The glycosphingolipid P$_1$ is an ovarian cancer-associated carbohydrate antigen involved in migration

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**Background:** The level of plasma-derived naturally circulating anti-glycan antibodies (AGA) to P$_1$ trisaccharide has previously been shown to significantly discriminate between ovarian cancer patients and healthy women. Here we aim to identify the Ig class that causes this discrimination, to identify on cancer cells the corresponding P$_1$ antigen recognised by circulating anti-P$_1$ antibodies and to shed light into the possible function of this glycosphingolipid.

**Methods:** An independent Australian cohort was assessed for the presence of anti-P$_1$ IgG and IgM class antibodies using suspension array. Monoclonal and human derived anti-glycan antibodies were verified using three independent glycan-based immunoassays and flow cytometry-based inhibition assay. The P$_1$ antigen was detected by LC-MS/MS and flow cytometry. FACS-sorted cell lines were studied on the cellular migration by colorimetric assay and real-time measurement using xCELLigence system.

**Results:** Here we show in a second independent cohort (n = 155) that the discrimination of cancer patients is mediated by the IgM class of anti-P$_1$ antibodies (P = 0.0002). The presence of corresponding antigen P$_1$ and structurally related epitopes in fresh tissue specimens and cultured cancer cells is demonstrated. We further link the antibody and antigen (P$_1$) by showing that human naturally circulating and affinity-purified anti-P$_1$ IgM isolated from patients ascites can bind to naturally expressed P$_1$ on the cell surface of ovarian cancer cells. Cell-sorted IGROV1 was used to obtain two study subpopulations (P$_1$-high, 66.1%; and P$_1$-low, 33.3%) and observed that cells expressing high P$_1$-levels migrate significantly faster than those with low P$_1$-levels.

**Conclusions:** This is the first report showing that P$_1$ antigen, known to be expressed on erythrocytes only, is also present on ovarian cancer cells. This suggests that P$_1$ is a novel tumour-associated carbohydrate antigen recognised by the immune system in patients and may have a role in cell migration. The clinical value of our data may be both diagnostic and prognostic; patients with low anti-P$_1$ IgM antibodies present with a more aggressive phenotype and earlier relapse.

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Glycosphingolipids ( GSLs ) have critical roles in embryonic development, signal transduction, cell signalling, apoptosis, receptor modulation, cell adhesion, growth and cell differentiation and carcinogenesis ( Jarvis et al , 1996 ; Hakomori, 1998 ; Kasahara and Sanai, 1999 ). The presence of tumour-associated GSLs antigens have been observed in epithelial ovarian cancer ( Pochechueva et al , 2012 ), which is the fifth most common cause of death from all cancers in women and the leading cause of death from gynaecological malignancies ( Ozols, 2006 ).

Printed glycan array technology (a glycan-based discovery approach) previously demonstrated that naturally occurring anti-glycan antibodies ( AGA ) in plasma of ovarian cancer patients exhibited specificities towards synthetic P1 trisaccharide. In our previous study, we have demonstrated using a printed glycan array that anti-P1 antibodies can discriminate healthy controls from ovarian cancer patients ( Jacob et al , 2012 ). This study (on a Swiss Discovery Cohort) showed that anti-P1 antibodies of IgM, IgG and IgA together were significantly lower in ovarian cancer patients, thereby discriminating them from healthy controls. The predictive value of the printed glycan array was validated by two independent glycan-based immunoassays, ELISA and suspension array ( Pochechueva et al , 2011b ).

The P b , P a , carbohydrate antigens, commonly expressed on GSL, are members of the P blood group system that differ in their specificity based on their oligosaccharide sequences. In cancer, the globo ( P b ) and neolacto ( P a ) series are precursor GSL that give rise to well-known tumour-associated carbohydrate antigens, such as Forssman antigen ( Hakomori et al , 1977 ; Taniguchi et al , 1981 ) and Globo H ( Gilewski et al , 2001 ; Chang et al , 2008 ; Wang et al , 2008 ). High levels of P a (Galβ1-4Glcβ1-4Glcβ1-1Ceramide; GB3, CD77), P b (GalNAcβ1-3Galβ1-4Glcβ1-1Ceramide; GB4) and Globo H were described in the past ( Wenk et al , 1994 ).

As shown previously, naturally occurring AGA to P 1 have the potential to be used diagnostically in plasma of ovarian cancer patients. However, it remains unknown whether P 1 -bearing GSL are present on ovarian cancer cells and whether naturally occurring anti-P 1 antibodies to chemically synthesised carbohydrates in glycan-based immunoassays bind to these GSL antigens. To our knowledge, no published reports regarding the role of P 1 in malignant transformation, particularly in ovarian cancer, are available, and the molecular mechanisms underlying GSL expression on the cell surface, as well as its function, have yet to be elucidated. Therefore, this study aims (A) to determine the responsible naturally occurring AGA immunoglobulin class discriminating cancer from normal; (B) to determine whether the level of these antibodies are predictive of patient outcome; (C) to investigate whether the related P 1 glycan epitopes are present on cells isolated from ovarian cancer tissues as well as on ovarian cancer cell lines; (D) to compare the AGA profiles in ascites and matched plasma; (E) to compare monoclonal anti-P 1 antibodies produced in humans and affinity purified anti-P 1 antibodies isolated from ascites; and finally (F) to investigate the functional role of the P 1 antigen in ovarian cancer.

MATERIALS AND METHODS

Biospecimens. Two independent patient cohorts from two different continents were used for the experiments: (A) matched plasma and ascites from 11 serous FIGO stage III/IV cancer patients from the previously described Swiss Discovery Cohort ( Jacob et al , 2012 ); (B) plasma from 155 Australian samples (Australian Validation Cohort) comprising healthy controls, borderline tumour and ovarian cancer patients. The Australian Validation Cohort was split into: (1) borderline tumours and adenocarcinomas of the ovary, tube and peritoneum (‘tumour group’), and (2) healthy control women (‘control group’). Patients were either admitted with an adnexal mass to the Gynaecological Cancer Centre of the Royal Hospital for Women, Randwick, Australia or were seen as outpatients to the Hereditary Cancer Centre of The Prince of Wales Hospital, Randwick, Australia. All patients were prospectively included after giving informed consent in accordance with ethical regulations (Hunter Area Research Ethics 04/04/07/3.04; South Eastern Sydney Illawarra HREC/ AURED Ref/08/09/17/3.02). The processing of blood plasma samples was performed constantly on ice within 3 h after collection as previously described ( Jacob et al , 2011a , 2012 ). All clinicopathological data (Supplementary Table S1) such as FIGO stage and grade were incorporated in a specifically designed in-house database (‘PEROV’), which was developed using Microsoft Access ( Microsoft Corporation, Redmond, WA, USA ). Diagnosis and histopathological features were independently re-evaluated by a pathologist specialised in gynaecological oncology (JS). Blood samples were stored in aliquots at −80 °C.

Glycan-based immunoassays ( printed glycan array, suspension array and ELISA ). The printed glycan array was performed as previously described ( Huljev et al , 2009 ; Bovin et al , 2012 ; Jacob et al , 2012 ). AGA were detected by ImmunoPure goat anti-human IgA + IgG + IgM conjugated to long chain biotin (1 : 100, ‘Combo’, Pierce, Rockford, IL, USA ). To detect the immunoglobulin class, developed printed glycan array slides were individually incubated with 1 : 50-diluted biotin-conjugated goat anti-human IgA, IgG or IgM ( ZYMED Laboratories, Invitrogen, Carlsbad, CA, USA ). The coupling procedures for end-biotinylated glycopolymers and antibody binding were described before ( Pochechueva et al , 2011a , b ). Experimental protocol was performed as described previously ( Pochechueva et al , 2011b ). Exceptions were made with respect to the use of goat anti-human IgG-R-PE or IgM-R-PE secondary antibodies ( Southern Biotech Ass. Inc., Birmingham, AL, USA ). ELISA was performed as described previously ( Pochechueva et al , 2011b ).

Extraction and identification of GSLs from cancer tissue samples and IGROV1 cell line. Fresh primary tissue samples (~100 mg) from a serous ovarian cancer and an endometrioid peritoneal cancer patient were collected to analyse glycolipids by negative ion electrospray ionisation mass spectrometry ( LC-ESI-MS/MS ). Detailed analysis of the procedure is described in Supplementary Information.

Affinity purification of anti-P 1 antibodies. Ascites fluid was collected from a late-stage serous ovarian cancer patient during primary surgery. The ascites was processed by centrifugation at 4 °C, 3000 g for 15 min. Supernatant was aliquoted and kept frozen at −80 °C. Thawed ascites (50 ml) was filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA) and diluted three times in PBS (ph 7.4). Glycan-polyacrylamide-Sepharose stored in 20% (v/v) ethanol was washed with 10 volumes 20% ethanol, 20 volumes milliQ water and equilibrated with 10 volumes of PBS. Preprocessed ascites affinity purified against Galβ1-4Galβ1-4GlcNAcβ1-polyacrylamide-Sepharose (P 1 -PAA-Sep; 10 ml). A constant flow rate of 1 ml min −1 was controlled by the use of an auxiliary pump (Model EP-1 Econo Pump, Bio-Rad, Hercules, CA, USA). Protein content and buffer composition was recorded by UV at 280 nm and conductivity, respectively (BioLogic DuoFlow Workstation, Bio-Rad). The column was washed with PBS containing 0.05% (v/v) Tween 20, unplugged and stored overnight at 4 °C. The next day, the column was inserted back into the chromatography system and washed until no protein was detected anymore. Bound anti-P 1 antibodies were eluted using 0.2 M TrisOH (pH 10.2) and neutralised by 2.0 M Glycine HCl (pH 2.5). Eluted anti-P 1 antibodies were concentrated using the Amicon Ultra-0.5
Flow cytometry. GSL expression on the cell surface membranes was analysed by flow cytometry (CyAn ADP Analyzer, Beckman Coulter, Nyon, CH, USA) prior to antibody labelling. Unconjugated antibodies included anti-P1 human IgM (clone P3NIL100; Immucor Gamma, Rödermark, Germany), anti-P1 murine monoclonal IgM (clone OSK17; Immucor Gamma) and anti-Gb3 monoclonal IgG2b (CD77, P\(^2\)) (clone BGR23; Seikagaku Biobusiness Corporation, Tokyo, Japan). Biotin-conjugated antibodies included anti-human mouse IgM (BD Bioscience, Basel, Switzerland), rat anti-mouse IgM and rat anti-mouse IgG2b (BD Bioscience). Streptavidin conjugated to FITC (BD Bioscience) was used for fluorescence detection. Dead and apoptotic cells were separated from live cells using propidium iodide (BD Bioscience). Matching isotype monoclonal antibodies conjugated to FITC were used as controls (BD Bioscience). All investigated cell lines were gated individually to exclude debris, followed by single cell gating to remove dead cells and doublets. Data acquisition was performed using Summit v4.3 (CyAn ADP Analyzer, Beckman Coulter). Data analysis was performed using FlowJo v9 (Tree Star Inc., Ashland, OR, USA).

FACS sorting. IGROV1 cells were grown to 80% confluence, washed twice in PBS and harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich, Buchs, Switzerland). Cells were then washed in PBS containing 1% FCS and resuspended to 10\(^6\) cells ml\(^{-1}\). Cell suspension (100 \(\mu\)l) was stained with human anti-P1 IgM (BD Bioscience) as mentioned above and run on a BD FACS Vantage SE DiVa Cell Sorter (BD Bioscience). Gated antibodies included anti-P1 human IgM (clone P3NIL100; Immucor Gamma, Rödermark, Germany), anti-P1 murine monoclonal IgM (clone OSK17; Immucor Gamma) and anti-Gb3 monoclonal IgG2b (CD77, P\(^2\)) (clone BGR23; Seikagaku Biobusiness Corporation, Tokyo, Japan). Biotin-conjugated antibodies included anti-human mouse IgM (BD Bioscience, Basel, Switzerland), rat anti-mouse IgM and rat anti-mouse IgG2b (BD Bioscience). Streptavidin conjugated to FITC (BD Bioscience) was used for fluorescence detection. Dead and apoptotic cells were separated from live cells using propidium iodide (BD Bioscience). Matching isotype monoclonal antibodies conjugated to FITC were used as controls (BD Bioscience). All investigated cell lines were gated individually to exclude debris, followed by single cell gating to remove dead cells and doublets. Data acquisition was performed using FlowJo v9 (Tree Star Inc., Ashland, OR, USA).

Flow cytometry-based inhibition assay. Monoclonal human IgM antibody directed to P1 (Immucor Gamma, Rödermark) was preincubated either with Sepharose-P1-PAA or Sepharose-Pk-PAA (Lectinity Holdings, Moscow, Russia) in different amounts ranging from 0.015 \(\mu\)mol to 0.06 \(\mu\)mol for 60 min at RT. The supernatant was further processed as described in the flow cytometry section.

Colorimetric cell migration assay. Sub-confluent tumour cells were ‘starved’ from serum by incubation in serum-free media for 24 h, before harvesting using a non-enzymatic cell dissociation buffer (Sigma Aldrich, Buchs, Switzerland), washed twice and resuspended in serum-free media containing 5\% (w/v) BSA. Tumour cells (7.5 \(\times\) 10\(^3\) in 300 \(\mu\)l) were loaded into cell culture inserts containing a polyethylene terephthalate membrane with 8-micron pores (Millipore). The insets were assembled into 24-well plates with each well containing 700 \(\mu\)l of media with 10\% supplemented with fetal calf serum, which was used as chemotactant. After incubation for 18 h at 37 °C, the media in the interior of the insert was removed, and the entire insert was immersed in 400 \(\mu\)l of 0.2\% crystal violet/10\% ethanol for 20 min. The insert was washed several times in water, and the non-migrated cells in the interior of the insert were removed using a cotton-tip swab. After air-drying, five random areas of the insert were photographed, and cell counts were performed. Colorometric cell migration assay was performed three times.

In addition, parental IGROV1 cells were preincubated with 1\% (w/v) BSA in PBS, the corresponding isotype control (ChromPure human IgM, Jackson ImmunoResearch Laboratories, Inc., MILAN Analytica AG, Rheinfelden, Switzerland) and human anti-P1 IgM (clone P3NIL100), both antibodies in a final concentration of 500 \(\mu\)g ml\(^{-1}\). After 1 h incubation, cells were processed according to previously described cell migration protocol.

MTT assay. Cultures were incubated with 500 \(\mu\)g ml\(^{-1}\) (final concentration) MTT dye (Sigma-Aldrich, Buchs, Switzerland) in PBS for 3 h, followed by removal of the medium and dissolution of the violet crystals in 200 \(\mu\)l of DMSO. The optical density (absorbance at 540 nm) was measured with a SynergyH1 Hybrid Reader (Biotek, Luzern, Switzerland). The data are given as absorbance at 540 nm, representing cell viability as a function of araC concentration. Each experiment was performed independently twice from multiple cultures.

Real-time cell migration analysis (xCELLigence). Real-time cell analysis (RTCA; xCELLigence System, Roche Diagnostics GmbH, Mannheim, Germany) was used to investigate cell migration in P1-low and -high serous ovarian cancer IGROV1 cells in a label-free environment (Solly et al., 2004; Ke et al., 2011). Migration was examined on 16-transwell plates (Roche Diagnostics GmbH) with microelectrodes attached to the underside bottom of the membrane for impedance-based detection of the migrated cells. Prior to each experiment, cells were deprived of FCS over a period of 24 h. Initially, 160 \(\mu\)l ‘chemoattractant’ media (RPMI 1640 containing 10\% FCS) and 50 \(\mu\)l RPMI 1640 containing 1\% FCS was added to the lower and upper chambers, respectively. Sterile PBS was loaded into the evaporation trophs. CIM-16 plates were further prepared according to the manufacture’s protocol. Background signals generated by the cell-free media were recorded. Cells were harvested using trypsin, counted and re-suspended in an appropriate volume of RPMI 1640 containing 1\% FCS. Cells (100 000 cells per 100 \(\mu\)l medium) were seeded onto the upper chamber of the CIM-16 plate and allowed to settle onto the membrane. Cell-free media was used as negative control. Each experiment was performed two times in duplicates. The programmed signal detection for quantification of the cell index was measured every 15 min over a period of 30 h. In an independent migration assay, 5 \(\mu\)M of 1-beta-D-arabinofuranosylcytosine (araC; Sigma-Aldrich), a DNA polymerase inhibitor, was added to avoid possible effects on migration caused by cell proliferation.

Statistical analysis. Detailed statistical procedure applied is described in Supplementary Information.

RESULTS

IgM antibodies in plasma against P\(_1\) trisaccharide are reduced in patients with tubal, peritoneal and ovarian cancer. In our previous study, the use of three glycans-based immunoassays (printed glycan array, ELISA and suspension array), detecting IgM, IgG and IgA together, revealed significant AGA interactions with the members of the P blood group system (Pochechueva et al., 2011a, b; Jacob et al., 2012). Overall, less AGA to P1, trisaccharide (printed glycan array, ELISA and suspension array) and P\(_K\) (printed glycan array) were observed in the plasma of the cancer patient group compared with the control group (Pochechueva et al., 2011b; Jacob et al., 2012).

In this study, we investigated the levels of IgM and IgG AGA in the plasma of an independent Australian Validation Cohort (n = 155). The cohort consisted of a ‘benign’ control group (healthy controls and benign gynaecological conditions; n = 81) and a ‘tumour’ group (ovarian borderline tumours, ovarian, tubal and peritoneal cancers; n = 74) (Supplementary Table S1). Based on suspension array data, AGA to the P1, trisaccharide belonged mainly to the IgM class (median, IQR; 9.948log(MFI), 9.351–10.909log(MFI)) in all the tested samples. Significantly lower IgM anti-P1 antibody levels were observed in the blood plasma samples of the tumour as compared with the control group (P = 0.0002) (Figure 1A). The tumour group revealed 15/74 (20.3\%) samples having lower AGA levels compared with the lowest control group sample. Logistic regression did not reveal any relationship between
clinopathological parameters and anti-P1 IgM antibody signatures in the tumour group. In contrast to IgM, generally lower IgG antibody levels (median, IQR: 7.35log(MFI), 6.726–7.858log(MFI)) were observed. Both the control and tumour groups were similar in IgG AGA levels, with no significant difference in between the groups (P = 0.7248, Figure 1A). We have compared various clinical parameters recorded along with collection of plasma samples for each patient. Statistical evaluation revealed a significant discrimination in FIGO stage of the non-mucinous cancer of ovary, tube and peritoneum group (FIGO Stage, non-mucinous cancer of the ovary, tube and peritoneum I/II vs III/IV, P = 0.01773, t-test). The remaining investigated clinical parameters were not significantly different in their anti-P1 IgM antibody levels mentioning Grade (G1 vs G2/3, P = 0.2883; t-test) or tumour origin (ovary vs tube vs peritoneum, P = 0.322; ANOVA). An increasing age has previously been demonstrated to be associated with reduction of AGA in a cohort of 48 control plasma samples using glycoprotein arrays (Oyelaran et al, 2009). To investigate the influence of age on anti-P1 antibodies (IgG and IgM), we have applied Pearson’s correlation to distribution of AGA levels of IgM and IgG to covalently attached P1 trisaccharide using suspension array. Decreased AGA levels in cancer patients (n = 23; lower than median fluorescence signal) have slightly earlier relapse (P = 0.055) than those with high antibody levels (n = 25, higher than median fluorescence signal).

The base peak chromatograms of the glycan alditols released from the GSLs extracted from serous ovarian (Figure 2A (a)) and peritoneal (Figure 2B (a)) cancer tissue showed several components. Globotriaosylceramide (Gb3, P5) was detected as [M-H]^-/C0 at m/z 505.31^- and low intensities, and the extracted ion chromatogram (EIC) showed it eluting at 15.4 min (Figures 2A b(i) and B b(i)). The MS^2 spectra of the precursor ion at m/z 505.31^- (Figure 2C (i)) showed prominent B- and C-type fragment ions (B1 at m/z 161.11^- , C1 at m/z 179.22^- and C2 at m/z 341.11^- ) corresponding to a (Hex3) or Gal-Gal-Glc sequence of Pk. Characteristic cross ring fragment ions corresponding to 2,4^/-A2 at m/z 221.21^- and 0,2A3 at m/z 281.01^- were also present in the spectrum, thereby confirming the presence of the 4-linked terminal Gal to the (Gal-Glc) disaccharide (Karlsson et al, 2010). A similar study indicated the absence of 0,2A2 fragment ion in the MS^2 spectrum of isoglobotriaosylceramide (Galz1-3Galβ1-4Glcβ1) (Karlsson et al, 2010) while previous reports have also noted that this characteristic cross ring cleavage was useful in distinguishing between Type 1 (Galβ1-3GlcNAc) and Type 2 (Galβ1-4GlcNAc) chains commonly found in mucins (Chai et al, 2001; Robbe et al, 2004; Everest-Duss et al, 2012).

Globotetraosylceramide (P antigen, Gb4) was detected at [M-H]^-/C0 m/z 708.31^- and the EIC showed it to elute at 17.0 min (Figures 2A (ii) and B (ii)). Despite appearing at low intensities, the MS^2 spectra of the precursor ion at m/z 708.31^- (Figure 2C (ii)) was indicated by the B- and C-type fragment ions (B1 at m/z 202.01^- , B2 at m/z 256.01^- , C1 at m/z 220.01^- and C2 at m/z 382.11^- ) which corresponded to the tetrasaccharide sequence, HexNAc-C1Hex2 or GalNac-Gal-Glc-Glc of the P antigen. Several Y-ion fragment ions occurring at m/z 341.01^- and m/z 708.31^- further confirmed the presence of 4-linked terminal Gal to the internal Galβ1-4Glcβ1 of the Gb4 tetrasaccharide. The pentasaccharide P1, HexNAcHex3 or GalNac-Gal-Glc-Glc-Glc of the P antigen, several Y-ion fragment ions seen from the reducing-end were also identified at m/z 343.21^- (Y3) and m/z 505.21^- (Y1) while the diagnostic cross ring cleavage corresponding to 2,4^/-A2 at m/z 424.11^- and 0,2A3 at m/z 476.11^- further confirmed the presence of 4-linked Gal to the internal Galβ1-4Glcβ1 of the Gb4 tetrasaccharide.

The pentasaccharide P1, HexNAcHex4 or Gal-Galα-Nac-Gal-Glc-Glc of the P antigen, a prominent 2,4^/-A2 fragment ion at m/z 476.11^- in the MS^2 spectra was also characteristic of the terminal Gal residue linked via a 4-linkage to the Gal-GlcNac-Gal-Glc tetrasaccharide. The cross ring cleavage at 0,2A3-H2O at m/z 425.11^- and the
absence of the $^{0,2}_{\text{A_4}}$ at m/z 646.1$^{1+}$ further demonstrates the 4-substitution of the GlcNAc residue and the 3-substitution of the internal Gal and thus tentatively identified this compound as Gal1-4Gal1-4GlcNAc$^{1-}$-3Gal1-4Glc, the P$_1$ antigen. The three P blood group antigens were thus shown to be expressed on the GSL extracted from the cancer tissue of these patients, we established an experimental cell culture model for the investigation into the functional role of P$_1$. Our panel of immortal cell lines, including human ovarian surface epithelial cells (HOSE6-3 and HOSE17-1) and various ovarian cancer cell lines ($n = 6$), were proliﬁed for P$_1$ and P$^b$ expression using flow cytometry (Figure 2D). Serous ovarian cancer cell line IGROV1 was heterogenic for P$_1$ expression (mean 34.1% ranging from 22.8% to 52.8%) based on human anti-P$_1$ IgM (clone P3NIL100) antibody. The second antibody used in this study, monoclonal murine anti-P$_1$ IgM (clone OSK17), conﬁrmed P$_1$ expression on IGROV1 (mean 22.3% ranging from 22.0% to 33.6%). IGROV1 cells were also positive for P$^b$ (mean 33.6% ranging from 12.0% to 54.0%). The normal control cell line HOSE6-3 was negative for both P$_1$ and P$^b$ as were the remaining ovarian cancer cell lines ($n = 5$).

To verify the presence of P$_1$ on IGROV1 cell line, we isolated GSLs from this cell line and analysed the released glycan aldolts using negative ion mode LC-ESI-MS/MS. The P$_1$ pentasaccharide at $m/z$ 870.3$^{1+}$ was shown to elute at 20.3 min, and the MS$^2$ spectra consisted of B$_2$ ($m/z$ 323.1$^{1+}$), Y$_3$ ($m/z$ 546.3$^{1+}$) and Y$_4$

Figure 2. P$_1$ is expressed on cancer tissue cells. (A, B) Base peak chromatograms shown for serous ovarian cancer (A (a)) and endometrioid peritoneal cancer tissue (B (a)). Selected mass peaks (red arrow) corresponding to the composition of the P$^b$ trisaccharide (Hex$^3$.HexNAc$^i$) (A and B b(i)); $m/z$ 505.3$^{3+}$, P tetrasaccharide (Hex$^3$.HexNAc$^i$) (A and B c (ii)); $m/z$ 708.3$^{1+}$, and P$_1$ pentasaccharide (Hex$^3$.HexNAc$^i$) (A and B d(iii)); $m/z$ 870.3$^{1+}$) are represented as an extracted ion chromatogram (EIC). (C) MS$^2$ spectrum of P$_1$ trisaccharide (Gal1-4Gal1-4Glc1-1) (i), P tetrasaccharide (GalNAc1-3Gal1-4Gal1-4Glc1-1) (ii) and P$_1$ pentasaccharide (Gal1-4Gal1-4GlcNAc1-3Gal1-4Glc1-1) (iii). (D) Representative flow cytometry results shown as contour plots with outliers demonstrate P$_1$ and P$^b$ negative cell lines HOSE6-3 and SKOV3. IGROV1 was detected positive with IgMs for both P$_1$ and P$^b$. Representative contour plots showing P$_1$ and P$^b$ expression (FITC; ordinate) and forward scatter (FSC; abscissa). Given percentage corresponds to P$_1$/P$^b$-positive cells.
(m/z 708.31⁻⁻⁻) fragment ions, which corresponded to the Gal-Gal-GlcNAc-Gal-Glc sequence. The terminal Galz1→4Gal linkage was also determined by the cross ring fragment ion at 586.31⁻⁻⁻ fragment ion observed at m/z 648.31⁻⁻⁻ (Supplementary Figure S2).

Monoclonal anti-P₁ IgM bind IGROV1 cells. Both P₁ and Pₖ share terminal disaccharide structure of composition Galz1→4Galβ1→4Glc(Nac). The monoclonal antibody to P₁ (clone P3NIL100) was used in a flow cytometry-based inhibition assay. Sepharose conjugated PAA-lactose, -N-acetyllactosamine (LacNAc), -P₁ trisaccharide, -Pₖ was incubated with the monoclonal antibody to P₁, before immunostaining of IGROV1 cells to observe the degree of inhibition of IgM antibody binding to the expressed P₁ on IGROV1 cell surface. In this experiment, repeated three times, flow cytometry of 52.8% P₁-positive IGROV1 cells revealed complete inhibition of monoclonal anti-P₁ antibody by preincubating with 0.015 μmol P₁ glycoabsorbents (0.04%). In contrast, less reduction (28.3% by 0.06 μmol Pₖ) of bound anti-P₁ antibodies to IGROV1 cells was observed in case of Pₖ glycoabsorbent (Figure 3). Anti-P₁ antibodies were only slightly inhibited by preincubation with glycoadsorbents Lactose (0.06 μmol; 46.8%) and LacNAc (0.06 μmol; 44.8%). This clearly demonstrates that IGROV1 cells express P₁ on their cell surface, and monoclonal antibody to P₁ shows clearly higher affinity to P₁ compared with Pₖ. It also demonstrates specifically that galactose z1→4 link is required for antibody binding and that lactose and LacNAc are not primarily epitopes of P₁.

Anti-glycan antibodies levels are similar in ascites and blood plasma. To determine whether anti-tumour antibodies were expressed in ascites as well as plasma of patients, we investigated the presence and distribution of AGA in matched ascites and blood plasma samples (n = 11 patients) for IgA, IgG and IgM together and separately using the printed glycan array. This was also done for epitope mapping of ascites-derived human anti-P₁ antibodies.

We detected a broad spectrum of AGA in both plasma and ascites (Figure 4). The fluorescence scan of the binding to the immobilised glycans on the array showed high amplitude of AGA in ascites of IgG, IgM and IgA in both 50 μM and 10 μM glycan-printed arrays (Supplementary Figure S3). We observed the highest median relative fluorescence signals in ascites (median, 32.75 × 10⁴ RFU) and in plasma (43.33 × 10⁴ RFU) for anti-glycan IgM antibodies. Anti-glycan IgG antibodies showed lower interaction with the glycans (ascites (8.11 × 10⁴ RFU), plasma (9.80 × 10⁴ RFU) as well as anti-glycan IgA (ascites (8.8 × 10⁴ RFU), plasma (10.4 × 10⁴ RFU)). Different levels of AGA to P₁, trisaccharide were detected in all the tested ascites and plasma samples (Figure 4). Highest binding to P₁ was observed for IgM (ascites (18.1 × 10⁴ RFU), plasma (14.3 × 10⁴ RFU)) compared with IgG and IgA.

Matched ascites and plasma AGA signals were not dependent on the volume of patient’s ascites. Based on raw data sets of 50 μM printed glycan arrays, strong correlation between matched ascites and blood plasma samples was observed (Figure 4): IgA + IgG + IgM (CCC = 0.889), IgA (CCC = 0.888), IgG (CCC = 0.961), and IgM (CCC = 0.950). Correlations were similar for 10 μM printed glycans. This demonstrates that detected AGA levels were independent of the volume of ascites.

Naturally occurring anti-P₁ antibodies in ascites bind to cancer cells expressing P₁ on their cell surface. The results achieved from the above experiments demonstrated that (A) anti-P₁ antibodies detected by glycan-based immunoassays are reduced in plasma of cancer patients, (B) P blood group-related glycans are detectable in tissue samples of cancer patients, (C) IGROV1
signals for 11 patients each with ascites and blood plasma. AGA to P1 trisaccharide are highlighted in black. Median signal intensity over all signals (glycan array slides) and independently for IgA (same scatterplot. Cutoff (5%) separating background signals from real AGA binding are indicated by a solid line for ascites and plasma in the same way as the plasma-derived autoantibodies.

We investigated AGA to P1 trisaccharide from an ascites sample of a late-stage serous ovarian cancer patient. The ascites fluid was first applied to the IGROV1 cell line in a 1:3 dilution, in which 24.3% of cells were stained positive with ascites-derived IgM antibodies (Figure 5A). Based on this result, we proceeded to affinity purify antibodies bound specifically to P1 trisaccharide conjugated to sepharose beads. Affinity-purified proteins yielded a maximum amount of IgM of 24 μg after concentration. Detection of purified anti-P1 antibodies of class IgM revealed binding to P1-expressing IGROV1 (15.1%) (Figure 5A). In contrast, the ovarian cancer cell line SKOV3, negative for P1 and Pk expression, had a positive staining of only 0.29% using the purified IgM-P1 antibodies. This demonstrates for the first time that ascites-derived AGA directed to P1 trisaccharide also bind to naturally expressed P1 antigen such as Gal\(^{1–4}\)GlcNAc and Gal\(^{1–3}\)GalNAc and Gal\(^{1–4}\)Glc(NAc) with 63.0% and Gal\(^{1–4}\)Gal with 36.3% (Gal\(^{1–3}\)Gal), and Pk, P\(_k\), andLe\(_\alpha\) and \(\alpha\)-rhamnose coupled beads revealed only minor binding. In the printed glycan array (Figure 5C), we applied a threshold of 5% to the highest median fluorescence signal (19 443 RFU; Gal\(^{1–4}\)Gal\(^{1–4}\)Glc-sp3) to eliminate potentially unspecific binding as described previously (Huflejt et al, 2009). We identified that affinity-purified IgM-P1 antibodies bound to P1 blood group-related structures as the top 10 glycans (Supplementary Table S2). Most of the identified low-affinity binding to glycan structures had substructures of the P1 antigen such as Gal\(^{1–4}\)Glc(NAc) with 63.0% and Gal\(^{1–4}\)Gal\(^{\beta}\) with 22.2% binding reactivity (Supplementary Table S2). This demonstrates that affinity-purified IgM-P1 antibodies preferentially bound to both Pk and P1 trisaccharide.

\(P_1\) expression leads to elevated migration rate in ovarian cancer cells. GSLs on the cell surface are described to have several functions in cellular processes, such as pathogen recognition, angiogenesis, cell motility and cell migration (Panjwani et al, 1995; Todeschini et al, 2008; Hakomori, 2010). Migration is a common feature in cancer cells, and therefore we investigated whether or not the presence of \(P_1\) affects cell migration by using a flow cytometry-based cell sorting of IGROV1 cells to separate \(P_1\)-high- from \(P_1\)-low-expressing subpopulations. After further sub-cultivation, \(P_1\) expression in \(P_1\)-low IGROV1 cells was determined by murine monoclonal anti-P1 IgM (OSK17; 18%) or human anti-P1 IgM (P3NIL100; 33.3%). In contrast, \(P_1\)-high-expressing IGROV1 cells were 50.0% and 66.1% positive for \(P_1\), respectively, for these antibodies (Figure 6A).

Utilising these \(P_1\)-sorted cell lines, we hypothesised that the presence of \(P_1\) affects cell migration. Significantly higher migration was detected in \(P_1\)-high-expressing when compared with \(P_1\)-low-expressing IGROV1 cells after an 18 h incubation period (\(P = 0.0006\)) (Figure 6B), as shown by colorimetric cell migration assay. In addition to end-point migration assay, real-time and label-free measurement of \(P_1\)-sorted IGROV1 cells was performed using the xCELLigence system. A number of 100 000 cells was seeded into each well of the upper chamber, and migration through the microporous membrane were recorded as cell index. IGROV1 cells of a late-stage serous ovarian cancer patient. The ascites fluid was initially bound to both Pk and P1 trisaccharide. Therefore the following series of experiments were aimed to investigate whether IgM antibodies derived from ascites bind to IGROV1 and in particular to P1 presented on its cell surface in the same way as the plasma-derived autoantibodies.

Next, we investigated whether purified IgM-P1 antibodies bind to glycan structures other than P1, (potential cross-reactivity). ELISA to a limited number of glycoconjugates revealed in the case of IgM specific binding to P1, trisaccharide, with cross-reactivity to Gal\(^{1–3}\)GalNAc and Gal\(^{1–4}\)GlcNAc (Figure 5B). Suspension and printed glycan array were additionally utilised to study the cross-reactivity to broader range of glycan structures, other than P1. In suspension array, the binding of ascsites-derived anti-P1 antibodies to P1, Pk, Le\(\alpha\) and \(\alpha\)-rhamnose was tested. No preferential binding was observed comparing P1 and Pk (P1 4.11 log(MFI); Pk 4.24 log(MFI), IgM class). In contrast, Le\(\alpha\) and \(\alpha\)-rhamnose coupled beads revealed only minor binding. In the printed glycan array (Figure 5C), we applied a threshold of 5% to the highest median fluorescence signal (19 443 RFU; Gal\(^{1–4}\)Gal\(^{1–4}\)Glc-sp3) to eliminate potentially unspecific binding as described previously (Huflejt et al, 2009). We identified that affinity-purified IgM-P1 antibodies bound to P1 blood group-related structures as the top 10 glycans (Supplementary Table S2). Most of the identified low-affinity binding to glycan structures had substructures of the P1 antigen such as Gal\(^{1–4}\)Glc(NAc) with 63.0% and Gal\(^{1–4}\)Gal\(^{\beta}\) with 22.2% binding reactivity (Supplementary Table S2). This demonstrates that affinity-purified IgM-P1 antibodies preferentially bound to both Pk and P1 trisaccharide.

Matched ascites and blood plasma samples (n = 11) were profiled for binding of IgA + IgG + IgM (n = 22 printed glycan array slides) and independently for IgA (n = 22), IgG (n = 22) and IgM (n = 22) to printed glycan array slides. One scatterplot represents signals for 11 patients each with ascites and blood plasma. AGA to P1 trisaccharide are highlighted in black. Median signal intensity over all signals calculated for ascites and plasma are shown by horizontal and vertical solid lines, respectively. Antibody signals (RFU x 10\(^3\)) are shown on ascites and blood plasma axis. Cutoff (5%) separating background signals from real AGA binding are indicated by a solid line for ascites and plasma in the same scatterplot.

Figure 4. Ascites and blood plasma from cancer patients contain comparable levels of anti-glycan antibodies independent of immunoglobulin class and volume of ascites. Matched ascites and blood plasma samples (n = 11) were profiled for binding of IgA + IgG + IgM (n = 22 printed glycan array slides) and independently for IgA (n = 22), IgG (n = 22) and IgM (n = 22) to printed glycan array slides. One scatterplot represents signals for 11 patients each with ascites and blood plasma. AGA to P1 trisaccharide are highlighted in black. Median signal intensity over all signals calculated for ascites and plasma are shown by horizontal and vertical solid lines, respectively. Antibody signals (RFU x 10\(^3\)) are shown on ascites and blood plasma axis. Cutoff (5%) separating background signals from real AGA binding are indicated by a solid line for ascites and plasma in the same scatterplot.
percentage of migrated cells was significantly lower (reduction by 64%; \( P < 0.001 \)) in cultures preincubated with the anti-\( P_1 \) IgM antibody. Pretreatment with the respective IgM isotype control did not affect the migration of IGROV1 cells.

**DISCUSSION**

Unlike \( P^k \), which has been extensively studied in the areas of verotoxin-mediated cytotoxicity, human immunodeficiency virus infection, immunology and epithelial carcinogenesis, there is, to our knowledge, no published study of \( P_1 \) and its potential presence, immunogenicity and its functional role in oncogenesis. Using glycan-based immunoassays, we have previously demonstrated that naturally occurring AGA significantly distinguish healthy controls from ovarian cancer patients (Pochechueva et al, 2011b; Jacob et al, 2012). The present study demonstrates that: (A) an unknown subpopulation of cancer patients show reduced levels of anti-\( P_1 \) IgM antibodies in an extended and independent cohort from another continent (Australian Validation Cohort), (B) \( P_1 \) carbohydrate antigens are detectable in cancer tissue as well as on the cell surface of cultured ovarian cancer cells, (C) ascites contains similar anti-glycan IgM-P1 antibody levels compared with blood plasma independent of its volume at the primary diagnosis, (D) naturally occurring AGA of IgM class derived from ascites of a
Figure 6. Elevated P₁ expression results in increased migration rate. (A) FACS-sorted subpopulations of P₁-low- and -high-expressing IGROV1 cells. Representative histograms showing P₁ expression (abscissa) on cell-sorted IGROV1 cells to normalised cell count (ordinate). P₁ distribution for unstained controls (red), P₁-positivity P3NIL100 antibody (green) and OSK17 antibody (blue). (B) Colorimetric cell migration assay showing enhanced migratory ability of IGROV1 cells expressing high compared with low levels of P₁. Stained cells counted in five fields and averaged. Representative image of cell sorted subpopulations of migrated cells (stained violet) after 18 h. (C) RTCA assay for P₁-sorted IGROV1 cells showing the migrated cells (cell index) in bar graphs at the time points 0 h, 10 h, 20 h and 30 h. Left bar graph shows RTCA experiment without araC, right bar graph with araC as proliferation inhibitor. Representative figure out of two independent experiments. (D) Bar chart showing the inhibition of cell migration of human IGROV1 cells incubated with anti-P₁ IgM compared with corresponding incubation with IgM isotype control. Number of migrated cells was normalised to control. Not significant (NS); *P<0.05, **P<0.01, ***P<0.001.

Ovarian cancer patient bind to naturally expressed and chemically synthesised P₁ on glycan arrays, and (E) the presence of P₁ on ovarian cancer cultured IGROV1 cells leads to enhanced migration.

Our results demonstrate that the significantly lower anti-P₁ antibody levels observed in cancer patients were primarily due to the reduction of IgM but not IgG antibodies. This is in full concordance with the literature on the IgM type-anti-Thomsen-Friedenreich antibodies (Desai et al, 1995) and anti-Lewis C antibodies in breast cancer (Bovin, 2013). A very recently published work demonstrated significantly reduced human IgM antibodies to another GSL (N-glyclyneuraminyl)-lactosylceramide (NeuGcGM3) in non-small cell lung cancer patients (Rodriquez-Zhurbenko et al, 2013). Therefore we can assume that IgM class of naturally occurring AGA was able to discriminate between cancer and healthy controls. Natural IgM antibodies belong to the innate immune system and are primarily produced by B1 or CD5+ cells (Boes, 2000; Martin and Kearney, 2001; Viau and Zouali, 2005). As part of the natural immunity, the binding of IgM to conserved carbohydrate structures acts as a first barrier to all invasive particles (’external’) and alterations on proteins and lipids within an organism (’internal’) (Vollmers and Brandlein, 2007). To date, several human monoclonal antibodies directed to tumour-associated carbohydrate antigens have been isolated. For instance, the monoclonal IgM antibody, SAM-6, specific for cancerous tissue was first derived from a gastric cancer patient (Pohle et al, 2004). Later, it was found that the receptor for SAM-6 is a tumour-specific O-linked carbohydrate epitope on GRP78, a central regulator of endoplasmatic reticulum in protein folding (Li and Lee, 2006). PAM-1, another monoclonal IgM antibody, binds to a tumour-specific N-linked carbohydrate epitope, a posttranslational modification on cysteine-rich fibroblast growth factor receptor, CFR-1. This antibody also inhibited tumour growth in vitro and in animal model systems by inducing apoptosis (Brandlein et al, 2003, 2004a, b). Consistent with the findings of Vollmers and colleagues regarding SAM-6 and PAM-1, our observation based on hierarchical clustering indicates that AGA preferentially bind to substructures of the glycan epitope (Jacob et al, 2012). Besides that the decrease of AGA mainly of the IgM class in gynaecological cancer patients (Jacob et al, 2012) also points to an involvement of naturally occurring IgM autoantibodies, which recognise GSL structures. We further propose that Galz1-4Galβ1-4Glc(Nac) (present in P₁, P₄ and P₁ antigens) are a result of malignant transformation and could serve as an ‘internal’ epitope on GSLs that is recognised by naturally circulating antiglycan IgM antibodies.

The observation of lower levels of anti-P₁ IgM compared with anti-P₁ IgG in cancer patients is very intriguing, and although exploring the underlying mechanisms was beyond the scope of the study, we speculate on a number of possibilities. The elevated presence of corresponding antigens on cancer cells, in our case P₁, GSL, may be bound by circulating anti-P₁ IgM and therefore no longer be freely floating in the plasma and ascites. Another potential mechanism may be tumour-induced immune suppression. It is known that ovarian cancers employ a range of strategies, such as secretion of immunosuppressive cytokines, to facilitate their escape from immune destruction (Lavoue et al, 2013). It is possible that this may lead to reduced antibody production against cancer-associated antigens, for example, P₁, conferring a survival advantage to cancer cells. The antibody repertoire in cancer patients would therefore be different. Another possible mechanism is the occupation of anti-P₁ IgMs by cancer cells (including circulating tumour cells) by virtue of their surface expression of
P₁, leading to a reduction in the amount of unbound antibody in plasma and/or ascites. The formation of immune complexes consisting of P₁ antigen shed from cancer cells, and anti-P₁ IgMs, may further contribute to this reduction. A prominent example in the literature for the formation of circulating immune complexes consisting of Lewis x and antibodies has been shown in *Helicobacter pylori*-infected human (Chmiela et al, 1998). With regards to no change being observed in the anti-P₁ IgG levels, we expected differences preferentially in the case of IgM rather than of IgG as glycan-based antigens are T-cell-independent antigens and are recognised by the innate immune system.

Our mass spectrometry results demonstrated that these P₁, Pₖ and P antigens are expressed on ovarian and peritoneal cancer tissues. The functional role of the GSL that carry these structures in carcinogenesis is poorly understood, especially in the case of P₁ and P. It was recently described that a monoclonal anti-Pₖ antibody (E3E2) inhibited angiogenesis and tumour development (Desselle et al, 2012). Enhanced expression of Pₖ has also been shown to cause doxorubicin resistance in breast cancer cells (Gupta et al, 2012). Cisplatin-resistant pleural mesothelioma cells were shown to be sensitised to cisplatin by the addition of sub-toxic concentrations of Verotoxin 1 (Johansson et al, 2010). In addition, we also demonstrate that P₁ and Pₖ antigens are also present on the surface of the IGROV1 serous ovarian cancer cells. IGROV1 is, therefore, to the best of our knowledge, the only immortal cell line from several investigated that expresses P₁. Using monoclonal antibodies against P₁, only minor cross-reactivity to other glycan structures was observed in glycan-based immunoassays (not shown). Furthermore, based on the flow cytometry inhibition assay using IGROV1 cells as an investigative model, the specificity of monoclonal antibodies to P₁ as shown by the full saturation of anti-P₁ antibodies by inhibition with 0.06 μmol glycans on sepharose beads indicates that IGROV1 expresses P₁ on the cell surface.

Serous pelvic masses commonly present with malignant ascites, a plasma–protein-rich intraperitoneal exudate of up to several litres. Several pathophysiological mechanisms are necessary for malignant ascites occur: (a) decreased lymphatic ascites absorption, (b) increased capillary permeability, (c) increased overall capillary membrane-surface area available for filtration, and consequently (d) an increased intraperitoneal oncotic pressure due to intraperitoneal protein concentration (Tamsma et al, 2001). Using the printed glycan array, we observed an unexpectedly rich spectrum of AGA with similar amounts in plasma and ascites. These findings suggested that there is an equilibrium between both body fluids. Early lymphatic obstruction is one of the first manifestations of an inflammatory reaction (Feldman, 1975). As IgM has an important role in inflammation with multivalent antigen binding due to its pentameric structure and as the first immunoglobulin class produced in a primary response to an antigen, lymphatic obstruction may result in a rapid increase in IgM secreting plasma cells. Natural circulating AGA have also been described in various inflammatory conditions, including diabetes mellitus type 1 (Gillard et al, 1989), chronic inflammatory bowel and Crohn’s disease (Malickova et al, 2006) and in patients with cancer (Young et al, 1979; Springer, 1984; Springer et al, 1988; Desai et al, 1995).

We also investigated the expression of A4GALT P₁- and Pₖ-profiled cell lines. As seen in our study, A4GALT is overexpressed in the P₁- and Pₖ-positive ovarian cancer cell line IGROV1 (Jacob et al, 2011b), suggesting that A4GALT is not only involved in Pₖ but also in P₁ synthesis in ovarian cancer cells. We propose that A4GALT is involved in the progression of various ovarian and peritoneal cancers; however, the molecular link between A4GALT mRNA levels and P₁ expression remains unknown. This is consistent with suspension array results in which no tested clinical parameters were shown to correlate with lower AGA levels to P₁ trisaccharide. A recently identified single nucleotide polymorphism (CT conversion) encoding an additional exon in the genomic region of A4GALT (Thuresson et al, 2011) could probably explain the presence of P₁ in cancer cells. However, it is unclear whether the overexpression of A4GALT is causative for the synthesis of P₁ or Pₖ in profiled cancer cell lines. The invasive phenotype of colon cells lacking Pₖ could be induced and inhibited by the transfection of Gb3 synthase (A4GALT) or RNA interference, respectively (Kovbasnjuk et al, 2005). Another immunohistochemistry-based study showed that Pₖ expression was elevated in colorectal cancers and their metastases; however, A4GALT mRNA levels, protein expression or galactosyltransferase activity were not investigated (Falguieres et al, 2008). The potential role of A4GALT in cancer initiation or progression needs to be elucidated in future studies with respect to all P blood group-related glycans.

For the first time, we also observed migration rate in P₁-high-expressing cells, and this was even more obvious when araC, a proliferation inhibitor, was added to the cultures (Roy et al, 2006). The addition of araC confirmed that the observed difference in migration was indeed due to altered cell migration and not cell proliferation. Our results suggest an involvement of P₁ in cell migration, but the underlying molecular mechanisms are unknown.

Finally, this study provides further evidence that P blood group-related antigens have a role in carcinogenesis and those naturally occurring anti-glycan IgM antibodies against them may have the potential to discriminate ovarian cancer patients from healthy individuals. If these surface antigens prove to be indeed tumour-specific, they may become candidate molecular targets for potential imaging tools for confocal fluorescence endoscopy and positron emission tomography and as tools in targeted immunotherapy (Janssen et al, 2006; Viel et al, 2008).

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