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Contribution of sialic acids to integrin α5β1 functioning in melanoma cells

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ABSTRACT

Purpose: To establish the relationship between sialylation of integrin α5β1 and possible alteration in the function of α5β1 receptor in melanoma cells.

Materials and methods: Integrin α5β1 was isolated from primary WM115 (RGP/VGP-like phenotype) and metastatic WM266-4 (lymph node metastasis) cells via affinity chromatography. Integrin α5β1 sialylation and the shift in relative masses of the enzymatically desialylated subunits were confirmed by confocal microscopy and SDS-PAGE, respectively. The ELISA assay was performed to evaluate sialic acid (SA) influence on integrin α5β1 binding to fibronectin (FN). Cell invasion was investigated by the Transwell invasion assay. The effect of neuraminidases treatment on melanoma cells was assessed by flow cytometry using *Maackia amurensis* and *Sambucus nigra* lectins.

Results: Both subunits of integrin α5β1 were found to be more abundantly sialylated in primary than in metastatic cells. The removal of SA had no effect on the purified integrin α5β1 binding to FN. Although metastatic cells underwent more pronounced desialylation than primary cells, invasion of primary WM115 cells was more dependent on the presence of α2-3 linked SA than it was in the case of metastatic WM266-4 cells. In both melanoma cell lines not only integrin α5β1 was involved in invasion, however simultaneous desialylation and usage of anti-integrin α5β1 antibodies resulted in lower invasion abilities of primary WM115 cells.

Conclusions: Our data suggest that in primary melanoma cells integrin α5β1 action is more likely dependent on its glycosylation profile, i.e. the presence of SA residues, which influence (decreased) their invasion properties and may facilitate malignant melanoma progression.

1. Introduction

Sialic acids (SAs) are monosaccharides commonly found at the outermost end of the sugar chains of various glycoconjugates. SA external position on cell surface molecules gives them the opportunity to strongly influence cell behavior because SAs may interact with other cell surface molecules, extracellular matrix (ECM) proteins as well as effector molecules. There is much evidence indicating that SAs either act as a biological target that allows recognition by a receptor [1] or mask recognition sites [2,3]. SA significance has been implicated in various diseases [4–7] including cancer, where they have been reported to play an important role in the colonization and metastatic potential of cancer cells [8–14].

Malignant melanoma is the sixth most commonly diagnosed cancer in the developed countries and one of the deadliest forms of cancer [15,16]. The lack of appropriate means for predicting the prognosis and effective treatments of melanoma at the diffuse stage requires development of new strategies for melanoma therapy [17]. The progress of melanoma from transformed melanocytes to metastatic lesions is the process taking place in 5 distinct steps [18], at each stage of melanoma progression it requires the involvement of cell adhesion proteins, among which integrins are of particular importance. Many studies have examined the role of SAs in the functionality of integrin receptors and SA impact on integrin mediated interactions [19–21]. Moreover, SA potential role in melanoma progression has also been investigated [22–26]. It is evident that melanoma progression was associated with the increased expression of α2-3 linked SA on the cell surface [25]. SA linked α2-3 monosaccharides were found to be an important factor increasing melanoma cell adhesiveness to fibronectin (FN) as well as melanoma cell migration rate on FN. Similarly, the association between high expression of α2-3-linked SA residues and metastatic potential has been observed in prostate [27] and gastric cancers [28] as well as oral carcinogenesis [29]. Integrin α5β1, which is the main FN receptor, was found to be modified by both α2-3 and α2-6 linked SA residues and its expression level on melanoma cells was positively associated with the progression of melanoma [25]. The present study was undertaken to

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Table 1
Neuraminidases used for integrin $\alpha_5\beta_1$ desialylation and conditions of digestion reactions.

Neuraminidase (origin)	Supplier	Specificity	Unit/10 µl	Desialylation conditions
α(2→3) *Streptococcus pneumoniae*	Sigma-Aldrich	SAα(2→3)Gal	0.01 U	1 h, 37 °C
α(2→3,6) *Clostridium perfringens*	New England Biolabs	SAα(2→3)Gal SAα(2→6)Gal/GalNAc	20 mU	16 h, 37 °C
α(2→3,6,8) *Vibrio cholerae*	Calbiochem	SAα(2→3)Gal SAα(2→6)Gal/GalNAc SAα(2→8)SA	6 mU	16 h, 37 °C

establish the relationship between sialylation of integrin α5β1 and possible alteration in this receptor function in melanoma primary WM115 and metastatic WM266-4 cells.

2. Materials and methods

2.1. Materials

The mouse monoclonal anti-α5 integrin antibody (clone SAM-1), mouse monoclonal anti-β1 integrin antibody (clone B3B11), rabbit polyclonal antisera against α_5 integrin subunit and Immobilon P membrane, were purchased from Merck Millipore (Darmstadt, Germany). Bovine serum albumin (BSA), trypsin/EDTA solution, penicillin/streptomycin solution, octyl-β-D-glucopyranoside, Cell Dissociation Solution, goat anti-mouse AP-conjugated antibody, ExtrAvidin-FITC, FN, normal goat serum, mouse monoclonal anti-vimentin antibody (clone VIM-13.2), high molecular mass standards and protease inhibitor cocktail were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Phosphate buffered saline (PBS), RPMI 1640 medium with Glutamax-I (RPMIG) and foetal bovine serum (FBS) were from LifeTechnologies/Gibco™ (Paisley, UK). Sheep anti-rabbit AP-conjugated immunoglobulin, 4-nitroblue-tetrazolium salt (NBT), and 5-bromo-4-chloro-3-indolylphosphate (BCIP) were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Secondary anti-mouse antibody Cy3-conjugated was from Jackson ImmunoResearch. Biotinylated and unbiotinylated *Maackia amurensis* lectin (MAL-II) and *Sambucus nigra* lectin (SNA), Vectashield were from Vector Laboratories U.K. All remaining chemicals were of analytical grade, commercially available.

2.2. Cell lines and culture conditions

Two human melanoma cell lines: primary WM115 (RGP/VGP-like phenotype) [30] and metastatic WM266-4 originating from lymph node metastasis [30] were obtained from ESTDAB Melanoma Cell Bank (Tübingen, Germany). Cells were maintained in 1640 RPMIG medium supplemented with 10% FBS and antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin) and were cultured as a monolayer in a humidified incubator under standard conditions (5% CO_2 and 37 °C). The cell cultures were routinely tested for mycoplasma. After reaching the confluence, the cells were harvested adequately for the next procedures as mentioned below. The conditioned media from cell cultures were collected, and stored at −70 °C for zymography analysis.

2.3. Cell extract preparation

The cells from the confluent culture were washed, harvested and pelleted by centrifugation. Then the cells were homogenised on ice by sonification (Sonics, Vibra-Cell) in 50 mM Tris/HCl, pH 7.4, containing 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and a protease inhibitor cocktail. The addition of protamine sulphate (final concentration 0.3%) and 25 mM octyl-β-D-glucopyranoside was followed by extraction for 1 h on ice. Finally, cell extracts were clarified by centrifugation at 18,000xg, 4 °C for 1 h. Protein concentration in the supernatants was determined [31].

2.4. Isolation of integrin α5β1 by affinity chromatography

Integrin α5β1 was isolated via affinity chromatography on the specific peptide-CH-Sepharose 4B column. Lipopharm (Zblewo, Poland) synthesized the specific peptide sequence: Ac-Gly-Ala-c-(Cys^{SS}-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys^{SS})-Gly-Ala-O-$(CH_2CH_2O)_2CH_2CH_2$-NH_2, according to Wobbe et al. [32]. The affinity column was prepared with the use of 2.6 g of CH-Sepharose 4B resin combined with 20 mg of a specific peptide. Prior to the homogenate separation the column was pre-equilibrated with 10 volumes of the extraction buffer (50 mM Tris-HCl, pH 7.4, 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$). The clarified cell lysate (2.5 mg of total protein) was passed over the column with 5 ml of resin in the extraction buffer with the addition of 25 mM octyl-β-D-glucopyranoside and allowed to interact with the immobilised ligand overnight at 4 °C. After that the column was extensively washed with the extraction buffer, and then the bound material was eluted with the extraction buffer lacking cations, but containing additionally 20 mM EDTA and 25 mM octyl-β-D-glucopyranoside. The column was subsequently eluted with 1 M NaCl to isolate any remaining bound proteins. Fractions eluted with EDTA and NaCl were dialysed against MiliQ water, and concentrated by lyophilisation.

2.5. Enzymatic desialylation of integrin α5β1and melanoma cells

The terminal SAs were released from the glycoproteins by specific neuraminidases (Table 1). 30 µg of purified integrin α5β1 was dried (SpeedVac Plus, Savant), resolved in 10 µl of the adequate buffer (according to the manufacturer) and incubated with specific neuraminidases (see Table 1). Next the samples were dried once again and after resolving they were split into two portions: for electrophoretic analysis and for the ELISA test. The enzymatic desialylation of cells was performed according to Markwell et al. [33] with minor modifications. After reaching 70% confluence the cells were harvested with Cell Dissociation Solution and centrifuged at 410xg, 4 °C, 5 min. 8.75×10^4 cells were resuspended in 0.5 ml of the culture medium supplemented with 10% FBS and specific neuraminidase, and incubated under conditions according to the manufacturer (details in Table 2). At the end of incubation time the cells were centrifuged, washed with a fresh culture medium and centrifuged again in order to remove residual neuraminidase. Then the cells were resuspended in a fresh culture medium and used for further experiments.

2.6. SDS-PAGE and immunodetection of integrin subunits

Cell lysates (10 µg) and the samples containing purified integrin α5β1 (wash fraction – 20 µg, elution fraction – 5 µg) were separated by 8% SDS-PAGE under non-reducing conditions. In the case of

Table 2
Neuraminidases used for cell desialylation and conditions of enzymatic reactions.

Neuraminidase (origin)	Units/0.5 ml	Desialylation conditions
α(2→3) *Streptococcus pneumoniae*	3 mU	4 h, 37 °C
α(2→3,6) *Clostridium perfringens*	12 U	1 h, 37 °C
α(2→3,6,8) *Vibrio cholerae*	27 mU	6 h, 37 °C

desialylation experiments 5 μg of purified (control) as well as desialylated (neuraminidase treated samples) integrin α5β1 were separated. Next, the proteins were transferred onto a PVDF membrane and tested with the use of specific antibodies against α_5 (rabbit polyclonal serum) and β_1 (mouse monoclonal antibody, clone B3B11) integrin subunits (1:2000 working dilution for both α_5 and β_1 subunits). The secondary, sheep anti-rabbit IgG (for α5 integrin subunits, 1:250 working dilution) or goat anti-mouse IgG (for β1 integrin subunit, 1:500 working dilution), both conjugated with AP, were used. The substrates for AP: BCIP and NBT were used for the colorimetric visualisation of specific proteins. The obtained results were archived with the use of ScionImage Software and then molecular masses of the observed protein bands were assigned with the use UVmap V.99 software.

2.7. ELISA test - binding of integrin α5β1 to FN

The ELISA assay was performed as described previously by Pocheć et al. [34] with modifications. Briefly, 3 μg of the material purified from affinity chromatography (neuraminidase-treated (see Table 1) or non-treated) was added into microtiter wells coated with FN (BD, Fibronectin 96 Well Cell Adhesion Assay) in 50 μl of the ELISA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mM octyl-β-D-glucopyranoside with the addition of 1% BSA), or in the same buffer, but lacking metal cations. The plate was incubated for 2 h at 37 °C, and then washed three times with 200 μl of the ELISA buffer. After that the material bound to FN was incubated for 1 h at 37 °C with rabbit polyclonal anti-α5 integrin subunit antibody in the ELISA buffer (working dilution 1:1000), and the secondary anti-rabbit antibody AP-conjugated (working dilution 1:500). Finally, the ELISA was developed with 10 mM pNpp (overnight incubation) and absorbance was measured at 405 nm. Values for non-specific binding, measured in the presence of 10 mM EDTA were subtracted from total binding to give values for specific binding of integrin α5β1 to immobilized ligands.

2.8. Confocal microscopy

The melanoma cells were seeded at the density of 5×10^4 cells/ml on coverslips coated with FN (5 μg/ml) overnight at 4 °C.

2.8.1. Staining for SNA and MAA positive oligosaccharides

Before staining, the cells were cultured overnight in the presence of biotinylated SNA (specific for α2-6 linked sialic acids) and MAL-II (specific for α2-3 linked sialic acids). Then the cells were washed three times with PBS, fixed in 2% PFA for 20 min at 37 °C, and incubated for 2 h with StreptAvidine-FITC in 2% BSA in PBS. At the end the slides were washed five times in PBS and one time in MiliQ water and mounted in Vectashield with DAPI.

2.8.2. Staining for integrin α5 or β1 subunits

Coverslips with cells were washed three times in PBS and incubated for 24 h with monoclonal antibodies against integrin α5 (clone SAM-1) or β1 subunit, both in 1:100 dilution in 2% BSA in PBS. Next, the cells were fixed in 2% PFA for 20 min at 37 °C, and blocked in 10% normal goat serum. After triple washing the Cy3-conjugated goat anti-mouse antibody (1:500 in 2% BSA in PBS) was added for 2 h, RT. Finally, the slides were washed five times in PBS and one time in MiliQ water, and mounted with Vectashield with DAPI.

2.8.3. Double-staining for SNA or MAA positive oligosaccharides and integrin α5 or β1 subunit

The cells were washed three times in PBS and incubated with specific biotinylated lectins, followed by the incubation with the StreptAvidine-FITC (as described above). Then the monoclonal antibodies against integrin α5 or β1 subunits were added and incubation was conducted (see above). After washing, the coverslips were mounted with Vectashield with DAPI, sealed and kept at 4 °C. In all cases cell fluorescence was observed with the use of a confocal microscope with the same setting conditions (Zeiss LSM 510 META, Carl Zeiss MicroImaging GmbH, Jena, Germany) and analyzed in LSM Image Browser.

2.9. Matrigel invasion test

Invasion assays were performed using BD BioCoat™ 96-Multiwell Tumor Invasion System (BD Bioscience) according to the supplier's instructions. The cells in the culture with 70% confluence were maintained for 24 h in FBS-free 1640 RPMIG and then they were stained with 1 μg/ml of fluorescent dye $DiIC_{12}(3)$ (BD Bioscience) in the same medium for 1 h in a culture incubator. The following steps were carried out avoiding direct light. Melanoma cells were harvested by Cell Dissociation Solution, counted, and 12,500 cells were seeded in the upper chamber in 1640 RPMIG supplemented with 10% FBS. 200 μl of phenol red-free 1640 RPMI supplemented with 25 μg/ml of FN were placed in the lower chambers and served as a chemoattractant. The assays were conducted for 24 h at 37 °C in a humidified CO_2 incubator. Fluorescence of the invaded cells was measured at 535/560 nm (excitation/emission) in a microplate reader with bottom excitation and reading mode (Bio-Tek). The experiments were performed in triplicate.

In some experiments cells were previously desialylated with SAα(2–3,6,8)Gal specific neuraminidase (as described above) or were pre-incubated with anti-integrin α5β1 antibody (final concentration 2 μl/ml).

2.10. Statistics

The significance of the differences between mean values was computed using one-way ANOVA with Post-hoc Tukey test. P values lower than 0.05 were considered significant.

2.11. Ethical issues

Ethical approval was not necessary for this study.

3. Results

3.1. Integrin α5β1 is abundantly and variously sialylated in melanoma cells

To determine the amount and type of the chemical bond of SA residues attached to glycans on integrin α5β1, we isolated this protein from two melanoma cell lines by affinity chromatography on the specific peptide (Ac-Gly-Ala-c-(Cys^{SS}-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys^{SS})-Gly-Ala-O-$(CH_2CH_2O)_2CH_2CH_2$-NH_2) bound to CH-Sepharose 4B column. The presence of integrin α5β1 in the eluted material was confirmed by SDS-PAGE under non-reducing conditions, followed by Western blotting with specific antibodies (Fig. 1). Next, the purified protein was desialylated with the use of specific sialidases, trimming the α2–3, α2–3,6 or α2–3,6,8 SA residues. Consequently, the shifts in relative molecular masses were observed in SDS-PAGE separation followed by Western blotting. The molecular masses of sialidases untreated integrin subunits in primary WM115 and metastatic WM266-4 were as follows: α5 subunit – 159 kDa and 160.6 kDa; β1 subunit – 140 kDa and 148.3 kDa, respectively. In all cases sialidases digestion caused the reduction of relative molecular masses of all analyzed integrin subunits (Table 3 and Fig. 2), however both integrin α5β1 subunits seemed to be more abundantly sialylated in primary WM115 cells than in metastatic WM266-4 cells. Integrin α5β1 subunit sialylation was further confirmed by merged immunostaining of integrin subunits and SNA or MAA lectin staining in confocal microscopy (Fig. 3).

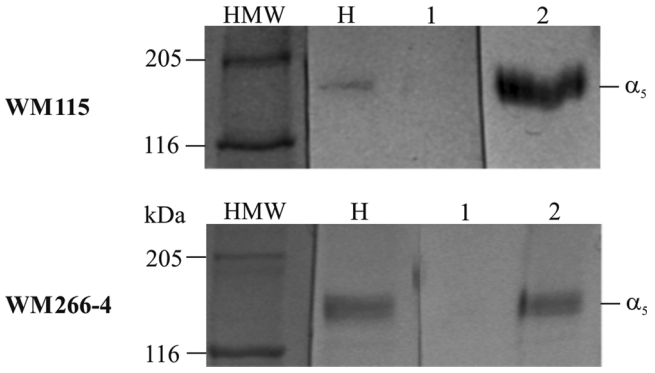

Fig. 1. Immunodetection of α5 integrin subunit in the material obtained after affinity chromatography. Material eluted on the specific peptide-CH-Sepharose4B column was separated by 8% SDS-PAGE under non-reducing conditions, transferred onto PVDF membrane and probed with specific antibodies against α5 integrin subunit. H – homogenate, 1 – wash fraction, 2 – elution fraction.

Table 3
Molecular masses of untreated and desialylated integrin α5β1 subunits. The shifts in molecular masses of integrin subunit digested with the use of specific SAα(2→3)Gal, SAα(2→6)Gal/GalNAc and SAα(2→8)SA sialidases were observed. Molecular masses of the observed bands were assigned with the use of UVmap V.99 software. Average results of two separate experiments are presented.

	integrin subunit	Control [kDa]	α(2-3) [kDa] (% of control)	α(2-3,6) [kDa] (% of control)	α(2-3,6,8) [kDa] (% of control)
WM115	**α5**	159	148	137	132
			(93)	**(86)**	**(83)**
	β1	140	134	129	124
			(96)	**(92)**	**(89)**
WM266-4	**α5**	161	159	145	143
			(99)	**(90)**	**(89)**
	β1	148	145	142	142
			(98)	**(96)**	**(96)**

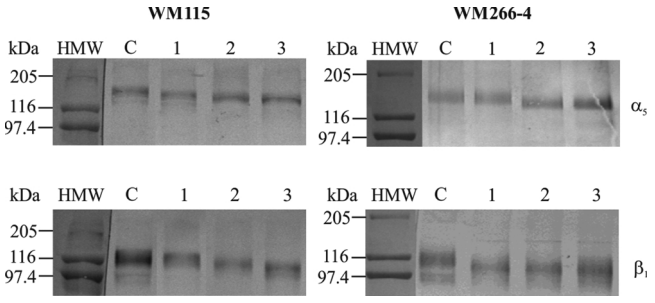

Fig. 2. Desialylation of α5β1 integrin subunits isolated from primary WM115 and metastatic WM266-4 cell lines. Sialic acid residues of α5β1 integrin were removed enzymatically with the use of specific sialidases. Western blot analysis showed the corresponding shift in molecular mass of integrin subunits. C – control, 1 – treated with neuraminidase α(2→3) (from *Streptococcus pneumonia*), 2 – treated with neuraminidase α(2→3,6) (from *Clostridium perfringens*), 3 – treated with neuraminidase α(2→3,6,8) (from *Vibrio cholera*).

3.2. SA linked to integrin α5β1 have no impact on the receptor interaction with FN

In order to assess the role of SA in integrin α5β1 interaction with FN, desialylated proteins purified by affinity chromatography from primary WM115 and metastatic WM266-4 cells were tested in the ELISA assay (Fig. 4). In most cases removal of specific SA residues did not affect integrin α5β1 binding to FN. There was nearly 30% stronger binding of integrin α5β1 to FN after deletion of α2-3 bound SA residues in primary WM115 cells, but the result was not statistically significant.

3.3. Melanoma cell invasion abilities depend rather on SA presence than integrin α5β1 action

In order to analyze the role of SA residues in melanoma cells invasion, primary WM155 and metastatic WM266-4 cells were desialylated prior to the Matrigel invasion assay. The yield of cell desialylation was confirmed by flow cytometry analysis after SNA and/or MAA staining (data not shown). The invasion assay revealed that invasion of primary WM115 cells was more dependent on the presence of SA than the invasion of metastatic WM266-4 cells (Fig. 5). In primary WM115 cells the use of α2–3,6 and α2–3,6,8 neuraminidases for desialylation resulted in a lower invasion potential, which was statistically significant. Surprisingly, in metastatic WM266-4 cells α2-3 and α2–3,6 neuraminidases specific treatment did not change or slightly decreased the invasion potential. For understanding the role of integrin α5β1 in invasion process, the specific anti-integrin α5β1 antibodies were used, however, no significant changes were observed in the invasion potential in both melanoma cell lines. Additionally, only in the case of primary WM115 cells simultaneous cell desialylation and the use of anti-integrin α5β1 antibodies resulted in lower invasion abilities of the tested cells. This effect was not observed in metastatic WM266-4 cells which was in line with the above mentioned lack of response in invasion assay of metastatic WM266-4 cells to sialic acids blockage through the use of specific lectins.

4. Discussion

Numerous studies have noted that in cancer the relative amount of sialylated glycoconjugates increases in the context of transformation and malignancy. In general, increased sialylation is known to promote tumor growth, facilitate the formation of metastasis, apoptosis evasion as well as resistance to cancer therapy [11,29,35–38]. In our previous studies on melanoma cell lines of a different origin we demonstrated that the elevated level of α2-3 sialylation was associated with a more aggressive phenotype of cancer [24,25]. In line with our results is the report showing that in B16 murine melanoma cell line and its sub-lines of a different metastatic potential, α2-3 linked SAs were predominantly found in a high metastatic sub-line, while α2-6 linked SAs were observed in a low-metastatic sub-line [22]. Our previous results also indicated that α2-3 linked SAs appeared to be a more important factor increasing melanoma cell adhesion to FN as well as their migration potential than α2-6 linked SAs [25]. In the present study we demonstrated that the invasion potential of primary WM115 and metastatic WM266-4 cells was to some extent dependent on the presence of SA residues. Surprisingly, SA dependence was more apparent in the case of primary WM115 cells, which expressed significantly lower amount of SA on their cell surface than metastatic WM266-4 cells did, as confirmed in the cells desialylation experiments. The primary WM115 melanoma cells showed significant invasion inhibition in the response to α2–3,6 and α2–3,6,8 linked SA cleavage, while metastatic WM266-4 melanoma cells showed the pronounced effect only in the case of 2–3,6,8 linked SA residues cleavage. The above described findings are in contrast with the results of other studies showing the association of α2-3 linked SA expression with a higher invasive and metastatic potential of gastric, colon, breast, skin and lung cancer cells [28,29,39–42].

A central role in malignancy is played by integrins, which are αβ heterodimeric transmembrane glycoproteins that perform structural roles [43,44], bidirectionally relay signals across the cell membrane [45] and participate in the growth factor receptor signaling pathways [46]. Several studies have reported the control of integrin activation by its glycosylation status, which in turn modulates cell adhesion to ECM proteins (i.e. FN) as well as has the impact on cancer cell invasion and

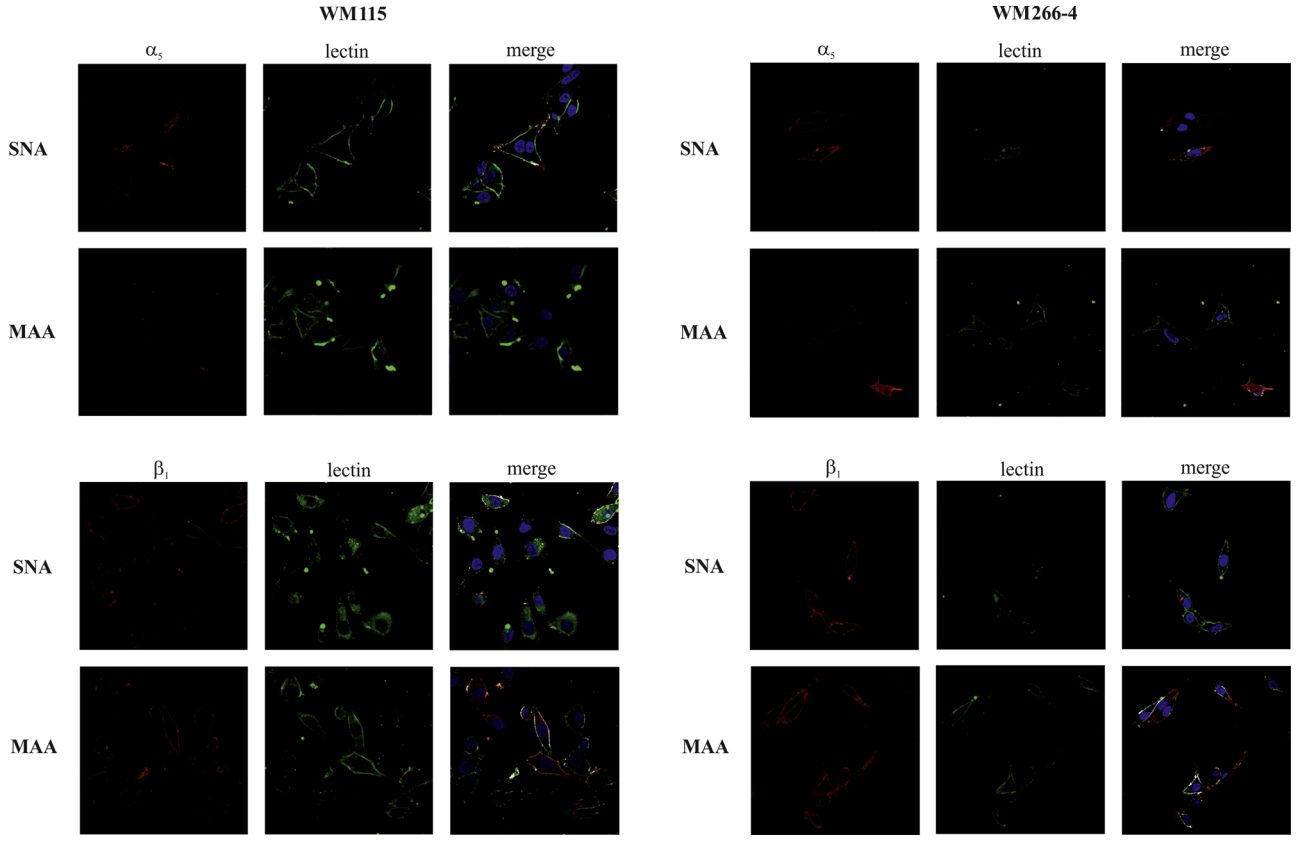

Fig. 3. Confocal microscopy analysis of sialic acids presence and α5β1 integrin expression in primary WM115 and metastatic WM266-4 melanoma cells. The localization and then the colocalization of SNA- or MAA-positive sialic acid structures and α5β1 integrin subunits were shown using specific lectins and antibodies.

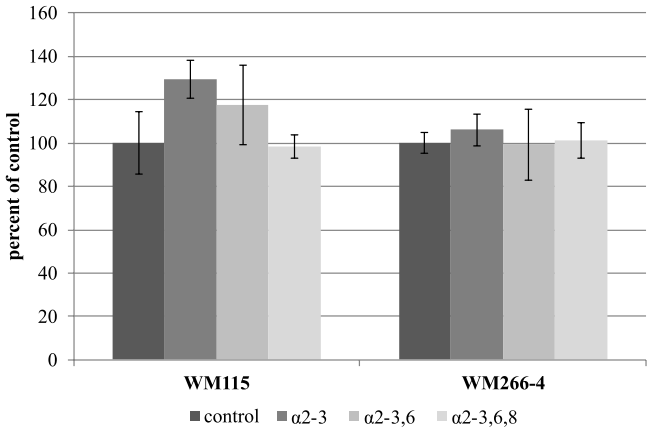

Fig. 4. Ligand binding specificity of purified neuraminidases-treated α5β1 integrin from primary WM115 and metastatic WM266-4 cells. α5β1 integrin after purification via affinity chromatography was treated with specific neuraminidases (see Table 1). Integrin α5β1 binding to FN was quantified using the modified ELISA test as described in 'Materials and methods' section. $P < 0.05$.

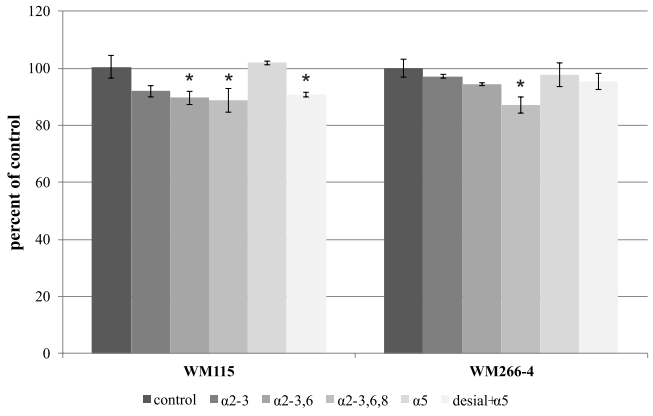

Fig. 5. Invasiveness of a desialylated and/or anti-α5β1 integrin antibody-treated melanoma cell. Primary WM115 and metastatic WM266-4 cells were desialylated with specific sialidases (see Table 1) and in some experiments subsequently treated with anti-α5β1 integrin antibody. The graph represents the percentage of desialylated and/or anti-α5β1 integrin antibody-treated invading cells relative to non-treated cells. Presented values are means ± standard deviation. * indicates $P < 0.05$.

metastasis [21,47,48]. It is noteworthy that the most variable properties of integrin glycans are β1-6 branching and sialylation [35]. It is possible that in the case of melanoma, β1-6 branched complex type *N*-glycans contribute to tumor invasion and metastasis more effectively than SA residues alone [26].

Integrin α5β1, the major FN receptor, is thought to play a fundamental role in a great number of cellular responses, including cell proliferation, survival, adhesion and migration, angiogenesis through interaction with FN and cross-talk between different cellular signaling pathways [21]. Although there are controversial reports concerning the role of integrin α5β1 in cancer [49–51], its overexpression was proved to positively correlate with melanoma metastasis [52]. In concordance with the fundamental role of integrin α5β1 in a great number of cellular responses are our previous results, where we have demonstrated that the level of integrin α5β1 expression on primary WM115 (RGP/VGP-like phenotype) was lower (25%) than on metastatic WM266-4 cells originating from lymph node metastasis (75%) [25]. Recently, it has been shown that a key factor regulating signal-competent and signal-incompetent integrin α5β1 conformational states is the *N*-glycosylation site position [53]. Integrin α5β1 within the polypeptide chains of α5

and β1 subunits has 15 and 14 potential *N*-glycosylations, respectively, and among them 10 (α5) and 8 (β1) are recognized as *N*-glycosylation sites, respectively. Additionally, both integrin subunits possess 13 potential *O*-glycosylation sites [21]. It was proved that *N*-glycosylation on the β-propeller domain on integrin α5 subunit functions as a key regulator of cell migration (putative sites 1–2) [54] and as a negative regulator of epidermal growth factor receptor and its cellular signaling of cell proliferation (putative sites 10–14) [55,56]. Other studies have indicated that alteration in the *N*-glycan portion of integrin α5β1 resulting from the overexpression of *N*-acetylglucosaminyltransferase V, *N*-acetylglucosaminyltransferase III and α2-3 galactoside transferase 1 played an important role in cell spreading and migration of FN [21,57,58].

Previously we demonstrated that the motility of primary WM115 and metastatic WM266-4 cells relied on the presence of α2-3 linked SA [25]. In the present study we proved that melanoma cell invasion did not involve integrin α5β1 action, however simultaneous desialylation and usage of anti-integrin α5β1 antibodies resulted in lower invasion abilities of primary WM115 cell in the comparison to untreated cells. This effect was not observed in WM266-4 cells that was in line with the above mentioned results of the Western blot analysis of purified and desialylated integrin α5 and β1 subunits. The results of our present study demonstrate that integrin α5β1 was abundantly, but variously sialylated in melanoma cells. Based on relative molecular weight loss of integrin α5β1 subunits after neuraminidase treatment, it could be stated that in both melanoma cell lines integrin α5 subunit possessed more sialylated N-glycans than integrin β1 subunit. In addition, the amount of α2-3 and α2-6 linked SA on integrin α5 subunit was comparable in primary WM115 melanoma cells, whereas α2-6 linked SA were found predominantly on integrin α5 subunit in metastatic WM266-4 melanoma cells. Several lines of evidence indicated the importance of integrin α5β1 sialylation in tumor dissemination. It was shown that hyposialylation or desialylation of the β1 integrin subunit enhanced the adhesion to FN and modified cell migration and metastatic potential [59,60]. Moreover, the elevated level of α2-6 linked SA on the α5 integrin subunit in highly metastatic mouse hepatocarcinoma cells increased integrin α5β1-dependent cell adhesion to FN and facilitated FN-mediated phosphorylation of FAK [48]. On the other hand, α2–3,6 sialylation on the integrin α5 subunit caused the increase in FN-induced cell motility by abolished integrin α5β1 interaction with the metastasis suppressor CD82 [61]. In turn, the presence of α2-8 linked SA on the integrin α5 subunit of human G361 melanoma cells was crucial for integrin α5β1-dependent cell adhesion to FN and it was believed that these oligosialic acids stabilize integrin α5β1 conformation in a form exhibiting high affinity to the ligand [62]. In our present study we did not observe any similar effects. Purified and desialylated integrin α5β1 adhesion to FN was not dependent on α2–3, α2–3,6 or α2–3,6,8 sialylation both in primary WM115 and metastatic WM266-4 cells. Therefore it was suggested that sialylation of α5β1 integrin did not interfere with FN binding capacity, but might influence α5β1 integrin participation in the cellular invasion process.

5. Conclusions

Our results indicated that integrin α5β1 subunits were more abundantly sialylated in primary than in metastatic melanoma cells with the higher amount of α2-3 linked SA in primary WM115 melanoma cells. In both analyzed cell lines SAs were not involved in integrin α5β1 binding to FN, nevertheless, the invasion of primary WM115 cells was SA-dependent. Moreover, integrin α5β1 was not involved in the invasion both in WM115 and WM266-4 cells, however simultaneous desialylation and the use of anti-integrin α5β1 antibodies resulted in lower invasion abilities of WM115 cells. Taken together, our findings demonstrated that primary rather than metastatic melanoma cells regulate their cellular activity i.e. invasion, by changes in the glycosylation profile of adhesion receptors, including α5β1 integrin. It should be considered that α2-6 sialylation may play different roles in the regulation of cell adhesion in various cancer cells when developing potential therapeutics targeting α2-6 sialylation [63].

Conflict of interest

The authors declare no conflict of interests.

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References

[1] Varki A. Glycan-based interaction involving vertebrate sialic-acid-recognizing proteins. Nature 2007;446:1023–9.
[2] Croci DO, Rabinovich GA. Linking tumor hypoxia with VEGFR2 signaling and compensatory angiogenesis: Glycans make the difference. Oncoimmunology 2014;25(3):e29380. https://doi.org/10.4161/onci.29380.
[3] Croci DO, Cerliani JP, Dalotto-Moreno T, Méndez-Huergo SP, Mascanfroni ID, Dergan-Dylon S, et al. Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. Cell 2014;156(4):744–58.
[4] Nigam PK, Narain VS, Kumar A. Sialic acid in cardiovascular diseases. Indian J Clin Biochem 2006;21(1):54–61.
[5] Wielgat P, Braszko JJ. Significance of the cell adhesion molecules and sialic acid in neurodegeneration. Adv Med Sci 2012;57(1):23–30.
[6] Gińdzieńska-Sieśkiewicz E, Radziejewska I, Domysławska I, Klimiuk PA, Sulik A, Rojewska J, et al. Changes of glycosylation of IgG in rheumatoid arthritis patients treated with methotrexate. Adv Med Sci 2016;61(2):193–7.
[7] Mahajan VS, Pillai S. Sialic acids and autoimmune disease. Immunol Rev 2016;269(1):145–61.
[8] Julien S, Adriaenssens E, Ottenberg K, Furlan A, Courtand G, Vercoutter-Edouart AS, et al. ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology 2006;16:54–64.
[9] Varki NM, Varki A. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest 2007;87:851–7.
[10] Bauer J, Osborn HM. Sialic acids in biological and therapeutic processes: opportunities and challenges. Future Med Chem 2015;7:2285–99.
[11] Lu J, Gu J. Significance of β-Galactoside α2,6 Sialyltranferase 1 in cancers. Molecules 2015;20:7509–27.
[12] Cagnoni AJ, Pérez Sáez JM, Rabinovich GA, Mariño KV. Turning-off signaling by siglecs, selectins, and galectins: chemical inhibition of glycan-dependent interactions in cancer. Front Oncol 2016;6:109.
[13] Pearce OM, Läubli H. Sialic acids in cancer biology and immunity. Glycobiology 2016;26:111–28.
[14] Vajaria BN, Patel KR, Begum R, Patel PS. Sialylation: an avenue to target cancer cells. Pathol Oncol Res 2016;22:443–7.
[15] Garbe C, Leiter U. Melanoma epidemiology and trends. Clin Dermatol 2009;27:3–9.
[16] Erdmann F, Lortet-Tieulent J, Schüz J, Zeeb H, Greinert R, Breitbart EW, et al. International trends in the incidence of malignant melanoma 1953-2008 - are recent generations at higher or lower risk? Int J Cancer 2013;132:385–400.
[17] Stadler S, Weina K, Gebhardt C, Utikal J. New therapeutic options for advanced non-resectable malignant melanoma. Adv Med Sci 2015;60(1):83–8.
[18] Li G, Herlyn M. Dynamics of intercellular communication during melanoma development. Mol Med Today 2000;6:163–9.
[19] Link-Lenczowski P, Lityńska A. Glycans in melanoma screening. Part 2. Towards the understanding of integrin N-glycosylation in melanoma. Biochem Soc Trans 2011;39:374–7.
[20] Pocheć E, Lityńska A. Glycosylation of integrins in melanoma progression. In: Blumenberg M, editor. Human skin cancer, potential biomarkers and therapeutic targets Rijeka: InTech; 2016. https://doi.org/10.5772/64287.
[21] Hoja-Łukowicz D, Przybyło M, Duda M, Pocheć E, Bubka M. On the trail of the glycan codes stored in cancer-related cell adhesion proteins. Biochim Biophys Acta 2017;1861:3237–57.
[22] Passaniti A, Hart GW. Cell surface sialylation and tumor metastasis. Metastatic

potential of B16 melanoma variants correlates with their relative number of specific penultimate oligosaccharide structures. J Biol Chem 1988;263:7591–603.
[23] Gorelik E, Duty L, Anakari F, Galili U. Alterations of cell surface carbohydrates and inhibition of metastatic property of murine melanomas by alpha 1,3 galactosyl-transferasegene expression. Melanoma Res 1995;55:4168–73.
[24] Lityńska A, Przybyło M, Pocheć E, Hoja-Łukowicz D, Ciołczyk D, Laidler P, et al. Comparison of the lectin-binding pattern in different human melanoma cell lines. Melanoma Res 2001;11:205–12.
[25] Kolasińska E, Przybyło M, Janik M, Lityńska A. Towards understanding the role of sialylation in melanoma progression. Acta Biochim Pol 2016;63:533–41.
[26] Przybyło M, Janik ME, Hoja-Łukowicz D. Bitter sweetness of malignant melanoma: deciphering the role of cell surface glycosylation in tumour progression and metastasis. In: Blumenberg M, editor. Human skin cancer, potential biomarkers and therapeutic targets Rijeka: InTech; 2016. https://doi.org/10.5772/64406.
[27] Saldova R, Fan Y, Fitzpatrick JM, Watson RW, Rudd PM. Core fucosylation and alpha2-3 sialylation in serum N-glycome is significantly increased in prostate cancer comparing to benign prostate hyperplasia. Glycobiology 2011;21:195–205.
[28] Wang FL, Cui SX, Sun LP, Qu XJ, Xie YY, Zhou L, et al. High expression of alpha 2, 3-linked sialic acid residues is associated with the metastatic potential of human gastric cancer. Cancer Detect Prev 2009;32:437–43.
[29] Shah MH, Telang SD, Shah PM, Patel PS. Tissue and serum alpha 2-3- and alpha 2-6-linkage specific sialylation changes in oral carcinogenesis. Glycoconj J 2008;25:279–90.
[30] Westermark B, Johnsson A, Betsholtz C, Heldin CH, Herlyn M, Rodeck U, et al. Human melanoma cell lines of primary and metastatic origin express the gene encoding the chains of platelet-derived growth factor (PDGF) and produce PDGF-like growth factor. Proc Natl Acad Sci USA 1986;83:7197–200.
[31] Peterson GL. A simplification of the protein assay method of Lowry et al. Which is more generally applicable. Anal Biochem 1977;83:346–56.
[32] Wobbe L, Zimmermann D, Wißbrock M, Urman S, Sewald K, Malešević M, et al. Integrin alpha 5 beta 1: a new purification strategy based on immobilised peptides. J Peptide Res 2006;66:22–9.
[33] Markwell MA, Paulson JC. Sendai virus utilizes specific sialyloligosaccharides as host cell receptor determinants. Proc Natl Acad Sci U S A 1980;77(10):5693–7.
[34] Pocheć E, Lityńska A, Amoresano A, Casbarra A. Glycosylation profile of integrin alpha 3 beta 1 changes with melanoma progression. Biochim Biophys Acta 2003;1643:113–23.
[35] Bellis SL. Variant glycosylation: an underappreciated regulatory mechanism for β1 integrins. Biochim Biophys Acta Biomembr 2004;1663:52–60.
[36] Schultz MJ, Swindal AF, Bellis SL. Regulation of the metastatic cell phenotype by sialylated glycans. Cancer Metastasis Rev 2012;31:501–18.
[37] Büll C, Stoel MA, den Brok MH, Adema GJ. Sialic acids sweeten a tumour's life. Cancer Res 2014;74:3199–204.
[38] Dall'Olio F, Malagolini N, Trinchera M, Chiricolo M. Sialosignaling: sialyltransferasea as engines of self-fueling loops in cancer progression. Biochim Biophys Acta 2014;1840:2752–64.
[39] Gomes C, Osorio H, Pinto MT, Campos D, Oliveira MJ, Reis CA. Expression of ST3GAL4 leads to SL(x) expression and induces c-Met activation and invasive phenotype in gastric carcinoma cells. PLoS One 2013;8:e66737.
[40] Cui H, Lin Y, Yue L, Zhao X, Liu J. Differential expression of the α2,3-sialic acid residues in breast cancer is associated with metastatic potential. Oncol Rep 2011;25:1365–71.
[41] Chen JY, Tang YA, Huang SM, Juan HF, Wu LW, Sun YC, et al. A novel sialyltransferase inhibitor suppresses FAK/paxillin signalling and cancer angiogenesis and metastatic pathways. Cancer Res 2011;71:473–83.
[42] Chovanec M, Plzak J, Betka J, Brabec J, Kodet R, Smetana Jr K. Comparative analysis of α2,3/2,6-linked N-acetyneuraminic acid and cytokeratin expression in head and neck squamous cell carcinoma. Oncol Rep 2004;12:297–301.
[43] Anderson LR, Owens TW, Naylor MJ. Structural and mechanical function of integrins. Biophys Res 2014;6:203–13.
[44] Pan L, Zhao Y, Yuan Z, Qin G. Research advances on structure and biological functions of integrins. Springerplus 2016;5:1094.
[45] Streuli CH, Akhtar N. Signal co-operation between integrins and other receptor systems. Biochem J 2009;418:491–506.
[46] Alam N, Goel HL, Zarif MJ, Butterfield JE, Perkins HM, Sansoucy BG, et al. The integrin-growth factor receptor duet. J Cell Physiol 2007;213:649–53.
[47] Gu J, Taniguchi N. Regulation of integrin functions by N-glycans. Glycoconj J 2004;21:9–15.
[48] Yu S, Fan J, Liu L, Zhang L, Wang S, Zhang J. Caveolin-1 up-regulates integrin α2,6-sialylation to promote integrin α5β1-dependent hepatocarcinoma cell adhesion. FEBS Lett 2013;587:782–7.
[49] Zhou GF, Ye F, Cao LH, Zha XL. Over expression of integrin α5β1 in human hepatocellular carcinoma cell line suppress cell proliferation in vitro and tumorigenicity in nude mice. Mol Cell Biochem 2000;207:49–55.
[50] Samandari E, Visarius T, Zingg MJ, Azzi A. The effect of γ-tocopherol on proliferation, integrin expression, adhesion, and migration of human glioma cells. Biochem Biophys Res Commun 2006;342:1329–33.
[51] Wang Y, Shenouda S, Barawal S, Rathinam R, Jain P, Bao L, et al. Integrin subunit α5 and α6 regulate cell cycle by modulating the chk1 and Rb/E2F to affect breast cancer metastasis. Mol Cancer 2011;10:84.
[52] Quian F, Zhang ZC, Wu XF, Li YP, Xu Q. Interaction between integrin α5 and fibronectin is required for metastasis of B16F10 melanoma cells. Biochem Biophys Res Commun 2005;333:1269–75.
[53] Li J, Su Y, Xia W, Qin Y, Humphries MJ, Vestweber D, et al. Conformational equilibria and intrinsic affinities define integrin activation. EMBO J 2017;36:629–45.
[54] Hang Q, Isaji T, Hou S, Wang Y, Fukuda T, Gu J. A key regulator of cell adhesion: identification and characterization of important *N*-glycosylation sites on integrin α5 for cell migration. Mol Cell Biol 2017;37(9):e00558–16.
[55] Hang Q, Isaji T, Hou S, Im S, Fukuda T, Gu J. Integrin α5 supresses the phosphorylation of epidermal growth factor receptor and its cellular signaling of cell proliferation *via N*-glycosylation. J Biol Chem 2015;200:29345–60.
[56] Hang Q, Isaji T, Hou S, Zhou Y, Fukuda T, Gu J. N-glycosylation of integrin α5 acts as a swich for EGFR-mediated complex formation of integrin α5β1 and α6β4. Sci Rep 2016;6:33507.
[57] Janik ME, Litynska A, Vereecken P. Cell migration-the role of integrin glycosylation. Biochim Biophys Acta 2010;1800:545–55.
[58] Takahashi M, Kizuka Y, Ohtsubo K, Gu J, Taniguchi N. Disease-associated glycans on cell surface proteins. Mol Asp Med 2016;51:56–70.
[59] Pretzlaff RK, Xue VW, Rowin ME. Sialidase treatment exposes the beta1-integrin active ligand binding site on HL60 cells and increases binding to fibronectin. Cell Adhes Commun 2000;7:491–500.
[60] Semel AC, Seales EC, Singhal A, Eklund EA, Colley KJ, Bellis SL. Hyposialylation of integrins stimulates the activity of myeloid fibronectin receptors. J Biol Chem 2002;277:32830–6.
[61] Ono M, Handa K, Withers DA, Hakomori S. Glycosylation effect on membrane domain (GEM) involved in cell adhesion and motility: a preliminary note on functional α3, α5-CD82 glycosylation complex in ldlD 14 cells. Biochem Biophys Res Commun 2000;279:744–50.
[62] Nadanaka S, Sato C, Kitajima K, Katagiri K, Irie S, Yamagata T. Occurrence of oligosialic acids on integrin α5 subunit and their involvement in cell adhesion to fibronectin. J Biol Chem 2001;276:33657–64.
[63] Yuan Y, Wu L, Shen S, Wu S, Burdick M. Effect of alpha 2,6 sialylation on integrin-mediated adhesion of breast cancer cells to fibronectin and collagen IV. Life Sci 2016;149:138–45.