

Materials and Methods

Antibodies and reagents

Mouse monoclonal anti-GFP antibody was purchased from Roche Applied Science, mouse anti-CD133 antibody (W6B3C1) was purchased from Miltenyi Biotec, and mouse anti-β-actin antibody was purchased from Sigma-Aldrich. Goat anti-mouse-HRP secondary antibody was purchased from Santa Cruz Biotechnology. α2,3-neuraminidase was purchased from New England Biolabs. α2-3,6,8-Neuraminidase was purchased from Sigma. Biotinylated MAA lectin was purchased from Vector Laboratories. Streptavidin-HRP was purchased from Southern Biotech. Peptide-N-glycosidase F (PNGase F) was purchased from sigma.

Cell culture and transfection

Human NSCs and human GICs were cultured in Dulbecco's modified Eagle's and F12 media supplemented with B27 (Invitrogen), 2 μg/ml heparin (Sigma), 20 ng/ml EGF (Chemicon) and 20 ng/ml FGF-2 (Chemicon). To establish U87MG that stably expressed CD133, U87MG were transfected with pEGFP-N1 vector or pEGFP-N1 containing a cDNA sequence encoding CD133 by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (*24*). After 72 h transfection, the cells were selected in the DMEM containing G418 (400 μg/ml). After 3 weeks of growth in the selection medium containing 400 μg/ml of G418, the individual G418-resistant clones were selected and analysed. The stablely transfected U87MG cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air).

Immunoblot, immunoprecipitation and lectin blot

Cell extracts were prepared using the lysis buffer containing 80 mM Tris pH 6.8, 2% SDS, 15% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol supplemented with a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). The cell lysates were resolved by SDS–PAGE under reducing conditions using 8% gels. After electrophoresis, proteins were transferred to PVDF membrane (BioRad) and were probed with the appropriate antibody followed by HRP-conjugated secondary antibody and an enhanced chemiluminescent substrate.

For immunoprecipitation of CD133, $\sim 1.0 \times 10^7$ U87MG/CD133-GFP cells were harvested in the lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% TritonX-100, 2 mM EDTA, 60 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF]. Then, immunoprecipitations were carried out with the indicated antibodies and protein G-agarose beads according to the manufacturer's protocol. The immunoprecipitates were subjected to western blot analysis according to the standard procedures. For lectin staining, the PVDF membrane was blocked in 5% BSA in TBS for 4 h at room temperature. The membrane was washed twice for 10 min with TBS, then once with lectin vehicle (1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ in TBS) before 1 h incubation with biotinylated MAA lectin (1 : 2000, Vector Laboratories). The membrane was washed three times for 10 min each in TBST (1% Tween-20 in TBS), and were then incubated 45 min with Streptavidin-HRP (1 : 2000, Southern Biotech) as described earlier (*25*).

Neuraminidase treatment of cell lysates

Hundred micrograms of cell lysates were treated with different concentrations of α2-3- or α2-3,6,8-neuraminidase according to the manufacturer's protocol at 37°C overnight. The cell lysates were subsequently boiled in 5× SDS sample buffer, resolved by SDS–PAGE, and immunoblotted for the CD133 protein.

Neuraminidase treatment of cells

To examine whether CD133 is sialylated at the terminal positions of its oligosaccharides, cells were incubated with α2-3,6,8-neuraminidase added to the culture media at concentration of 10 mU/ml or 100 mU/ml for 3 h at 37°C. Then the cells were harvested, and proceeding with western blot analysis.

To examine the effect of desialylation on CD133 protein stability, U87MG/CD133-GFP cells were treated with or without α2-3--neuraminidase added to the culture medium for 3 h at 37°C. The CHX was added to the culture medium (100 μg/ml) to inhibit protein synthesis. Protein extracts were prepared at the indicated time points following CHX treatment.

Immunofluorescent staining

Cells were plated on coverslips coated with poly-L-lysine overnight at 37°C and then fixed with 4% formaldehyde at room temperature for 30 min. The cells were treated with 0.2% Triton X-100 in PBS on ice for 5 min and blocked with 1% bovine serum albumin (BSA). For nuclear staining 50 μg/ml Hoechst 33258 (Invitrogen) was used. Cells were viewed under 60× oil objective of a fluorescent microscope (Nikon, Tokyo, Japan). Images were taken by a Nikon CoolSNAP camera.

Results

CD133 is sialylated in NSC and GIC

To determine whether CD133 is sialylated at the terminal positions of its oligosaccharides, the alteration of CD133 molecular weight was investigated in various cells which were treated with neuraminidase to remove the terminal sialyl residues of surface glycans. The electrophoretic mobility of CD133 in NSCs treated with or without neuraminidase was monitored by blotting with CD133-specific antibody W6B3C1. As shown in Fig. 1A, neuraminidase treatment of NSCs caused an increase in the electrophoretic mobility of CD133 protein, consistent with the enzymatic removal of SAs. We obtained the same results in GICs (Fig. 1B). Thus, these data indicated that endogenous CD133 protein in NSCs and GICs is sialylated.

To further explore whether exogenous CD133 is sialylated, stable cell lines U87MG/GFP or U87MG/CD133-GFP were established by transfecting the recombinant plasmid of GFP or GFP-fused CD133 into U87MG cells. CD133 was localized predominantly on the plasma membrane and pre-nuclear (Fig. 1**C**), which was consistent with previous report (*26*). Western blot assay showed that U87MG/CD133-GFP cells expressed the moderate levels of ectopic CD133 (Fig. 1D). The lysates of U87MG/CD133-GFP cells treated with different concentrations of neuraminidase were analysed by western blotting using anti-GFP or W6B3C1 antibody. Neuraminidase treatment caused a gradually decrease in the molecular weight of CD133-GFP in a dose-dependent manner, suggesting that ectopic CD133 is sialylated (Fig. 1E). Thus, these data confirmed the existence of SAs residues on the polysaccharide chains of CD133 protein.

Identification of α2,3-linked SA in the polysaccharide chains of CD133

SA is commonly attached to the termini of complex carbohydrates through an α2→3 or an α2→6 linkage

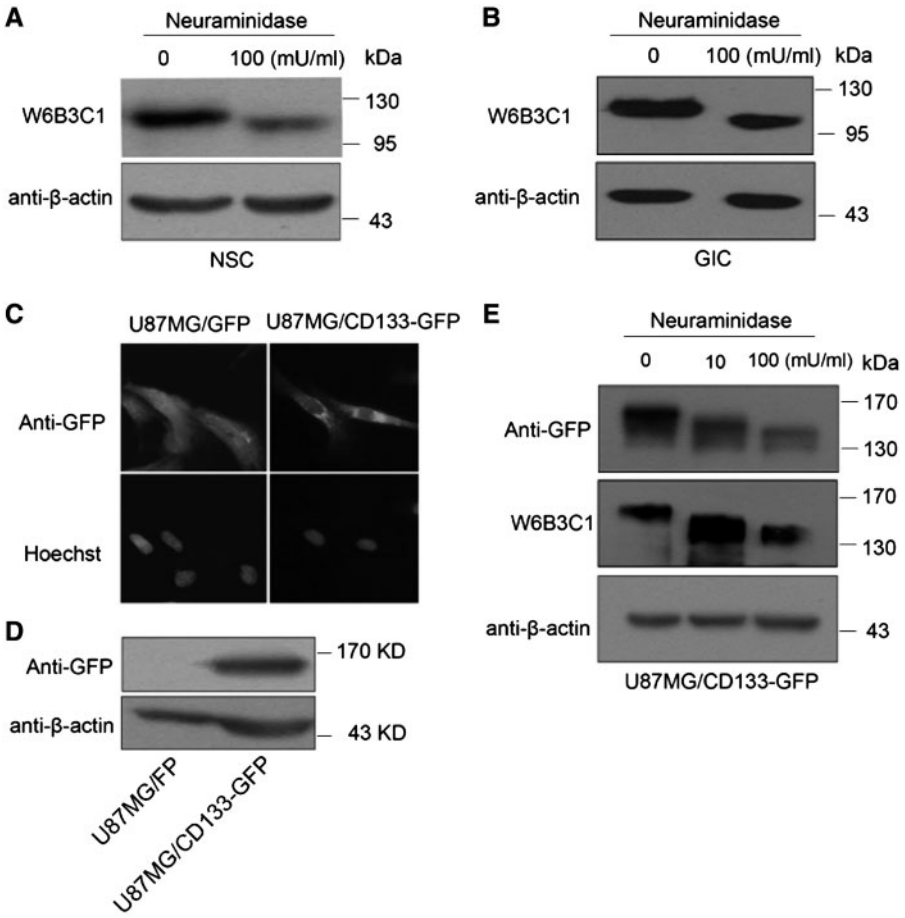

Fig. 1 Desialylation of CD133 in cells after neuraminidase treatment. (A) and (B) Human NSCs (A) and GICs (B) were treated with neuraminidase at 100 mU/ml for 3 h at 37°C, then cells were harvested and lysates were analysed by SDS–PAGE followed by immunobloting for CD133 with W6B3C1 antibody. β-Actin expression served as a loading control. (C) Expression of CD133 in U87MG/GFP and U87MG/CD133-GFP cells was confirmed by immunofluorescent staining with anti-GFP antibody. Hoechst 33258 was used for nuclear staining. Images were captured at 60× magnification. (D) Expression of CD133 in U87MG/GFP and U87MG/CD133-GFP cells was confirmed by western blot using anti-GFP antibody. β-Actin expression served as a loading control. (E) U87MG/CD133-GFP cells were treated with the indicated concentrations of neuraminidase for 3 h at 37°C. Cell lysates were immunoblotted with anti-GFP and W6B3C1 antibodies. β-Actin expression served as a loading control.

or is poly-merized in the form of an α2→8 or an α2→9 linkage (*27–29*). In order to investigate the characteristic of CD133 sialylation, the lysates of U87MG/CD133-GFP cells were treated with α2,3-neuraminidase, which specifically cleaves α2,3-linked terminal SA residues. α2,3-Neuraminidase treatment caused a gradually decrease in the molecular weight of CD133-GFP, confirming the α2,3-sialylation of CD133 (Fig. 2A). To further investigate the characteristic of CD133 sialylation, the lysates of U87MG/CD133-GFP cells were subject to mock, α2,3-neuraminidase or α2-3,6,8-neuraminidase hydrolysis. In response to treatment with different neuraminidases to remove α2-3- or α2-3,6,8-linked SAs residues, CD133-GFP protein showed the same alteration of electrophoretic mobility as compared to that of control-treated example (Fig. 2A), suggesting that SAs at the termini of complex carbohydrates attaching to CD133 are mainly via a α2,3-linkage.

α2,3-Sialylation of CD133 was further confirmed using biotin-labeled lectins: *Maackia amurensis* lectin (MAA) which is specific for carbohydrate structures containing α2,3-linked SAs, and *Sambucus nigra* lectin (SNA) which is specific for carbohydrate structures containing α2,6-linked SAs (*30*). We precipitated CD133-GFP protein from the cell lysates of U87MG/CD133-GFP cells using anti-GFP antibody, and then MAA and SNA lectins were detected by immunoblotting. As shown in Fig. 2B, MAA, but not SNA (data not shown), was precipitated by CD133 protein. We then did the same type of assay using anti-poly-α2,8-linked SAs antibody and found that there was no detectable amount poly-α2,8-linked SAs in CD133 precipitates (data not shown). Thus, these data further demonstrated that SAs at the termini of complex carbohydrates attaching to CD133 are mainly via α2, 3-linkage.

CD133 carries SAs that are predominantly attached to the N-linked sugar chains

Proteins can be glycosylated on certain amino acid side chains, and these modifications are designated as N- and O-glycosylation. N-glycosylated species are modified at Asn-residues and O-glycosylation occurs

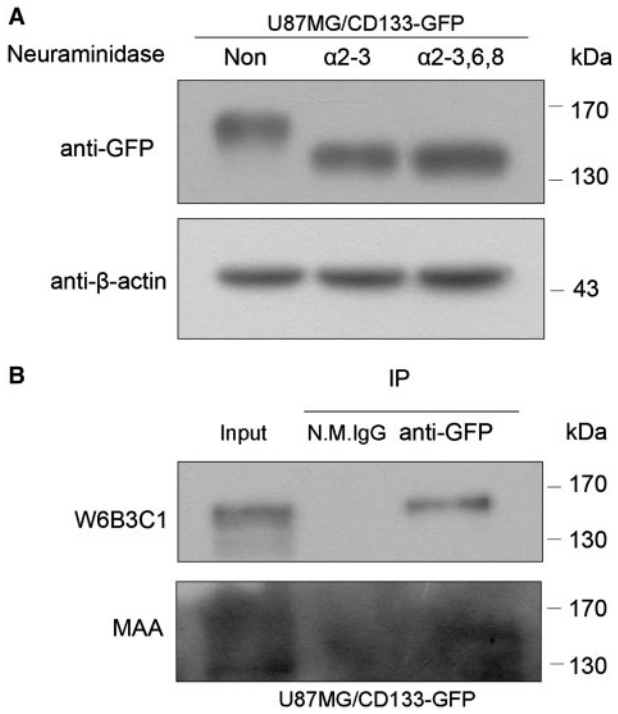

Fig. 2 SA residues are linked to CD133 glycan via α2,3-linkage. (A) Lysates from U87MG/CD133-GFP cells were subject to mock, α2-3-neuraminidase or α2-3,6,8-neuraminidase hydrolysis at 37°C overnight. Then the lysates were boiled with 5× SDS sample buffer and immunoblotted for CD133 with anti-GFP antibody. β-Actin expression served as a loading control. (B) GFP-fused CD133 proteins were immunoprecipitated (IP) from U87MG/CD133-GFP cells, resolved by SDS–PAGE and transferred to PVDF membrane. The membrane was overlaid with antibody W6B3C1 to verify the IP efficiency and with lectin MAA to confirm α2,3-linked SA residues.

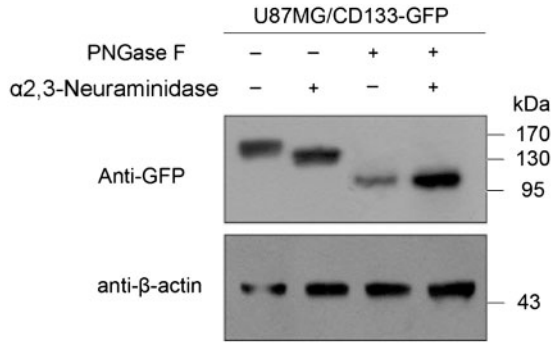

Fig. 3 SA residues are linked to CD133 *N*-glycan. Cell lysates were treated with α2-3-neuraminidase with or without pre-treatment with PNGase F at 37°C overnight. The lysates were then subjected to SDS–PAGE analysis. β-Actin expression served as a loading control.

at Ser- or Thr-residues. An enzyme, PNGase F, removes unaltered most of the common *N*-linked carbohydrates from proteins while hydrolyzing the originally glycosylated Asn residue to Asp. The lysates of U87MG/CD133-GFP cells were treated with PNGase F to remove *N*-linked glycans, resulting in a mobility shift equaling around 30 kDa (Fig. 3). These data indicated that CD133 is heavily N-glycosylated, which was consistent with previous reports (*12*).

To further explore the characteristic of CD133 sialylation, the lysates of U87MG/CD133-GFP cells pre-treated PNGase F to removal N-linked carbohydrates were performed with α2-3-neuraminidase hydrolysis. SDS–PAGE analysis was then used to identify the shift in the apparent size of treated CD133 protein. However, there was no difference in apparent size between CD133 protein treated with PNGase F alone and CD133 protein treated with PNGase F and α2-3-neuraminidase (Fig. 3). These results suggested that CD133 carries SAs that are predominantly or entirely attached to the N-linked sugar chains.

Sialylation of CD133 regulates its protein stability

SA, the most abundant terminal monosaccharide of glycoconjugates, has been found to be involved in a variety of cellular functions, such as cell–cell interaction, angiogenesis and the biological stability of glycoproteins (*31–36*). Therefore, we supposed that there might be a direct connection between α2-3-sialylation and CD133 protein stability. To test this hypothesis, we first evaluated whether α2-3-neuraminidase added to cell medium effectively removed the α2,3-linked SA residues of CD133. The alteration of CD133 molecular weight was investigated in U87MG/CD133-GFP cells treated with mock, α2,3-neuraminidase or α2-3,6,8-neuraminidase. In response to treatment with different neuraminidases, CD133-GFP protein showed the same alteration of electrophoretic mobility as compared to that of control-treated example (Fig. 4A, lanes 1-3). Furthermore, α2,3-neuraminidase treatment in U87MG/CD133-GFP cells resulted in the same alteration of electrophoretic mobility of CD133-GFP protein as compared to that of lysates treated with α2,3-neuraminidase or α2-3,6,8-neuraminidase (Fig. 4A, lanes 2 and 4–6). Next, the half-lives of CD133 protein were measured by CHX chase experiments in U87MG/CD133-GFP cells pre-treated with or without α2-3-neuraminidase to remove the terminal SAs. As shown in Fig. 4B, the half-life of CD133 protein was significantly decreased to ~3 h in U87MG/CD133-GFP cells after treatment with α2-3-neuraminidase to remove the terminal α2-3-linked SAs. CD133 protein decreased to only 80% of the initial amount after 6 h in U87MG/CD133-GFP cells without treatment of α2-3-neuraminidase, suggesting that desialylation of CD133 leads to a remarkable decrease in its protein stability. To investigate whether CD133 is specifically stabilized by the α2,3-sialylation, we examined the contribution of the α2,3-sialylation to Na^+/H^+ exchanger isoform 1 (NHE1) protein stability. NHE1 is an ubiquitous protein that plays a central role in the regulation of intracellular pH and is N-glycosylated (*37*, *38*). α2,3-Neuraminidase treatment caused a gradually decrease in the molecular weight of NHE1, confirming the α2,3-sialylation of NHE1. However, treatment with α2-3-neuraminidase to remove the terminal α2-3-linked SAs did not obviously decrease the half-life of NHE1 protein (Fig. 4**C**).

These data raised the question of how α2,3-sialylation specifically regulates the CD133 protein stability. Eukaryotic cells contain two major systems for

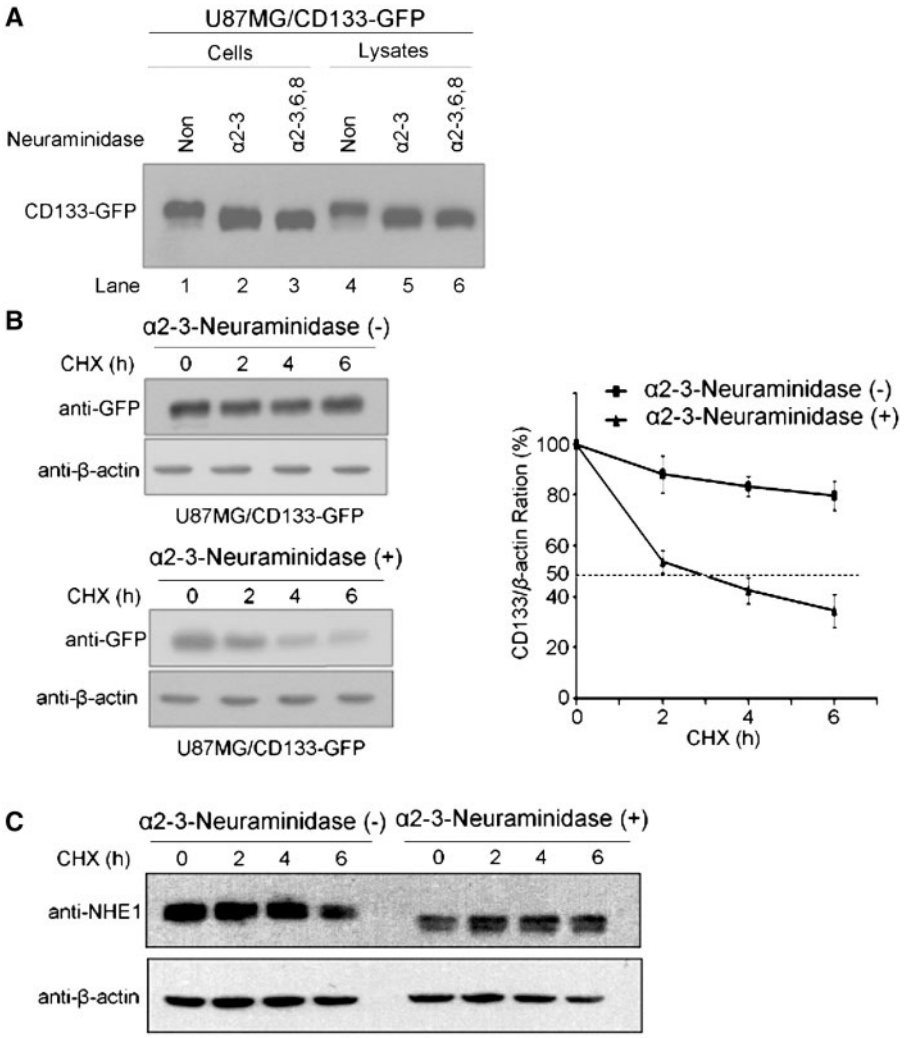

Fig. 4 Desialylation of CD133 decreases its stability. (A) U87MG/CD133-GFP cells were treated with mock, α2-3-neuraminidase or α2-3,6,8-neuraminidase added to cell medium for 3 h at 37°C. The cell lysates were immunoblotted with anti-GFP antibody (lanes 1–3). The lysates from U87MG/CD133-GFP cells were subject to mock, α2-3-neuraminidase or α2-3,6,8-neuraminidase hydrolysis at 37°C overnight. Then the lysates were boiled with 5× SDS sample buffer and were immunoblotted for CD133 with anti-GFP antibody (Lanes 4–6). (B) After treatment with or without α2,3-neuraminidase for 3 h at 37°C, the half-lives of CD133 were measured by cycloheximide (CHX) chase experiment in U87MG/CD133-GFP cells. Protein extracts were prepared at the indicated time points following CHX treatment and were immunoblotted using the CD133 antibody W6B3C1. β-Actin expression served as a loading control. The graph represented CD133/β-actin ratio level as measured by densitometry. The value of CD133/β-actin at 0 h was taken as 100%. The data was shown as the means ± SD of at least three experiments. (**C**) After treatment with or without α2,3-neuraminidase for 3 h at 37°C, the half-lives of NHE1 were measured by CHX chase experiment in U87MG/CD133-GFP cells. Protein extracts were prepared at the indicated time points following CHX treatment and were immunoblotted using the NHE1 antibody. β-Actin expression served as a loading control.

protein degradation: the lysosomal apparatus, a membrane-enclosed vacuole containing multiple acid proteases and the proteasome, an ATP-dependent proteolytic complex that mostly degrades the ubiquitinated proteins (*39*, *40*). Chloroquine and NH4Cl are known to inhibit lysosomal hydrolases by reducing the acidification of the endosomal/lysosomal compartments. MG132 is a widely used inhibitor of the proteasome (*39*, *40*). To explore the mechanism of α2,3-sialylation-regulating CD133 protein stability, we first determined the level of CD133 protein following inhibition of proteasomal or lysosomal degradation. We did not observe the significant alteration of CD133 protein level in response to MG132 treatment (Fig. 5A, lanes 1 and 2). However, both chloroquine and NH4Cl treatments induced an obvious increase in CD133 protein level (Fig. 5A, lanes 3–6). These data indicated that CD133 degradation mainly requires the activity of lysosomal hydrolases but not the proteasome. Thus, we examined next whether α2,3-sialylation regulates CD133 protein stability through lysosomal pathway-mediated CD133 degradation. To test this hypothesis, U87MG/CD133-GFP cells pre-treated with or without α2-3-neuraminidase to remove the terminal SAs were treated with CHX and vehicle or lysosomal hydrolases inhibitor chloroquine. We examined the levels of CD133 proteins using western blot and observed that the negative effect of desialylation on the level of CD133 protein was inhibited by lysosomal degradation inhibitor chloroquine (Fig. 5B).

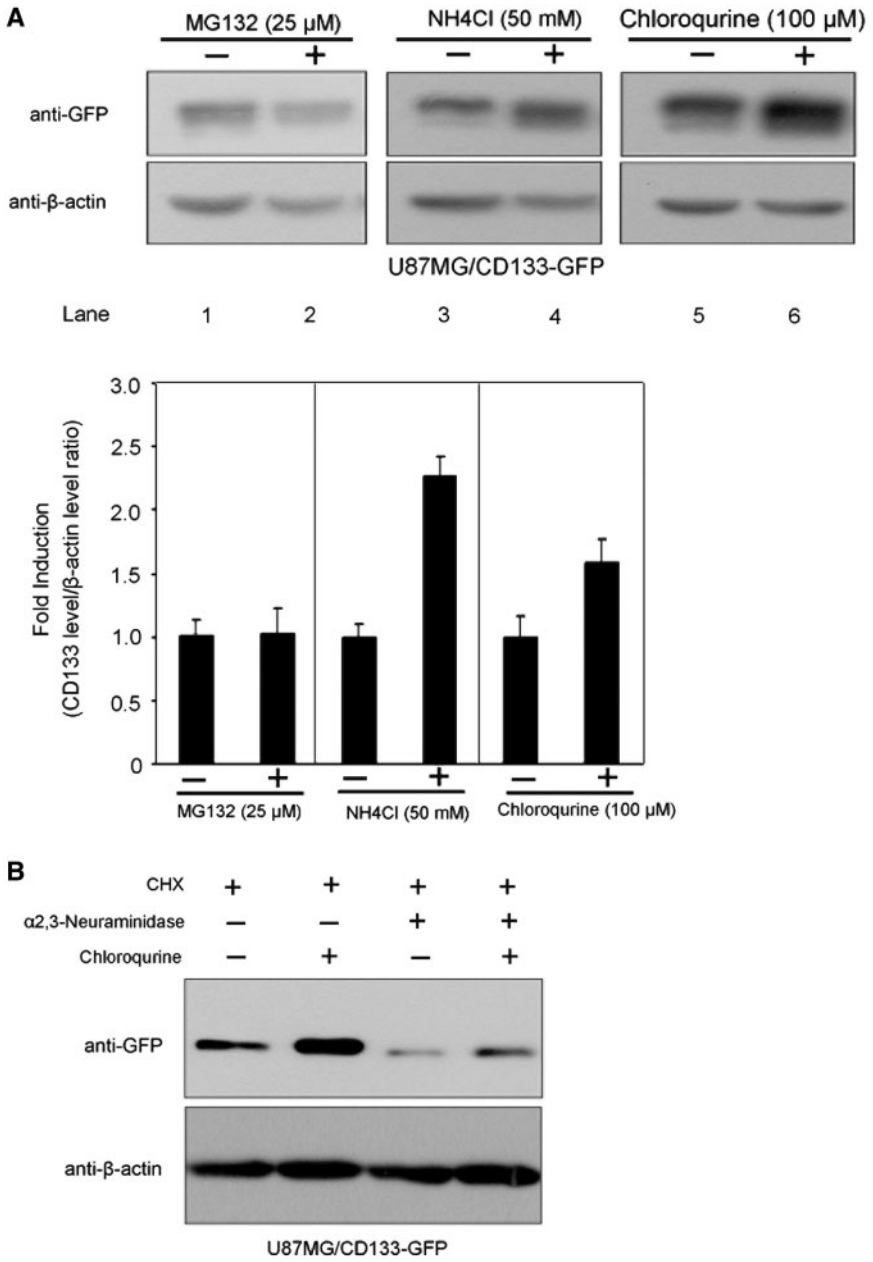

Fig. 5 CD133 is degraded by lysosomes-dependent pathway. (A) U87MG/CD133-GFP cells were incubated with vehicle (lanes 1, 3 and 5), various protease inhibitors MG132 (2 μM) (lane 2), NH4Cl (50 mM) (lane 4) or chloroquine (100 μM) (lane 6) for 24 h. Equivalent amounts of protein from each sample were immunoblotted with anti-GFP antibody and β-actin expression served as a loading control. The graph represented CD133/β-actin ratio level as measured by densitometry. The value of CD133/β-actin in U87MG/CD133-GFP cells treated with vehicle was taken as 100%. The data was shown as the means ± SD of at least three experiments. (B) U87MG/CD133-GFP cells pretreated with or without α2-3-neuraminidase to remove the terminal SAs were treated with CHX and vehicle or chloroquine (100 μM) for 12 h. Protein extracts were immunoblotted using GFP antibody. β-Actin expression served as a loading control.

Collectively, these findings indicated that α2,3-sialylation might protect CD133 from lysosomal pathway-mediated degradation.

Discussion

CD133 is a cholesterol-interacting pentaspan membrane glycoprotein specifically associated with plasma membrane protrusions (*1*, *2*). Although the biological function of CD133 remains largely unknown, CD133 has received interest due to its widespread use as a marker for the isolation and characterization of normal and CSCs, especially in brain tumours (*3*). The glycosylation status of CD133 is supposed to be associated with cell differentiation, and might play a role in the recognition of CD133 by the AC133 antibody which is frequently used to isolate normal and CSCs (*13*, *14*, *41*). Although CD133 has been identified as a N-glycosylated protein, the specific glycosylation status of CD133 remains unclear. Here, we reported that SAs could be linked to CD133 N-glycan terminal through α2,3-linkage, which in turn contributes to CD133 protein stability.

SAs are known to be ubiquitously expressed on the non-reducing ends of the sugar chains of glycoproteins and glycolipids in tissues and are known to be key determinants for a large variety of biological processes, including cell–cell communication, immune defense, tumour cell metastasis and inflammation (*31–36*). Our important finding of this study is that CD133 carries SAs that are predominantly or entirely attached to the N-linked sugar chains through α2,3-linkage, which was suggested by several evidences: (i) the molecular weight of CD133 was decreased in response to the treatment of α2-3-neuraminidase or α2-3,6,8-neuraminidase; (ii) MAA, which is specific for carbohydrate structures containing α2,3-linked SAs, was precipitated by CD133 protein and (iii) there was no obvious difference in the apparent size between CD133 protein treated with PNGase F alone and CD133 protein treated with PNGase F and α2-3-neuraminidase. Changes in the expression of specific SAs during differentiation may be useful markers for the degree of cell maturation (*42*, *43*). The alternation of CD133 molecular weight in response to neuraminidase was coincident with the decrease in molecular weight of CD133 during CSC differentiation. Taken all of these facts into account, we conclude that CD133 is an α2,3-sialylated glycoprotein, which might contribute to CD133 biological functions.

Another important finding of this study is that α2,3-sialylation might protect CD133 protein from lysosomal pathway-mediated degradation. Desialylation by α2-3-neuraminidase decreased the half-life of CD133, but had no obvious effect on the half-life of NHE1 protein. Interestingly, the negative effect of desialylation on the protein level of CD133 was inhibited by lysosomal degradation inhibitor chloroquine. Several studies support a role for sialylation in regulating the protein stability (*44*, *45*). Although these data indicated the relationship between sialylation-regulating CD133 protein stability and lysosomal pathway-mediated degradation, the mechanism of how α2,3-sialylation protects CD133 from lysosomal pathway-mediated degradation should be further investigated.

Thus, when all of the data presented here are taken in whole, it suggests that: (i) SAs could be linked to CD133 N-glycan terminal through α2,3-linkage and (ii) α2,3-sialylation might protect CD133 from lysosomal pathway-mediated degradation. Our findings may provide clues to understanding the utility of glycosylated CD133 epitopes in defining NSCs and TICs and to understanding CD133 molecular function.

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Conflict of interest

None declared.

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