Glycans in melanoma screening. Part 1. The role of β1,6-branched N-linked oligosaccharides in melanoma

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Abstract
Melanoma, which is one of the most aggressive human tumours, originates from melanin-producing melanocytes. As no effective systemic therapy exists for advanced-stage melanoma, the best chance of recovery remains surgical removal of thin early-stage melanoma. Aberrant glycosylation is a hallmark of malignancy and a well-studied class of β1,6-branched oligosaccharides is associated with malignant transformation of rodent and human cells, and poor prognosis in cancer patients. It is evident that increased β1,6 branching significantly contributes to the phenotype of melanoma cells, influencing the adhesion to extracellular matrix components and motility as well as invasive and metastatic potential. Despite the considerable success in establishing the role of β1,6-branched N-linked oligosaccharides in melanoma biology, there is virtually no progress in using these glycans as a screening tool for the early diagnosis of the disease, or a target-specific therapeutic agent.

Melanoma: a challenge for glycomics
Melanoma (melanoma malignum) is one of the most aggressive human tumours and originates from melanocytes that are responsible for the production of melanin. As such cells are found predominantly in the skin, melanoma usually develops in the skin (over 95% of tumours are found in the skin), but very rarely it can also start to develop in other parts of the body, for example in the eyes (where the most common is uveal melanoma), membrane covering the brain and spinal cord, the digestive tract and lymph nodes. Primary skin melanoma (cutaneous melanoma) may develop in precursor melanocytic nevi, but more than 50% of cases are believed to arise de novo without pre-existing pigment lesions. In primary cutaneous melanoma four major clinical/histopathological subtypes have been identified. These include: superficial spreading, nodular, lentigo maligna and acral lentiginous melanoma. Other very rare types of melanoma of the skin include amelanotic melanoma and desmoplastic melanomas. It is commonly accepted that both environmental factors and genetic predisposition play an important role in tumour development and progression [1–4]. The progression of cutaneous melanoma from normal melanocytes to metastatic disease is believed to proceed through multiple stages. This process involves formation of nevi from normal melanocytes (common acquired nevus), dysplastic nevus, RGP (radial growth phase) primary melanoma, a subsequent VGP (vertical growth phase) primary melanoma, and finally the development of metastatic melanoma [5,6]. Nowadays, the most important prognostic factors in melanoma are TNM (tumour/node/metastasis) stage, Breslow depth (tumour thickness) and ulceration [7–9]. Although cutaneous melanoma accounts only for approx. 4% of all dermatological cancers, it is responsible for over 80% of skin cancer deaths, and only 14% of patients with metastatic melanoma survive over 5 years [6,10]. Despite the improvements in diagnosis, the best chance of melanoma recovery remains surgical removal of thin early-stage melanoma tissue [11]. Therefore one of the major goals of melanoma research is to better understand cancer biology, which in turn might result in the development or improvement of diagnostic techniques that could better and earlier detect melanocytic lesions, in order to give patients suffering from melanoma the best chance of prolonged survival.

Glycosylation and melanoma
Glycosylation changes are one of the multiple molecular alterations associated with the neoplastic process and clinical characteristics of cutaneous melanoma and this is currently the subject of intensive investigation. Glycosylation is one of the most abundant post-translational modifications of proteins, as nearly 50% of all proteins are thought to be glycosylated. Glycosylation is involved in several physiological and pathological events, including protein folding and stability, signal transduction, cell growth, migration, differentiation, tumour invasion and metastasis [12–15]. Based on the extensive analysis of various human and rodent tumours, it is well known that differences in glycan structures are associated with transition from the normal to the transformed phenotype [13,16–21]. The most frequently observed alterations during tumorigenesis include the extensive synthesis of highly branched and sialylated glycans, premature termination of biosynthesis and re-expression of foetal type antigens.
Comparative lectin-binding studies on cell extracts obtained from different melanoma cell lines (primary site, WM35; metastatic sites, WM9, WM239 and A375) performed by our group revealed that acquisition of the metastatic potential was associated with an increase in staining and/or in the number of glycoproteins that reacted with *Sambucus nigra* (a lectin specific for α2,6-linked sialic acids), *Maackia amurensis* (a lectin specific for α2,3-linked sialic acids) and *Phaseolus vulgaris* (PHAL, a lectin specific for β1,6-branched complex-type N-glycans) agglutinins [22]. In WM9, WM239 and A375 cell lines, additional bands with the apparent molecular mass of 160–100 kDa were stained with PHAL, suggesting that cells from metastatic sites contained more β1,6-branched N-linked oligosaccharides. Among these proteins, N-cadherin was identified by specific antibodies as one of the proteins undergoing changes in oligosaccharide composition in different melanoma cells. N-cadherin is one of the classical cadherin subtypes that is expressed on neuronal, endothelial and muscle cells, but is rarely found on normal human melanocytes in contrast with E-cadherin and P-cadherin [23]. Interestingly, the cadherin superfamily has been shown to have a critical role in maintaining homeostasis in the skin by regulating the interaction between melanocytes and epidermal keratinocytes, dermal fibroblasts and vascular endothelial cells [24,25]. During melanoma development, the progressive loss of E-cadherin expression causes a disruption in keratinocyte-mediated regulation of melanoma cells and it appears to be one of the critical steps in progression of melanoma, as it could trigger the release of cancer cells from the primary focus. Moreover, the loss of functional E-cadherin is paralleled by up-regulation in expression of N-cadherin on melanoma cells. N-cadherin mediates homotypic adhesion between melanoma cells and facilitates melanoma cell interaction with other N-cadherin-expressing cells in the skin, i.e. fibroblasts and endothelial cells, resulting in the increased motility, proliferation and invasive potential of melanoma cells. N-cadherin has also been shown to prevent apoptosis, and to play a key role in transendothelial migration of melanoma cells.

### Functions of β1,6-branched N-linked oligosaccharides

The β1,6-branched complex-type N-glycans are synthesized due to the action of GnT-V (N-acetylglucosaminyltransferase V, EC 2.4.1.155), which catalyses the addition of GlcNAc (N-acetylglucosamine) to α6- and/or tetra-antennary oligosaccharides [26]. In malignant transformation the increased β1,6 branching of N-linked oligosaccharides is a result of the enhanced activity of GnT-V associated with the increased expression of the GnT-V gene (*Mgat5*), which is in turn regulated by Ras/Raf/MAPK (mitogen-activated protein kinase), the signalling pathway commonly activated in tumour cells. In oncogenic transformation, *Mgat5* expression is also regulated by the Ets family including the Ets-1 transcription factor, which additionally regulates several molecules associated with cell invasion and metastasis, such as cyclin D (cell-cycle progression), VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) (potent angiogenic factors), MMP (matrix metalloproteinases)-2, -3 and -9 and Rho/Cdc42/rac-1 (motility) [13]. The last mentioned method of GnT-V regulation supports the hypothesis that the observed changes in tumour cell glycosylation could be a by-product of the changes associated with the alterations in gene expression during malignant transformation. There is also a hypothesis that the increased GnT-V activity and β1,6 branching in melanoma could reflect previous fusion of tumour-associated macrophages with cells of the primary tumour [27,28].

Tissue invasion and metastasis are highly dependent on alteration in the ECM (extracellular matrix), cell–cell and cell–ECM interactions. These interactions, in turn, are largely dependent on the surface properties of cells. Thus quantitative changes in a few key aspects of global glycosylation may be able to make their presence biologically potent features of the malignant phenotype. Our previous studies with the use of swainsonine, a competitive inhibitor of Golgi mannosidase II, provided evidence that β1,6-branched N-linked oligosaccharides on the cell surface of WM35, WM9, WM239 and A375 cells were quite important for the biological properties of these melanoma cells [29,30]. Treatment with swainsonine resulted in the increased adhesion to FN (fibronectin), reduced migration on FN and inhibited invasiveness through Matrigel™ in the cell lines tested.

The importance of β1,6-branched oligosaccharides in mammray tumour cells, including melanoma, has been clearly demonstrated, not only with the use of specific glycosylation inhibitors, but also by transfection of those cells with cDNA encoding GnT-V or GnT-III (N-acetylgalactosaminyltransferase III), which competes with GnT-V for the same substrate (once a bisecting GlcNAc residue is added to the core mannose by GnT-III, it prevents the formation of β1,6-branched glycans by GnT-V since it cannot utilize the bisected oligosaccharides as a substrate); as well as by using genetically altered mice [13,19,20,31]. Functionally, the increased β1,6 branching of N-linked oligosaccharides has been found to correlate with the loss of contact inhibition, reduction in cell–substratum adhesion, enhancement of cellular motility, reconstruction of the vascular system and an increase in the metastatic potential in murine models [13,26,31–33]. Although mechanisms by which oligosaccharides might control these properties of tumour cells remain unclear, some data suggest that this mostly occurs due to the changes in the function of cell-adhesion molecules, particularly those involved in cell adhesion and motility. β1,6-Branched oligosaccharides have also been shown to be associated with the increased melanin production and autophagy in macrophage–melanoma fusion hybrids [34,35].

### GnT-V substrates

It was well accepted that tumour progression to a metastatic phenotype is directly associated with the increased level of...
β1,6-branched N-glycans, but only a few proteins have been identified to possess this type of glycan. To identify candidate proteins that bear β1,6-branched N-glycans in WM35, WM9, WM239 and A375 cell lines, comparative analysis on PHAL-bound material obtained from clarified lysates was performed by MS/MS (tandem MS) [36,37]. This procedure indicated that WM35 cells had the lowest number of PHAL-reactive proteins in comparison with WM9, WM239 and A375 cells. Generally, serum-free medium had the highest number of PHAL-reactive proteins [36,37]. Regardless of melanoma progression, αv and β1 integrin subunits, LAMP-1 (lysosomal-associated membrane protein 1), Mel-CAM (melanoma cell-adhesion molecule, CD146), intercellular adhesion molecule (CD54) and Mac-2 BP were always substrates for GnT-V in the cell lines tested. Integrin α3, α5 and β3 subunits had no β1,6-branched N-glycans in WM35 cells being an RGP primary melanoma, α4 integrin subunit, CD44 and N-cadherin appeared to possess these glycans only in the A375 cell line, which is the most aggressive melanoma cell line among the ones studied. The variations in the number of PHAL-positive proteins in the cell lines tested did not arise from differences in the level of GnT-V transcript as measured by real-time RT (reverse transcription)–PCR [38], nor seem to be associated with cell-adhesion molecule expression [39].

Some of these proteins, being a substrate for GnT-V, are particularly important in view of melanoma biology, as various cell-adhesion molecules belonging to the integrin, cadherin and immunoglobulin superfamily have been implicated in tumour progression in cutaneous melanoma. In melanoma cells an increase in expression of cell-adhesion receptors of the immunoglobulin superfamily, such as L1, Mel-CAM and ALCAM (activated leucocyte cell-adhesion molecule), as well as N-cadherin and αvβ3 and α5β1 integrins, was found [25]. Interestingly, integrin expression, along with histo/pathological criteria, is a prognostic marker for malignant melanoma and may indicate the site of subsequent metastasis [40,41]. The relationship between β1,6-branched N-glycans added to cell-surface proteins and their function in interactions is not fully understood, but a growing body of evidence indicates that post-translational events, such as glycosylation, are important determining factors. It is believed that these bulky oligosaccharides may slow down receptor movement in the plane of the membrane, exert an influence on residency time on the cell surface and the ligand binding properties, as well as affecting the interaction with membrane-associated proteins including EGF (epidermal growth factor) receptor and various members of the tetraspanin family [37,42–46].

Conclusions

It is well known that changes in the expression and structure of carbohydrates can be considered as a universal feature of malignant transformation on melanoma cells. In view of the findings presented in this review, it is evident that the increased β1,6 branching via catalysis by GnT-V significantly contributes to the phenotype of melanoma cells, influencing the adhesion to ECM components and motility, as well as invasive and metastatic potential. It was expected that with the advance of glycomics, it would be possible to defeat the deadliest forms of tumours, including malignant melanoma, but in the past years this expectation has not been fulfilled. Despite the success in establishing the role of β1,6-branched N-linked oligosaccharides in melanoma biology, there is virtually no progression in using these glycans as a screening tool for the early diagnosis of the disease, or a target-specific therapeutic agent that may be useful in the treatment of malignancy by interfering with the metastatic process. Thus this intriguing problem merits further study.

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