Serum galectins -2, -4 and -8 are greatly increased in colon and breast cancer patients and promote cancer cell adhesion to blood vascular endothelium

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**Translational Relevance**

Adhesion of invaded tumour cells to the blood vascular endothelium is a pivotal step in metastasis. This study revealed that the levels of several galectin members were greatly increased in the sera of colon and breast cancer patients and that galectins promote cancer cell adhesion to blood vascular endothelium *in vitro* by interaction with cancer-associated TF/MUC1. Elevated circulating galectin-2 levels are a marker of poor prognosis in colon cancer whilst higher levels of circulating auto anti-MUC1 antibodies with specificity for the TF-epitope of MUC1 protected against the poor prognosis associated with increased circulating galectin-2. Targeting the actions of circulating galectins may therefore represent a promising therapeutic approach to reduce metastasis.
Abstract

**Purpose:** Adhesion of disseminating tumour cells to the blood vascular endothelium is a pivotal step in metastasis. Previous investigations have shown that galectin-3 concentrations are increased in the bloodstream of cancer patients and that galectin-3 promotes adhesion of disseminating tumour cells to vascular endothelium *in vitro* and experimental metastasis in vivo. This study determined the levels of galectin-1, -2, -3, -4, -8 and -9 in the sera of healthy people and colon and breast cancer patients and assessed the influence of these galectins on cancer-endothelial adhesion *in vitro*.

**Experimental design:** Serum galectins and auto anti-MUC1 antibodies were assessed using ELISA and MUC1 glycan microarrays and cancer-endothelial adhesion was determined using monolayers of human micro-vascular lung endothelial cells.

**Results:** The levels of serum galectin-2, -3, -4, and -8 were increased up to 31-fold in cancer patients and in particular those with metastases. As previously shown for galectin-3, the presence of these galectins enhances cancer-endothelial adhesion by interaction with the TF (Galβ1,3GalNAca-) disaccharide on cancer-associated MUC1. This causes MUC1 cell surface polarization, thus exposing underlying adhesion molecules that promote cancer-endothelial adhesion. Elevated circulating galectin-2 levels were associated with increased mortality in colorectal cancer patients but this association was suppressed when anti-MUC1 antibodies with specificity for the TF epitope of MUC1 were also present in the circulation.

**Conclusions:** Increased circulation of several members of the galectin family is common in cancer patients and these may, like circulating galectin-3, be involved in metastasis promotion.
**Introduction:**

Adhesion of disseminating tumour cells to the blood vascular endothelium is a crucial step in cancer metastasis and is regulated by various cell surface adhesion molecules/ligands on cancer cells as well as on vascular endothelial cells (1).

Galectins form a family of 15 mammalian galactoside-binding proteins expressed by many types of human cells. They are synthesized in the cell cytoplasm from where they can be transported into the cell nucleus, possibly via both active transport and passive diffusion (2), or secreted through a non-classical pathway (3). Cytoplasmic galectins are involved in the regulation of cellular apoptosis (4, 5). The presence of galectin-1 and -3 in the cell nucleus promotes mRNA splicing (6). The cell-surface associated galectins act as cell adhesion molecules and promote cell-cell and cell-matrix interactions during cancer development and progression (7, 8).

Earlier studies have reported that the concentration of galectin-3 is significantly increased in the sera of patients with colorectal, lung (9), bladder (10), head and neck cancers (11) and melanoma (12-14). Our recent studies have revealed that an increased circulation of galectin-3 promotes cancer cell blood-borne metastasis in nude mice (15). This effect is a result of the galectin-3 interaction with the oncofetal Thomsen-Friedenreich (Galβ1,3GalNAc, TF) disaccharide expressed by the cancer-associated transmembrane mucin protein MUC1(16). The galectin-3-TF/MUC1 interaction induces MUC1 cell surface polarization and exposure of the underlying adhesion molecules that increases cancer cell heterotypic adhesion to vascular endothelium and cancer cell homotypic aggregation to form micro-tumour emboli (17).
There is limited information concerning the expression or role of the other galectin members in the circulation. Subcutaneous injection of mouse breast tumour NeuTL cells to FVB/N mice has been shown to increase plasma levels of galectin-1 (18). Serum galectin-1 level is increased in thyroid cancer (11), but not in head and neck squamous cell carcinomas (19). As members of the galectin family share similar carbohydrate binding properties and many are often found to be co-expressed in the same cell types and tissues, we speculated that other galectin members may also be altered in the bloodstream of cancer patients and, like galectin-3, may also be involved in cancer cell adhesion to vascular endothelium in metastasis.

This study shows that the concentrations of free circulating galectin-2, -3, -4, and -8 are all markedly increased in the blood circulation of colon and breast cancer patients and in particular those with metastasis. The presence of these galectins promotes cancer cell adhesion to vascular endothelial cells by interaction with the TF disaccharide on cancer-associated MUC1, an effect that is diminished when anti-MUC1 antibodies against the MUC1 TF epitope are also present in the circulation, probably as a result of their competitive binding to cancer-associated TF/MUC1.
Materials and Methods

Materials

Recombinant human galectin-1, -2, -3, -4 and -8, all the anti-galectin antibodies and biotinylated anti-galectin antibodies were from R&D Systems (Abingdon, UK). B27.29 anti-MUC1 mAb was kindly provided by Dr. Mark Reddish (Biomira Inc, Canada). Biotin-conjugated peanut agglutinin (PNA) was purchased from Vector Laboratories Ltd (Peterborough, UK). Endo-N-acetyl-galactosaminidase (EC 3.2.1.97), O-glycanase, was obtained from Prozyme Inc (Oxford, UK). The Calcein AM cell labelling solution was from Invitrogen and the Non-Enzymatic Cell Dissociation Solution (NECDS) was from Sigma.

Cell Culture

Human colon cancer HT29-5F7 cells, kindly provided by Dr. Thecla Lesuffleur (INSERM U560, Lille, France), are enterocyte-like subpopulations of HT29 cells that express mainly MUC1 and MUC5B and were isolated as a consequence of their resistance to 5-fluorouracil (20). Human colon cancer SW620 cells were obtained from the European Cell Culture Collections via the Public health Laboratory Service (Porton Down, Wiltshire, UK). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) as described in our previous studies (16). Human macro-vascular umbilical vein endothelial cells (HUVECs) and human micro-vascular lung endothelial cells (HMVEC-Ls) were obtained from Lonza (Verviers, Belgium) and were cultured in EGM and EGM-2 endothelial growth media and supplements (EGM Bulletkits and EGM-2 Bulletkits, respectively). MUC1 transfection of HBL-100 human breast epithelial cells with full length cDNA encoding MUC1 and the subsequent selection of the MUC1 positive transfectant HCA1.7+ and the negative revertant HCA1.7- was described previously(21).
Serum samples

Fifty one serum samples from colorectal cancer patients, 40 without clinically detectable metastasis (26 males and 14 females) and 11 with liver metastasis (7 males and 4 females) and 40 serum samples from breast cancer patients (all females) were obtained from CTBRC cancer tissue bank (Liverpool, UK). These serum samples were obtained by CTBRC from patients at time when their primary tumours were removed by surgery at the Royal Liverpool Hospital. The length of survival of these patients in the next 10 years was followed up. Thirty-one serum samples from healthy people (13 Male and 19 female) were obtained from Sera Laboratories International (Haywards Heath, UK). The patients’ ages were from 25 to 91 (mean age 63) and healthy people from 20 to 51 (mean age 37) (Supplementary Material, Table S1). Although the healthy controls were younger than the patients, there was no association between age and concentration of any of the galectins (Spearman’s rho correlation coefficient: -0.24, -0.21, 0.02, -0.034 and -0.18, with p=0.19, 0.27, 0.92, 0.86 and 0.29 for galectin-1, -2, -3, -4 and -8, respectively).

Determination of serum galectins

High-binding 96-well plates were coated with anti-galectin antibody at 2.5μg/ml in coating buffer (15mM Na2CO3, 17mM NaHCO3, pH 9.6) overnight at 4°C. The plate was washed with washing buffer (0.05% Tween-20 in PBS) and incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 hr at room temperature. Serum samples or standard recombinant galectins were introduced to the plates for 2 hr before application of biotinylated anti-galectin antibody (1.25μg/ml in blocking buffer) (Supplementary Material, Table S2) for 1 hr at room temperature. After introduction of peroxidase-ExtrAvidin (1:10,000 dilution in blocking buffer) for 1 hr, the plates were developed with SigmaFAST OPD for 10 min. The
reaction was stopped by adding 4M sulphuric acid and the absorbance was read at 492nm by a microplate reader.

Assessments of galectin binding to TF-expressing glycans

High-binding 96-well plates were coated with 5μg/ml TF-expressing glycoproteins (Antarctic fish anti-freeze protein, asialofetuin or asialo bovine mucin) in coating buffer overnight at 4°C. The plates were washed and incubated with blocking buffer for 1 hr before introduction of recombinant galectins (1 μg/ml) for 2 hr at room temperature. Biotinylated anti-galectin antibodies (1.25μg/ml in blocking buffer) were introduced for 1 hr at room temperature. After application of peroxidase-ExtrAvidin for 1 hr, the plates developed with SigmaFAST OPD and the absorbance was read at 492nm as above.

In some experiments, the plates were coated overnight at 4°C with 5μg/ml asialo bovine mucin pre-treated without or with 1.25 U/ml O-glycanase for 2 hr at 37°C before application of recombinant galectin (1μg/ml) and subsequent measurements of TF expression or galectin binding by ELISA as above.

Assessments of the effects of galectins on cancer cell adhesion to endothelial monolayers.

HUVECs or HMVEC-Ls were released from the culture flasks by trypsinization and re-suspended at 1x10^5/ml in EGM or EGM-2 endothelial culture medium. 1x10^4 cells were applied to white walled, clear bottomed 96-well plates at 37°C for 2-3 days for the formation of tight endothelial monolayers.

HT29-5F7 or SW620 cells detached from culture flasks with NECDS were suspended in serum-free DMEM to 1x10^5 cells/ml and incubated with 10μl/ml Calcein AM cell labelling
solution at 37°C for 30 min on a shaking waterbed at 80rpm. The cells were washed with serum-free DMEM and re-suspended in fresh serum-free DMEM to 5x10^4/ml. Ten μl of 100μg/ml recombinant galectin was pre-incubated with 10μl of 10μM lactose for 30 mins before mixed with 960μl cell suspension for 1 hr at 37°C. One hundred μl cell suspension was then introduced to the endothelial monolayers without or with the addition of 10μg/ml (final concentration) anti-TF (TF5) antibody (22) for 30 min at 37°C. After washing with PBS, the cell-associated fluorescence was measured by a fluorescence microplate reader.

In some experiments, the HT29-5F7 cells were pre-treated with or without 0.02U/ml O-glycanase for 3 hr at 37ºC before incubation with each galectin (1μg/ml) and subsequent analysis of cell adhesion to HMVEC-L monolayers as above.

In some other experiments, the Calcein AM-labelled cancer cells were applied to endothelial monolayers cultured in 24-well plates inserted with glass coverslips. The numbers of adherent cells at the end of experiments were counted under fluorescence microscope and the cell adhesion was expressed as number of cells/FOV (Field of View) as previously described (15).

**Assessment of MUC1 cell surface localization.**

MUC1 positively-transfected (HCA1.7+) human breast epithelial HBL-100 cells were released from the culture flasks by NECDS and re-suspended at 5x10^5/ml in serum-free DMEM. The cells were incubated with or without 1μg/ml galectin for 1 hr at 37°C. One hundred and 50μl of cell suspension was applied to a poly-lysine coated side for 30 min before fixed with 2% paraformaldehyde for 15 min at room temperature. The slides were incubated with 5% goat serum in PBS for 30 min before application of anti-MUC1 B27.29
(2.5µg/ml in 1% goat serum/PBS) for 1 hr. After washing, the slides were incubated with Texas-Red conjugated secondary antibody (2.5µg/ml in 1% normal goat serum) for 1 hr before mounted with Vectorshield Mounting Medium (Vector Laboratories). The slides were then blinded and the localisation of cell surface MUC1 was analysed by the fluorescent microscopy with x25 objective.

**MUC1 siRNA knock-down**

HT29-5F7 cells were released from the culture flasks by trypsinization and re-suspended at 5x10⁴/ml in antibiotic-free DMEM. The cell suspension was seeded in 96-well plates and incubated at 37°C overnight. MUC1 siRNA or non-targeting control siRNA (100nM) were applied at 37°C for 72 hr. The cells were either lysed for protein assessments by immunoblotting with anti-MUC1 antibody (B27.29), or released with NECDS for subsequent cell adhesion assessments.

**Determination of auto anti-MUC1 antibodies in serum**

MUC1 peptides were synthesized, O-glycosylated *in vitro* using various recombinant glycosyltransferases and purified exactly as previously described (23, 24). The glycopeptides were printed on Schott Nexterion® Slide H (Schott AG, Mainz, Germany) on a BioRobotics MicroGrid II spotter (Genomics Solution) with a 0.21 mm pitch using Stealth 3B Micro Spotting Pins (Telechem International ArrayIt Division). The Slides were incubated in a humidified hybridization chamber with 75% relative humidity for 1 h before the unspotted areas of the slides were blocked with 25 mM ethanolamine in 100 mM sodium borate pH 8.5 for 1 hr. Serum samples (1:25 serial dilution) or monoclonal antibodies (1 µg/ml) were introduced to the slides in a closed container with gentle agitation for 1 h. After washing with 0.05% Tween-20/PBS, the slides were incubated with Cy3-conjugated goat anti-human IgG
(Fc specific, 1:5000 dilution in PBS-T) for 1 hr. After washing, the slides were dried and scanned in a ProScanArray HT Microarray Scanner (PerkinElmer) followed by image analysis with ProScanArray Express 4.0 software (PerkinElmer). Each spot was done in 4 replicates and the mean value of relative fluorescence intensity (RFU) was obtained. For comparison, slides were scanned with identical scanning parameters. Data were analyzed using GraphPad Prism software (23).

Statistical analysis

One-way ANOVA followed by Dunnett or Kruskal-Wallis tests for multiple comparisons or Fisher’s exact test were used where appropriate.

For assessing the relationship between serum galectin concentrations and mortality risk in cancer patients, Cox proportional hazards analysis (STATA, version 10.0, StataCorp, College Station, TX) was used. The analyses were adjusted for age, gender and disease stage. Hazard ratios were used to describe the change of mortality risk when the galectin levels are increased by 2- or 10-fold. P<0.05 from 2-sided tests were considered to be statistical significance.
Results

The concentrations of several galectin members are significantly altered in the sera of colorectal and breast cancer patients in comparison to healthy people

The concentrations of 6 galectins (galectin-1, -2, -3, -4, -8 and -9) were analysed in the sera from 31 healthy people, 51 colorectal cancer (40 without detectable metastasis and 11 with liver metastasis) and 40 breast cancer patients. In comparison to healthy people, the concentrations of four galectins, galectin-2, -3, -4 and -8, were significantly increased in both colorectal and breast cancer patients (Fig 1A-F). The median concentrations of galectin-2 were 1.9-fold higher (p<0.001) in colorectal cancer patients and 2.3-fold higher (p<0.001) in those who also had liver metastases (Supplementary Material, Table S3). The galectin-3 concentration was 11.3-fold higher (p<0.001) in colorectal cancer patients and 31.6-fold higher (p<0.001) in those with metastases. Galectin-4 was 11.1-fold higher (p<0.001) in colorectal cancer patients and 25.3-fold higher (p<0.001) in those with metastases. Galectin-8 was 1.8-fold higher (p<0.01) in colorectal cancer patients and 5.6-fold (p<0.01) higher in those with metastases. The levels of serum galectin -2, -3, -4 and -8 were also significantly increased (1.2-, 11.3-, 11.0- and 1.8-fold higher, respectively) in the serum of breast cancer patients. The concentration of serum galectin-1 was significantly lower in breast cancer patients than in healthy people and galectin-9 was unchanged but both of these galectins showed significant increase in colon cancer patients with metastasis. Spearman correlation analysis showed trends of correlations between cancer stages and the levels of galectin-2, -3 and -8 (p=0.15, 0.27 and 0.12, respectively) in colorectal cancer and of galectin-2 and -4 (p=0.14 and 0.12, respectively) in breast cancer, although none of those reached statistical significance.

As earlier studies have suggested that circulating clotting factors carry both N- and O-linked
glycans and are recognized by some galectins (25, 26), we assessed possible differences between serum and plasma levels of these galectins by spiking freshly obtained blood samples before clotting or addition of heparin and separation of serum or plasma. We found no significant differences in the galectin-1 and -2 levels in the plasma in comparison to serum but galectin-3 and -4 levels were significantly (3.0- and 1.9-fold) higher in plasma than in serum. This suggests that the real circulating concentrations of galectin-3 and -4 for healthy people and the cancer patients are higher than those reported here in sera.

**Galectins increase cancer cell adhesion to endothelial cells**

Our previous investigations have shown that an increased circulation of galectin-3 enhances cancer cell adhesion to vascular endothelial cells in metastasis (15). As the concentrations of galectin-2, -4 and -8 were also shown to be increased in the circulation of cancer patients and in particularly those with metastasis, we assessed the effects of those galectin members on cancer cell adhesion to vascular endothelium.

Pre-incubation of human colon cancer HT29-5F7 cells with recombinant galectin-2, -4 or -8 at concentrations similar to those observed in cancer patients all induced a dose-dependent increase of HT29-5F7 cell adhesion to HUVEC monolayers (Fig 2A). At 1 μg/ml, galectin-2, -4 and -8 caused 168±73% (p<0.001, ANOVA, Dunnett’s), 189±32% (p<0.001) and 180±21% (p<0.001), respectively, increase in cell adhesion. The effects of these galectins on cancer cell adhesion were completely abolished by the presence of 10 μM lactose (Fig 2B). Furthermore, each galectin at 1 μg/ml also induced significant increase of adhesion to human micro-vascular lung endothelial cells (HMVEC-Ls) of HT29-5F7 as well as SW620 cells, but not HT29 cells (Fig 2C). HT29-5F7 cells were a subpopulation of the parental HT29 cells selected for their resistance to 5-fluorouracil and have been shown previously to have higher
MUC1 expression than the parent HT29 cells (17, 20).

The expression of TF-expressing MUC1 is important in galectin-mediated cancer-endothelial adhesion

Our previous studies have shown that galectin-3 increases cancer cell adhesion to endothelial cells by interaction with the TF disaccharide on cancer associated MUC1 (16), the discovery that galectin -2, -4 and -8 could each induce cancer cell adhesion to endothelial cells led us to investigate whether these galectin effects were also, like that of galectin-3, associated with interaction of the galectins with the TF disaccharide on MUC1.

To test this, we first determined by direct galectin ELISA whether these galectin members recognized the TF disaccharides. It was found that all these galectins recognized the TF-expressing Antarctic fish antifreeze glycoprotein (AFG), asialo-fetuin (ASF) and asialo bovine mucin (ABM) although with different binding affinities (Fig 3A). ABM is the strongest ligand for galectin-4 while ASF is the strongest ligand for galectin-2. Mucin proteins, such as ABM, are known to carry TF as well as many other carbohydrate structures (27). Treatment of ABM with O-glycanase specific for removal of unsubstituted TF, caused 4.2-fold reduction of TF expression (Fig 3B) and markedly reduced the binding (by 50 to 80%) of all the galectin members (Fig 3C). These results indicate binding of all those galectin members to terminal TF disaccharides.

To assess any involvement of cancer-associated TF disaccharide in galectin-mediated cell adhesion, we pre-treated the HT29-5F7 cells with O-glycanase and assessed the influence of this on galectin-mediated cell adhesion. O-glycanase treatment of HT29-5F7 cells resulted in 70% reduction of cell surface TF expression when assessed by TF-binding peanut agglutinin
analysed by flow cytometry (Fig 3D). Removal of the cell surface TF by O-glycanase treatment markedly reduced HT29-5F7 cell adhesion to HMVEC-Ls induced by each of these galectin members (Fig 3E). This suggests that the terminal TF disaccharide on cancer cell surface is involved in galectin-mediated cancer cell adhesion to endothelium.

Having demonstrated the involvement of cancer-associated TF in galectin-mediated cell adhesion, we then assessed the role of MUC1 in galectin-mediated cell adhesion. It was found that treatment of HT29-5F7 cells with MUC1 siRNA caused 87% reduction of MUC1 expression (Fig 4A) and this significantly attenuated the increased cell adhesion to HMVEC-Ls mediated by each of the galectins (Fig 4B). Together, these results indicate that the MUC1-associated TF contributes to galectin-mediated cancer-endothelium adhesion. This conclusion is further supported by the observations that the presence of each of these galectins induced significant increase of adhesion to HMVEC-Ls of the MUC1 positively-transfected (also TF-expressing) (15) HCA1.7+ cells but not of the MUC1 negative revertants HCA1.7- of human breast epithelial HBL-100 cells (Fig 4C).

**Galectin cell surface binding induces change in MUC1 cell surface localization.**

As the galectin-3-mediated cancer cell adhesion is associated with change in MUC1 cell surface localization and exposure of the smaller cell surface adhesion molecules (15), we then analysed whether the interaction between TF/MUC1 and galectin-2, -4 and -8 also induced changes in MUC1 cell surface localization. Treatment of the HCA1.7+ cells with each of these galectin members (1 µg/ml) resulted in a significant increase in the proportion of cells showing discontinuous MUC1 cell surface localization in comparison to the cells treated with control BSA (Fig 4D and Supplementary Table S4). Thus, as previously shown for galectin-3, the influence of these galectins on cancer cell adhesion is associated with
polarisation of MUC1 cell surface localization, which allows exposure of the underlying adhesion molecules.

**Increased circulation of galectin-2 correlates with increased mortality risk in colorectal cancer patients**

The discovery that several galectin members are all increased in the circulation of colon and breast cancer patients and that the presence of these galectins enhances cancer cell adhesion to vascular endothelium led us to investigate whether the elevated levels of these galectins in the circulation have direct links with patient’s survival.

To test this hypothesis, we used a Cox regression model to analyse the relationship between circulating galectin levels at the time of surgical removal of the primary tumours and subsequent 10-year survival for colorectal and breast cancer patients. This survival regression analysis, which was adjusted for age, gender and disease stage, showed no significant correlation between the serum concentrations of galectin-1, -3, -4 and -8 and patients survival for either colorectal or breast cancer patients (Table 1). However, the increased level of circulating galectin-2 correlated with a significantly increased mortality in colorectal cancer patients. A 2-fold increase of serum galectin-2 level was associated with an 18% increase of mortality risk while a 10-fold increase associated with a 75% increased risk of 10-year mortality (p=0.013).

**Serum galectin-2-associated reduction in survival is reduced by co-existence of auto-antibodies against TF/MUC1**

It is known that various auto anti-MUC1 antibodies exist in the blood circulation of cancer patients (23). As the influence of circulating galectins on cancer cell adhesion to vascular
endothelium is attributed by their interaction with cancer-associated TF/MUC1, we further speculated that the presence of auto anti-MUC1 antibodies specifically against the TF epitope of MUC1 may produce a preventive effect on galectin-mediated adhesion hence metastasis as a result of their competitive binding to TF/MUC1 on disseminating tumour cells in the bloodstream.

To test this possibility, we determined the levels of auto anti-MUC1 antibodies against the TF as well as sialyl-TF epitope of MUC1 in the sera of 51 colorectal cancer patients using an O-glyco-peptide microarray (23). The serum levels of the auto anti-MUC1 antibodies against its TF epitope and sialyl-TF epitope in these patients ranged from 0 to 19444 and from 28 to 20763 units respectively (Supplementary Fig S1A and 1B). When the patient samples were divided into two subgroups with equal numbers of high and low (above and below the median 1290 units) levels of auto-antibodies against TF/MUC1, the galectin-2-associated increase of mortality risk remained significant only in the low level of auto anti-TF/MUC1 group but not in the high level of auto-anti-TF/MUC1 group (Table 2). On the other hand, when the patient samples were divided into two subgroups with equal numbers of high and low (above and below the median 2055 units) levels of auto-antibodies against the sialylated-TF/MUC1, the galectin-2-associated increase of mortality risk remained significant in both groups (p=0.043 in the high group and p=0.004 in the low group). No correlation was seen between the levels of auto-anti-TF/MUC1 and anti-sialyl-TF/MUC1 antibodies themselves and mortality risk (not shown). It is known that sialylation of the TF residue of MUC1 reduces galectin binding to TF/MUC1 (16). Collectively, these discoveries suggest that the presence antibodies against the TF-glycosylated MUC1 epitope prevents the interaction of circulating galectins with cancer cell-associated TF/MUC1 and hence blocks their effect on cancer-endothelial adhesion in metastasis as a result of competitive binding to cancer-
associated TF/MUC1. This conclusion is supported by the demonstration that the presence of a human anti-TF antibody (TF5) resulted in significant inhibition of the increase of HT29-5F7 cell adhesion to HMVEC-Ls induced by each of these galectins (Supplementary Fig S1C).
Discussion

This study shows that the concentrations of serum galectin-2, -3, -4, and -8 levels are all significantly higher in colon and breast cancer patients than in healthy people. Patients with metastasis also have higher levels of circulating galectins than those without metastasis. The presence of these galectins promotes cancer cell adhesion to vascular endothelium in vitro as a result of the galectin interactions with cancer-associated TF/MUC1. Such a metastasis promoting effect of circulating galectins is supported by a direct association between increased circulating galectin-2 level and increased mortality risk in colorectal cancer patients, an association that is diminished when a subgroup of auto anti-MUC1 antibodies with specificity for TF-glycosylated MUC1 is also present in the circulation, as a result of their competitive binding to cancer-associated TF/MUC1.

The mechanism for the increased circulating galectin in cancer patients is unclear. Members of the galectin family are expressed by many types of human cells including epithelial, endothelial and immune cells. Earlier reports have shown that the plasma or serum levels of galectin-3 (9), -1 and -4 (28) were significantly reduced following surgical removal of the primary tumours in colorectal cancer patients. This indicates that tumour cells may make significant contributions to the increased circulation of those galectin members. On the other hand, cellular expressions of galectin-8 and -4 have been reported to be lower in colorectal cancer than in healthy colonic epithelium (29, 30). It seems likely therefore that other cells, eg stromal or immune cells, may be the major contributor to the increased circulation of these galectins, in particularly that of galectin-8 and -4. This is in keeping with a recent report showing lack of correlation between galectin-3 serum level and corresponding cancer tissues in thyroid cancer (31).
Several galectin members e.g. galectin-1 and -3, are over-expressed in the tissues surrounding tumours. In colorectal cancer, galectin-1 expression is stronger in the peri-tumour stromal cells than in the tumours (32). It is possible therefore the peri-tumour stromal cells may make important contribution to the increased circulation of galectins in cancer. All immune cells including monocytes, macrophages and lymphocytes express galectins. The expressions of galectins in immune cells are heavily influenced by inflammatory regulators and also by differentiation (33) and activation (34). When stimulated with cytokine GM-CSF, monocytes have shown to secrete more galectin-3 in cell culture condition (35). Many pro-inflammatory cytokines, such as TNF-α, IL-1, IL-8 and GM-CSF, are up-regulated in cancerous conditions (1), and their presence may cause the immune cells to secret more galectins into the bloodstream. Thus, the increased circulation of the members of galectins in cancer patients is likely come from the peri-tumour stromal tissues, the immune cells as well as the tumour cells themselves.

Interestingly, although the serum concentrations of several galectin members are shown in this study to be higher in colon cancer patients and all of these galectins at pathological concentrations increase cancer-endothelial adhesion *in vitro*, only the increased level of circulating galectin-2 shows a direct correlation with increased mortality risk in the patients studied here. Although it is possible that the sample size of this study is not large enough to elucidate all the potential relationship of these galectins with survival, the effects of some galectin members on cancer-endothelial adhesion and metastasis are highly likely to be influenced by the presence of different galectin binding ligands or antibodies in the circulation. Members of the galectin family have shown to bind serum proteins with very different affinities. For example, serum IgGA1 binds strongly to galectin-1 (36) while a haptoglobin-related serum glycoprotein (37) and the 90K/Mac-2bp (38) are serum ligands of
galectin-3. A systematic analysis of the galectin binding proteins in human serum using galectin affinity purification followed by electrophoresis and mass spectrometry has shown that galectin-3, -8 and -9 each recognizes a much broader range of serum ligands while galectin-2, -4 and -7 each show binding to only trace or no serum ligands (39). Thus, the influence on metastasis and patient survival of circulating galectin-2 demonstrated in this study may reflect the relative lack of circulating competitive ligands for this galectin.

It is not clear why the increased galectin-2 level in breast cancer patients was not found to be associated with increased mortality risk as in colorectal cancer. The increase of circulating galectin-2 in breast cancer patients was less marked than in colorectal cancer. Further studies performed in larger patient cohorts may clarify the role of galectin-2 in breast cancer. In contrast to the findings here, one recent study found no increase in plasma galectin-2 concentration in colorectal cancer (28). It is possible that the different use of the testing antibodies in that study might contribute to this discrepancy.

The discovery of a protective effect of circulating auto-antibodies against specifically the TF epitope of MUC1 on galectin-mediated metastasis is very interesting. This may provide some explanation for reported inconsistencies in the relationship between the level of general anti-MUC1 antibodies and cancer survival. The levels of auto anti-MUC1 antibodies have shown to correlate with survival in breast (40) and gastric (41) cancer but not in colorectal cancer (42). It now seems likely that there is a complex relationship between circulating galectin concentration, the presence of circulating auto-antibodies against TF-glycosylated MUC1, and cancer survival.
The results presented here also imply that the low efficacy of many of the MUC1-associated immunotherapeutic approaches (either antibody based- or peptide vaccine-based) (43) may be partly due to the varying glycosylation of MUC1. A more selective targeting of TF-glycosylated MUC1 might be more effective.

Galectins bind to various galactoside-terminal carbohydrate structures that are expressed by various human cells including different immune cells. Galectins are important immune regulators and regulate cell activation, apoptosis, adhesion, migration and chemotaxis (44-47). An increased circulation of several galectin members in cancer patients may alter immune surveillance, which, in turn, could contribute to cancer progression and metastasis. Although the concentrations of serum galectins in cancer patients demonstrated in this study is lower than those typically used in the in vitro galectin studies for immune cells (usually at concentrations >10-20μg/ml), galectin binding can induce ligand clustering which, in the case of galectin-3, can enhance the galectin binding affinity by as much as 10,000-fold (48). Functionally important galectin concentrations may therefore be achieved in the micro-environment and produce a significant influence on immune surveillance in cancer patients.

Watanabe et al have recently reported a similar increase of circulating galectin-3 and -4 in colorectal cancer patients (28). It is noted that the Watanabe study also reported a significant increase of plasma galectin-1 level in colorectal cancer patients while the present study showed a significant increase of serum galectin-1 in colon cancer patients that only have liver metastasis. It is possible that the use of plasma samples in the Watanabe study and the use of serum samples in this study may account for this discrepancy as a consequence of some binding of galectins to activated clotting factors.
Thus, increased circulation of members of the galectin family is a common feature in cancer patients and may, like that of circulating galectin-3, also promote disseminating tumour cell adhesion to blood vascular endothelium in metastasis as a result of their interactions with the cancer-associated TF/MUC1. Such a metastasis-promoting effect of circulating galectins is, however, prevented when a subgroup of auto anti-MUC1 antibodies against the TF-epitope of MUC1 is also present in the blood circulation as a result of their competitive binding to the cancer-associated TF/MUC1. Elevated circulating galectin-2 concentrations are a marker of poor prognosis in colorectal cancer whilst higher levels of circulating auto anti-MUC1 antibodies with specificity for the TF-epitope of MUC1 abolished circulating galectin-2-associated poor prognosis. This implies that circulating galectins may represent promising therapeutic targets for the development of effective agents to reduce metastasis.

**Abbreviations:**

AFG, Antarctic fish antifreeze glycoprotein; ABM, Asialo (bovine) submaxillary mucin; ASF, asialo fetuin; EGM-2, endothelial growth medium-2; FOV, field of view; HMVEC-Ls, human micro-vascular lung endothelial cells; NECDS, Non-enzymatic cell dissociation solution; TF disaccharide, galactose-\(\beta_1,3\)N-acetyl-galactosamine\(\alpha\)-) Thomsen-Friedenreich antigen.

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Figure legends

Figure 1. Concentrations of circulating galectins in the sera of colorectal and breast cancer patients and healthy people. Serum galectin-1, -2, -3, -4, -8 and -9 levels were assessed by individual galectin ELISA and calculated from standard curves derived using recombinant galectins run in parallel in each assessment (galectin-9 level is shown as OD reading as no recombinant galectin-9 is commercially available as a standard) (note different y scales are used in the figures). *P <0.05, **P <0.01, ***<0.001.

Figure 2. Galectins increase human colon cancer cell adhesion to monolayers of macro- and micro-vascular endothelial cells.

A. The presence of recombinant galectin-2, -4 or -8 each induced a dose-dependent increase of HT29-5F7 cell adhesion to monolayers of HUVECs (human umbilical vein endothelial cells). Data are expressed as mean ± SD of triplicate determinations of two independent experiments. B. The presence of galectin inhibitors abolishes galectin-induced HT29-5F7 cell adhesion to HUVECs. Data are expressed as mean ± SD of 10 randomly-selected FOV of two independent experiments. C. The presence of each galectins induces cell adhesion to HMVEC-Ls of HT29-5F7 and SW620 cells but not parent HT29 cells. Data are expressed as mean ± SD of triplicate determinations of two independent experiments. *** <0.001.

Figure 3. Galectin-mediated cancer cell adhesion is associated with galectin binding to TF disaccharide on cancer cells.

A. Direct galectin ELISA shows binding of recombinant galectin-2, -4 and -8 to TF-expressing- asialo bovine mucin (ABM), asialo fetuin (ASF), Antarctic fish anti-freeze glycoprotein (AFG). Data are expressed as mean ± SD of triplicate determinations. B. O-glycanase treatment of ABM reduces the expression of TF. ABM (5μg/ml) was treated with
or without 1.25 U/ml O-glycanase (OG) before it was used as substrate for the detection of TF expression by ELISA with TF-binding peanut agglutinin (PNA). C. Removal of the TF on ABM by O-glycanase reduces galectin binding. Data are expressed as mean ± SD of triplicate determinations. D. Treatment of HT29-5F7 cells with O-glycanase reduces cell surface TF expression. The cell surface TF expression was assessed with TF-binding PNA (peanut agglutinin) and analysed by flow cytometry after treatment of the cells O-glycanase (Solid line histogram: without O-glycanase treatment; dotted line histogram: after O-glycanase treatment; shaded histogram: isotype control). A shift of the histogram to the left after O-glycanase treatment indicates a reduction of the cell surface TF expression. E. Removal of the cell surface TF by O-glycanase reduces galectin-mediated HT29-5F7 cell adhesion to HMVEC-Ls. Data are expressed as mean ± SD of absolute number of cells per FOV from least 10 randomly-selected FOVs of triplicate determinations of two independent experiments. *P<0.05, **P <0.01, ***<0.001.

**Figure 4.** Galectin-mediated cancer cell adhesion is associated with binding of the galectins to MUC1.

A. Suppression of MUC1 expression by siRNA. HT29-5F7 cells were treated with or without MUC1 siRNA or control siRNA before the expressions of MUC1 or β-actin were assessed by anti-MUC1 (B27.29) or anti- β-actin immunoblotting. Representative blots are shown. B. siRNA MUC1 suppression abolishes galectin-mediated cell adhesion to HMVEC-Ls. Data are expressed as mean ± SD of triplicate determinations of two independent experiments. C. Galectins induce adhesion to HMVEC-Ls of MUC1-positively transfected but not negatively-transfected HBL-100 cells. Data are expressed as mean ± SD of triplicate determinations of two independent experiments. D. Representative images of MUC1 cell surface localization of
HCA1.7+ cells without or with treatment of each galectin (1μg/ml).  *P <0.05, **P <0.01, *** <0.001

**Table 1.** Relationship between serum galectin levels and 10-year mortality risk in colon and breast cancer patients. Cox hazard analysis of the serum galectin levels and patients’ survival within 10 years after primary tumour removal were conducted in 51 colorectal and 40 breast cancer patients. The results (hazard ratios) describe the change of mortality risk when the galectin levels are increased by 2-fold or 10-fold.

**Table 2.** Influence of the presence of auto antibodies against TF and sialyl-TF epitope of MUC1 on serum galectin-2-associated mortality risk in colorectal cancer. The serum auto anti-MUC1 antibodies against the TF and sialyl-TF epitopes of MUC1 in the 51 colorectal cancer patients were assessed by glycan microarray coated with 20 μM MUC1 glyco-peptides (60mer) as described in the method. The serum samples were then divided into two groups of above and below the median concentrations of either anti-TF/MUC1 or anti-sialyl-TF/MUC1 antibody. The serum galectin-2-associated mortality risk in patients’ survival in the 10-year period was then analysed by Cox hazards analysis.
References


36. Sangeetha SR, Appukuttan PS. IgA1 is the premier serum glycoprotein recognized by human galectin-1 since T antigen (Galbeta1-->3GalNAc-) is far superior to non-repeating N-acetyl lactosamine as ligand. Int J Biol Macromol. 2005;35:269-76.
Barrow et al, Fig 1

**A**

Graph showing Galectin-1 levels (ng/ml) across healthy, colorectal, colorectal/L. Mets, and breast tissues.

**B**

Graph showing Galectin-2 levels (ng/ml) across healthy, colorectal, colorectal/L. Mets, and breast tissues.

**C**

Graph showing Galectin-3 levels (ng/ml) across healthy, colorectal, colorectal/L. Mets, and breast tissues.

**D**

Graph showing Galectin-4 levels (ng/ml) across healthy, colorectal, colorectal/L. Mets, and breast tissues.

**E**

Graph showing Galectin-8 levels (ng/ml) across healthy, colorectal, colorectal/L. Mets, and breast tissues.

**F**

Graph showing Galectin-9 (OD492) levels across healthy, colorectal, colorectal/L. Mets, and breast tissues.
Hannah et al, Fig 2

A

HT29-5F7 adhesion to HUVECs (Fluorescence intensity)

Gal-2, Gal-4, Gal-8, BSA

B

HT29-5F7 adhesion to HUVECs (Cells/FOV)

Gal-2, Gal-4, Gal-8

C

Cancer cell adhesion to HMVEC-Ls (fluorescence intensity)

HT29, HT29-5F7, SW620

Control (BSA), Gal, Gal+Lac, Lac
Hannah et al, Fig 3

A

Galactin binding (OD492)

Gal-2

Gal-4

Gal-8

B

TF expression by ABM (OD492)

Untreated

OG-treated

C

Galactin binding to ABM (OD492)

Gal-2

Gal-4

Gal-8

D

Cell counts

FLH

E

HT29-5F7 adhesion to HMVECs (Cells/FOV)

Control (BSA)

Gal-2

Gal-4

Gal-8

Untreated

OG-treated
Hannah et al, Fig 4

(A) Western blot showing MUC1 expression in control vs. MUC1 siRNA-treated cells, with Actin as a loading control. MUC1 migration is indicated with an arrow.

(B) Graph showing HT29-5F7 adhesion to HMVECs with BSA, Gal-2, Gal-4, and Gal-8, comparing control, con-siRNA, and MUC1 siRNA treatments. Significant differences are indicated with asterisks (*, **, ***).

(C) Bar graph showing cell adhesion to HMVEC-Ls with HCA1.7- and HCA1.7+ cells, with ** and *** indicating significant differences.

(D) Representative images showing adhesion of control and Gal-2 treated cells to HMVEC-Ls.
Table 1. Serum galectin levels and 10-years mortality risk in colorectal and breast cancer patients

<table>
<thead>
<tr>
<th></th>
<th>Hazard Ratio</th>
<th>SE</th>
<th>P value</th>
<th>95% CI</th>
</tr>
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<tr>
<td><strong>Colorectal cancer (n=51)</strong></td>
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<tr>
<td>Gal-1 (2-fold)</td>
<td>0.96</td>
<td>0.074</td>
<td>0.60</td>
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<td>0.88</td>
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<td>0.40</td>
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<td>1.12-2.75</td>
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<td>Gal-3 (2-fold)</td>
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<td>0.07</td>
<td>0.97</td>
<td>0.87-1.14</td>
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<td>0.23</td>
<td>0.97</td>
<td>0.63-1.57</td>
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<td>Gal-4 (2-fold)</td>
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<td>0.54</td>
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<td>0.79-1.00</td>
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<td>0.68</td>
<td>0.14</td>
<td>0.06</td>
<td>0.46-1.02</td>
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<tr>
<td>Gal-8 (2-fold)</td>
<td>1.01</td>
<td>0.98</td>
<td>0.94</td>
<td>0.82-1.22</td>
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<td>1.03</td>
<td>0.33</td>
<td>0.94</td>
<td>0.54-1.93</td>
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<td><strong>Breast cancer (n=40)</strong></td>
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<tr>
<td>Gal-1 (2-fold)</td>
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<td>0.11</td>
<td>0.82</td>
<td>0.83-1.26</td>
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<tr>
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<td>1.08</td>
<td>0.38</td>
<td>0.82</td>
<td>0.55-2.14</td>
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<tr>
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<td>0.11</td>
<td>0.82</td>
<td>0.83-1.27</td>
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<td>(10-fold)</td>
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<td>0.53-2.22</td>
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<td>0.45</td>
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<td>0.70</td>
<td>0.33</td>
<td>0.45</td>
<td>0.28-1.76</td>
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### Table 2. Influence of the presence of circulating auto antibodies against the TF and sialyl-TF epitope of MUC1 on serum galectin-2-associated mortality risk in colorectal cancer

<table>
<thead>
<tr>
<th></th>
<th>Hazard Ratio</th>
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<th>P value</th>
<th>95% CI</th>
</tr>
</thead>
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<td></td>
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<tr>
<td>High (n=26)</td>
<td>1.75</td>
<td>0.87</td>
<td>0.26</td>
<td>0.67-4.63</td>
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<td>2.15</td>
<td>0.73</td>
<td>0.024</td>
<td>1.10-4.17</td>
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<tr>
<td><strong>Anti-Sialyl-TF/MUC1</strong></td>
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<tr>
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<td>2.06</td>
<td>0.73</td>
<td>0.043</td>
<td>1.02-4.15</td>
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<tr>
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<td>1.13</td>
<td>0.004</td>
<td>1.44-6.31</td>
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Serum galectins -2, -4 and -8 are greatly increased in colon and breast cancer patients and promote cancer cell adhesion to blood vascular endothelium

Hannah Barrow, Xiuli Guo, Hans H Wandall, et al.

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