

The CD151-midkine pathway regulates the immune microenvironment in inflammatory breast cancer

Hayward, Steven; Gachechiladze, Mariam; Badr, Nahla; Andrijes, Regina; Molostvov, Guerman; Paniushkina, Liliia; Sopikova, Barbora; Slobodova, Zuzana; Mgebrishvili, Giorgi; Sharma, Nisha; Horimoto, Yoshiya; Burg, Dominic; Robertson, Graham; Hanby, Andrew; Hoar, Fiona; Rea, Daniel; Eckhardt, Bedrich; Ueno, Naoto; Nazarenko, Irina; Long, Heather

DOI:

[10.1002/path.5415](https://doi.org/10.1002/path.5415)

License:

Other (please specify with Rights Statement)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Hayward, S, Gachechiladze, M, Badr, N, Andrijes, R, Molostvov, G, Paniushkina, L, Sopikova, B, Slobodova, Z, Mgebrishvili, G, Sharma, N, Horimoto, Y, Burg, D, Robertson, G, Hanby, A, Hoar, F, Rea, D, Eckhardt, B, Ueno, N, Nazarenko, I, Long, H, van Laere, S, Shaaban, A & Berditchevski, F 2020, 'The CD151-midkine pathway regulates the immune microenvironment in inflammatory breast cancer', *Journal of Pathology*, vol. 251, no. 1, pp. 63-73. <https://doi.org/10.1002/path.5415>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

This is the peer reviewed version of the following article: Hayward, S., Gachechiladze, M., Badr, N., Andrijes, R., Molostvov, G., Paniushkina, L., Sopikova, B., Slobodova, Z., Mgebrishvili, G., Sharma, N., Horimoto, Y., Burg, D., Robertson, G., Hanby, A., Hoar, F., Rea, D., Eckhardt, B., Ueno, N., Nazarenko, I., Long, H., van Laere, S., Shaaban, A. M. and Berditchevski, F. (2020), 'The CD151-midkine pathway regulates the immune microenvironment in inflammatory breast cancer', *J. Pathol.*, 251: 63-73., which has been published in final form at <https://doi.org/10.1002/path.5415>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

The CD151-midkine pathway regulates the immune microenvironment in inflammatory breast cancer.

Steven Hayward¹, Mariam Gachehiladze², Nahla Badr^{1,14}, Regina Andrijes¹, Guerman Molostvov¹, Liliia Paniushkina³, Barbora Sopikova², Zuzana Slobodová², Giorgi Mgebrishvili², Nisha Sharma⁴, Yoshiya Horimoto⁵, Dominic Burg⁶, Graham Robertson⁶, Andrew Hanby⁷, Fiona Hoar⁸, Daniel Rea¹, Bedrich L. Eckhardt^{9,10,11}, Naoto T Ueno^{10,11}, Irina Nazarenko^{3,12}, Heather M. Long¹, Steven van Laere¹³, Abeer M. Shaaban¹, Fedor Berditchevski^{1*}

¹Institute of Cancer and Genomic Sciences, The University of Birmingham, Edgbaston, Birmingham, UK, ²Department of Clinical and Molecular Pathology, Palacky University, Olomouc, Czech Republic, ³Institute for Infection Prevention and Hospital Epidemiology, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Germany, ⁴Breast Unit, Level 1 Chancellor Wing, St James Hospital, Leeds Teaching Hospitals NHS Trust, ⁵Department of Breast Surgical Oncology, Juntendo University School of Medicine, Tokyo, Japan, ⁶Lyramid, 2/55 Clarence St, Sydney Australia, ⁷University of Leeds, Leeds Institute of Cancer and Pathology (LICAP) Leeds, ⁸Hospital, Sandwell and West Birmingham Hospitals, Department of General and Breast Surgery, Birmingham, UK, ⁹Olivia Newton-John Cancer Research Institute, Heidelberg, Australia, ¹⁰Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA, ¹¹Morgan Welch Inflammatory Breast Cancer Research

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi:10.1002/path.5415

Program and Clinic, ¹²German Cancer Consortium (DKTK), Partner Site Freiburg and German Cancer Research Center (DKFZ), Heidelberg, Germany, ¹³Translational Cancer Research Unit Center for Oncological Research, University Antwerp, Belgium, ¹⁴Department of Pathology, Menoufia University School of Medicine, Menoufia, Egypt.

*Correspondence to: F Berditchevski, Institute of Cancer and Genomic Sciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom. E-mail: f.berditchevski@bham.ac.uk

Conflict of interest: GR and DB are currently employees of Cellmid and Lynamid. No other conflicts were disclosed.

Running title: CD151 and immune microenvironment in inflammatory breast cancer

Abstract

The immune microenvironment in inflammatory breast cancer (IBC) is poorly characterised, and molecular and cellular pathways that control accumulation of various immune cells in IBC tissues remain largely unknown. Here, we discovered a novel pathway linking the expression of the tetraspanin protein CD151 in tumour cells with increased accumulation of macrophages in cancerous tissues. Importantly, elevated expression of CD151 and higher number of tumour-infiltrating macrophages correlated with better patient responses to chemotherapy. Accordingly, CD151-expressing IBC xenografts were characterised by the increased infiltration of macrophages. *In vitro* migration experiments demonstrated that CD151 stimulates the chemoattractive potential of IBC cells for monocytes via mechanisms involving midkine (a heparin-binding growth factor), integrin $\alpha 6\beta 1$ and production of extracellular vesicles (EVs). Profiling of chemokines secreted by IBC cells demonstrated that CD151 increases production of midkine. Purified midkine specifically stimulated migration of monocytes, but not other immune cells. Further experiments demonstrated that the chemoattractive potential of IBC-derived EVs is blocked by anti-midkine antibodies. These results demonstrate for the first time that changes in the expression of a tetraspanin protein by tumour cells can affect the formation of the immune microenvironment by modulating recruitment of effector cells to cancerous tissues. Therefore, a CD151-midkine pathway can be considered as a novel target for controlled changes of the immune landscape in IBC.

Keywords: IBC, tumour microenvironment, macrophages, tetraspanins, midkine

INTRODUCTION

Inflammatory breast cancer (IBC) is rare but is one of the deadliest forms of locally advanced breast cancer. Whilst IBC displays distinct clinical features, the underlying molecular pathways involved in IBC pathology remain poorly understood.

Immunotherapy is considered to be a promising new approach in treatment of IBC [1]. However, the immune landscape of IBC tissues remains poorly investigated. In a recent study no association was found between the infiltration of FoxP3-positive regulatory T cells and IBC phenotype [2]. Others reported a more pronounced infiltration of CD14⁺ monocytes/macrophages into post-treatment IBC tissues than into non-IBC tissues [3], and more M2 macrophages were present in treatment-naïve cancerous tissues as compared to normal breast tissues [4]. Interestingly, the frequency of macrophage infiltration into non-cancerous normal tissues of IBC patients was higher than that of non-IBC breast cancer patients [5]. Furthermore, increased infiltration of B-cells was found to correlate with a better prognosis [6] and increased number of stromal tumour infiltrating lymphocytes was a significant predictor of better overall survival in IBC [7]. However, the molecular mechanisms of immune cell recruitment into IBC tissues are unknown.

Tetraspanin proteins play an important role in regulation of tumour cell migration, proliferation and apoptosis [8]. Recently, various tetraspanins have emerged as mediators of cell communication between tumour cells and surrounding tumour microenvironment (TME). Specifically, it has been proposed that tetraspanins regulate TME via exosomes [9-12]. Exosomes are nanovesicles that are proposed to function as short- and long-distance communication vehicles between various cell types [13].

We, and others, have previously reported that the elevated expression of the tetraspanin CD151 in non-IBC invasive ductal carcinoma correlated with decreased survival of patients with Luminal A and triple-negative subtypes of breast cancer [14,15]. By contrast, we found no correlation between the expression of CD151 and any of the clinico-pathological parameters in invasive lobular carcinomas [16], suggesting that CD151 may play distinct roles in pathogenesis of different subtypes of breast cancer.

Here, we report elevated expression of CD151 in ~74% of IBC patients and demonstrate that high levels of CD151 predict better response to treatment. Elevated expression of CD151 also correlated with increased infiltration of CD68⁺ macrophages into IBC tissues. *In vitro* experiments demonstrated that CD151 increases the chemoattractive potential of IBC cells for monocytes via production of extracellular vesicles and the growth factor midkine. These results identify CD151 and midkine as important new regulators of the immune microenvironment in IBC.

Materials and methods

Details of cell culture protocols, antibody information, ELISA protocol, analysis of EVs and animal experiments are provided in supplementary material, Supplementary materials and methods.

Patients and ethics statement. Patients clinically proven to have IBC according to published international guidelines [17], were managed by neoadjuvant chemotherapy followed by surgery, and the pathological complete response was assessed using the

classification in accordance with published criteria. All experiments were approved by the West Midlands – Black Country NRES Committee (07/Q2702/24), Ethics committee of the Juntendo University Hospital (14-144) or Leeds Research Ethical Committee (06/Q1206/180). All donors provided written informed consent for the collection of blood samples and subsequent analysis. Clinical details and treatment regimens are shown in supplementary material, Tables S1 and S2.

Western blotting. Cells were lysed in Laemmli buffer supplemented with protease and phosphatase inhibitors. Equal amounts of protein per lane were loaded and resolved on SDS–PAGE and then transferred onto nitrocellulose membrane. The membranes were incubated with specific antibodies as listed in supplementary material, Table S3. Infrared fluorescence-tagged secondary antibodies were used to visualise the signals, and the images were capture and quantified using a LI-COR Odyssey scanning system.

Peripheral blood mononuclear cells (PBMC) transmigration assay. PBMCs were isolated from heparinised whole blood samples of consented healthy donors (07/Q2702/24) using density gradient centrifugation (Ficoll-Paque; GE Healthcare, Little Chalfont, UK). Growth medium conditioned for 3 days by IBC cells (CM) were centrifuged at 1000 rpm for 3 min then used as chemoattractant. PBMCs (2×10^6 /200 μ l) were allowed to migrate towards CM for 4 h. Migrated cells were counted, stained, and analysed using flow cytometry.

siRNA-based knockdown. BCX010 cells were transfected with one of two siRNAs (20 nM) targeting $\alpha 6$ integrin subunit or a control siRNA using Lipofectamine®RNAiMAX transfection reagent (ThermoFisher). All siRNAs were purchased from Qiagen in the

Flexitube format; targeted sequences were: GGTCGTGACATGTGCTCAC and CAAGACAGCTCATATTGAT (referred to as si- α 6/1 and si- α 6/2, respectively). Medium was changed 24 h post-transfection and cells were left to condition freshly-added growth medium for an additional 72 h. Conditioned medium was used for PBMC transmigration assays as described above.

Flow cytometry. Cells were washed twice in PBS and stained for surface markers by incubation for 30 min on ice with saturating amounts of various combinations of the following antibodies: AmCyan-conjugated CD3 mAb, PE-Texas Red-conjugated CD4 mAb (Beckman Coulter), PE-conjugated CD8 mAb, APC-conjugated CD14 mAb, PE-Cy7-conjugated CD19 mAb (listed in supplementary material, Table S3). After subsequent washes, stained cells were either analysed immediately or fixed in 4% paraformaldehyde (for later analysis, using an LSRII flow cytometer (Beckman Coulter). The gating strategy is shown in supplementary material, Figure S1. Data were analysed using FlowJo software (Ashland, OR, USA).

Gene set enrichment analysis. Gene set enrichment analysis (GSEA) was performed on an existing gene expression dataset of 50 patients with IBC (accession number in the ArrayExpress E-MTAB-1006) [18]. Patients were dichotomized according to the median CD151 expression levels, and differential gene expression analysis was performed between the “CD151-high” and “CD151-low” group in the cohort of IBC patients. Resulting fold-changes were used as input for GSEA using gene sets that were reported by Angelova, *et al* [19] and that are characteristic for 56 different immune cell types. An enrichment score (ES) for each immune gene set was defined as the maximum value of

Accepted Article

a running sum, calculated by starting at the top of the ranked list (i.e. genes overexpressed in the “CD151-high” group), considering each gene in succession and incrementing the sum if the gene is present in the immune gene set and decrementing the sum otherwise. Significance of the ES was assessed using 1000 random gene label permutations and P values less than 5% were considered significant.

Isolation of extracellular vesicles (EVs) and depletion of EVs. EVs were purified from the culture supernatant of cells growing in complete medium supplemented with vesicles-depleted FCS (ThermoFisher Scientific) for 72 h. Conditioned medium was centrifuged sequentially at 600×g for 10 min, followed by 10,000×g for 45 min and finally at 70,000×g for 2 h. In experiments involving depletion of EVs, conditioned medium was collected after final centrifugation and filtered before use in migration experiments. CD151-positive EVs were depleted after two cycles of incubation of conditioned medium with anti-CD151 mAb (5C11) pre-bound to G-protein conjugated agarose. Mouse isotype control IgG1 antibody with no known specificity (Biolegend) was used as a control in these experiments.

Immunohistochemistry. Seventy seven inflammatory breast cancer treatment naïve core biopsies were collected from three centres in the UK and Japan in the period between 2006 and 2014). Tissue specimens were fixed with 10% neutral-buffered formalin and embedded in paraffin wax. FFPE tissue blocks were processed for staining using a standard protocol. Sections were incubated with primary mouse mAb to CD151 (RLM30, Leica). Tumour infiltrating macrophages were identified using anti-CD68 (PG-M1, Dako). Antibodies used are listed in supplementary material, Table S3

Evaluation and scoring systems. Immunostained slides were evaluated and scored independently by two pathologists (AMS and MG). Membranous CD151 immunoreactivity was scored semi-quantitatively as negative (0), weak (1+), moderate (2+) or strong (3+) expression. Tumours without any detectable CD151 positivity or with faint membranous positivity in $\leq 10\%$ of tumour cells was considered as expression negative (0). Incomplete membranous staining, which was barely noticeable but in $>10\%$ tumour cells was considered as weak expression (1+). Circumferential membranous staining that was incomplete and/or moderate and in $>10\%$ tumour cells or complete membranous staining in $\leq 10\%$ tumour cells was considered as moderate expression (2+). Circumferential membranous staining, that was complete, strong and in $>10\%$ of cells was considered as strong expression (3+). Finally, for statistical purposes, cases scored as negative or weak expression were considered as negative (0) and cases with moderate or strong expression were considered as positive (1). For CD68 staining, cells with granular cytoplasmic and membranous positivity were counted per high power field (x40 objective) in three “hot spots”. Areas of necrosis were avoided and excluded from the evaluation of both markers.

Statistical analysis. Overall survival (OS) was defined as the period from the date of surgery until tumour-related death. Cases lost during follow-up and those ending in death from non-tumour related causes were censored. For time-to-event data, Kaplan–Meier curves were calculated, and tests of statistical significance were based on log-rank statistics. Comparison between patient subgroups was done using the Mann–Whitney U test. The correlation between quantitative data including CD68 and CD151 immunoreactivity was assessed by using Spearman’s rank test. All tests of significance

were two-sided; $p < 0.05$ was considered significant. All conventional statistical analyses were performed using IBM SPSS statistics software version 20.00 (IBM, Chicago, IL, USA).

RESULTS

Expression of tetraspanin CD151 and recruitment of macrophages correlate with better responses to chemotherapy in IBC patients. Previous studies suggested that CD151 is likely to play contrasting roles in different subtypes of breast cancer. It was also proposed that CD151 affects communication between cancer cells and surrounding stroma leading to changes in its composition and function. As a first step towards understanding whether cancer cell associated CD151 is involved in shaping the tumour microenvironment in IBC, gene set enrichment analysis (GSEA) was performed on an existing gene expression data set from 50 patients. Patients were dichotomized according to the median *CD151* expression levels, and differential gene expression analysis was performed between the “CD151-high” and “CD151-low” groups. Resulting fold-changes were used as input for GSEA using gene sets that were reported by Angelova, *et al* [19] and that are characteristic for 56 different immune cell types. Three immune gene sets reflecting monocytes (ES=4880; $P=0.018$), plasma cells (ES=4064; $P=0.027$) and resting dendritic cells (ES=3494; $P=0.032$) were enriched in IBC tumours that expressed high levels of *CD151*.

Circulating monocytes mobilised to the tumour are subsequently polarised into macrophages. Thus, we analysed whether expression of CD151 in cancer cells correlates

with accumulation of macrophages in IBC. Accumulation of tumour associated macrophages in IBC samples was analysed by immunohistochemistry using anti-CD68 mAb (supplementary material, Figure S2A). Median count of CD68-positive macrophages in three high power fields was 30. A low CD68 count (≤ 30) was seen in 40 (52%) and high CD68 count (>30) was seen in 37 (48%) patients. Accumulation of macrophages into IBC tissues did not correlate with the tumour grade, ER or HER2 status (Table 1). However, increased infiltration of CD68⁺ macrophages positively correlated with pathological responses. More CD68⁺ macrophages were seen in patients with complete pathological response ($r=0.27$, $p=0.023$, Table 1).

Using the same cohort of patients, we observed high levels of CD151 in 57 (~74%) samples (supplementary material, Figure S2B). Although, there was no correlation between CD151-positivity and grade, ER or HER2 status of the tumour, Spearman's rank correlation analysis demonstrated a significant positive relationship between CD151 expression and macrophage abundance in IBC tissues ($r=.308$, $p=0.006$). Importantly, elevated CD151 expression was also positively associated with probability of partial or complete pathological response ($r=.44$, $p<0.0001$) (Table 1). Thus, the expression of CD151 on cancer cells may contribute to specific changes in the immune microenvironment in IBC.

CD151 regulates the chemoattractive potential of IBC cells for monocytes. To further examine the relationship between tissue accumulation of macrophages and CD151 expression in IBC tumours, we initially examined whether conditioned medium (CM) from cultured IBC cells could influence migration of monocytes and lymphocytes

present in peripheral blood mononuclear cells (PBMCs). These experiments demonstrated that CM from BCX010 cells, a recently established triple-negative IBC model [20] (BCX-CM), was more chemotactic for PBMCs when compared to the control growth media ($p=0.07$) (Figure 1A). When analysed in more detail, we found that there was a significant increase in migration of CD14⁺ monocytes towards BCX-CM ($p<0.01$), whereas migration of CD3⁺ T-cells was not consistently affected (Figure 1A). In contrast, CM from SUM149 and MD-IBC3 cells, two well-established IBC cell models, had no effect on migration of either PBMCs, monocytes or T cells (Figure 1B,C).

Interestingly, out of three cell lines tested, BCX010 cells expressed the highest level of CD151 when compared to SUM149 and MDA-IBC3 (Figure 2A). To investigate whether differences in CD151 expression were responsible for the higher chemoattractive potential of BCX010 cells, we generated a stable BCX010/CD151KD cell line in which expression of the tetraspanin was decreased by ~10-fold (supplementary material, Figure S3). Conversely, we established a stable MD-IBC/CD151 cell line which expressed CD151 at the level similar to that seen in BCX010 cells (supplementary material, Figure S3). Migration of monocytes towards media conditioned by the BCX010/CD151KD was decreased ($p=0.02$) to the levels seen in the control experiment (i.e. migration towards the control growth medium) (Figure 2B). Conversely, CM of MD-IBC/CD151 cells was more chemotactic for monocytes than CM of MD-IBC/pLVx ($p=0.01$) (Figure 2C). These results demonstrated that elevated expression of CD151 increases the chemoattractive potential of IBC cells for monocytes. In agreement with these results, we found that the number of macrophages associated with tumours formed by BCX010/pLVx cells in mice

was consistently higher than that associated with BCX010/CD151KD tumours ($p=0.025$) (Figure 2D).

To examine whether CD151 also affects cancer cell induced polarization of monocytes to macrophages, PBMC-derived monocytes were incubated with CM-BCX010/pLVx and CM-BCX010/CD151KD for 72 h and subsequently analysed for the expression of CD68 and CD163, two well established macrophage markers. While these experiments demonstrated that both cell lines are able to induce polarization of monocytes, we found no noticeable differences between the control and CD151-depleted cells within this time-frame (supplementary material, Figure S4).

The effect of CD151 on production of chemokines and growth factors. To examine the mechanisms underlying the role of CD151 in regulation of monocyte migration by IBC cells we analysed the CM of BCX010 cells for the presence of 31 chemokines using a chemokine antibody array. Only CXCL8/IL-8 and midkine were detected in media conditioned by BCX010/pLVx and BCX010/151KD cells (Figure 3A). While there were no differences in signal intensities for CXCL8/IL-8, the level of midkine in CM-BCX010/pLVx appeared to be higher when compared to CM-BCX010/CD151KD. To measure the level of these chemokines more precisely, we carried out ELISA. As shown in Figure 3B the expression levels of CXCL8/IL-8 was ~100pg/ml for both BCX010/pLVx and BCX010/CD151KD cells. Similarly, there were only marginal differences between secretion of CXCL8/IL-8 by MD-IBC/pLVx and MD-IBC/CD151 cells (Figure 3B). By contrast, we found that BCX010/pLVx cells produced more than 580 pg/ml of midkine, and depletion of CD151 resulted in a consistent decrease of midkine production by ~25–

30% (Figure 3C). Accordingly, ectopic expression of CD151 cells resulted in increased production of midkine by MDA-IBC3 cells (Figure 3C). Furthermore, while recombinant midkine specifically increased chemotactic migration of monocytes in a concentration-dependent manner, migration of neither T-cells nor B-cells was affected (Figure 3D). Conversely, antibodies to midkine specifically blocked migration of monocytes towards CM-CD151(+) but not T cells (Figure 3E). These results demonstrated the CD151-dependent increase in the chemoattractive potential of media conditioned by IBC cells is caused by the higher level of midkine production.

Production of midkine by IBC cells is regulated by the CD151-integrin complex. To investigate molecular mechanisms underlying the effect of CD151 depletion on production of midkine, we first compared expression levels of the growth factor in cells. As shown in Figure 4A, CD151 knockdown did not change the total level of midkine in BCX010 cells, thus suggesting that the effect was mediated by post-translational mechanisms. The integrin $\alpha 6\beta 1$, one of the reported midkine receptors [21], is also known to be a principal partner for CD151 [22]. Thus, we investigated whether the CD151– $\alpha 6\beta 1$ complex is involved in the regulation of midkine production by IBC cells. Initially, we analysed the activity of the CD151 mutant which is unable to directly associate with integrins (CD151-QRD) (25). Re-expression of the CD151-QRD protein in BCX010/CD151KD cells at the level seen in the control BCX010/pLVx cells (supplementary material, Figure S5) failed to restore the chemoattractive potential of the CM for monocytes (Figure 4B). Accordingly, the level of midkine in CM-BCX010/CD151QRD was similar to that seen in the CM-BCX010/CD151KD (Figure 4C).

Accepted Article

In control experiments, re-expression of the wild-type CD151 recovered both the level of midkine in CM and its chemoattractive potential (Figure 4B,C). In complementary experiments we demonstrated that knockdown of the $\alpha 6$ integrin subunit decreased the level of midkine in CM-BCX010 and its chemoattractive potential (Figure 4D,E). These results demonstrated that the CD151– $\alpha 6\beta 1$ complex plays a key role in the regulation of midkine secretion by IBC cells.

The role of extracellular vesicles in CD151-dependent migration of monocytes. It has been proposed that cancer cell associated tetraspanins may regulate the TME through secretion of extracellular vesicles (EVs) [23]. Our preliminary experiments demonstrated that the depletion of CD151 did not affect production of extracellular vesicles (supplementary material, Figure S6). Next, we investigated whether EVs contribute to the increased chemoattractive potential of CD151-expressing IBC cells by analysing migration of monocytes towards BCX-CM depleted of EVs by centrifugation. These experiments demonstrated that CM-BCX010/pLVx completely lost its chemoattractive potential for monocytes upon depletion of EVs (Figure 5A). By contrast, there was only a negligible effect of EVs-depletion on migration of CD14⁺ monocytes towards CM-BCX010/CD151KD (Figure 5B). These results demonstrate that most of the chemoattractive activity of BCX010/pLVx-CM for monocytes was associated with EVs.

Tetraspanins are known to influence the production level and composition of EVs [24,25]. To examine if midkine is associated with vesicles secreted by IBC cells we measured the concentration of midkine in EV-depleted CM. Strikingly, the level of midkine in EV-depleted CM was decreased by 2.5-fold (Figure 6A). By contrast, the levels of IL-8 in CM

Accepted Article

were only marginally affected by EVs-depletion (supplementary material, Figure S7). Importantly, the increase in monocyte migration towards EVs secreted by CD151-expressing IBC cells was completely negated by anti-midkine antibodies (Figure 6B). Conversely, neither EVs nor inhibitory anti-midkine mAb affected migration of CD3-positive lymphocytes (Figure 6B). To illustrate further the role of CD151 in the association of midkine with EVs, we measured the concentration of the chemokine after CM-BCX010/pLVx was immunodepleted of CD151-positive vesicles. These experiments show that the level of midkine was consistently decreased after the depletion. We also found that the level of midkine was consistently decreased when CM-BCX010/pLVx was depleted of CD63-positive vesicles; these results are in agreement with an earlier observation showing that CD63 is present on all types of cell-derived vesicles [26]. By contrast, and as expected, immune-depletion did not change the concentration of midkine in CM-BCX010/151KD (Figure 6C). Accordingly, we found that depletion of CD151-containing vesicles decreased the chemoattractive potential of CM-BCX010/pLVx cells relative to that of CM-BCX010/151KD cells (Figure 6D). Therefore, the biological activity of midkine was associated with CD151-positive EVs indicating the importance of EV-associated midkine in CD151-dependent regulation of the chemoattractive potential of IBC cells for CD14⁺ monocytes (Figure 6E).

DISCUSSION

Here we demonstrated for the first time that the expression of a tetraspanin protein by tumour cells can influence the composition of the immune microenvironment in cancerous

tissues. Specifically, we found that accumulation of CD68-positive macrophages in IBC tissues correlated with the elevated expression of tetraspanin CD151 by tumour cells. Furthermore, our data reveal a novel pathway whereby CD151-dependent production of midkine and its association with tumour cell-derived vesicles regulate migration of monocytes, and could therefore be an important factor in accumulation of macrophages in CD151-positive tumours. Importantly, both increased infiltration of CD68⁺ macrophages and the elevated expression of CD151 correlated with better clinical responses to neoadjuvant chemotherapy treatment in IBC patients, as judged by pathological examination of the tissues (i.e. correlated with partial or complete pathological response (PR)).

Our finding of a positive correlation between expression of CD151 and response to chemotherapy in breast cancer is somewhat counter-intuitive given that in most common breast cancers, high expression of CD151 correlates with poor survival. On the other hand, our earlier work and more recent results suggest that CD151 may, in fact, improve responses of HER2⁺ breast cancer cells to HER2-targeting therapies [14,27]. These observations suggest that the predictive value of CD151 expression in breast cancer including IBC is likely to be dependent on distinct molecular subtypes of patient tumours as well as treatment regimens.

We observed correlation between expression of CD151 and patient responses to therapy, but there was no association with overall survival (OS). This may not be surprising given that we found no correlation between pathological responses and OS in our cohort of IBC patients. A link between pathological complete response (pCR) and survival in IBC

Accepted Article

remains a contentious topic. In earlier studies, a higher rate of pCR in an unselected IBC cohort significantly correlated with better disease-free and OS [28]. However, when examined in IBC subtypes, significance was lost in hormone receptor positive patients. Furthermore, a more recent study demonstrated that despite a significant correlation between pCR and metastasis-free survival, the correlation with overall survival was not observed [29]. Whilst the underlying differences between IBC and non-IBC require further investigation, it has been proposed that the lack of correlation between PR and OS in IBC may be due to a higher metastatic risk rather than chemosensitivity of the tumours [30].

Extensive infiltration of CD68⁺ macrophages into cancerous tissues has been previously associated with poor prognosis in various types of cancer including breast cancer [31]. Furthermore, a recent study by Valeta-Magara, *et al* demonstrated that IBCs are characterized with high infiltration by M2 macrophages which strongly express IL-8 and GRO (growth-related oncogene) chemokines [5]. By contrast, we observed that high infiltration of CD68⁺ macrophages correlated with better pathological responses to the treatment in IBC patients, albeit this was not linked to improved overall survival. Similar findings were recently reported for large and locally advanced breast cancer in which high levels of CD163⁺ tumour-associated macrophages in the tumour tissues were a good predictor for pCR but not for OS [32].

Our results point towards a novel mechanism that controls accumulation of CD68⁺ macrophages in cancerous tissues wherein CD151 regulates production of midkine by tumour cells (Figure 6E). Several studies report elevated expression of midkine (both at mRNA and protein levels) in breast cancer tissues, that was associated with poor survival

Accepted Article

and increased microvascular density of the tumours [33]. Our results suggest that midkine produced by tumour cells can also stimulate recruitment of monocytes/macrophages to breast cancer tissues. Accordingly, we found that increased expression of midkine correlated with monocyte and macrophage signatures in breast cancer. Whilst the links between expression of midkine and the tumour immune microenvironment have not been investigated previously, midkine was shown to be a key factor in infiltration of leukocytes in various inflammatory conditions [34].

Interestingly, the chemotactic potential of midkine was enhanced when it was associated with cancer cell-derived extracellular vesicles thus suggesting that EVs serve as effective platforms for presentation of midkine to cell surface proteins on target cells. Surface immobilization and/or clustering of midkine appears to be an important factor which regulates its biological activities [35]. Our experiments also demonstrated that the chemotactic activity of midkine for monocytes is associated with CD151-positive vesicles. This suggests that CD151 functions as a cargo for the recruitment of a midkine-binding protein(s) to the EVs. Three out of four classes of surface receptors for midkine (i.e. syndecans, integrins and LRP1) were previously detected on extracellular vesicles thus making them potential targets for CD151-mediated immobilization of midkine on vesicular surfaces.

In summary, our work identifies the CD151-midkine pathway as an important new regulator of the immune microenvironment in IBC. CD151-dependent elevated production of midkine and ensuing recruitment of monocyte/macrophages are likely to have broader consequences for the formation of the immune microenvironment in IBC and progression

of the disease. This warrants further, more detailed investigation of this phenomenon.

Acknowledgements

This work was supported by Breast Cancer Campaign grant 2012PR113 (to FB), the Inflammatory Breast Cancer Network UK (to FB) and Ministry of Higher Education and Research, Egypt (NB). AMS is supported by the Birmingham CRUK Centre. We are grateful to Dr. James Arnold for his advice.

Author contributions statement

AMS, DR, HML, MG, IN, SvL and FB conceived and designed the study. SH, MG, NB, GMo, LP, BS, ZS, NS, YM, FH, and IN collected the data. SH, MG, NB, GMo, LP, RA, BS, ZS, YM, IN, GM, HL, SvL and FB performed analysis and interpretation of the data. AMS, HML, MG, SvL, GR and FB drafted the article. AMS, NB and FB obtained the funding. NS, YM, FH, DB, GR, EB and NU provided study material. MG, MG, and NB contributed to the statistical analysis. All authors had final approval of the submitted and published versions.

Data availability statement

Reagents and materials generated for this study are available on reasonable request. The Array Express entry E-MTAB-1006 [18] is publicly available.

References

1. Costa R, Santa-Maria CA, Rossi G, *et al.* Developmental therapeutics for inflammatory breast cancer: Biology and translational directions. *Oncotarget* 2017; **8**: 12417-12432.
2. Demir L, Yigit S, Ellidokuz H, *et al.* Predictive and prognostic factors in locally advanced breast cancer: effect of intratumoral FOXP3+ Tregs. *Clin Exp Metastasis* 2013; **30**: 1047-1062.
3. Mohamed MM, El-Ghonaimy EA, Nouh MA, *et al.* Cytokines secreted by macrophages isolated from tumor microenvironment of inflammatory breast cancer patients possess chemotactic properties. *Int J Biochem Cell Biol* 2014; **46**: 138-147.
4. Reddy JP, Atkinson RL, Larson R, *et al.* Mammary stem cell and macrophage markers are enriched in normal tissue adjacent to inflammatory breast cancer. *Breast Cancer Res Treat* 2018; **171**: 283-293.
5. Valeta-Magara A, Gadi A, Volta V, *et al.* Inflammatory Breast Cancer Promotes Development of M2 Tumor-Associated Macrophages and Cancer Mesenchymal Cells through a Complex Chemokine Network. *Cancer Res* 2019; **79**: 3360-3371.
6. Arias-Pulido H, Cimino-Mathews A, Chaher N, *et al.* The combined presence of CD20 + B cells and PD-L1 + tumor-infiltrating lymphocytes in inflammatory breast cancer is prognostic of improved patient outcome. *Breast Cancer Res Treat* 2018; **171**: 273-282.

- Accepted Article
7. Van BC, Rypens C, Van DP, *et al.* Infiltrating stromal immune cells in inflammatory breast cancer are associated with an improved outcome and increased PD-L1 expression. *Breast Cancer Res* 2019; **21**: 28.
 8. Hemler ME. Tetraspanin proteins promote multiple cancer stages. *Nat Rev Cancer* 2014; **14**: 49-60.
 9. Zoller M. Tetraspanins: push and pull in suppressing and promoting metastasis. *Nat Rev Cancer* 2009; **9**: 40-55.
 10. Nazarenko I, Rana S, Baumann A, *et al.* Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. *Cancer Res* 2010; **70**: 1668-1678.
 11. Zech D, Rana S, Buchler MW, *et al.* Tumor-exosomes and leukocyte activation: an ambivalent crosstalk. *Cell Commun Signal* 2012; **10**: 37.
 12. Detchokul S, Newell B, Williams ED, *et al.* CD151 is associated with prostate cancer cell invasion and lymphangiogenesis in vivo. *Oncol Rep* 2014; **31**: 241-247.
 13. Steinbichler TB, Dudas J, Riechelmann H, *et al.* The Role of Exosomes in Cancer Metastasis. *Semin Cancer Biol* 2017.
 14. Novitskaya V, Romanska H, Kordek R, *et al.* Integrin alpha3beta1-CD151 complex regulates dimerization of ErbB2 via RhoA. *Oncogene* 2014; **33**: 2779-2789.
 15. Kwon MJ, Park S, Choi JY, *et al.* Clinical significance of CD151 overexpression in subtypes of invasive breast cancer. *Br J Cancer* 2012; **106**: 923-930.
 16. Romanska HM, Potemski P, Krakowska M, *et al.* Lack of CD151/integrin alpha3beta1 complex is predictive of poor outcome in node-negative lobular breast carcinoma:

- opposing roles of CD151 in invasive lobular and ductal breast cancers. *Br J Cancer* 2015; **113**: 1350-1357.
17. Dawood S, Merajver SD, Viens P, *et al.* International expert panel on inflammatory breast cancer: consensus statement for standardized diagnosis and treatment. *Ann Oncol* 2011; **22**: 515-523.
18. Van der Auwera I, Limame R, van Dam P, *et al.* Integrated miRNA and mRNA expression profiling of the inflammatory breast cancer subtype. *Br J Cancer* 2010; **103**: 532-541.
19. Angelova M, Charoentong P, Hackl H, *et al.* Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. *Genome Biol* 2015; **16**: 64.
20. McAuliffe PF, Evans KW, Akcakanat A, *et al.* Ability to Generate Patient-Derived Breast Cancer Xenografts Is Enhanced in Chemoresistant Disease and Predicts Poor Patient Outcomes. *PLoS ONE* 2015; **10**: e0136851.
21. Muramatsu H, Zou P, Suzuki H, *et al.* alpha4beta1- and alpha6beta1-integrins are functional receptors for midkine, a heparin-binding growth factor. *J Cell Sci* 2004; **117**: 5405-5415.
22. Lammerding J, Kazarov AR, Huang H, *et al.* Tetraspanin CD151 regulates alpha6beta1 integrin adhesion strengthening. *Proc Natl Acad Sci U S A* 2003; **100**: 7616-7621.
23. Rana S, Zoller M. Exosome target cell selection and the importance of exosomal tetraspanins: a hypothesis. *Biochem Soc Trans* 2011; **39**: 559-562.
24. Andreu Z, Yanez-Mo M. Tetraspanins in extracellular vesicle formation and function. *Front Immunol* 2014; **5**: 442.

- Accepted Article
25. Zha QB, Yao YF, Ren ZJ, *et al.* Extracellular vesicles: An overview of biogenesis, function, and role in breast cancer. *Tumour Biol* 2017; **39**: 1010428317691182.
 26. Kowal J, Arras G, Colombo M, *et al.* Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* 2016; **113**: E968-977.
 27. Mieszkowska M, Piasecka D, Potemski P, *et al.* Tetraspanin CD151 impairs heterodimerization of ErbB2/ErbB3 in breast cancer cells. *Transl Res* 2019; **207**: 44-55.
 28. Masuda H, Brewer TM, Liu DD, *et al.* Long-term treatment efficacy in primary inflammatory breast cancer by hormonal receptor- and HER2-defined subtypes. *Ann Oncol* 2014; **25**: 384-391.
 29. Manai M, Finetti P, Mejri N, *et al.* Inflammatory breast cancer in 210 patients: A retrospective study on epidemiological, anatomo-clinical features and therapeutic results. *Mol Clin Oncol* 2019; **10**: 223-230.
 30. Monneur A, Goncalves A, Gilabert M, *et al.* Similar response profile to neoadjuvant chemotherapy, but different survival, in inflammatory versus locally advanced breast cancers. *Oncotarget* 2017; **8**: 66019-66032.
 31. Choi J, Gyamfi J, Jang H, *et al.* The role of tumor-associated macrophage in breast cancer biology. *Histol Histopathol* 2018; **33**: 133-145.
 32. Kaewkangsadan V, Verma C, Eremin JM, *et al.* Tumour-draining axillary lymph nodes in patients with large and locally advanced breast cancers undergoing neoadjuvant chemotherapy (NAC): the crucial contribution of immune cells (effector, regulatory) and cytokines (Th1, Th2) to immune-mediated tumour cell death induced by NAC. *BMC Cancer* 2018; **18**: 123.

33. Li F, Tian P, Zhang J, *et al.* The clinical and prognostic significance of midkine in breast cancer patients. *Tumour Biol* 2015; **36**: 9789-9794.
34. Sorrelle N, Dominguez ATA, Brekken RA. From top to bottom: midkine and pleiotrophin as emerging players in immune regulation. *J Leukoc Biol* 2017; **102**: 277-286.
35. Takada T, Toriyama K, Muramatsu H, *et al.* Midkine, a retinoic acid-inducible heparin-binding cytokine in inflammatory responses: chemotactic activity to neutrophils and association with inflammatory synovitis. *J Biochem* 1997; **122**: 453-458.

Table 1. Association between infiltration of CD68+ macrophages and CD151 expression and clinicopathological features in IBC.

		Correlations					
<i>Spearman's rho</i>		ER	HER2	Grade	Path. Response	OS	CD68
CD151	Correlation Coefficient	0.005	-0.002	-0.002	0.440**	0.018	0.308**
	Significance (2-tailed)	0.963	0.988	0.990	0.000	0.882	0.006
	N	77	77	76	70	71	77
ER	Correlation Coefficient		-0.140	0.343**	-0.083	0.116	-0.072
	Significance (2-tailed)		0.225	0.002	0.492	0.334	0.532
	N		77	76	70	71	77
HER2	Correlation Coefficient			-0.148	-0.013	0.253**	-0.073
	Significance (2-tailed)			0.203	0.916	0.033	0.525
	N			76	70	71	77
Grade	Correlation Coefficient				0.084	-0.130	0.207
	Significance (2-tailed)				0.495	0.284	0.073
	N				69	70	76
Path.	Correlation Coefficient					-0.010	0.271*
Response	Significance (2-tailed)					0.940	0.023
	N					64	70
OS	Correlation Coefficient						-0.130
	Significance (2-tailed)						0.280
	N						71

** Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05 level (2-tailed)

Figure legends

Figure 1. Differences in chemoattractive potential of media conditioned by IBC cells. Conditioned medium (CM) from (A) BCX010, (B) SUM149, or (C) MD-IBC3 cells was used as a chemoattractant for PBMCs. Purified PBMCs were allowed to migrate for 4–5 h and subsequently profiled by flow cytometry. “Control”- growth medium. Shown are results of experiments using blood from 3–7 donors. Numbers observed in separate experiments are connected by dotted lines. P values were calculated using two-tailed *t*-tests and are indicated on graphs.

Figure 2. CD151 is a regulator of the chemoattractive potential of IBC cells for monocytes. (A) Expression of CD151 in IBC cells was analysed by Western blotting. (B) Knockdown of CD151 completely inhibits the chemoattractive potential of BCX010 cells. (C) Ectopic expression of CD151 stimulates the chemoattractive potential of MD-IBC03 cells. (B,C) Migration experiments were carried out using purified PBMCs as described in the legend to Figure 1. Shown are results of the experiments using blood from 4–5 donors. Numbers observed in separate experiments are connected by dotted lines. P values were calculated using two-tailed *t*-tests and are indicated on graphs. (D) Accumulation of F4/80-positive macrophages in BCX010 xenografts. BCX010/pLVx and BCX010/CD151KD cells were injected bilaterally into mammary fat pads of five NSG mice. Phenotyping of tumour-infiltrating leukocytes was carried out using flow cytometry.

Figure 3. CD151 stimulates secretion of Midkine by IBC cells. (A) Profiling of conditioned medium (CM) from BCX010/pLVx and BCX010/CD151KD cells for chemokine production using human chemokine antibody array membranes. (B)

Concentrations of CXCL8/IL-8 in CN from BCX or MD-IBC03 cells were measured by ELISA. Shown are means of the results of 3 independent experiments. (C) Concentrations of midkine in CM from BCX and MD-IBC03 cells were measured by ELISA. Shown are means of the results of 3 independent experiments. (D) Midkine specifically stimulates migration of monocytes. Purified PBMCs were allowed to migrate for 4 h towards growth medium supplemented with midkine at the concentrations shown. Migrated cells were profiled by flow cytometry. Shown are means of two experiments each performed in duplicates. (E) Antibodies to midkine (IP14) specifically inhibit migration of monocytes. Purified PBMCs were allowed to migrate for 4 h towards CM from BCX010-pLVx supplemented with various concentrations of anti-midkine mAb. Isotype control mouse mAb were IgG2a. Migrated cells were profiled by flow cytometry. Shown are means of two experiments each performed in duplicates.

Figure 4. Production of midkine is controlled by the CD151- $\alpha 6\beta 1$ complex. (A) Expression of midkine by BCX010/pLVx and BCX010/CD151KD. (B) CM from BCX010/CD151KD, BCX010/CD151QRD or BCX010/CD151rec cells was used as a chemoattractant for PBMCs. Purified PBMCs were allowed to migrate for 4 h and subsequently profiled by flow cytometry. Shown are results of experiments using blood from two donors. Numbers observed in separate experiments are connected by dotted lines. (C) Concentrations of midkine in CM from BCX010/CD151KD, BCX010/CD151QRD or BCX010/CD151rec cells were measured by ELISA. Shown are means of the results of two independent experiments each performed in duplicate. (D,E) Knockdown of $\alpha 6$ integrin subunit suppressed (D) the chemoattractive potential of CM-

BCX010 and (E) decreased production of midkine by the cells. Chemotactic migration of PBMCs and midkine concentrations in CM-BCX010 were assessed as described in (B) and (C), respectively.

Figure 5. Critical role of EVs in the CD151-dependent chemoattractive potential of IBC cells. CM from (A) BCX010/pLVx or (B) BCX010/CD151KD cells was depleted of EVs (“-EVs”) by centrifugation. Purified PBMCs were allowed to migrate for 4 h and subsequently profiled by flow cytometry. “Control”- growth media. CM not depleted of EVs was included as a positive control. Shown are results of the experiments using blood from four donors. Numbers observed in separate experiments are connected by dotted lines. P values were calculated using two-tailed *t*-tests and are indicated on graphs. *P-value below 0.02.

Figure 6. Midkine is associated with EVs secreted by IBC cells. (A) Concentrations of midkine in EVs-depleted CM-BCX010/pLVx and CM-BCX010/CD151KD cells as measured by ELISA. Shown are means of the results of three independent experiments. (B) Migration towards vesicles produced by BCX010/pLVx cells was inhibited by anti-midkine mAb. Purified PBMCs were allowed to migrate for 4 h towards growth medium supplemented with EVs (~10 µg) produced by BCX010-pLVx and anti-midkine (or isotype control) mAb (20 µg/ml). Migrated cells were profiled by flow cytometry. Data generated using blood from an individual donor (separate experiments) are connected by dotted lines. The diamonds show the total number of CD14-positive cells migrated towards the control growth medium; triangles – migration towards the control medium supplemented with extracellular vesicles (EVs); circles – migration towards the control medium with EVs

Accepted Article

additionally supplemented with the anti-midkine blocking antibodies; squares – migration towards the control medium with EVs additionally supplemented with the isotype control antibodies (C) Concentration of midkine in tetraspanin-depleted CM-BCX010/pLVx and CM-BCX010/CD151KD cells as measured by ELISA. Shown are means of the results of two independent experiments. (D) CM from BCX010/pLVx cells was immunodepleted of CD151-positive EVs or incubated with IgG1 control mAb. Purified PBMCs were allowed to migrate for 4 h and subsequently profiled by flow cytometry. CM from BCX010/CD151KD was included for reference. Shown are results of two experiments (i.e. using blood from 2 donors), each in duplicate. Numbers observed in separate experiments are connected by dotted lines. (E) Schematic diagram showing how expression of the $\alpha_6\beta_1$ -CD151 complex in IBC cells regulates migration of monocytes.











