Abstract. Heparan sulphate is a sulphated glycosaminoglycan and is able to bind to and regulate the activity of many growth and signalling factors. We have previously shown that its expression is correlated with tumour grade and cell proliferation in breast phyllodes tumours. In this study, we examined the use of heparan sulphate as a biomarker of invasive ductal carcinoma and the effects of differentially sulphated heparan species on breast cancer cell behaviour. Immunohistochemistry using the 10E4 monoclonal antibody was carried out on 32 paraffin-embedded breast cancer specimens and paired non-cancerous breast tissues to compare the expression patterns of heparan sulphate. Upregulated expression of the sulphated 10E4 epitope in heparan sulphate was detected in both epithelial and stromal compartments of breast cancer compared with normal mammary tissues, with a 2.8X increase in immunoreactivity score. To determine the effects of differentially sulphated heparan sulphate molecules on breast cancer behaviour, cultured breast carcinoma cells were treated with chlorate, a competitive inhibitor of glycosaminoglycan sulphation, and two different heparan sulphate species. Inhibition of glycosaminoglycan sulphation resulted in a significant increase in cancer cell adhesion and a reduction in cell migration, together with upregulated expression of focal adhesion kinase and paxillin. Both porcine intestine- and bovine kidney-derived heparan sulphate species could block the change in cell adhesion. However, the former heparan sulphate species completely abolished, while the latter exacerbated, the chlorate-induced decrease in cell migration. The results show that heparan sulphate is a useful biomarker of breast invasive ductal carcinoma. Different sulphation patterns of heparan sulphate residues have differential effects in regulating breast cancer cellular behaviour, and this may be exploited to develop heparan sulphate into a useful target for treatment of breast carcinoma.

Introduction

Breast carcinoma is the most widespread form of malignancy in women worldwide, and the commonest cause of cancer-related deaths (1,2). Besides clinical and histopathological staging and hormonal receptor status, cancer cell adhesion and migration are important parameters that affect patient prognosis (3,4). Thus, understanding the molecular regulators of cancer cellular behaviour and determination of their effects on tumour cell adhesion and migration are essential to a better comprehension of cancer biology, and is of fundamental importance in the development of therapeutic targets.

Heparan sulphate is an unbranched, polyanionic glycosaminoglycan composed of alternating repeats of glucosamine and glucuronic/iduronic acid residues (5,6). Heparan sulphate chains are attached to core protein backbones to form heparan sulphate proteoglycans, which can be found attached to the cell surface or within the extracellular matrix (7). The physiological function of heparan sulphate is highly dependent on the presence of sulphate groups, which modulate the ability of heparan sulphate to bind to and interact with different growth and signalling factors (7-9). The importance of differential sulphation of heparan sulphate has been highlighted by recent studies on knockout mice, in which loss of heparan 2-O-sulphation resulted in renal, ocular and skeletal defects whereas absence of N-sulphation led to pulmonary hypoplasia and respiratory distress in newborn pups (10-12).

Studies on the core proteins of heparan sulphate proteoglycans using breast cancer tissue samples have shown that these molecules may be important prognostic indicators. Several authors have reported increased expression of the core protein of syndecan-1, a transmembrane heparan...
sulphate proteoglycan, in women with aggressive forms of breast cancer associated with a poorer prognosis (13-15). Indeed, expression of syndecan-1 in breast ductal carcinoma in situ was found to be associated with the presence of angiogenic and lymphangiogenic factors, and correlated with the response of primary breast cancer to neoadjuvant chemotherapy (16,17). Upregulated expression of the core protein of glypican-1, a glycosyolphosphatidylinositol-linked heparan sulphate proteoglycan, was also noted in human breast cancer and influenced the response of cancer cells to growth factors (18).

In contrast to the heparan sulphate proteoglycan core proteins, relatively less is known about the potential use of the heparan sulphate glycosaminoglycan chain as a biomarker of invasive ductal carcinoma in clinical samples, and the effects of differentially sulphated heparan sulphate species on breast cancer cellular behaviour. We have recently shown that expression of the heparan sulphate glycosaminoglycan chain is correlated with tumour grade and cell proliferation in phyllodes tumours (19). In the current study, we present evidence that sulphated heparan is upregulated in human breast invasive carcinoma tissues, and that the level of sulphation influences tumour cellular behaviour. We also show that differentially sulphated porcine intestine- and bovine kidney-derived heparan sulphate species have dissimilar effects on breast carcinoma cells.

Materials and methods

Clinical samples. A total of 32 archived, formalin-fixed paraffin-embedded breast cancer specimens and paired non-cancerous breast tissues from the corresponding patients were obtained from the Department of Pathology, Singapore General Hospital for this study. Ethics approval was obtained from the Institutional Review Board, Singapore General Hospital.

Immunohistochemistry. Immunohistochemical staining of clinical samples using the 10E4 antibody was performed as previously described (19). Briefly, 4-μm thick tissue sections were deparaffinised and rehydrated. Antigen retrieval using 0.1 mg/ml testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO) in PBS was carried out at room temperature for 10 min. The sections were deparaffinised and rehydrated. Antigen retrieval using 0.1 mg/ml testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO) in PBS was carried out at room temperature for 10 min. The sections were then washed with PBS and the unbound sites blocked using 1% bovine serum albumin for 1 h at room temperature. The wells were then washed with PBS and dried.

Cells were pre-cultured for 48 h in serum-containing DMEM supplemented with PBS (control group), 30 mM chloride or 30 mM chloride plus 100 ng/ml heparan sulphate. The cells were then collected and seeded at a density of 1x10^6 cells per well in the above fibronectin-coated culture plates and allowed to attach for 30 min at 37˚C. The attached cells were washed with PBS, fixed for 15 min in 4% paraformaldehyde, and stained for 30 min using 0.25% crystal violet in 20% methanol. After washing, the number of attached cells was determined by releasing the crystal violet with 1% sodium dodecyl sulphate and measuring the absorbance at 595 nm.

Cell adhesion assay. Coating of 96-well culture plates with 20 μg/ml fibronectin (BD Biosciences, San Jose, CA) in phosphate-buffered saline (PBS) was carried out overnight at 4˚C (21). The wells were washed with PBS and the unbound sites blocked using 1% bovine serum albumin for 1 h at room temperature. The wells were then washed with PBS and dried.

Cultured cells were seeded at a density of 1x10^5 cells per well in the above fibronectin-coated culture plates and allowed to attach for 30 min at 37˚C. The attached cells were washed with PBS, fixed for 15 min in 4% paraformaldehyde, and stained for 30 min using 0.25% crystal violet in 20% methanol. After washing, the number of attached cells was determined by releasing the crystal violet with 1% sodium dodecyl sulphate and measuring the absorbance at 595 nm.

Cell migration assay. Cancer cells were cultured in serum-containing DMEM in 6-well plates until they reached 90% confluence. A horizontal line was then scraped across the bottom of each well using a sterile 100-μl plastic pipette tip, after which the culture was continued and the culture medium was supplemented with PBS (control group), 30 mM chloride or 30 mM chloride plus 100 ng/ml heparan sulphate. The average distance between the wound edges in each well was determined by measurement at five randomly selected sites along the length of the wound. The difference in the wound gap distance at 0 and 18 h after scraping was calculated to determine the distance migrated.

Cell proliferation assay. Cells were seeded in 96-well plates at a density of 4x10^3 cells per well and cultured for 72 h in serum-containing DMEM supplemented with PBS (control group), 30 mM chloride or 30 mM chloride plus 100 ng/ml heparan sulphate. At the end of the culture period, the cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min, and stained using 0.25% crystal violet in 20% methanol for 30 min. After washing, 1% sodium dodecyl sulphate was added for 1 h to release the crystal violet, and
specificity of the amplification, and the size of the PCR step of 60˚C for 25 sec, and extension step of 72˚C for 18 sec described: denaturation step of 94˚C for 15 sec, annealing for 15 min, 45 PCR cycles were performed as previously listed in Table I. After an initial activation step of 95˚C (Roche, Indianapolis, IN) with the intron-spanning primers hexamers, real-time PCR was carried out in a LightCycler of cDNA using Superscript III (Invitrogen) and random according to the manufacturer's protocol. After synthesis cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) Real-time RT-PCR. Fluorescence immunocytochemistry. Cover slips were coated with fibronectin as described above. Cells were pre-cultured for 48 h in serum-containing DMEM supplemented with PBS (control group) or 30 mM chlorate. They were seeded at a density of 1x10^6 cells per coverslip and allowed to attach for 2 h to form adhesion. Untagged cells were then washed off. Attached cells were fixed in 4% paraformaldehyde for 10 min and washed with PBS containing 0.2% Triton X-100. After blocking, the cells were incubated with a 1:100 dilution of either mouse anti-paxillin IgG, antibody, clone 165 (BD Biosciences) or rabbit anti-F AK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4˚C overnight as previously described (22,23). After washing, the signal was detected using an Alexa Fl ouor 568 goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) or an Alexa Fl uor 568 goat anti-rabbit secondary antibody respectively. To achieve double fluorescence labelling for F-actin, the cells were then incubated with Alexa Fluor 488 phalloidin (1:50 dilution) for 1 h at room temperature. The samples were examined using a Fluoview FV1000 laser scanning confocal microscope (Olympus, Melville, NY). For immunocytochemical detection of heparan sulphate, cells were fixed using Sainte-Marie's fixative as this gives better preservation of glycosaminoglycans (24). After blocking, cells were incubated with the anti-heparan sulphate antibody, and requires the presence of either mouse anti-paxillin IgG1 antibody, clone 165, mouse anti-FAK IgG1 antibody clone 165, mouse anti-FAK IgG1 antibody clone 165, mouse anti-FAK IgG1 antibody clone 77, and mouse anti-β1-integrin IgG1 antibody clone 18 (all from BD Biosciences). The relative protein expression level was determined by densitometry measurement of the band intensity and normalisation to β-actin. Statistical analysis. All experiments consisted of at least three replicates. Statistical comparison between two groups was performed by the Student's t-test, and among three groups by one-way analysis of variance (ANOVA) with Tukey's post test using GraphPad Prism v4.03 for Windows (GraphPad Software, San Diego, CA). The Wilcoxon matched pairs test was used for comparison of clinical samples. Statistical significance was defined as a p-value of <0.05.

Results

Expression of heparan sulphate in breast cancer tissues. To determine if heparan sulphate is differentially expressed between breast carcinoma and non-cancerous breast tissues, we examined the expression of the 10E4 epitope in both epithelial and stromal compartments of 32 samples of invasive ductal carcinoma and paired non-cancerous mammary tissues from the corresponding patients. The mouse monoclonal antibody 10E4 is a well-established anti-heparan sulphate antibody, and requires the presence

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of the sulphate moiety on heparan sulphate for binding to its epitope (20,27,28). The mean age of the patients in the sample population was 60.4 years. As shown in Fig. 1, upregulated expression of the sulphated 10E4 epitope was detected in both the epithelial and stromal compartments of breast cancer compared with normal mammary tissues. Significantly, the mean immunoreactivity score of the cancer samples was 2.8 times higher than that of normal breast tissues, suggesting that heparan sulphate would be a useful biomarker for breast invasive ductal carcinoma.

**Sulphate group in heparan sulphate is involved in regulating breast cancer cell adhesion.** To determine if the sulphate group of heparan sulphate affects breast cancer cell adhesion, we cultured MCF-7 breast cancer cells in the presence of 30 mM chlorate. Chlorate is a widely used and well-known competitive inhibitor of glycosaminoglycan sulphation. It acts as a sulphate analogue in the cellular synthesis of the sulphate donor 3’-phosphoadenosine 5’-phosphosulphate, which is required for glycosaminoglycan sulphation (5,20,29-32). Previous studies involving breast cancer cell cultures and other biological systems have shown no significant effects on glycosaminoglycan chain or protein synthesis or on cell viability when chlorate was used at concentrations of up to 30 mM.

When 30 mM chlorate was added to the culture medium, MCF-7 cells showed an increase in cell adhesion, suggesting that glycosaminoglycan sulphation regulates breast cancer cell adhesion (Fig. 2A). A similar increase was also seen when MDA-MB-231 human breast cancer cells were cultured in medium containing 30 mM chlorate (Fig. 2B). Furthermore, supplementation of chlorate-containing culture medium with exogenous sulphate blocked the increase in cell adhesion, thus confirming that the increase was indeed due to competitive inhibition of glycosaminoglycan sulphation (Fig. 2A). To further verify that glycosaminoglycan sulphation was reduced by chlorate treatment, we extracted and measured the amount of sulphated glycosaminoglycans produced by the cancer cells using the Blyscan dye-binding assay. As shown in Fig. 2C, chlorate administration resulted in 38.4% reduction in glycosaminoglycan sulphation. A decrease in sulphation of heparan sulphate was confirmed by fluorescence immunocytochemical staining of chlorate-treated cells using the 10E4 anti-heparan sulphate antibody (Fig. 2D).

**Comparative effects of differentially sulphated heparan sulphate species on cancer cell adhesion.** Chlorate inhibits sulphation of all glycosaminoglycans and does not act specifically on heparan sulphate. Thus, to determine if the chlorate-induced increase in cancer cell adhesion was due to a reduction in sulphation of heparan sulphate molecules, bovine kidney-derived heparan sulphate was added to MCF-7 cells grown in chlorate-containing medium. As shown in Fig. 3A, supplementation of the culture medium with adequately sulphated heparan sulphate molecules completely abolished the effect of chlorate on cell adhesion, suggesting that the sulphation status of heparan sulphate is important in regulating cancer cell adhesion. To investigate this further, we repeated the above experiment but added porcine intestine-derived heparan sulphate to the chlorate-containing culture medium instead of the bovine kidney-derived species.
Heparan sulphate from porcine intestines possesses a lower degree of sulphation compared to that obtained from bovine kidney (33, 34). We hypothesised that it may thus be less effective in blocking the effect of chlorate on cell adhesion. Indeed, as shown in Fig. 3B, addition of porcine intestine-derived heparan sulphate resulted in only a partial, instead of complete, block of the chlorate-induced increase in cell adhesion.

We also examined the effect of inhibiting glycosaminoglycan sulphation on the expression of paxillin (PAX), focal adhesion kinase (FAK) and β1-integrin (ITGB1) in chlorate-treated MCF-7 cells. Paxillin and FAK are key regulatory components in cell adhesion and cell movement (35-37). We have previously shown that β1-integrin (ITGB1)

Figure 2. Effect of chlorate treatment on breast cancer cell adhesion. (A) MCF-7 cells were cultured in DMEM with 7.5% FBS in the presence of PBS (control), 30 mM chlorate (ClO₃⁻) or 30 mM chlorate plus 10 mM sulphate (SO₄²⁻). Comparison among all three groups using one-way ANOVA showed a statistically significant difference (p<0.0001), with the chlorate-alone treated group having greater cell adhesion compared to the control and the chlorate plus sulphate groups (Tukey’s post test; p<0.001). (B) MDA-MB-231 cells cultured in chlorate-containing medium also showed an increase in cell adhesion (Student’s t-test; p<0.0001). (C) Chlorate treatment resulted in a significant reduction in sulphation of glycosaminoglycans produced by MCF-7 cells (Student’s t-test; p<0.0005). (D) Immunofluorescence staining using the 10E4 anti-heparan sulphate antibody showed reduced staining intensity of chlorate-treated MCF-7 cells, demonstrating a decrease in sulphation of heparan sulphate molecules. Values represent mean ± SEM of at least three replicates. Scale bars, 20 μm.

Figure 3. Sulphation status of heparan sulphate regulates breast cancer cell adhesion. MCF-7 cells were cultured in serum-containing medium supplemented with PBS (control), 30 mM chlorate (ClO₃⁻), (A) 30 mM chlorate plus 100 ng/ml bovine kidney-derived heparan sulphate (HS-BK), or (B) 30 mM chlorate plus 100 ng/ml porcine intestine-derived heparan sulphate (HS-PI). Comparison among all three groups in each panel using one-way ANOVA showed a statistically significant difference (p<0.0001), with an increase in cell adhesion in the group treated with chlorate alone compared against the control group (Tukey’s post test; p<0.001). Supplementation with HS-BK completely abolished the effect of chlorate on cell adhesion, with cells in this group possessing a similar degree of adhesion compared with those in the control group (p>0.05). In contrast, HS-PI was only able to partially block the effect of chlorate, and cells in this group were more adherent compared against the control group (p<0.01). Values represent mean ± SEM of at least three replicates.

Figure 4. Effects of reduced glycosaminoglycan sulphation on ITGB1, FAK and paxillin expression. MCF-7 cells were cultured for 48 h in medium supplemented with PBS (control group) or 30 mM chlorate. (A) Gene transcript levels of ITGB1, FAK and PXN were measured using real-time RT-PCR. Chlorate treatment significantly upregulated the expression of FAK (p=0.0375) and PXN (p=0.0440). (B) Representative Western blot of three independent experiments. (C) Analysis by densitometry measurements showed increased levels of ITGB1 (p=0.0016), FAK (p=0.0002) and PXN (p=0.0003). Values represent mean ± SEM of three replicates.
influences the activity of FAK and paxillin, with resultant effects on breast cancer cell adhesion and spreading (23). Using real-time RT-PCR, reduction in glycosaminoglycan sulphation was shown to significantly increase the expression of FAK and PXN by 1.9X and 1.7X respectively (Fig. 4A). The expression of ITGB1 was also upregulated, although this did not reach statistical significance. The changes in gene transcript levels were accompanied by similar up-regulation in protein levels, as determined by Western blotting (Fig. 4B and C). Expressions of ITGB1, FAK and PXN proteins in chlorate-treated cells were increased by 1.3X, 3.1X and 1.6X respectively. Using fluorescence immuno-cytochemistry, cells in the treatment group were found to possess an increase in focal adhesion formation together with stronger staining intensities for PXN and FAK proteins compared against those in the control group (Fig. 5).

Contrasting effects of different heparan sulphate species on cancer cell migration. To determine if the increase in cell adhesion due to reduced glycosaminoglycan sulphation would affect cancer cell migration, we cultured MCF-7 cells (Fig. 6A) and MDA-MB-231 cells (Fig. 6B) in the presence of chlorate, and measured the distance migrated by the cells across a wound gap over an 18-h period. In both cases, inhibition of glycosaminoglycan sulphation resulted in a significant decrease in cell migration. This chlorate-induced reduction in cell migration could be completely ablished by supplementation of the culture medium with porcine intestine-derived heparan sulphate (HS-PI) (Fig. 6C). However, in contrast to what was observed in the cell adhesion experiments (Fig. 3), addition of bovine kidney-derived heparan sulphate to the chlorate-containing culture medium led to a significant further decrease in cell migration instead of blocking the effect of chlorate (p<0.05). This suggests that differentially sulphated heparan sulphate species have diametrically opposite effects on breast cancer cell migration.

To examine if changes in cell proliferation as a result of chlorate treatment would confound the interpretation of the wound gap cell migration data, we cultured MCF-7 cells in medium supplemented with chlorate and measured the effect on cell growth. Despite treating the cells with chlorate for 72 h, cell proliferation was reduced by only 11% (Fig. 6D). Although this was statistically significant, the small change in cell proliferation makes it unlikely to be an important confounding factor in interpreting the 42% decrease in cell migration resulting from chlorate treatment (Fig. 6A). Chlorate-treated cells did not show any chromatin condensation, suggesting that reducing glycosaminoglycan sulphation did not result in apoptosis (Fig. 6E).
Discussion

We have investigated the use of heparan sulphate glycosaminoglycan as a biomarker of invasive breast ductal carcinoma in patient samples, and compared the effects of differentially sulphated porcine intestine- and bovine kidney-derived heparan sulphate species on cancer cell adhesion and migration. Adhesion of cancer cells to the extracellular matrix is an important determinant of local tumour invasion and distant metastasis (38-41). Changes in cell adhesion enable tumour cells to invade into surrounding tissues and spread to distant sites, contributing significantly to patient morbidity and mortality.

Studies on heparanase, an endoglucuronidase that hydrolyses heparan sulphate, suggest that degradation of heparan sulphate leads to enhanced breast cancer growth and invasion (42,43). Upregulated expression of heparanase, with a consequential reduction in heparan sulphate level, has been reported in breast cancer with a larger primary tumour size and distant metastasis (44). These studies suggest that heparan sulphate glycosaminoglycan chains are involved in breast carcinogenesis and that their presence implies a poorer prognosis for patients. Indeed, in the present study, upregulated expression of the sulphated 10E4 epitope in heparan sulphate was seen in the epithelial and stromal compartments of invasive ductal carcinoma compared with the corresponding normal breast tissues.

However, recent studies have shown that not all heparan sulphate chains are bad, and that the sulphation status of different heparan sulphate species is an important determinant of the biological effects of these molecules on cancer cells. This has been highlighted in the past few years in reports on the SULF-1 gene, which codes for the enzyme sulfatase-1 that removes 6-O-sulphate groups from heparan sulphate (45-47). Loss of 6-O-sulphate groups was shown to inhibit growth of breast, pancreatic and hepatocellular cancers. In contrast, absence of SULF-1 resulted in chemoresistance. Our experiments with chlorate, which competitively inhibits glycosaminoglycan sulphation and thus mimics overexpression of sulphatase-1 in the loss of 6-O-sulphate groups, showed an increase in cell adhesion and a reduction in cell proliferation. This phenomenon could be blocked by addition of adequately sulphated heparan sulphate to the culture medium, with bovine kidney-derived heparan sulphate containing a larger number of sulphate groups being more efficacious than porcine intestine-derived heparan sulphate (Fig. 3).

Interestingly, heparan sulphate regulation of breast cancer cell migration appears to be affected not only by the number of sulphate groups present but also by the position of the sulphate groups. Thus, inhibition of heparan sulphation, as well as the presence of highly sulphated bovine kidney-derived heparan sulphate, both led to a significant reduction in cell migration (Fig. 6). In contrast, optimally sulphated porcine intestine-derived heparan sulphate was able to block the effect of chlorate treatment. The results are in agreement with the recently proposed concept of a ‘heparanome’, in which differentially sulphated sugar sequences regulate the biological activities of different heparan sulphate species (8,48).

Heparan sulphate has been shown to affect cancer cellular behaviour through several mechanisms (9,49). It is able to bind to and interact with a multitude of growth factors and signalling proteins, resulting in stimulation of growth and metastasis of cancer cells (7). Heparan sulphate in the extracellular matrix also acts as a reservoir for aggregation of growth and angiogenic factors. Furthermore, binding of heparan sulphate to these molecules could protect them from degradation and thus prolong their effects on cancer cells. In breast cancer cells, fibroblast growth factor-2 (FGF-2) binds to heparan sulphate and stimulates cellular proliferation. Degradation of heparan sulphate by heparinase treatment abolished binding of the growth factor as well as the FGF-2-induced tumour growth (30). Down-regulation of SULF-1 in MDA-MB-468 breast cancer cells, with the resultant persistent presence of 6-O-sulphate groups on heparan sulphate molecules, increases autocrine activation of the epidermal growth factor receptor-extracellular signal-regulated kinase (EGFR-ERK) pathway, mediated via amphiregulin and heparin-binding EGF-like growth factor (HB-EGF) (50). In addition, loss of SULF-1 has been shown to increase cell proliferation in tumour-associated angiogenesis through FGF-2, hepatocyte growth factor, and vascular endothelial growth factor (VEGF) signalling.

Heparan sulphate is capable of binding to fibronectin and many other components of the extracellular matrix, and helps to regulate cell adhesion (7). Integrin-mediated cell adhesion leads to recruitment of the cytoplasmic protein tyrosine kinase FAK to focal adhesion sites, and the phosphorylation of both FAK and paxillin (36,51-55). The transmembrane heparan sulphate proteoglycan syndecan-4 is an essential element in the formation of focal adhesions (56-58). Expression of syndecan-4 in Chinese hamster ovary (CHO) cells resulted in increased numbers of focal adhesion complexes (56). On the other hand, CHO cell mutants deficient in glycosaminoglycans showed reduced focal adhesion formation when cultured on a fibronectin substrate (59). In our experiments, reduction in heparan sulphation in MCF-7 breast cancer cells was shown to increase cancer cell adhesion and upregulate FAK and paxillin at both gene transcript and protein levels, and this could be completely blocked by exogenous heparan sulphate.

Heparan sulphate has been shown to be a key player in a number of signalling pathways in cell migration. It has been reported to modulate transendothelial migration of monocytes by regulating G-protein-dependent signalling (60). HT1080 fibrosarcoma cells that overexpressed the heparan sulphate proteoglycan syndecan-2 showed activation of the small GTPase Rac and increased cell migration (61). Furthermore, in addition to acting as a co-receptor in FGF signalling, heparan sulphate may act as a direct receptor in the FGF-2 activation of ERK1/2, which is required for bronchial epithelial and corneal epithelial cell migration (62-64).

In conclusion, we have shown that heparan sulphate is a useful biomarker of breast invasive ductal carcinoma, and is involved in regulating cancer cell adhesion, migration and focal adhesion complex formation through different sulphation patterns on its sugar residues. A better understanding of the effects of differentially sulphated heparan sulphate species
on cancer cell behaviour is important for the development of these molecules into therapeutic targets for breast cancer.

Acknowledgements

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References


