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Serum Neu5Gc biomarkers are elevated in primary cutaneous melanoma

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A B S T R A C T

Cutaneous melanoma is one of the most aggressive and deadly types of skin cancer and rates of disease are continuing to increase worldwide. Currently, no serum biomarkers exist for the early detection of cutaneous melanoma. Normal human cells cannot make the sialic acid sugar, Neu5Gc, yet human tumor cells express Neu5Gc and Neu5Gc-containing glycoconjugates have been proposed as tumor biomarkers. We engineered a Neu5Gc-specific lectin based on the pentameric B-subunit of the Shiga toxigenic *Escherichia coli* subtilase cytotoxin, termed SubB2M. We have detected elevated Neu5Gc-containing biomarkers in the sera of ovarian and breast cancer patients in a highly sensitive surface plasmon resonance (SPR)-based assay using our SubB2M lectin. Here, we used the SubB2M-SPR assay to investigate Neu5Gc-containing glycoconjugates in the serum of cutaneous melanoma patients. We found elevated total serum Neu5Gc levels in primary (n = 24) and metastatic (n = 38) patients compared to cancer-free controls (n = 34). Serum Neu5Gc levels detected with SubB2M can distinguish cutaneous melanoma patients from cancer-free controls with high sensitivity and specificity as determined by ROC curve analysis. These data indicate that serum Neu5Gc-containing glycoconjugates are a novel class of biomarkers for cutaneous melanoma, particularly for primary melanoma, and have the potential to contribute to the early diagnosis of this disease.

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1. Introduction

Cutaneous melanoma is one of the most aggressive and deadly types of skin cancer worldwide. Although there has been progress in treatment methods, the 5-year survival rate for patients diagnosed with distant metastatic melanoma is less than 30% [1]. The continual increase in the worldwide incidence of melanoma [2] highlights the need for effective and accessible screening methods for this disease.

The most common screening method for cutaneous melanoma is a total body skin examination performed by an experienced physician supported by dermoscopy or other imaging techniques. However, even experienced physicians achieve just over 80% in their ability to differentiate between benign melanocytic lesions and melanoma [3]. If melanoma is suspected, a biopsy is taken from the patient, but tumor biopsies are not suitable for continuous disease monitoring. The only circulating serum biomarker used clinically for melanoma is lactate dehydrogenase (LDH). Measurement of serum LDH levels is included in the American Joint Committee on Cancer melanoma staging system and is used in stage IV patients only as a predictor of survival outcomes [4]. There are no FDA approved serum biomarkers for clinical use as adjuncts in the early detection and diagnosis of primary cutaneous melanoma.

Aberrant glycosylation is a feature of all cancer cells. Glycans terminating with the sialic acid *N*-glycolylneuraminic acid (Neu5Gc) are found at extremely low levels on healthy human tissues [5]. Humans express an inactive cytidine monophosphate *N*-acetylneuraminic acid (Neu5Ac) hydroxylase (CMAH) enzyme [6]. CMAH is the only known enzyme responsible for the conversion of Neu5Ac to Neu5Gc, and thus human cells are thought to be unable to synthesize Neu5Gc [5,6]. Regardless, human tumor cells express Neu5Gc [7–11], and Neu5Gc-containing glycoconjugates have been proposed as tumor biomarkers for decades [8,10,12].

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Using structure-aided design, we engineered a Neu5Gc-specific lectin [13,14] based on the pentameric B-subunit of the Shiga toxigenic *Escherichia coli* (STEC) subtilase cytotoxin (SubAB); SubB preferentially recognizes α2-3 linked Neu5Gc [15,16]. The SubB lectin was mutated to reduce background recognition of Neu5Ac and to expand the recognition from only α2-3-linked Neu5Gc to include both α2-3 and α2-6 Neu5Gc linkages to substituent sugars [13]. We have used this engineered lectin, termed SubB2M, to examine Neu5Gc-containing biomarkers in the sera of ovarian and breast cancer patients in a highly sensitive surface plasmon resonance (SPR)-based assay, and detect elevated levels of Neu5Gc-biomarkers in both of these cancers [17,18]. In the current study, we use the SubB2M SPR-based assay [18] to investigate Neu5Gc-containing glycoconjugates in the serum of cutaneous melanoma patients to determine its potential as an adjunct in the detection, diagnosis or monitoring of cutaneous melanoma.

2. Materials and methods

2.1. Human serum samples

Serum samples from cancer-free (normal) individuals (non-malignant diagnosis, no history of cancer) ($n = 34$), patients with primary cutaneous (diagnosed by tumor excised from the skin) ($n = 24$) and metastatic melanoma (diagnosed as metastatic melanoma by tumor/s excised from a secondary site) ($n = 38$) were obtained from the Victorian Cancer Biobank, Australia, under Project No. 17020. As described in one of our previous studies [17], 'normal' controls are defined as individuals with a non-cancer condition at the time the sample is taken. The melanoma serum samples were collected immediately pre-operatively, or for some of the primary cases, within 1 month of excision of the primary lesion, using Serum Separation Tubes (BD) and were processed and stored at −80 °C within 2 h of collection. The patient data and serum samples used in this project were provided by the Victorian Cancer Biobank with informed consent from all donors and use of the samples was approved by the Griffith University HREC (GU Ref No: 2017/732) in accordance with the National Statement on Ethical Conduct in Human Research. Detailed sample information can be found in Supplementary Table 1.

2.2. SubB2M-$_{A12}$-SPR assay

The recombinant SubB2M and SubB$_{A12}$ proteins were expressed and purified as previously described [13,16]. Briefly, SubB2M and SubB$_{A12}$ were expressed in *E. coli* BL21 (DE3) cells transformed with the SubB2M or SubB$_{A12}$ expression constructs, respectively, as His$_6$-tagged fusion proteins, which were then purified by Ni-NTA affinity chromatography.

SPR was conducted using the Biacore S200 system (Cytiva) with immobilization of SubB2M and SubB$_{A12}$ performed essentially as described previously [17]. SubB2M was immobilized through flow cells 2 and 3 and SubB$_{A12}$ was immobilized through flow cell 4 (capture levels: 5000–6000 Response Units (RU)) onto a series S sensor chip CM5 (GE) using the EDC/NHS capture kit. After immobilization, a start-up cycle of 0.5% normal human serum (Sigma-Aldrich, Cat No. H4522) was run over the immobilized SubB proteins for 10 steps of 30 s at 30 μL/min flow rate to condition the chip. A final wash of 10 mM Tris/1 mM EDTA was run for 30 s at a 30 μL/min flow rate prior to beginning the data collection. SPR analysis was performed using multi-cycle analysis and reference subtraction (values for 0.5% normal human serum only) using the Biacore S200 evaluation software. At least two independent SPR runs were performed for each sample set. Human serum samples were diluted 1:200 in PBS and analyzed in duplicate in each SPR run as described above. RU values obtained for each serum sample with SubB$_{A12}$ (flow cell 4) were subtracted from the RU values obtained with SubB2M from flow cells 2 and 3 and averaged to obtain the final RUs used for conversion to glycoprotein units (GPUs). The conversion to GPUs, i.e. Neu5Gc glycoprotein units, was based on an internal calibration curve consisting of the Neu5Gc-containing glycoproteins, bAGP and CA125, combined at starting concentrations of 15 μg/ml and 15 units/ml, respectively, in 0.5% normal human serum, as previously described [18].

2.3. Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0. The mean GPUs between normal serum samples compared to cancer patient serum samples were analyzed by two-tailed, unpaired *t*-tests, with a P value of <0.05 considered significant. Optimal cut-off values from Receiver operating characteristics (ROC) curve analyses were determined by maximizing the sum of specificity and sensitivity.

3. Results

3.1. Serum Neu5Gc glycoconjugates are elevated in patients with cutaneous melanoma relative to cancer-free controls

We acquired a collection of cutaneous melanoma patient sera from the Victorian Cancer Biobank, along with cancer-free controls, to screen for total Neu5Gc glycoconjugate levels using our established SubB2M-$_{A12}$-SPR assay [18]. This collection of samples consists of serum taken from 24 primary cutaneous melanoma patients, 38 metastatic melanoma patients and 34 cancer-free controls (Table 1, see Supplementary Table 1 for full details of donors). The SubB2M-$_{A12}$-SPR assay uses a parallel analysis of all serum samples with SubB2M and with a non-sialic acid binding version of SubB, called SubB$_{A12}$ [15], to control for any non-specific, non-Neu5Gc-dependent binding of serum components to the SubB protein [18]. Using this assay, we detected significantly elevated total serum Neu5Gc levels (reported as glycoprotein units (GPUs)) in primary and metastatic patients compared to cancer-free controls (Fig. 1A). ROC curve analysis showed that Neu5Gc biomarker levels detected with our SPR-based assay can distinguish cancer-free individuals from melanoma patients (primary and metastatic combined) with a sensitivity of 85.48% and a specificity of 94.12% when a cut-off of >15.56 GPUs was applied (Fig. 1B). When ROC curve analysis was applied to only the primary melanoma patients, the ability of Neu5Gc serum biomarker levels to distinguish melanoma patients from cancer-free individuals increased to a remarkable 100% specificity and 100% sensitivity (Fig. 2).

3.2. Neu5Gc serum glycoconjugates show variation between primary and metastatic patients

There was a significant difference in mean SPR response units (converted to GPUs) detected in the metastatic patients compared to the primary melanoma patients (Fig. 1A). The basic principle of SPR is the recognition of an analyte in solution by a biomolecule immobilized onto the surface of a metal biosensor chip (SubB2M and SubB$_{A12}$ in our assay) that results in an increase in the local refractive index at the metal surface, which is measured as response units (RUs). This change in the refractive index is proportional to the mass concentration at the surface of the biosensor chip, therefore the significant reduction in RUs, and thereby apparent GPUs, detected between primary and metastatic melanoma patients (Fig. 1A), may be due to a decrease in the total concentration of serum Neu5Gc glycoconjugates or by a shift in the

Table 1
Summary of the serum sample donors used in this study.

	Normal	Primary	Metastatic
Age range	18–93	35–94	26–88
Total samples	34	24	38
No. of males	12	6	18
No. of females	22	18	20
Clinical features	Non-malignant diagnosis, no history of cancer	Primary cutaneous melanoma, lesion excised from the skin and diagnosed	Metastatic melanoma, tumor excised from a secondary site and diagnosed
Pathological stage	N/A	Stage I = 5 Stage II = 9 Stage III = 8 Stage IV = 1 Unknown = 1	Stage III/IV
Tumor subtype (if known)	N/A	Acral lentiginous = 3 Desmoplastic = 1 Lentigo maligna = 3 Nodular = 9 Spindle cell = 1 Superficial spreading = 5 Unknown = 2	N/A
Mean GPUs (SD)	6.94 (5.72)	41.5 (14.9)	20.1 (9.19)

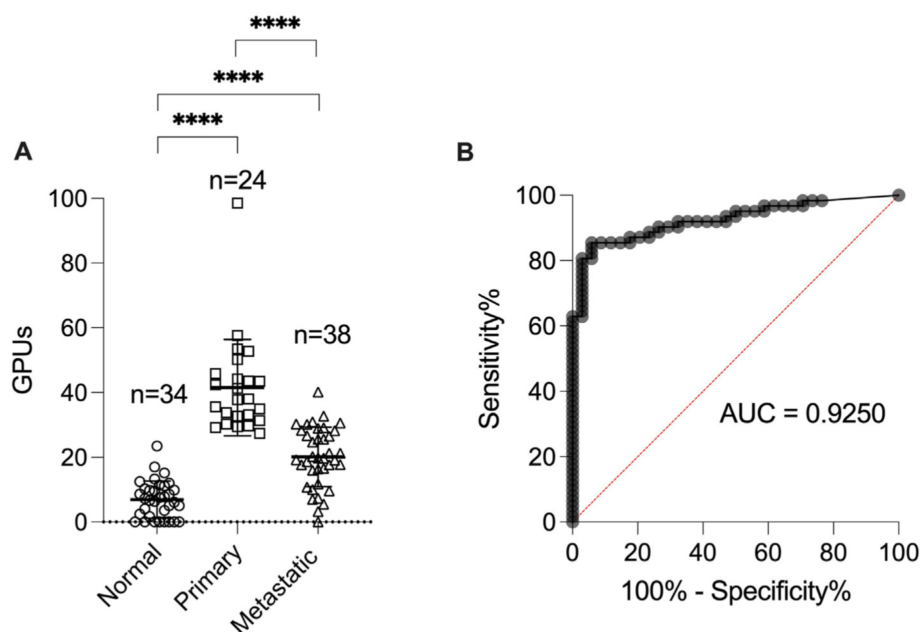

Fig. 1. Analysis of Neu5Gc serum glycoconjugates in cutaneous melanoma patients compared to cancer-free controls. A) 34 cancer-free (Normal), 24 primary cutaneous melanoma and 38 metastatic melanoma serum samples were screened for total Neu5Gc levels, expressed as glycoprotein units (GPUs), using our previously described SubB2M-$_{A12}$-SPR assay [18] (see Supplementary Table 1 for details of each donor). The mean GPUs from duplicate analyses of each serum sample are shown. Error bars = ± 1 SD from the mean for each group. Statistical analysis was performed using two-tailed unpaired *t*-tests. ****: *P*-value = <0.0001 compared to Normal. Duplicate, independent assays were performed with both showing the same trends. One representative assay is shown. **B)** ROC curve depicting the ability of serum Neu5Gc levels determined by the SubB2M-$_{A12}$-SPR assay to distinguish melanoma patients from cancer-free (normal) individuals. Sensitivity% (true positive rate; ability to detect disease) is plotted against 100 %-specificity% (false positive rate or 100 %-true negative rate; ability to detect lack of disease). ROC analyses were performed with the data shown in Fig. 1A using Graphpad Prism 9.0. AUC = area under the curve.

molecular weight of the Neu5Gc serum glycoconjugate/s being detected.

4. Discussion

The worldwide incidence of cutaneous melanoma is increasing faster than any other solid tumor type [19]. The ability of cutaneous melanoma to metastasize rapidly to distant organs, particularly the head and neck, liver and lungs, makes it the most lethal skin cancer [20]. Early detection of primary tumors before metastasis occurs is crucial for increasing survival rates [21]. Using our established SPR-based assay (17,18), in this study we detected significantly elevated levels of serum Neu5Gc glycoconjugates in primary cutaneous and metastatic melanoma patients compared to cancer-free controls. Our assay can distinguish cancer-free individuals from melanoma patients (primary + metastatic) with a sensitivity of 85.48% and a specificity of 94.12%, and a remarkable 100% sensitivity and 100% specificity for cancer-free individuals from primary melanoma patients. Our cohort of primary melanoma patients included 5 individuals with stage I disease (Supplementary Table 1), and our assay was able to distinguish these early-stage patients from cancer-free controls. Furthermore, our cohort of primary melanoma patients included patients with all tumor subtypes (acral lentiginous, desmoplastic, lentigo maligna, nodular, spindle cell and the

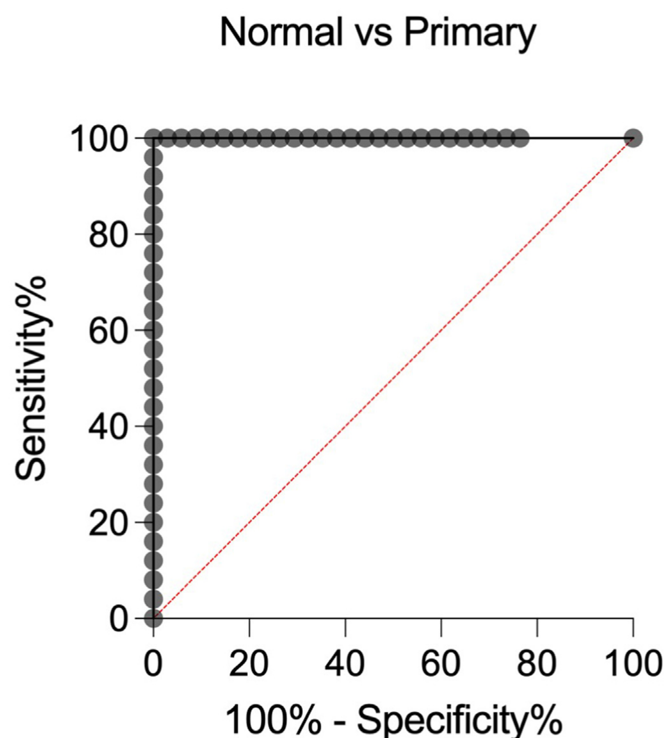

Fig. 2. Receiver operating characteristics (ROC) curve depicting the ability of serum Neu5Gc levels determined by the SubB2M-$_{A12}$-SPR assay to distinguish primary melanoma patients from cancer-free (normal) individuals. Sensitivity% (true positive rate; ability to detect disease) is plotted against 100 %-specificity% (false positive rate or 100 %-true negative rate; ability to detect lack of disease). ROC analyses were performed with a selection of the data shown in Fig. 1A using Graphpad Prism 9.0.

more common superficial spreading (also known as low-cumulative solar damage melanoma) [22]), indicating that our assay can detect Neu5Gc biomarkers derived from all lesion subtypes. These data suggest that serum Neu5Gc-containing glycoconjugates are a novel class of biomarkers for cutaneous melanoma and have the potential for early detection of this disease.

Compared to our previous studies investigating serum Neu5Gc biomarkers in breast and ovarian cancers [17,18], where we observed an increase in SPR signal as disease progressed, we noted that the SPR RUs detected in the serum from melanoma patients with confirmed metastatic disease (late-stage) decreased compared to primary melanoma patients. These data suggest that the composition of Neu5Gc-containing serum biomarkers changes in the transition from primary to metastatic disease. This change is due to either a reduction in the concentration of Neu5Gc serum glycoconjugates, or due to a decrease in the molecular weight of the molecules carrying the Neu5Gc glycosylation, for example, a glycolipid rather than a high molecular weight molecule, such as a mucin. Mucin biomarkers are used for disease monitoring in breast and ovarian cancer. CA15-3, also known as MUC1, is used for monitoring in breast cancer and CA125, also known as MUC16, is used for monitoring in ovarian cancer. On the other hand, particular gangliosides, sialic acid-containing, cell surface glycosphingolipids, are known to be overexpressed and shed by melanoma cells [23]. The expression of the three major cell surface gangliosides, GM3, GD3 and GD2, are altered during melanoma development and progression [24]. For example, an increase in the expression of GD3 relative to GM3 in tissue samples and cultured human cells as melanoma progresses has been reported by multiple groups [24–27] and may contribute to the malignant properties of melanoma cells [28].

The ganglioside Neu5Gc-GM3, a variant of GM3 that contains Neu5Gc instead of the typical Neu5Ac, is known to be expressed in melanoma tumor samples [29–31] and on the surface of cultured human melanoma cells [32], but not in or on their normal counterparts. As we observed higher levels of Neu5Gc glycoconjugates in serum from primary melanoma patients, and previous reports have shown a shift in GM3 to GD3 gangliosides as melanoma progresses, we hypothesize that the Neu5Gc serum biomarker we are detecting in the melanoma patients is Neu5Gc-GM3, with higher levels of this ganglioside present in primary patients, and lower levels in metastatic patients, as the expression of GD3 relative to GM3 increases. Alternatively, in the primary melanoma patients, the circulating Neu5Gc glycoconjugates may exist as larger molecular weight structures, such as an aggregate or micelle, or in a membrane bound vesicle, accounting for the higher RUs detected in the primary patients compared to the metastatic patients. Future studies in our lab will focus on the identification and complete characterisation of the Neu5Gc biomarkers being detected in melanoma patient serum and will investigate the potential contribution of Neu5Gc-GM3 to total serum Neu5Gc glycoconjugates detected in our assay.

In summary, our data indicate that Neu5Gc-containing glycoconjugates are being shed by melanoma cells into the circulation. Current studies are working to identify the Neu5Gc-containing glycoconjugates detected by our assay, to resolve the observed differences between primary and metastatic patients, and to further support the development of a melanoma-specific, Neu5Gc-biomarker assay that can detect disease early and potentially predict clinical outcome.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Michael P Jennings reports financial support was provided by National Health and Medical Research Council. Michael P Jennings has patent #WO2018085888A1 licensed to Inoviq. Christopher J Day has patent #WO2018085888A1 licensed to Inoviq. James C. Paton has patent #WO2018085888A1 licensed to Inoviq. Adrienne W. Paton has patent #WO2018085888A1 licensed to Inoviq. Michael P Jennings has patent #2021901444 licensed to Inoviq. Lucy K Shewell has patent #2021901444 licensed to Inoviq. Christopher J Day has patent #2021901444 licensed to Inoviq. James C Paton has patent #2021901444 licensed to Inoviq. Adrienne W. Paton has patent #2021901444 licensed to Inoviq.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.12.053.

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