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Diverse expression of N-acetylglucosaminyltransferase V and complex-type β1,6-branched N-glycans in uveal and cutaneous melanoma cells

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Although both uveal (UM) and cutaneous (CM) melanoma cells derive from the transformed melanocytes, their biology varies significantly in several aspects. Malignant transformation is frequently associated with alternations in cell glycosylation, in particular those concerning branched complex-type N-glycans. These changes occur principally in β1,4-N-acetylglucosaminyltransferase III (GnT-III) that catalyzes the synthesis of glycans with bisected N-acetylglucosamine (GlcNAc) and β1,6-N-acetylglucosaminyltransferase V (GnT-V) that is involved in forming β1,6-branched antenna in complex-type glycans. We searched for the reasons of a different behavior of CM and UM cells in the expression of GnT-III and GnT-V and their oligosaccharide products. Our study showed that UM cells have more β1,6-branched glycans than CM cells, what results from a higher expression of *MGAT5* gene encoding GnT-V. The higher β1,6-branching of glycans in UM may contribute to their higher potential to migrate on fibronectin and weaker binding to main extracellular matrix proteins, observed in our previous studies.

Key words: β1,4-N-acetylglucosaminyltransferase III, β1,6-N-acetylglucosaminyltransferase V, bisected GlcNAc, β1,6-branched N-glycans, uveal melanoma, cutaneous melanoma

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INTRODUCTION

Complex-type N-glycans present on the cell surface are necessary for cellular interactions (Yoshimura *et al.*, 1995b; Guo *et al.*, 2001; Zhang *et al.*, 2004). Changes in the amount and composition of complex-type structures have been frequently observed in the transformation and progression of different types of tumors (Laidler & Lityńska, 1997; Taniguchi & Korekane, 2011), including melanoma (Pocheć *et al.*, 2003; Przybyło *et al.*, 2007; Pocheć *et al.*, 2013). The increase in the size of N-glycans is the result of increased branching of complex-type structures (Kim & Varki, 1997; Karaçali *et al.*, 2014). One of the most commonly observed changes in the glycosylation profile of tumor cells is an enhanced synthesis of the antenna linked β1,6 to the core structure of N-glycans (Dennis *et al.*, 1987; Lityńska *et al.*, 2008). It has been shown that β1,6-branching is inhibited when bisected N-acetylglucosamine (GlcNAc) is added to the complex-type structures during the previous step of oligosaccharide processing pathway (Zhao *et al.*, 2006). The key factor that determines the structure of the complex-type N-glycans is cell-specific expression of Golgi glycosyltransferases (Taniguchi & Korekane, 2011). The formation of β1,6-branching in N-glycans is catalyzed by β1,6-N-acetylglucosaminyltransferase V (GnT-V) encoded by *MGAT5* gene, while bisected GlcNAc is bound to the core structure by β1,4-N-acetylglucosaminyltransferase III (GnT-III) encoded by *MGAT3* gene (Taniguchi *et al.*, 1996). The expressions of GnT-III and GnT-V and their products are strongly associated with carcinogenesis (Taniguchi *et al.*, 1999), and therefore the altered expression of glycosyltranferases is extensively investigated in the human surgery resected sections and *in vitro* models for use as prognostic markers in cancer diagnosis.

Although uveal (UM) and cutaneous (CM) melanoma cells derive from the transformed melanocytes, their biological properties vary significantly in several aspects (ten Berge *et al.*, 1994; Jovanovic *et al.*, 2013). The development of UM and CM has a different genetic background; about 50% of CM have functional mutations in *BRAF* gene that activate Ras-Raf-MEK-ERK pathway, whereas in UM cells this mutation was not found (Belmar-Lopez *et al.*, 2008; Shtivelman *et al.*, 2014). In turn, ocular melanoma cells are characterized by mutations in *GNAQ* and *GNA11* genes, encoding the α-subunit of G protein (Nikolaou *et al.*, 2012). UM and CM progression also differs remarkably; the well-defined stages of progression can be distinguished in CM in contrast to UM (Seftor *et al.*, 1999; Chudnovsky *et al.*, 2005). Additionally, CM and UM melanoma cells metastasize preferentially to various organs, due to different ways of dissemination (Folberg, 1993; van den Bosch *et al.*, 2010).

Our group has investigated the glycosylation profiles of melanoma cells for over fifteen years. We have clearly demonstrated that the glycosylation profile of adhesion proteins (mainly integrins) depends on the stage of tumor development (Pocheć *et al.*, 2003, Przybyło *et al.*, 2007; Pocheć *et al.*, 2013) and the location of metastasis (Kremser *et al.*, 2008; Janik *et al.*, 2010). Here we searched for the reasons of a different behavior of UM and CM cells in the surface glycosylation. The present study was designed to compare the expression of GnT-III and GnT-V enzymes and their products in primary melanoma cells of different origin, uveal *vs.* cutaneous. The results of the previous studies have suggested that the increase in β1,6-branching begins in the early stage

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Abbreviations: CM, cutaneous melanoma; ECM, extracellular matrix; FN, fibronectin; GlcNAc, N-acetylglucosamine; GnT-III, β1,4-N-acetylglucosaminyltransferase III; GnT-V, β1,6-N-acetylglucosaminyltransferase V; PHA-E, *Phaseolus vulgaris* erythroagglutinin; PHA-L, *Phaseolus vulgaris* leucoagglutinin; UM, uveal melanoma

of oncogenic transformation (Demetriou *et al.,* 1995; Ito *et al.*, 2001; Pocheć *et al.*, 2015). Our study showed that UM cells are more abundant in β1,6-branched glycans than CM cells and this results from a higher expression of *MGAT5* gene.

MATERIALS AND METHODS

Cell lines and reagents. Primary human melanoma cell lines, two cutaneous (FM-55-P, IGR-39) and two uveal (92-1, mel-202) were obtained from the ESTDAB (European Searchable Tumour Cell Line Database) Melanoma Cell Bank (Tűbingen, Germany). RNeasy Plus Mini Kit (74134) and Omniscript RT Kit (205113) were purchased from Qiagen (Hilden, Germany). RPMI1640 medium (72400) and fetal bovine serum (FBS, 10270) were procured from Life Technologies (Gibco, Paisley, UK). Penicillin-streptomycin solution (P4333), protease inhibitor cocktail (P2714), Total Protein Kit, Micro Lowry, Peterson's Modification (TP0300), High Molecular Weight Markers (HMW, SDS6H), polyclonal rabbit anti-GAPDH IgG (G9545) and alkaline phosphatase-conjugated goat anti-mouse IgG (A1682) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal primary antibodies against GnT-III and GnT-V were kindly provided by Prof. Naoyuki Taniguchi and Prof. Eiji Miyoshi (Osaka University Graduate School of Medicine, Osaka, Japan). Alkaline phosphatase (AP)-conjugated sheep anti-rabbit IgG (AP322A) was obtained from Millipore (Chemicon, Temecula, CA, USA). FITC-conjugated (fluorescein-labeled *Phaseolus vulgaris* erythroagglutinin, PHA-E, FL-1121 and fluorescein-labeled *Phaseolus vulgaris* leucoagglutinin, PHA-L, FL-1111) and biotinylated lectins (PHA-E, B-1125 and PHA-L, B-1115), Carbo-Free blocking solution (SP-5040) and Vectashild with DAPI (H-1200) were provided by Vector Laboratories (Burlingame, CA, USA). RIPA buffer (89900) and PageRuler Prestained Protein Ladder (26616) were ordered from Thermo Scientific (Carlsbad, CA, USA). Laemmli sample buffer (LSB, 161-0737) and β-mercaptoethanol (161-0710) were procured from Bio-Rad (Hercules, CA, USA). Substrates for alkaline phosphatase (AP), 5-bromo,4-chloro,3-indolyphosphate (BCIP, 11383221001) and nitroblue tetrazolium (NBT, 11383213001) were from Roche (Mannheim, Germany).

Cell culture and cell lysate preparation. Melanoma cells were cultured in RPMI1640 with 10% FBS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37°C in 5% CO_2 humid atmosphere (CO_2 incubator, Lab Line Instruments). The cells were lysed in RIPA buffer containing protease inhibitors. The lysates were centrifuged (15000 rpm) for 20 min at 4°C and the protein concentration was measured in the collected supernatants using the Total Protein Kit (Micro Lowry, Peterson's Modification) and BSA as a standard.

Real-time PCR. *MGAT3* and *MGAT5* expressions were analyzed quantitatively using real-time PCR (qRT-PCR). RNA was extracted using RNeasy Protect Mini Kit and cDNA was synthesized in reverse transcription using the Omniscript RT Kit according to the manufacturer's protocols. qRT-PCR was performed as described previously (Bubka *et al.*, 2014) with minor modification. To assess the expression of *MGAT3* and *MGAT5* genes the values of Ct (cycle threshold) were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an endogenous control according to the equation:

$$\Delta Ct = Ct_{\text{tested gene}} - Ct_{\text{endogenous control}}$$

Sequence of GAPDH primers were as follows, forward: 5'-CAGCCTCAAGATCATCAGCA-3', reverse: 5'-GTCTTCTGGGTGGCAGTGAT-3'.

Western blotting. GnT-III and GnT-V expression was analyzed by Western blotting. Thirty μg of total cell lysate protein were boiled in LSB with 5% β-mercaptoethanol for 5 min, separated on 8% SDS/PAGE gel, transferred to a polyvinylidene difluoride membrane (PVDF), and detected using the monoclonal antibodies. After overnight blocking in 1% BSA in TBST, the membranes were incubated for 1 h at RT with mouse anti-GnT-III, mouse anti-GnT-V and rabbit anti-GAPDH antibodies diluted 1:100 (anti-GnT-III, anti-GnT-V) or 1:4000 (anti-GAPDH) in 1% BSA in TBST. Then, AP-conjugated goat anti-mouse IgG was applied in a 1:4000 dilution, followed by incubation at RT for 1 h. Tested enzymes and GAPDH as an endogenous control were visualized by colorimetric reaction using BCIP and NBT substrates for AP. HMW protein markers were used to calculate the relative molecular weights of GnT-III and GnT-V. Band intensities were measured densitometrically in relation to GAPDH in UVImap analysis software (UVItec, Cambridge, UK).

Lectin blotting. The expression of complex-type N-glycans with bisecting GlcNAc and β1,6-branched was detected using biotinylated PHA-E and PHA-L lectins, respectively. Protein extracts (10 μg) were SDS/PAGE separated on 8% gels and electroblotted. The PVDF membranes were blocked with Carbo-Free blocking solution in TBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) overnight at 4°C to prevent non-specific binding of lectins. Then, the membranes were probed with PHA-E and PHA-L lectins (1:4000, in TBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$) for 1 h at RT followed by incubation with streptavidin-conjugated AP (1:4000, in TBS) for 1 h at RT. Glycans-bound lectins were detected by AP colorimetric reaction.

Fluorescent staining. The cells were cultured on coverslips placed in 4-well plates overnight. Before staining, the cells were washed three times with PBS, fixed in 2% paraformaldehyde for 10 min at RT and incubated with FITC-conjugated PHA-E and PHA-L lectins (diluted 1:100 in 2% BSA in PBS) overnight at RT. The excess of lectins was removed by washing with PBS, the slides were mounted with Vectashield with DAPI and the coverslips were sealed. Images were captured in a Zeiss confocal microscope (LSM 510) and analyzed in LSM Image Browser (Carl Zeiss MicroImaging GmbH, Germany).

Statistical analysis. The results are represented as means of three measurement ± standard deviation (S.D.). Duncan test was used to determine statistical significance ($*p < 0.05$) between samples of melanoma cells.

RESULTS

The expression of GnT-V is higher in UM

The expression of GnT-III and GnT-V was analyzed on gene and protein levels. To determine *MGAT3* and *MGAT5* expression, RNA isolated from melanoma cells was subjected to real-time PCR and the relative abundance of transcripts was assessed using *GAPDH* as a reference gene. Our data showed that both 92-1 and mel-202 UM cells expressed more transcript for GnT-V than FM-55-P and IGR-39 CM cells. The level of *MGAT3* transcript was lower than *MGAT5* in all ana-

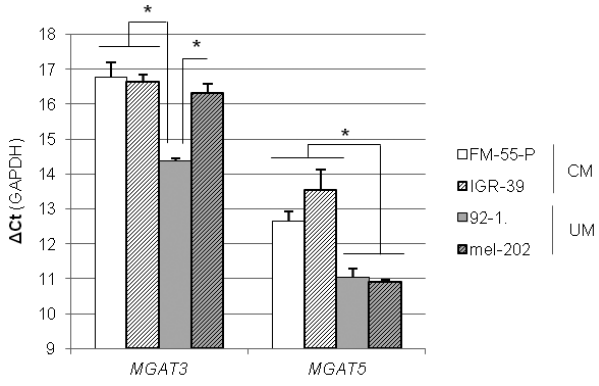


Figure 1. The expression of MGAT5 gene is higher in uveal (92-1, mel-202) than cutaneous (FM-55-P, IGR-39) melanoma cells.
The isolated RNA was reverse transcribed and subjected to real-time PCR. The expression of *MGAT3* and *MGAT5* genes was normalized to the expression leśvel of GAPDH according to the equation: $\Delta Ct = Ct_{\text{tested gene}} - Ct_{\text{endogenous control}}$. A high Ct value corresponds to low gene expression. *indicates $p < 0.05$. CM, cutaneous melanoma; UM, uveal melanoma.

lyzed cell lines, but we did not observe any differences related to the origin of melanomas (Fig. 1).

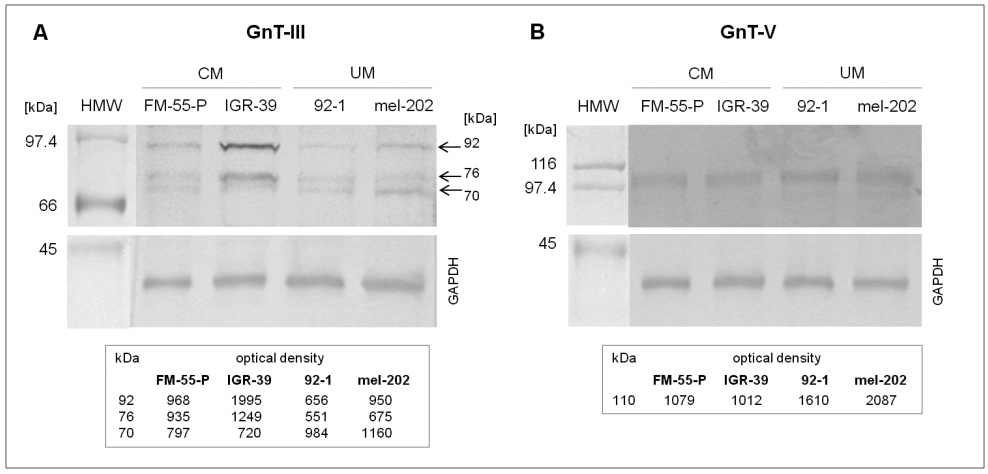
Immunoblot analysis of GnT-III and GnT-V expression on protein level showed a difference related to melanoma origin in the case of the second enzyme. The higher content of GnT-V protein in 92-1 and mel-202 UM cell lines (Fig. 2B) resulted probably from a higher transcription of *MGAT5* gene. We observed three isoforms of GnT-III enzyme with different molecular weights, 92, 76 and 70 kDa. The significantly highest expression of two 92 and 76 kDa GnT-III isoforms was noticed in IGR-39 cutaneous cells. The study did not reveal any relationship between GnT-III expression and the location of melanoma origin (Fig. 2A).

GlcNAc β1,6-branched N-glycans are more abundant in UM

PHA-E and PHA-L lectins specifically recognizing structures with bisecting GlcNAc and β1,6-branched complex-type glycans, respectively, were used to detect these products of GnT-III and GnT-V catalytic activities. We have applied two methods for the detection of signals from glycoprotein-bound lectins, AP-based colorimetric reaction in lectin blotting and fluorescence detection in confocal microscopy. Lectin blotting studies showed that PHA-E was bound to bisecting GlcNAc bearing glycoproteins ranging from 40 to 200 kDa, while PHA-L staining of β1,6-branched glycoproteins was observed within the whole range of used molecular weight standards. PHA-E-binding glycoprotein pattern was similar for both CM cell lines, while the intensity of PHA-E staining differed within tested UM cells and the glycoprotein pattern recognized by this lectin varied significantly between UM and CM cells (Fig. 3A). What is interesting, 92-1 cells showed the highest expression of N-glycans with bisecting GlcNAc (Fig. 3A) and the highest expression of *MGAT3* among all cells tested (Fig. 1), but not the expression of GnT-III at protein level (Fig. 2A). The amount of β1,6-branched N-glycans was proportionally higher in UM cells than in CM cells (Fig. 3B) and it was consistent with the higher expression of *MGAT5* gene (Fig. 1) and GnT-V protein (Fig. 2B). Lectin fluorescence in confocal microscopy studies confirmed the differences in glycan expression levels obtained in lectin blotting (Fig. 4).

DISCUSSION

Many previous studies have shown that glycosylation affects intracellular interactions and contact with ECM proteins (Gu *et al.*, 2012; Christiansen *et al.*, 2014) *via* regulation of signaling pathways (Zhao *et al.*, 2008). Differences in the glycan structure may contribute to functional dissimilarity of melanoma cells from eye and skin despite both types of melanomas develop from the same type of cells. Our previous study, performed on the same set of uveal and cutaneous melanoma cell lines, demonstrated different adhesion and migration abilities of UM and CM cells. Cutaneous cells bound strongly to FN (Przybyło *et al.*, 2008), vitronectin (Janik *et al.*, 2014), collagen (COL) and laminin (LN) (Laidler *et al.*, 2006), but migrated poorly on FN (Przybyło *et al.*, 2008) in comparison to uveal cells. Swainsonine



kDa	optical density			
	FM-55-P	IGR-39	92-1	mel-202
92	968	1995	656	950
76	935	1249	551	675
70	797	720	984	1160

kDa	optical density			
	FM-55-P	IGR-39	92-1	mel-202
110	1079	1012	1610	2087

Figure 2. The amount of GnT-III (A) and GnT-V (B) enzymes detected in cutaneous (FM-55-P, IGR-39) and uveal (92-1, mel-202) melanoma cells.
Protein extracts (30 μg) were SDS/PAGE separated on 8% gel in reducing conditions, electroblotted and probed with monoclonal antibodies against GnT-III and GnT-V. After incubation with AP-conjugated anti-mouse IgG, the bands corresponding to GnT-III and GnT-V enzymes were visualized by colorimetric reaction and the intensity of bands was measured densitometrically. CM, cutaneous melanoma; UM, uveal melanoma; HMW, High Molecular Weight Markers (Sigma-Aldrich, SDS6H).

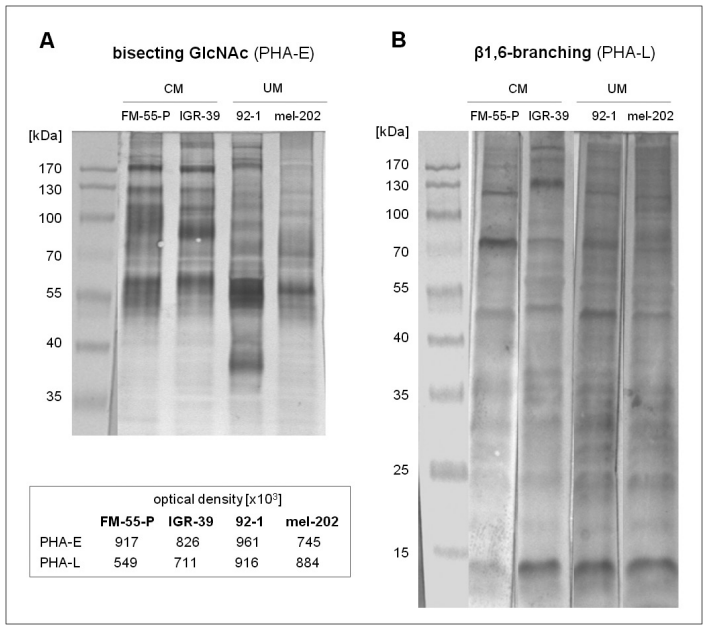


Figure 3. Glycosylation profiles of complex-type N-glycans with bisecting GlcNAc (A) and β1,6-branched (B) in cutaneous (FM-55-P, IGR-39) and uveal (92-1, mel-202) melanoma cells.
Protein extracts (10 μg) were SDS/PAGE separated on 8% gel in reducing conditions, electroblotted and probed with biotinylated PHA-E for bisecting GlcNAc bearing glycans **(A)** and PHA-L for β1,6-branched glycans **(B)**. After incubation with streptavidin-conjugated AP, glycosylation profiles were visualized by colorimetric reaction. Densitometric signal from all glycoproteins recognized by the lectin in each cell line was measured and the values are given below in the frame. CM, cutaneous melanoma; UM, uveal melanoma

that reduces formation of complex-type glycans, including β1,6-branched ones, affected more efficiently uveal than cutaneous cell migration on FN (Przybylo *et al.*, 2008). We assume that even though surface glycans are not the only one, they are an important element influencing the differences in cell migration on FN. Here we present the characterization of complex-type N-glycans β1,6-branched and with bisecting GlcNAc in melanoma cells derived from primary tumors of ocular and cutaneous origins. Our analysis showed a significantly higher expression of GnT-V enzyme (Figs. 1, 2B) and its products (Figs. 3B, 4B) in UM in comparison to CM. GnT-V is an enzyme that is the most strongly associated with cancer progression and metastasis of other glycosyltransferases involved in creation of multi-antennary N-glycans (Miyoshi *et al.*, 2012). Results of many previous studies showed that the amount of β1,6-branched glycans increases with progression of various cancers (Taniguchi *et al.*, 1999) and promotes metastasis (Couldrey & Green, 2000). Immunohistochemical analysis of over hundred human colorectal cancer cases surgically resected showed that the expression of GnT-V correlated with metastasis of this cancer (Murata *et al.*, 2000). Also other immunohistochemical studies performed on clinical samples collected postoperatively provided the evidence for positive correlation between GnT-V level and metastatic potential of gastric cancers (Tian *et al.*, 2008; Huang *et al.*, 2014), endometrial cancer (Yamamoto *et al.*, 2007) and mucinous ovarian cancers (Takahashi *et al.*, 2009). On the other hand, there is a group of cancers that shows a favorable prognosis when the expression of GnT-V is higher. This inversely proportional relationship was observed in tissue sections of oral squamous cell carcinoma (Seto *et al.*, 2013), neuroblastoma (Inamori *et al.*, 2006), bladder cancers (Ishimura *et al.*, 2006), non-small cell lung cancers (Dosaka-Akita *et al.*, 2004) and testicular germ

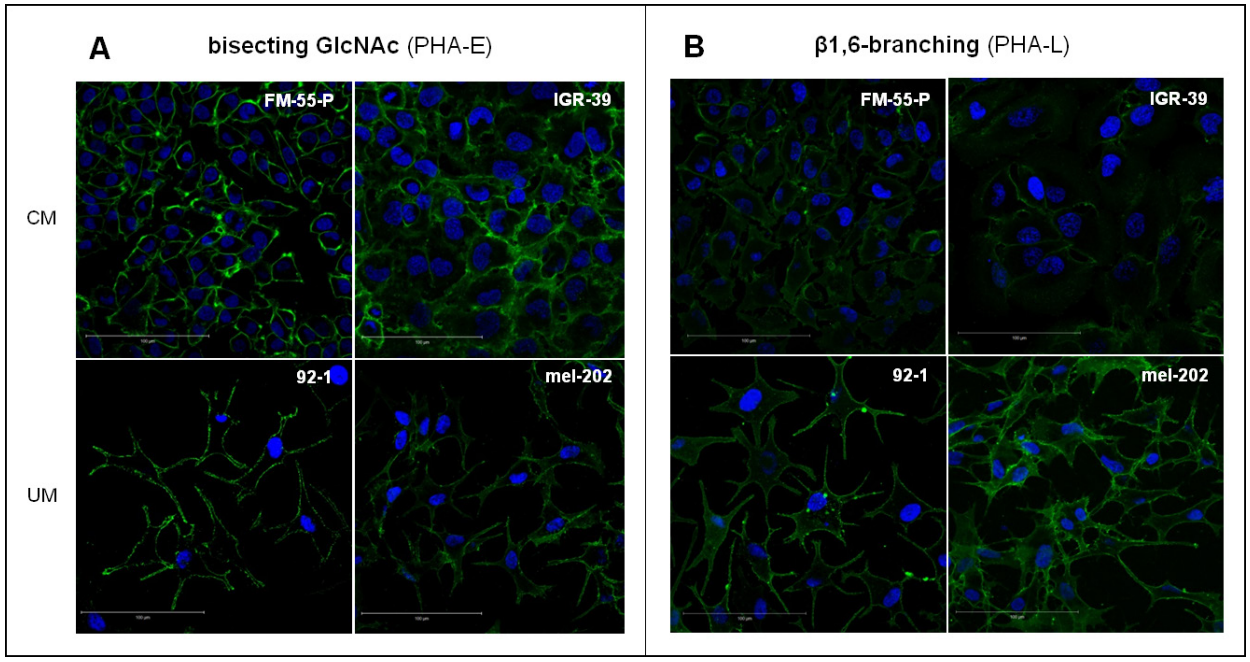


Figure 4. Surface expression of β1,6-branched complex-type N-glycans is higher in uveal melanoma (UM) than in cutaneous melanoma (CM).
Cells growing on coverslips were fixed in 2% paraformaldehyde, incubated with FITC-conjugated PHA-E for detection of bisecting GlcNAc bearing glycans **(A)** and FITC-conjugated PHA-L for detection of β1,6-branched glycans **(B)** and lectin staining (green) was visualized in a Zeiss LSM 510 META confocal microscope. DAPI was used to stain cell nuclei (blue). Scale bar — 100 mm

cell tumors (Kyan *et al.*, 2008). In view of above data, using GnT-V expression level as a prognostic marker seems to be promising, but cancer-specific. To the best of our knowledge, GnT-V expression was not analyzed in clinical section of melanoma tumors, but it has been studied on *in vitro* models. Positive correlation between the expression of GnT-V and its products *vs.* melanoma progression was shown in our previous studies for cell lines derived from vertical growth phase and metastatic lesion of the same patients (Pocheć *et al.*, 2013). Here we proved that the expression of GnT-V and its products at the early stage of melanoma progression is also dependant on the origin of melanoma.

Our present study did not show any correlation between the expression of GnT-III (Fig. 1, 2A) and N-glycans with bisecting GlcNAc (Fig. 3A, 4A) with respect to the place of melanoma origin. The expression of GnT-III was demonstrated frequently in chemically induced tumors in animal models (Narasimhan *et al.*, 1988) and in cancer cell lines (Bubka *et al.*, 2014). But the number of clinical data on the relationship between GnT-III and cancer progression is much lower than those describing GnT-V expression in cancers. The elevated level of GnT-III was observed in sera of patients with hepatomas and liver cirrhosis (Ishibashi *et al.*, 1989), while the activity of GnT-III was increased in patients with two hematological cancers, myelogenous leukemia and multiple myeloma (Yoshimura *et al.*, 1995a). Based on the studies on animal and cell line models it is known that GnT-III and its products have a suppressive effect on tumor progression (Isaji *et al.*, 2010). The transfection of cDNA encoding GnT-III to highly metastatic subclone of B16-F1 murine melanoma cell line exhibited a higher expression of GnT-V products resulted in the reduced invasion into Matrigel and weakened adhesion to COL and LN. The injection of GnT-III-transfected melanoma cells into nude mice suppressed lung metastasis (Yoshimura *et al.*, 1995b). The ability to reduce cancer by GnT-III was confirmed in several further studies on different models, among them through intracellular pathways induced by epidermal growth factor receptor excessively modified by overexpressed GnT-III (Gu *et al.*, 2009). In our study, the glycosylation pattern obtained for bisecting GlcNAc glycans was quite different from β1,6-branching (Fig. 3), indicating that different proteins are substrates for GnT-III and GnT-V and/or the proteins are modified by these enzymes with various intensity. What is important, both CM cells displayed the higher expression of bisecting GlcNAc than β1,6-branched glycans though the reaction with PHA-E was observed in a less narrow range of molecular weight markers than PHA-L (Fig. 3). Taking into account suppressive properties of GnT-III action, the higher expression of bisected GlcNAc on CM glycoproteins may contribute to their lower ability to migrate on FN and stronger binding to ECM proteins than in case of UM cells showed in our previous studies (Laidler *et al.*, 2006; Przybyło *et al.*, 2008; Janik *et al.*, 2014).

Determining glycosylation pattern and searching for features specific for melanoma cells of different origins is a crucial stage in understanding biology of melanoma and a reason for differences in the development and progression of UM and CM. The higher level of GnT-V and β1,6-branched glycans in UM than CM cells may be one of the reasons for lower adhesion and higher migration exhibited by UM cells.

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