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The lectin *Helix pomatia* agglutinin recognizes O-GlcNAc containing glycoproteins in human breast cancer

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There has been considerable interest in understanding the epitopes that bind the lectin *Helix pomatia* agglutinin (HPA) in breast cancer as the lectin has been shown to identify glycosylation changes associated with the development of metastatic disease. HPA has previously been shown to recognize aberrant O-linked α-*N*-acetylgalactosamine (GalNAcα)/mucin glycosylation in cancer, including exposed Tn epitopes. However, recent glycan-array analysis reported that diverse epitopes are also recognized by the lectin, e.g. consortium for functional glycomics (CFG) data: GalNAcα1,3Gal; β-GalNAc; GlcNAcβ1,4Gal. The intriguing observations from the CFG array led to this study, in which HPA-binding epitopes were localized and characterized in an in vitro model of breast cancer metastasis. HMT3522 (benign disease), BT474 (primary cancer) and T47D/MCF7 (metastatic cancer) cells were assessed in confocal microscopy-based co-localization studies and a glycoproteomic analysis based on 2-dimensional electrophoresis (2DE), western blotting and mass spectrometry was adopted. HPA binding correlated with levels of integrin α6, transcription factors heterogeneous nuclear ribonuclear protein (HnRNP) H1, HnRNP D-like, HnRNP A2/B1 as well as heat shock protein 27 (Hsp27), glial fibrillary acidic protein and enolase 1 (ENO1). These glycoproteins were non-detectable in the non-metastatic breast cancer cell lines. The recognition of HnRNPs, Hsp27 and ENO1 by HPA correlated with O-GlcNAcylation of these proteins. Integrin α6 was the most abundant HPA glycoprotein in the breast cancer cells with a metastatic phenotype; this concurred with previous findings in colorectal cancer. This is the first report in which HPA has been shown to bind O-GlcNAcylated transcription factors. This class of proteins represents a new means by which HPA differentiates cancer cells with an aggressive metastatic phenotype.

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Introduction

Breast cancer remains a major clinical problem worldwide with most women succumbing to the disease as a result of the metastatic spread of their primary tumor (Chambers et al. 2001; Steeg 2006). Although prognosis and treatment of primary breast cancer have improved over the past few decades, largely as a result of hormonal and biological treatments, identification of a marker for early prediction of metastatic breast cancer has remained elusive.

It is widely accepted that altered glycosylation of cell surface glycoproteins accompanies malignant transformation. Glycoproteins with such altered glycoforms may offer scope as biomarkers for monitoring the metastatic spread of breast and other cancers. Many studies have used lectins—carbohydrate-binding proteins of non-immune origin to identify changes in glycosylation in cancer cells and tissues (Baldus and Hanisch 2000; Takano et al. 2000; Handerson et al. 2005; Blonski et al. 2007). Among others, the lectin from the Roman snail *Helix pomatia* has emerged as a promising lectin in this regard (Brooks 2000). The predictive value of *H. pomatia* agglutinin (HPA) was first shown when breast cancers from patients with poor prognosis were found to bind the lectin (Leathem and Brooks 1987). HPA has since been reported to be a useful tool for prognostication of breast (Brooks and Leathem 1991), gastric (Kakeji et al. 1991), colorectal (Schumacher et al. 1994) and other solid tumors. The HPA-binding characteristics of cancer cells have been correlated with the migration of breast and colon cancer cells to secondary sites in vivo (Schumacher and Adam 1997; Kohler et al. 2010). Alterations in cellular glycosylation may facilitate cancer cell migration to secondary sites by favoring the binding of cancer cells to the endothelia (Blood and Zetter 1990) and transmigration into the organ of metastasis (Naumov et al. 2001).

One of the main difficulties in the area of glycobiology, however, is the identification of the precise glycoproteins exhibiting cancer-associated glycoforms. It was initially thought that HPA bound to α-*N*-acetylgalactosamine (GalNAcα) of tumor-associated antigens such as the Tn antigen (GalNAcα1-3-O-Ser/Thr; Springer 1989; Piller et al. 1990), the Forssman antigen (GalNAcα1-3GalNAc; Baker

et al. 1983) or blood group A substance (GalNAcα1-3Galβ1-4GlcNAcβ1-Fucα1-2; Mourali et al. 1980; Anderson and Haas 1984), but reports have also shown that HPA has affinity for *N*-acetylglucosamine (GlcNAc; Wu and Sugii 1991) and sialic acid (Dwek et al. 2001). Glycan array analysis also showed that HPA binds a range of diverse glycans (Markiv et al. 2010).

Recently, a glycoproteomic study showed that HPA recognizes a multitude of proteins bearing aberrant glycan structures in colorectal cancer (Saint-Guirons et al. 2007). Increased levels of HPA-binding glycoproteins have also been observed in metastatic cancer, but it remains unclear whether the same glycoproteins bind HPA in cancer cells from different tissues. We previously observed that HPA bound integrin and annexin in metastatic colorectal cancer (Saint-Guirons et al. 2007) and hypothesized that HPA may identify the same glycoproteins in metastatic breast cancer. To evaluate this possibility, the proteins recognized by HPA in metastatic breast cancer cell lines were assessed using cytochemical and glycoproteomic-based approaches.

Results

Cellular localization of HPA binding in breast cells

Human breast cell lines were used as the in vitro model to represent phenotypes ranging from benign disease (HMT3522), primary cancer (BT474), to metastatic cancer (MCF7 and T47D). The cells have defined HPA-binding properties and known metastatic behaviors when implanted in severe combined immunodeficient mice, with HMT3522, BT474 exhibiting non-metastatic and MCF7/T47D exhibiting metastatic capabilities (Schumacher and Adam 1997). Confocal microscopy facilitated detailed analysis of HPA binding (Figure 1). HPA bound intensely to the T47D cell surface, with some granular and perinuclear staining also observed. Intense granular and punctuate perinuclear staining was noted in the MCF7 cells, whereas very low or "negligible" HPA binding was detected in the BT474 and HMT3522 cells.

HPA interaction with cytoplasmic and membrane protein of breast cells

Cytoplasmic and membrane proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed following transfer by Western blotting and probing with HPA (Figure 2). Protein gels stained with Coomassie Brilliant Blue (CBB) showed a similar distribution and intensity of bands for the cytoplasmic preparations, whereas there was considerable cell-to-cell heterogeneity across membrane protein separations. Lectin blotting revealed eight bands that were common to both T47D and MCF7 in the cytoplasmic fraction and four bands that were common to all the four breast cell lines. When Western blots of membrane proteins were probed with HPA, six bands were observed in T47D and five bands in MCF7, all of which were common to T47D. Two major HPA bands were observed across all the cell lines including HMT3522 and BT474. HPA binding was most intense in the membrane fraction of proteins from T47D which exhibited the lowest protein banding pattern in the CBB stained gel, we assume that these proteins represent heavily glycosylated species.

Characterization and analysis of HPA-binding membrane glycoproteins

In order to further characterize the HPA-binding proteins in the cell lines, the proteins were separated by 2DE and stained with CBB. A replicate gel was prepared, transferred to nitrocellulose and probed with HPA. Again, the proteins of the four breast cancer cell lines were compared, to identify proteins that were altered in the cells with the metastatic phenotype. The reproducibility of the system was evaluated using an ANOVA test in 6 replicate gels. In all cases a coefficient of variation of ≤ 0.06 was observed. HPA-binding species were observed amongst the higher molecular weight proteins notably at 130 and 80 kDa (Figure 3B). The protein species with a fold difference ≥ 2 and $P \leq 0.0005$, which showed the highest abundance were subjected to matrix assisted laser desorption ionization (MALDI)-mass spectrometry (MS) and tandem mass spectrometry (MS-MS) (Table I). HPA-binding proteins were identified as: heterogeneous nuclear ribonuclear protein H1 (HnRNP H1), HnRNP D-like, HnRNP A2/B1: isoform 1 (HnRNP A2/B1), enolase 1 (ENO1), heat shock protein 27 (Hsp27) and glial fibrillary acidic protein (GFAP) isoform 1. These proteins have been shown to reside in the cytoplasm and they are therefore assumed to be present in our preparation by virtue of being in complex with the membrane proteins.

The HPA-binding glycoproteins, except HnRNP H1, were found to be elevated in the metastatic MCF7 and T47D cells, with the most notable changes in levels observed in ENO1, HnRNP D-like, HnRNP A2/B1 (data not shown). Other non-HPA-binding proteins that showed increased levels in the MCF7/T47D cells (metastatic phenotype) have been identified and characterized. Some of these proteins have already been shown in previous studies to correlate with poor prognosis cancer including those of the breast (see references in Table I).

Anti-CD49f (integrin α6)

A predominant HPA-binding protein was observed in T47D with an Mr of ~130 kDa, this was not identified by MS-MS due to its relatively low abundance. To facilitate its characterization, T47D membrane proteins were separated by SDS–PAGE, blotted to nitrocellulose and probed with an anti-CD49f antibody. In this analysis, the 130-kDa protein that had been observed to bind HPA was also recognized by the anti-CD49f antibody (Figure 4A). A blot in which the lectin was omitted and streptavidin alone was used confirmed that the antibody binding was not due to interaction between streptavidin and endogenous biotinylated T47D proteins.

HPA recognition of the blood group A epitope

HPA is known to bind blood group A substance (Mourali et al. 1980; Anderson and Haas 1984). Recently, proteomic studies have also shown that HPA recognizes glycoproteins that contain the blood group A epitope (Saint-Guirons et al. 2007; Welinder et al. 2009). To determine if the HPA binding

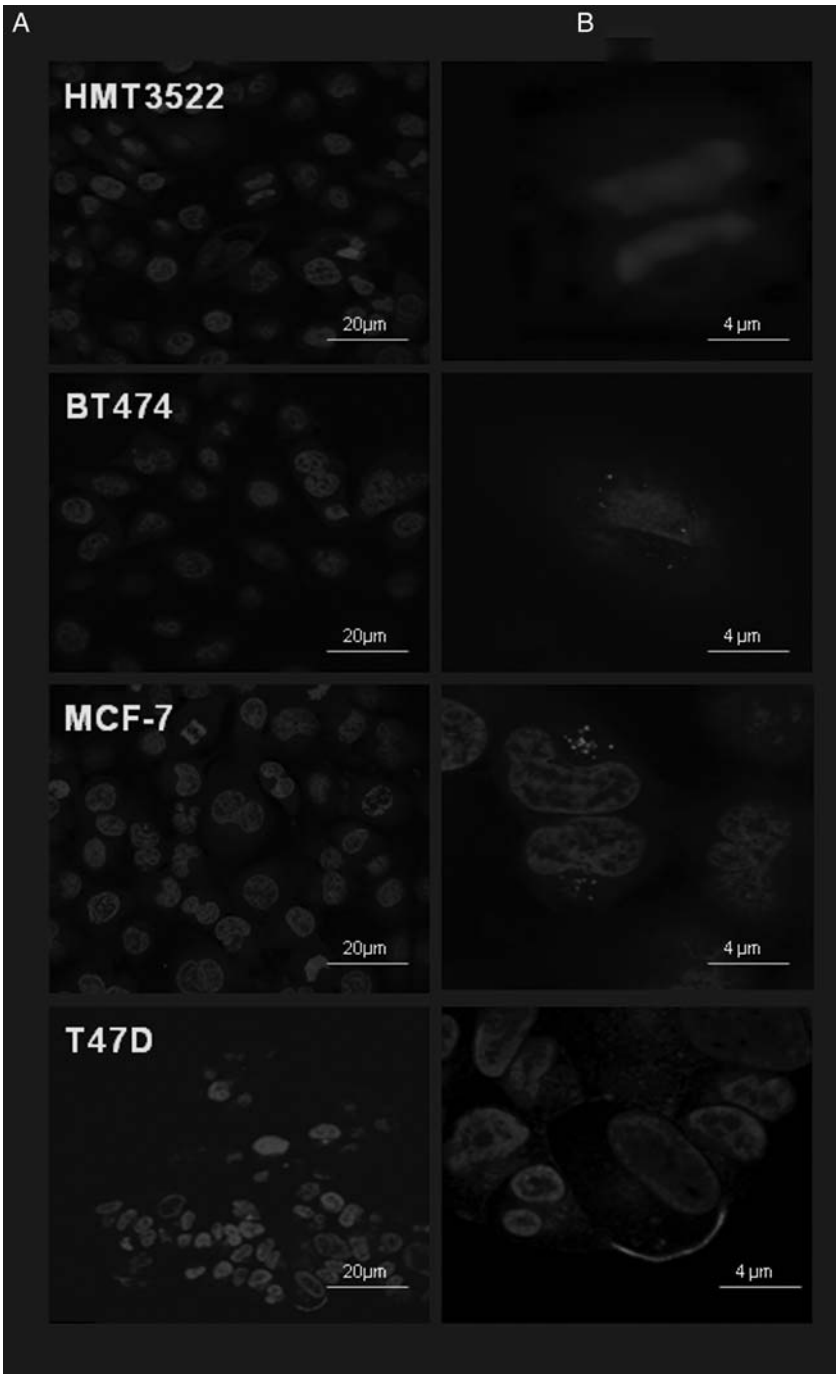

Fig. 1. Confocal images showing the binding of TRITC-labeled HPA to breast cancer cell lines, counter-stained with the nuclear label To-Pro-3. Images showing HPA binding in HMT3522, BT474, MCF7 and T47D, (**A**) scale bars = 20 μm and (**B**) scale bars = 4 μm. HMT3522 and BT474 showed occasional or "negligible" granular staining, whereas T47D showed intense binding on the membrane surface with some granular intracellular staining. MCF7 showed intense granular staining in the perinuclear region perhaps representing the binding of lectin to glycoproteins transiting through the Golgi apparatus.

in T47D was due to the recognition of the blood group A antigen, membrane proteins were separated and blotted as above and probed with an anti-human blood group A antibody. The most predominant protein species observed to bind HPA in T47D did not interact with the antibody, but a protein band at ~85 kDa bound to the antibody (Figure 4A).

Recognition of O-GlcNAc-containing glycoproteins by HPA

In addition to mucin-type O-glycosylation, other forms of O-linked glycosylation have been shown to be increased in cancer cells and tissues. In particular, the attachment of O-GlcNAc to Ser/Thr on the protein backbone has been shown to be an important in regulating protein signaling, by

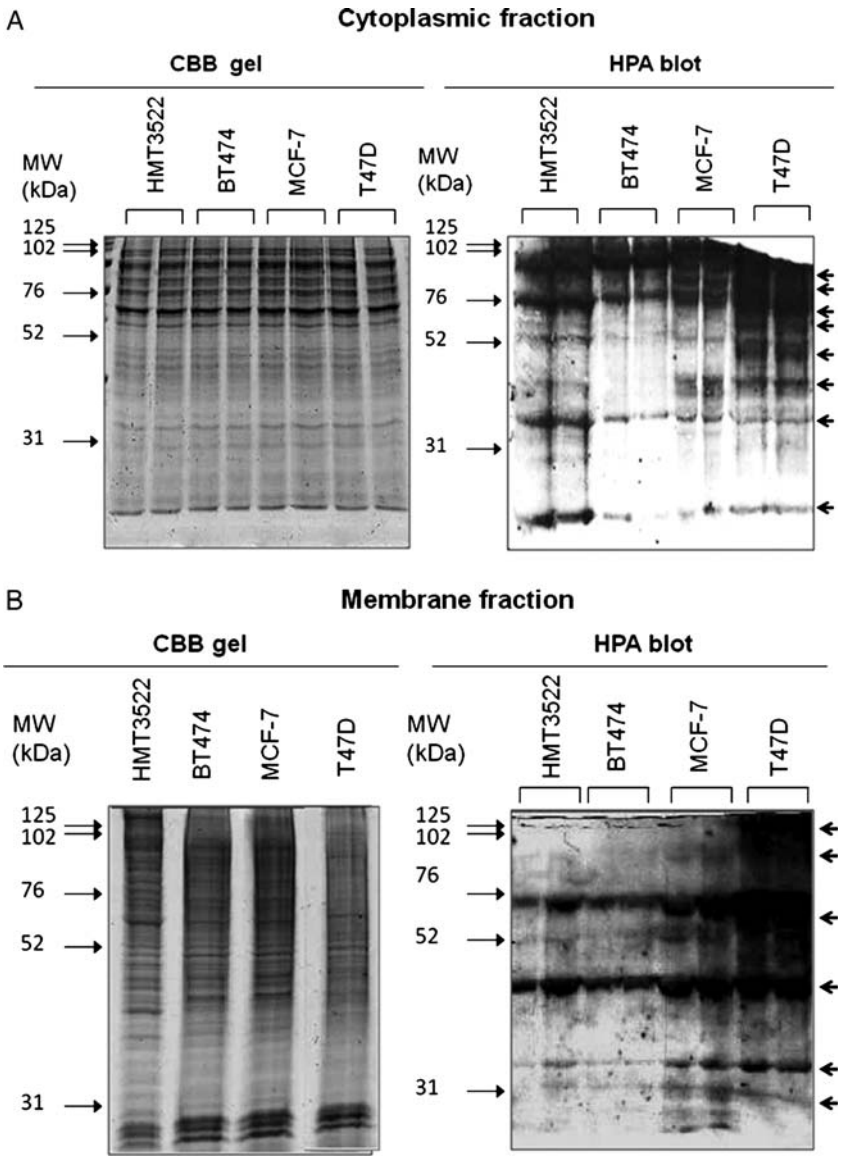

Fig. 2. Representative SDS–PAGE separations and lectin blot of cytoplasmic and membrane proteins. MW markers (kDa) are indicated on the left of the gels and blots. Twenty micrograms of proteins were loaded in each lane. (**A**) and (**B**) shows CBB-stained gel with the respective lectin blot of cytoplasmic and membrane proteins, respectively. Detection was performed using ECL. (**A**) Eight major HPA bands were observed in T47D and MCF7. The bands in HMT3522 and BT474 were noted. (**B**) Six HPA bands were observed in T47D and five in MCF7. Two bands were observed in HMT3522 and BT474, which were also observed in T47D and MCF7.

competing with the substrate(s) for protein kinases and modulating protein phosphorylation (Slawson et al. 2008, 2010).

The crystal structure of HPA has shown that both GlcNAc and GalNAc residues could fit into the binding site of the lectin in an α-linkage, and in addition, recent lectin glycan array studies have showed that GlcNAc in a β-linkage can also fit in the groove of the lectin-binding site (Sanchez et al. 2006; Lescar et al. 2007; Markiv et al. 2010). An investigation was performed to determine the O-GlcNAc status of the HPA-binding T47D proteins. Membrane proteins were separated, blotted as before and probed with an anti-O-GlcNAc antibody. Three major protein species interacted with the antibody, the Mr of these closely corresponded to the predominant HPA-binding proteins (∼75, 50 and 35 kDa; Figure 4A). Treatment with 50 mM GlcNAc resulted in significantly reduced binding of the anti-O-GlcNAc antibody to all three proteins. Western blots of 2DE-separated proteins revealed that the antibody binding to the O-GlcNAc species at 75 kDa correlated with the position on the 2DE gel of separation of transcription factors HnRNP H1 (74 kDa) and ENO1 (75 kDa) (Figure 4B). The proteins with the lower Mr of ∼50 kDa, corresponded to the HnRNP D-like (49 kDa), HnRNP A2/B1 (45 kDa) and the protein with Mr of ∼35 kDa corresponded to Hsp27.

HPA recognition of O-GlcNAc was also examined using fluorescence microscopy. In this approach, an anti O-GlcNAc antibody and a secondary, FITC-labeled, goat anti-mouse

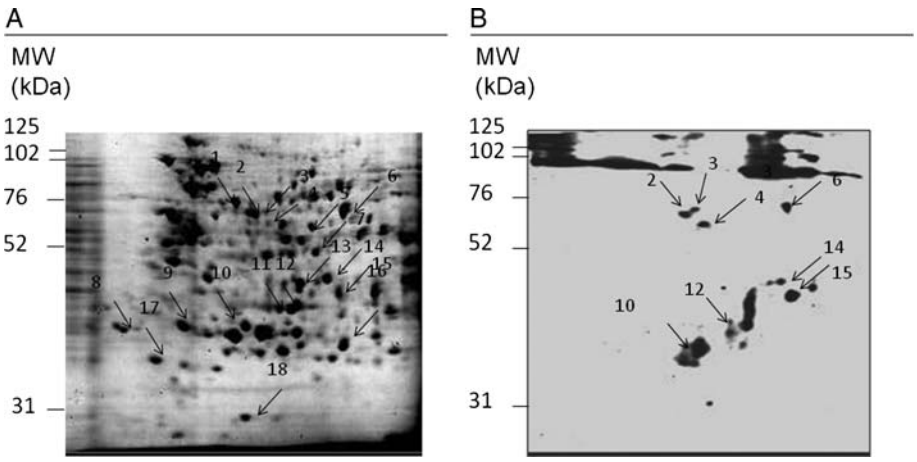

Fig. 3. Representative 2DE gel and the respective lectin blot of T47D membrane proteins. MW markers (kDa) are indicated on the left of the gels and blots; 100 µg of protein was loaded for each gel. Lectin detection was performed using ECL. Western blot revealed numerous proteins in T47D that interacted with HPA; these proteins were also increased in levels in the metastatic cell lines compared with the non-metastatic cell lines. See Table I for proteins identity.

Table I. Proteins identification with accession number, mascot score, MW and predicted pI

Spot no.	Protein ID	HPA bound	Protein levels	Accession no.	Mascot score	Mr (kDa)	Predicted pI	References
1	Glutamate synthetase	−	+	gi|31831	82	42.8	6.61	Liver cancer, Christa et al. (1994)
2	HnRNP H1	+	−	gi|5031753	73	49.5	5.89	Breast cancer, Zhang et al. (2008)
3	HnRNP H1	+	−	gi|5031753	124	49.5	5.89	N/A
4	HnRNP H1	+	−	gi|5031753	134	49.5	5.89	N/A
5	Elongation factor Tu	−	+	gi|704416	139	49.9	7.7	N/A
6	ENO1	+	+	gi|4503571	50	47.5	7.01	Head and neck cancer, Tsai et al. (2010)
7	Elongation factor Tu	−	+	gi|704416	151	49.9	7.7	N/A
8	Macropain subunit delta	−	+	gi|296734	44	19.6	5.01	N/A
9	Proteasome subunit alpha type 2	−	+	gi|4506181	44	25.9	6.9	Colorectal cancer, Arlt et al. (2009)
10	Hsp27	+	+	gi|662841	75	22.4	7.83	Breast cancer, Storm et al. (1996)
11	Enoyl Coenzyme A hydratase 1 peroxisomal	−	+	gi|16924265	165	36.1	8.5	N/A
12	GFAP	+	+	gi|4503979	66	49.9	5.42	Malignant myoepithelial of the breast, Shiraishi et al. (1999)
13	Prosomal protein P30-33K	−	+	gi|190447	97	30.4	6.5	Breast cancer, Bhui-Kaur et al. (1998)
14	HnRNP D-like	+	+	gi|14110407	126	46.5	9.6	N/A
15	HnRNP A2/B1 (isoform A2)	+	+	gi|4504497	62	36.0	8.67	Breast and lung cancer , Zhou et al. (2001)
16	Chaperonin containing TCP1 subunit alpha type 2	−	+	gi|4504497	62	36.0	8.67	Colorectal cancer, Coghlin et al. (2006)
17	Proteasome alpha 6 subunit	−	+	gi|8394076	219	27.8	6.34	Colorectal cancer, Arlt et al. (2009)
18	Nm23	−	+	gi|35068	101	20.7	7.07	Breast cancer, Steeg (2006)

Minus sign (−) denotes no HPA binding or no overexpression of proteins in metastatic T47D and MCF-7 cell lines; Plus sign (+) denotes HPA binding and/or overexpression of proteins in metastatic T47D cell lines.

antibody were incubated with the cancer cells, and following this, the cells were incubated with TRITC-labeled HPA (Figure 5A). The lectin bound the same epitopes that were observed to interact with the O-GlcNAc antibody, but, in addition, HPA recognized other intracellular and membrane resident glycoproteins. ENO1, HnRNP A2/B1 and Hsp27 were immunoprecipitated (Figure 5B). All three proteins were recognized by both HPA and succinylated wheat germ agglutinin (sWGA). Treatment with recombinant β-*N*-acetylglucosaminidase (O-GlcNAcase) reduced significantly, or abolished, both HPA and sWGA interaction with the three proteins.

To identify if the HPA binding observed in the perinuclear region of the metastatic cell lines (T47D/MCF7) was due to the lectin recognizing glycoproteins transiting through, or resident in, the Golgi apparatus, the cells were stained with HPA-TRITC and a Golgi apparatus (NBD-C_6 ceramide) "tracker dye" (Pagano et al. 1989). HPA was observed to co-localize, in part, with the Golgi tracker dye (Figure 6), but other epitopes were also observed to bind the lectin, presumably the O-GlcNAc containing glycoproteins in Figure 4.

Discussion

The main aim of this study was to determine if the HPA-binding glycoproteins in colorectal cancer are the same as those that bind the lectin in breast cancer. The initial findings with confocal microscopy revealed intense HPA binding in T47D and MCF7 cancer cells, which exhibit a metastatic

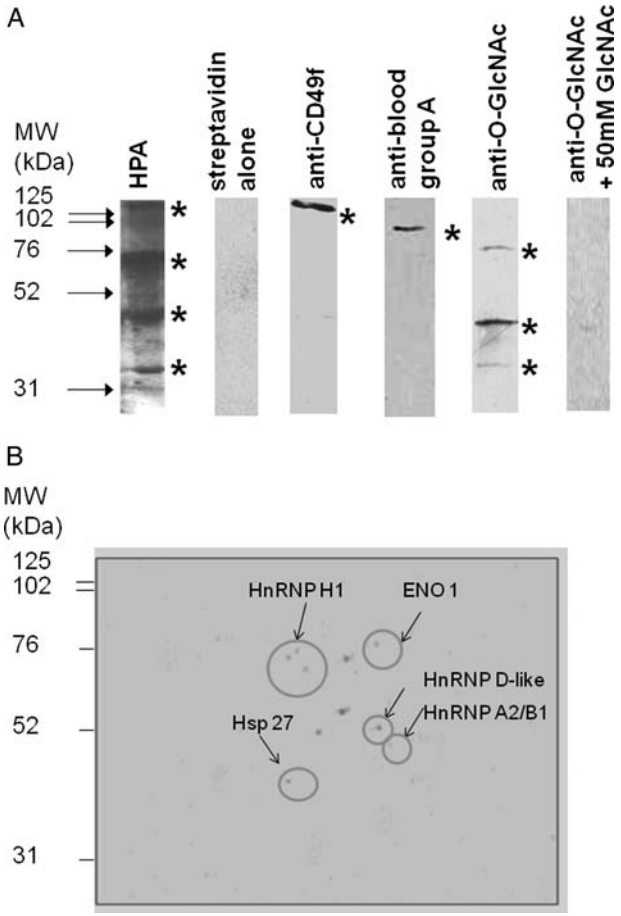

Fig. 4. (**A**) Representative 1DE blots of T47D membrane proteins probed with streptavidin, anti-CD49f, anti-blood group A, anti-O-GlcNAc antibody and anti-O-GlcNAc with 50 mM GlcNAc. These blots were compared with a reference HPA blot. MW markers (kDa) are indicated on the left of the HPA blot. No bands were observed when streptavidin was incubated alone. The antibody directed against CD49f interacted with one protein species at ~130 kDa, the blood group A antibody interacted with one protein species at ~85 kDa and the O-GlcNAc antibody interacted with three protein species at ~75, ~50 and ~35 kDa. Treatment with 50 mM GlcNAc almost completely abolished interaction with the O-GlcNAc antibody. (**B**) Western blots of 2DE-separated proteins revealed that O-GlcNAcylated species at ~75 kDa correlated with the position of transcription factors HnRNP H1 (~74 kDa) and the ENO1 (~75 kDa) and those at the lower MW ~50 corresponded to HnRNP D-like (49 kDa) and HnRNP A2/B1 (45 kDa).

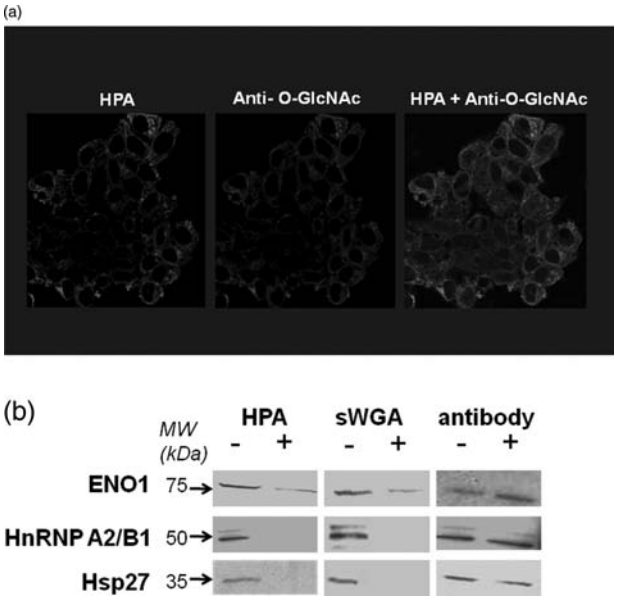

Fig. 5. (**A**) Co-localization of HPA binding and O-GlcNAc epitope in T47D cells. Confocal images showed strong binding in T47D and O-GlcNAc binding and strong co-localization in the cytoplasmic area showed with white arrows; scale bars = 30 µm. (**B**) Representative western blots of immunoprecipitated HPA-binding proteins (ENO1, HnRNP A2/B1, Hsp27) from T47D cells. Immunoprecipitated proteins were treated as follows. Lanes 1, 3 and 5, proteins untreated; lanes 2, 4 and 6, proteins were treated with recombinant O-GlcNAcase. All the three proteins interacted both with HPA and sWGA and binding was reduced or almost completely abolished when the proteins were treated with O-GlcNAcase suggesting that HPA recognizes ENO1, HnRNP A2/B1 and Hsp27 via O-GlcNAcylation. The immunoprecipitated proteins were also probed with the respective antibodies to ensure equal loading of treated and untreated proteins.

phenotype in mice (Schumacher and Adam 1997). HPA bound both the cell surface and the cytoplasm, the punctuate HPA binding observed in the perinuclear region of MCF7 cells, was thought to correspond to a disruption in the glycosylation pathways in the Golgi apparatus, or in transport pathways, which have been shown to result in higher concentrations of immature *N*-acetylgalactosaminylated glycoforms in these cellular compartments (Roth 1984; Laitinen et al. 1990; Mitchell et al. 1995; Brooks et al. 2001). Similar phenotypic observations have been made using the colorectal cancer cells SW480, SW620 and HT29 (Saint-Guirons et al. 2007). Co-localization studies using a Golgi dye tracker revealed that some of the HPA binding was located to the Golgi apparatus in the T47D and MCF7 cells. Western blot analysis of proteins probed with HPA demonstrated that increased binding was associated with a metastatic phenotype; a similar observation was reported in colorectal cancer (Saint-Guirons et al. 2007). Considering the results obtained using confocal microscopy and the lectin blotting, this study confirmed the earlier data showing that the development of a metastatic phenotype is accompanied by alterations in glycosylation. The localization of HPA-binding epitopes to cell membrane proteins may contribute to the metastatic behavior of the cancer cells in vivo.

A 2DE-based proteomic approach was used to characterize the HPA-binding glycoproteins of the T47D cells. HPA-binding glycoproteins identified by MS included molecules involved in apoptotic pathways (Hsp27), pre-mRNA splicing (HnRNP H1, HnRNP D-like and HnRNP A2/B1), cellular remodeling (GFAP) and cell migration (ENO1). We assume that these HPA-binding glycoproteins form complexes with proteins in the membrane fraction. The HPA-binding proteins were increased in the T47D cells and were the most abundant proteins in the lectin blots of T47D. The proteins identified in this proteomic study have been observed in other cancers: for example, Hsp27 in brain, breast and prostate cancer (Kato et al. 1992; Andrieu et al. 2010); HnRNP H1 in breast (Zhang et al. 2008); HnRNP A2/B1 in breast and lung

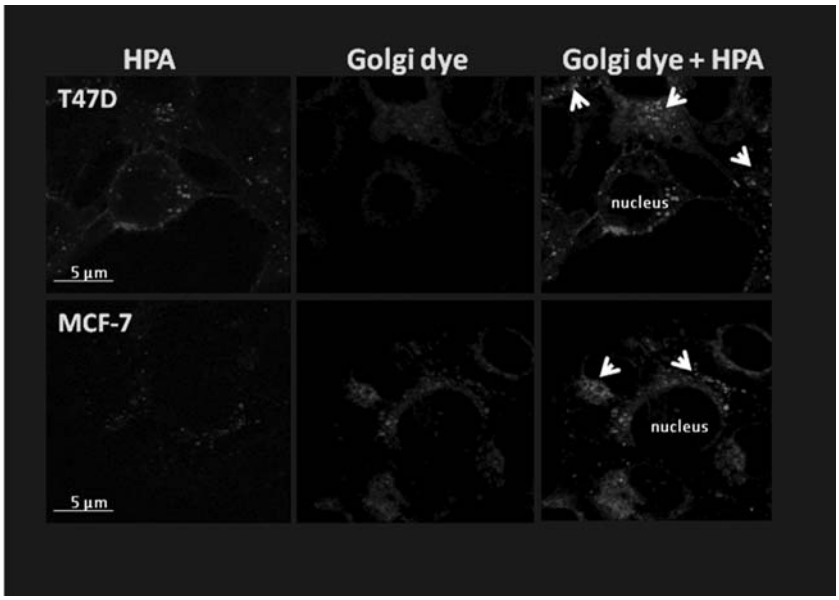

Fig. 6. Confocal images showing the binding of TRITC-labeled HPA and the Golgi tracker dye (green) to MCF7 and T47D cell lines. Images showing the strong association of HPA labeling in the perinuclear region corresponding to the Golgi apparatus (orange), scale bars = 5 µm.

(Zhou et al. 2001); GFAP in breast (Shiraishi et al. 1999) and ENO1 in head and neck cancer (Tsai et al. 2010).

Analysis of the membrane proteins of T47D using the anti-CD49f antibody (against integrin α6) showed that HPA recognizes a protein band of the same apparent molecular weight as the integrin α6 subunit. This finding was consistent with a previous report in which integrin α6 has previously been detected as a HPA-binding protein in colorectal cancer cells (Saint-Guirons et al. 2007).

To check whether the glycan epitope(s) recognized by HPA in T47D includes the blood group A antigen, immunoblots were performed using an anti-blood group A antibody directed against a terminal GalNAcα containing the blood group A epitope. In this analysis, the predominant HPA-binding glycoproteins were of a different molecular weight compared with the glycoproteins that bound the anti-blood group A antibody. One protein, HPA-binding protein, at ~85 kDa interacted with the antibody, this correlated with the molecular weight of a protein previously reported in breast and ductal adenocarcinoma (Hirota et al. 1992; Welinder et al. 2009), suggesting that most of HPA binding epitopes in T47D encompasses antigens other than the blood group A sugars. These might, for example, include the Tn antigen (Springer 1989; Piller et al. 1990) or sialyl tn antigen (Dwek et al. 2001).

The modification of proteins by the attachment of O-GlcNAc residues is a common form of glycosylation among nuclear pore and cytoplasmic proteins. This form of glycosylation has been shown to act as an alternative pathway to phosphorylation for intracellular signaling and is a notable feature accompanied with chronic diseases such as diabetes and cancer (Slawson et al. 2008, 2010). To determine if the HPA-binding antigens detected in the 2DE analysis were O-GlcNAcylated, the proteins that bound HPA were compared with those that were recognized by an anti-O-GlcNAc antibody. The 1-dimensional electrophoresis (1DE) immunoblots showed three protein species which interacted with the anti-O-GlcNAc antibody, these had Mr of 75, 50 and 35 kDa and were observed in the membrane fraction of T47D; these proteins were not detected by the anti-O-GlcNAc antibody when the antibody was pre-incubated with 50 mM GlcNAc, suggesting that these proteins were O-GlcNAcylated. Saint-Guirons et al. (2007), and others, have previously shown that the interaction of HPA with colorectal cancer-derived glycoproteins may be inhibited using GlcNAc, but this had not been correlated with the O-GlcNAcylation of the proteins. In our study, immunoblot analysis of 2DE-separated membrane proteins of T47D with both the HPA and the anti-O-GlcNAc antibody revealed the HPA-binding glycoproteins at 75 kDa separated at the same pI and molecular weight (MW) marker as ENO1 and HnRNP H1, the proteins at 50 kDa corresponded to the separation position of HnRNP D-like and HnRNP A2/B1 and the protein at 35 kDa corresponded to Hsp27 in this system. Some proteins, which were not detected in HPA blot, also interacted with the anti-O-GlcNAc antibody. We assume that these proteins also exist as O-phosphorylated proteins and it is possible that they are transiting between O-phosphorylation and O-GlcNAcylation phases. Another interesting observation was that the HPA-binding proteins were of a higher (than predicted) theoretical molecular weight; for example, estimated and theoretical MW marker of ENO1 is 76 and 47.5 kDa, respectively, and here, we assume that these proteins are heavily glycosylated isoforms. In further studies, ENO1, HnRNP A2/B1 and Hsp27 were immunoprecipitated; the results of this approach confirmed the MS analysis of the 2DE separations. In addition, the interaction of HPA with these proteins was O-GlcNAc-dependent as treatment of the protein with O-GlcNAcase enzyme reduced the reactivity with HPA in a manner similar to that observed with sWGA; further supporting the observation that HPA recognizes these proteins via O-GlcNAcylation.

It has been showed that the transition between the glycosylation and phosphorylation status of a protein exerts control

on cell regulatory mechanisms and disruption in this cross-talk might be implicated in many disease states (Bork et al. 2008; Copeland et al. 2008). It will be interesting to examine whether the glycosylation status of these proteins bears any relation to cellular function.

Piecing together evidence from the proteomic, immunoblotting and immunocytochemistry work, we conclude that integrin α6 is an HPA-binding protein recognized in both colorectal and breast cancer cells. HPA also recognizes O-GlcNAcylated cytoplasmic proteins (Hsp27, ENO1, HnRNP H1, HnRNP A2/B1 and HnRNP D-like). The O-GlcNAcylation of the HnRNP, transcription factors opens up the possibility of glycosylation affecting the regulation and, therefore, functions of these proteins and suggests a new mechanism by which HPA may detect poor prognosis cancers.

Materials and methods

Cell culture

Human breast cancer cell lines HMT3522, BT474, MCF7 and T47D were kindly provided by Dr Susan Brooks (Oxford Brookes University, UK). The cells were grown in Dulbecco's modified Eagle's medium (Lonza Ltd, UK) supplemented with 10% v/v fetal calf serum (Biosera, UK), in 25 or 175 cm^2 flasks at 37°C, in 5% v/v CO_2 and in a humidified atmosphere. The cells were grown to 70–80% confluence, washed thrice in phosphate-buffered saline (PBS) and mechanically removed from the tissue culture flask using a sterile plastic scraper. The cells were disrupted by sonication using an ultrasonic probe (MS73 Status 200) at 40% power, five times for 10 s with intermittent cooling on ice cold water and used for membrane protein extraction.

Confocal microscopy

Cells were seeded into 6-well plates and maintained as above for 24 h. Cells were fixed 30 min with 10% w/v formaldehyde, trypsinized (1 mg/mL; Sigma, UK) and blocked in l0% v/v bovine serum albumin (BSA). The cells were treated with 100 µg/mL of DNase-free ribonuclease A for 20 min at 37°C and the nuclei were counter-stained using 1 mM To-Pro-3 (Invitrogen, UK) prior to lectin or antibody incubation. Cells were incubated with HPA-TRITC (Sigma) in the dark at 5 µg/mL for 1 h.

A monoclonal mouse anti-O-GlcNAc antibody (sc-81483, Santa Cruz Biotech, UK) was used at 10 µg/mL for 1 h followed by 20 min incubation with monoclonal polyclonal IgG-FITC at 5 µg/mL (Santa Cruz Biotech). A control was included in which the anti-O-GlcNAc step was omitted. To determine if the lectin HPA recognizes glycoproteins resident in the Golgi apparatus, a dye that locates to this organelle (NBD-C_6-ceramide, B-34400, Invitrogen) was applied to the cells at 10 µM for 1 h at 4°C in 4-(2-hydroxylethyl-1-piperrazineethane sulfuric acid) buffer (Invitrogen). Both the O-GlcNAc antibody and Golgi apparatus dye steps were performed following the lectin incubation steps.

Images were collected using a Leica TCS SP2 confocal microscope with ×63 ceramic dipping objective and by sequential scanning. Images were acquired at a scanning speed of 400 Hz, 1024 × 1024 pixel resolution and a line average of 4. A 466-nm laser was used for the excitation of NBD C_6-ceramide (intensity 35%, emission bandwidth 500–536 nm), 488 nm for IgG-FITC (intensity 40%, emission bandwidth 500–550 nm), 543 nm for HPA-TRITC (intensity 40%, emission bandwidth 550–630 nm) and 633 nm laser for the To-Pro-3 (intensity 35%, emission bandwidth 650–720 nm).

Sodium dodecyl sulfate–PAGE and 2DE

Membrane proteins were extracted and separated from the cytoplasmic proteins using a centrifugal protein extraction method (Saint-Guirons et al. 2007). The membrane proteins and cytoplasmic proteins were prepared on at least six separate occasions using each cell line: HMT3522, BT474, MCF7 and T47D. For 1DE SDS–PAGE, 20 µg of cytoplasmic and membrane proteins were separated, on at least six separate occasions, on a 12% acrylamide gel, run at 200 V for 60 min (Saint-Guirons et al. 2007). For 2DE experiments between 90 and 100 µg of membrane proteins from each of the cell lines was used. Proteins were prepared in 8 M urea, 3 M thiourea, 4% w/v 3-3-cholamidopropyl dimethylammonia-1-propane sulphonate hydrate, 2% v/v carrier ampholytes (GE Healthcare, UK), 1% w/v dithiothreitol (DTT). pH gradient strips (linear pH 3–10, GE Healthcare) were rehydrated overnight at room temperature, isoelectric focusing was performed at 20°C using the following conditions: 300 V for 30 min; 600 V for 30 min and 3500 V for 2 h 50 min. Focused strips were run in the second dimension by sealing onto a 12% SDS–PAGE gel using molten agarose (0.5% w/v agarose, 25 mM Tris, 192 mM glycine, 0.1% w/v SDS, trace of bromophenol blue). In all the procedures, the gels of membrane and cytoplasmic proteins were stained using 0.025% w/v Coomassie Brilliant Blue (CBB) R-250 in 10% v/v acetic acid for 60 min, followed by destaining in 10% v/v acetic acid for 120 min. Six replicates of the cell membrane preparations from each of the cell lines were run on the 2DE system.

Analysis of 2DE data

The 1DE and 2DE gel images were obtained from the CBB-stained gels using a BioRad GS-800 densitometer. The data were analyzed using the BioRad Quantity One computer software package. Images in the TIFF format were imported into the Progenesis Same Spots version 3.0 systems (Non-Linear Dynamics, UK) and processed by background subtraction, spot detection, landmarking and overlay analysis.

MS analysis for protein identification

The HPA-binding proteins identified on the X-ray film [exposed to enhanced chemiluminescence (ECL) reagent following the western blotting step] were used as a template to locate the equivalent protein species on a CBB-stained gel; run at the same time as the western blot. The CBB-stained gel was used to manually excise the HPA-binding proteins of interest. Protein identification was performed by commercial arrangement with Dr Thomas, Department of Biology, University of York, using a MALDI-TOF/TOF 4700 analyser (Applied Biosystems).

Briefly, the proteins were reduced using DTT, *S*-carbamidomethylation and iodoacetamide prior to tryptic

digestion. The excised protein gel pieces were washed three times in 50% v/v acetonitrile/25 mM ammonium bicarbonate and air-dried before rehydration in 10 mL of 20 μg/mL sequencing-grade, modified porcine trypsin (Promega, UK). Protein digestion was performed overnight at 37°C. A 0.5-μL aliquot of each tryptic digest and 0.5 μL of a solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% v/v acetonitrile containing 0.1% v/v trifluoroacetic acid was applied to the MALDI target plate. Mass spectra were obtained in the reflection mode with an accelerating voltage of 20 kV. The peptide mass fingerprint was compared with the masses of all theoretical tryptic peptides generated in silico by the MASCOT search program updated in 2010 (Pappin et al. 1993). Collision-induced dissociation MS/MS was also performed to corroborate the significant matches from the MALDI/MS.

Immunoprecipitation and removal of O-GlcNAc

Cells were lysed as above. Immunoprecipitation was performed overnight using the μMACS™ streptavidin kit and according to the manufacturer's instructions. Approximately 500 μL of T47D cell lysate was incubated with the specific biotinylated antibody: anti-ENO1 (ARP34376-T100; Aviva Systems Biology, UK); anti-HnRNP A2/B1 (AB6102; Abcam, UK) and anti-Hsp27 (AF5E5; Abfrontier) and 100 μL of streptavidin microbeads. After mixing, the lysate was incubated for 1 h on ice. The magnetic μMACS™ separator column was prepared by rinsing with the 200-μL lysis buffer. The lysate–antibody–microbead complex was applied to the column and allowed to run through the column. The μ column was washed with 4 × 200 μL of wash buffer and the immunoprecipitated protein was eluted with 100 μL of 1× SDS–PAGE sample buffer. The immunoprecipitated proteins were treated with *Bacteroides thetaiotaomicron* O-GlcNAcase (6779-GH, R&D Systems, UK) following the manufacturer's protocol, the enzymatic treatment was monitored by assessing the digest mixture by separation using SDS–PAGE and western blotting with sWGA.

Western blotting

Proteins from the cell membrane and cytoplasm preparations were separated by SDS–PAGE as detailed above and transferred to nitrocellulose membranes (Whatman, UK) using a wet blotting system with transfer buffer (25 mM Tris, 192 mM glycine 20% v/v methanol) at 150 V for 1 h 30 min. The blots were blocked with 5% w/v BSA in PBS/Tween 0.05% v/v at 4°C for 60 min, incubated on a rocking platform with 0.5 μg/mL of biotinylated HPA (Sigma) or biotinylated sWGA (Vector Laboratories, UK) for 120 min and 0.125 μg/mL streptavidin horseradish peroxidase (HRP) conjugate for 60 min (Piercenet, UK). The blots were visualized using an ECL capture system with the SuperSignal WestPico reagent (Piercenet). Western blots in which the lectin step was omitted were also prepared. Blots were also prepared and probed with an anti-blood group A antibody (Ortho-Reagent, Johnson and Johnson, UK); 50 μg/mL; for 2 h followed by 1 h incubation with a HRP-labeled goat anti-mouse antibody at 10 μg/mL (Sigma). An anti-O-GlcNAc (sc-81483, Santa Cruz Biotech) and an anti-integrin α6 (sc-59971, Santa Cruz Biotech) antibody, 20 μg/mL, were used followed by detection with goat anti-mouse IgG-HRP, 10 μg/mL (Santa Cruz Biotech). The chemiluminescent signal was captured onto the X-ray film using exposure times ranging from 30 s to 1 min.

Inhibition studies

Membrane proteins from T47D were separated by SDS–PAGE and blotted as detailed above. Prior to probing with an antibody against O-GlcNAc, the antibody was incubated with 50 mM GlcNAc for 1 h. The blots were visualized using an ECL capture system as above.

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Conflict of interest

None declared.

Abbreviations

CBB, Coomassie Brilliant Blue; CFG, consortium for functional glycomics; ENO, enolase; GalNAc, *N*-acetylgalactosamine; GFAP, glial fibrillary acidic protein; GlcNAc, *N*-acetylglucosamine; HnRNP, heterogeneous nuclear ribonuclear protein; HPA, *Helix pomatia* agglutinin; Hsp27, heat shock protein 27; MALDI, matrix assisted laser desorption ionization; MS-MS, tandem mass spectrometry; MW, molecular weight; O-GlcNAcase, β-*N*-acetylglucosaminidase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sWGA, succinylated wheat germ agglutinin; 2DE, 2-dimensional electrophoresis.

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