EUROPEAN JOURNAL OF CANCER 47 (2011) 1669–1675

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Relationship between CCL5 and transforming growth factor-β1 (TGFβ1) in breast cancer

M.C. Hartmann [a], R.M. Dwyer [a,b,*], M. Costello [a], S.M. Potter [a], C. Curran [a], E. Hennessy [a], J. Newell [c], D.G. Griffin [d], M.J. Kerin [a]

[a] Division of Surgery, School of Medicine, National University of Ireland Galway, Ireland
[b] Regenerative Medicine Institute, National University of Ireland Galway, Ireland
[c] Clinical Research Facility and School of Mathematics, Statistics, and Applied Mathematics, National University of Ireland Galway, Ireland
[d] Department of Clinical Biochemistry, University Hospital Galway, Ireland

ARTICLE INFO

Article history:
Available online 12 June 2011

Keywords:
CCL5
TGFβ1
Breast cancer
Menstrual hormones
Stromal–epithelial interaction
Tumour
Microenvironment

ABSTRACT

Purpose: Investigate circulating CCL5 in breast cancer patients and healthy controls, along with gene expression levels in corresponding tumour tissue and isolated primary stromal cells. Hormonal control of CCL5, and a potential relationship with TGFβ1, was also investigated.

Methods: Circulating levels of CCL5 and TGFβ1 were measured in 102 breast cancer patients and 66 controls using ELISA. Gene expression levels (CCL5, CCR5, TGFβ1, TGFβRII) were quantified in corresponding tumour tissue ($n = 43$), normal tissue ($n = 16$), and isolated tumour ($n = 22$) and normal ($n = 3$) stromal cells using RQ-PCR. CCL5 and circulating menstrual hormones (LH, FSH, Oestradiol, Progesterone) were analysed in serum samples from healthy, premenopausal volunteers ($n = 60$).

Results: TGFβ1 was significantly higher in breast cancer patients (Mean(SEM) 27.4(0.9) ng/ml) compared to controls (14.9(0.9) ng/ml). CCL5 levels decreased in the transition from node negative (59.6(3.7) ng/ml) to node positive disease (40.5(6.3) ng/ml) and increased again as the number of positive lymph nodes increased ($\geqslant 3$ positive 50.95(9.8) ng/ml). A significant positive correlation between circulating CCL5 and TGFβ1 ($r = 0.423$, $p < 0.0001$) was observed, and mirrored at the gene expression level in tumour tissue from the same patients ($r = 0.44$, $p < 0.001$). CCL5, CCR5 and TGFβ1 expression was significantly higher in tumour compared to normal breast tissue ($p < 0.001$). A significant negative correlation was observed between circulating CCL5, Oestradiol and Progesterone ($r = -0.50$, $r = -0.39$, respectively, $p < 0.05$).

Conclusion: CCL5 expression is elevated in the tumour microenvironment. The data support a role for hormonal control of circulating CCL5 and also highlight a potentially important relationship between CCL5 and TGFβ1 in breast cancer.

1. Introduction

A connection between inflammation and cancer was reported as early as 1863 by Virchow, who observed cancer developing in sites of chronic inflammation.[1] Tumours seem to seize molecular pathways seen in wound healing and as a consequence appear as 'wounds that do not heal'.[2] However, the underlying molecular mechanisms facilitating the interconnection of inflammation and cancer remain poorly understood.

* *Corresponding author:* Tel.: +353 91 544637; fax: +353 91 494509.
E-mail address: roisin.dwyer@nuigalway.ie (R.M. Dwyer).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2011.05.001

CCL5(RANTES) is a chemotactic cytokine that plays an important part in inflammation through activation of T cells, monocytes, dendritic cells, natural killer cells, eosinophils and basophils.[3] An association between CCL5 and multiple cancer types has been reported, with the most striking findings reported in relation to breast cancer.[4] CCL5 binds to multiple receptors including CCR1, CCR3 and CCR4, with CCR5 recognised as its principal receptor.[5]

Conflicting reports exist in relation to the role of CCL5 in breast cancer progression. On a systemic level, Niwa et al. found elevated plasma levels of the chemokine using ELISA in breast cancer patients compared to healthy controls, and reported a correlation with disease stage.[6] Another group also observed significantly elevated serum CCL5 in breast cancer patients compared to healthy controls, although no significant change was detected between patients with metastatic and non-metastatic disease.[7]

Using immunohistochemistry, tissue from patients with advanced breast carcinoma was reported to have elevated levels of CCL5,[8] with the protein rarely found in biopsies taken from healthy patients. Also joint CCL5 and CCL2 expression in the same breast tumour tissues has been correlated with more advanced disease.[9] In an *in vivo* model of breast cancer, tumours secreting lower levels of CCL5 were shown to have reduced metastatic potential.[10] Tumour derived CCL5 has also been implicated in reduced T-cell response and shown to support *in vivo* growth of murine mammary carcinoma.[11] In contrast, Kurt et al. reported that tumour-derived CCL5 on its own had no role in breast cancer progression.[12]

CCL5 protein expression at diagnosis has been correlated to clinical outcome in stage II patients, suggesting a potential role for the chemokine as a biomarker for the disease independent of oestrogen receptor-α (ER-α) status.[13] In combination with ER status the prognostic strength of CCL5 was greatly improved in Stage II patients.[13] Another study also reported an interesting link between oestrogen and CCL5 in the context of atherosclerosis,[14] where a significant decrease in circulating CCL5 was found after three months of Estradiol replacement and raloxifene therapy.[14] This is interesting considering raloxifene has been shown to reduce risk of invasive breast cancer.[15,16] Given the pivotal role of Oestradiol in disease progression, a potential link between circulating hormone levels and CCL5 warrants further investigation.

Transforming growth factor-β1(TGFβ1) is a well-established factor in tumourigenesis.[17] TGFβ1 signalling is involved in epithelial to mesenchymal transition, angiogenesis, suppression of the immune response and metastasis.[18] It is thought to act as a tumour suppressor in early stage disease and switch to tumour promotion in later stage breast cancer.[19,20] Elevated systemic levels of TGFβ1 were shown to drop in breast cancer patients following removal of the tumour[21] and a significant relationship between systemic levels prior to cancer treatment and overall survival has also been reported.[22]

The aim of this study was to further investigate a potential relationship between CCL5 and breast cancer, both at the systemic and tumour tissue level. Serum CCL5 levels were observed to decrease in the switch from node negative to node positive disease, and increase again as the number of positive lymph nodes increased. This pattern has previously been described for TGFβ1,[19] and led to further investigations into a potential relationship between the two factors. Investigation of a relationship between CCL5 and circulating menstrual hormones was also performed.

2. Materials and methods

2.1. Study cohort

The study was approved by the institutional ethical committee. Preoperative blood samples were obtained with informed consent from 102 breast cancer patients (Table 1). Blood samples from healthy female volunteers ($n = 66$) with no past or present history of malignant or inflammatory conditions were collected in an outpatient facility.

Serum samples were also obtained from 15 healthy premenopausal volunteers every week for four consecutive weeks ($n = 60$) to investigate the relationship between CCL5 and circulating menstrual hormones.

All blood samples were collected in Vacutainer Serum Separator Tubes II (Becton Dickinson), allowed to clot for 30 min and centrifuged at 2000 rpm @ 4 °C for 10 min. Serum was then stored at −80 °C until required.

2.2. Chemokine detection

Circulating levels of CCL5 and TGFβ1 were measured in 168 samples (102 breast cancer, 66 healthy controls) using Quantikine® Enzyme Linked Immunosorbent Assay (ELISA) kits (R&D Systems).

Table 1 – Breast cancer patient details.

	Breast cancer patients	Control group
Number of patients	*n*(%)	*n*(%)
Total	102	66
Premenopausal	40(39.2)	26(39.4)
Postmenopausal	62(80.8)	40(60.6)
Tumour characteristics	*n*(%)	
Histology		
Ductal	66(64.7)	
Lobular	15(14.7)	
Other	10(9.8)	
Unknown	11(10.8)	
Epithelial subtype		
Luminal A	68(66.7)	
Luminal B	9(8.8)	
Her-2/neu	6(5.9)	
Basal	8(7.8)	
Unknown	11(10.8)	
Grade		
1	10(9.8)	
2	36(35.3)	
3	37(36.3)	
Unknown	19(18.6)	

2.3. Analysis of circulating menstrual hormones

Luteinizing Hormone (LH), Follicular Stimulating Hormone (FSH), Oestradiol and Progesterone were measured in serum samples ($n = 60$) by direct chemiluminescence, using Siemens ADVIA® Centaur™ Immunoassay System. The mid-cycle phase was determined by an LH peak, and the mid-luteal phase established by a peak in Progesterone.

2.4. Analysis of gene expression

Corresponding tissue specimens ($n = 43$) were available on a subset of the breast cancer patients from whom serum samples had been obtained. Breast tissue obtained from breast reduction mammaplasty ($n = 16$) served as normal controls. At the time of harvest breast tissue was snap frozen in liquid nitrogen and homogenised in 1 ml QIAzol reagent (Qiagen Ltd.). Total RNA was then isolated using RNeasy® tissue mini kit (Qiagen Ltd) according to manufacturer's instructions, including an on-column DNase treatment step.

RNA was reverse transcribed using SuperScript III reverse transcriptase enzyme (Invitrogen). Real-time quantitative PCR (RQ-PCR) was carried out using an ABI Prism 7000 (Applied Biosystems) targeting CCL5, CCR5, TGFβ1 and TGFβRII. Results were normalised to endogenous control genes MRPL19 and PPIA[23] and expression levels of the respective genes in tumour tissues compared to normal tissues.

2.5. Culture of primary stromal cells

To investigate gene expression on a cellular level, fresh breast tissue specimens were harvested from patients undergoing surgery with prior informed consent. Breast tissues obtained from reduction mammoplasty served as normal controls. All tissue specimens were washed twice in phosphate buffered saline (PBS), supplemented with 200U penicillin/200 μg/ml streptomycin. They were minced finely using crossed scalpels and digested for 12–18 h in 0.1% Collagenase Type III (Biochem Corp) at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated FBS. Following differential centrifugation of digested tissue, the stromal fraction was cultured in stromal medium selective for fibroblast growth as described previously.[24] Cells were confirmed to be positive for the stromal marker CD 90 and negative for epithelial pancytokeratin (MNF116, Dako).

2.6. Statistical analysis

Continuous variables of interest are summarised numerically by Mean(SEM), and graphically using boxplots and scatterplots. Two sample t-test, one way ANOVA and repeated measures ANOVA were used to compare mean responses as appropriate. The degree of relationship between pairs of response variables was assessed using the Pearson or Spearman correlation coefficient as appropriate. Scatterplot smoothers were employed to indicate the likely relationship between variables in a population. All analyses were performed using Minitab 15.

3. Results

3.1. Systemic CCL5 and TGFβ1 levels

Patient demographics and clinicopathological details are shown in Table 1. There was no significant difference detected in CCL5 serum levels of breast cancer patients (Mean(SEM) 46.1(4.0) ng/ml) compared to healthy controls (45.4(3.4) ng/ml, Fig. 1A). When divided on the basis of lymph node status CCL5 levels were found to decrease in the transition from node negative (59.6(3.7) ng/ml) to node positive disease ($\leqslant 2$ lymph nodes positive 40.5(6.3) ng/ml) and increase again as the number of positive lymph nodes increased ($\geqslant 3$ lymph nodes positive 50.95(9.8) ng/ml, Fig. 1B). When correlated with other clinicopathological characteristics including menopausal status, tumour epithelial subtype, tumour stage or tumour grade, no significant relationships were identified.

Serum TGFβ1 levels were found to be significantly higher in the breast cancer cohort (27.4(1.5) ng/ml) compared to healthy controls (14.9(0.9) ng/ml, Fig. 1C). When grouped on the basis of nodal status, TGFβ1 levels, similar to the pattern observed in CCL5, were found to decrease from node negative (27.3(2.2) ng/ml) to node positive disease ($\leqslant 2$ lymph nodes positive 22.0(2.7) ng/ml), and then increase again as the number of nodes positive increased ($\geqslant 3$ lymph nodes positive 30.7(3.5) ng/ml, Fig. 1D).

Further investigation revealed a significant positive correlation between systemic CCL5 and TGFβ1 across all serum samples examined ($r = 0.42$, $p < 0.0001$, Fig. 2).

3.2. CCL5 and circulating menstrual hormones

A potential relationship between circulating hormones and CCL5 was examined in serum samples from healthy premenopausal volunteers ($n = 15 \times 4$ weekly samples, total $n = 60$). CCL5 levels were stratified based on phase of the menstrual cycle (Fig. 3A). A significant drop in CCL5 levels in the transition from late luteal/early follicular to mid follicular phase of the menstrual cycle was observed ($p < 0.05$). This corresponds with an increase in Oestradiol levels in the first two phases of the menstrual cycle. Further investigation revealed a significant negative correlation between Oestradiol (Fig. 3B), Progesterone (Fig. 3C) and circulating CCL5, with the strongest correlation detected with Oestradiol ($n = 60$, $r_s = -0.502$, $p < 0.0001$). No relationship between circulating LH or FSH and CCL5 was detected.

3.3. Analysis of tissue gene expression

Gene expression analysis was carried out on corresponding tissue from breast cancer patients ($n = 43$), on whom circulating levels had been measured, and compared to normal tissue obtained at reduction mammoplasty ($n = 16$). Results were normalised to endogenous control genes and expressed as Relative Quantity (Log_{10}).[23] Expression of CCL5 and CCR5 was significantly elevated in tissue from breast cancer patients compared to controls ($p < 0.001$, Fig. 4A). A significant positive correlation between expression of the CCL5 ligand and its principle receptor CCR5 was detected ($n = 43$, $r = 0.562$, $p < 0.0001$). TGFβ1 expression was significantly

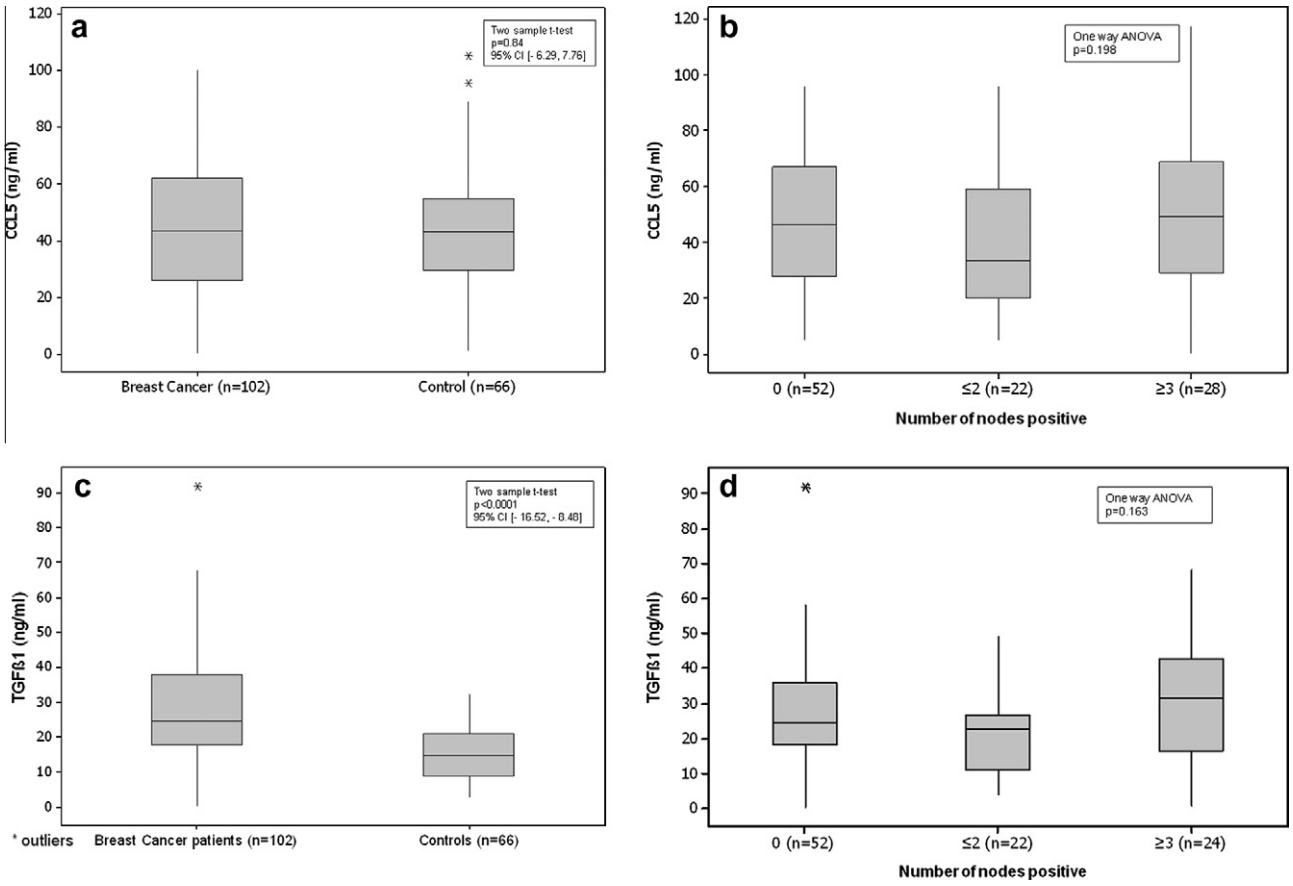

Fig. 1 – (a) Circulating levels of CCL5 in breast cancer patients (n = 102) and age matched controls (n = 66). Data are presented as boxplots showing the range of circulating CCL5 in ng/ml. The interquartile range box represents CCL5 levels from the 25th percentile to the 75th percentile, with the line representing the median. There was no significant difference detected in serum levels of CCL5 in breast cancer patients compared to controls (* represents outliers). (b) Circulating serum CCL5 levels grouped based on lymph node status. Graph depicts drop in CCL5 serum levels from node negative (59.6 (3.7) ng/ml) to node positive disease (⩽2 lymph nodes positive 40.5 (6.3) ng/ml). CCL5 serum levels increased again as number of positive lymph nodes increased (⩾3 positive lymph nodes 50.95 (6.2) ng/ml). Data are presented as boxplots. (c) Circulating serum levels of TGFβ1 in breast cancer patients (n = 102) and age matched controls (n = 66). Significantly higher levels of TGFβ1 were detected in breast cancer patients compared to normal controls ($p < 0.0001$). (d) TGFβ1 serum levels stratified based on lymph node status.

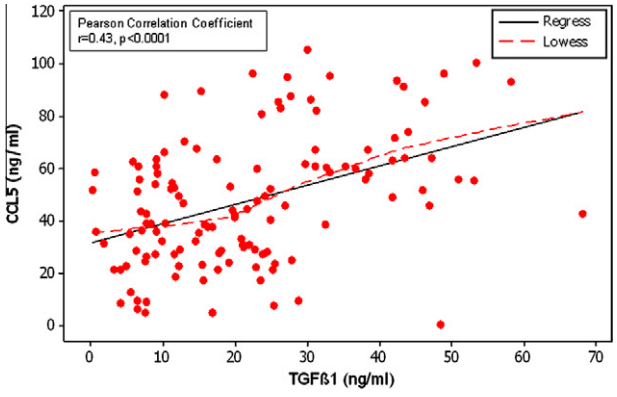

Fig. 2 – Scatterplot depicting significant positive correlation between serum CCL5 and TGFβ1 (n = 127, r = 0.43, $p < 0.0001$).

increased in tumour compared to normal breast tissue ($p < 0.0001$) whilst expression of TGFβRII remained unchanged (Fig. 4B). A significant positive correlation between CCL5 and TGFβ1 gene expression was observed across all tissue samples (n = 59, r = 0.435, $p < 0.001$, Fig. 4C).

3.4. *Analysis of primary breast tumour stromal cells*

Gene expression analysis was carried out on primary tumour stromal cells isolated from tumour (n = 22) and normal breast tissue (n = 3) harvested at reduction mammoplasty. Results were normalised using the endogenous control gene PPIA and expressed relative to normal stromal cell gene expression. CCL5 expression was increased (Mean(SEM) 0.8(0.2) Log_{10} Relative Quantity(RQ)), and TGFβ1 expression decreased (−0.12(0.05) Log_{10} RQ) in tumour stromal cells compared to normal stromal cells (Fig. 5). CCR5 was not detected in any

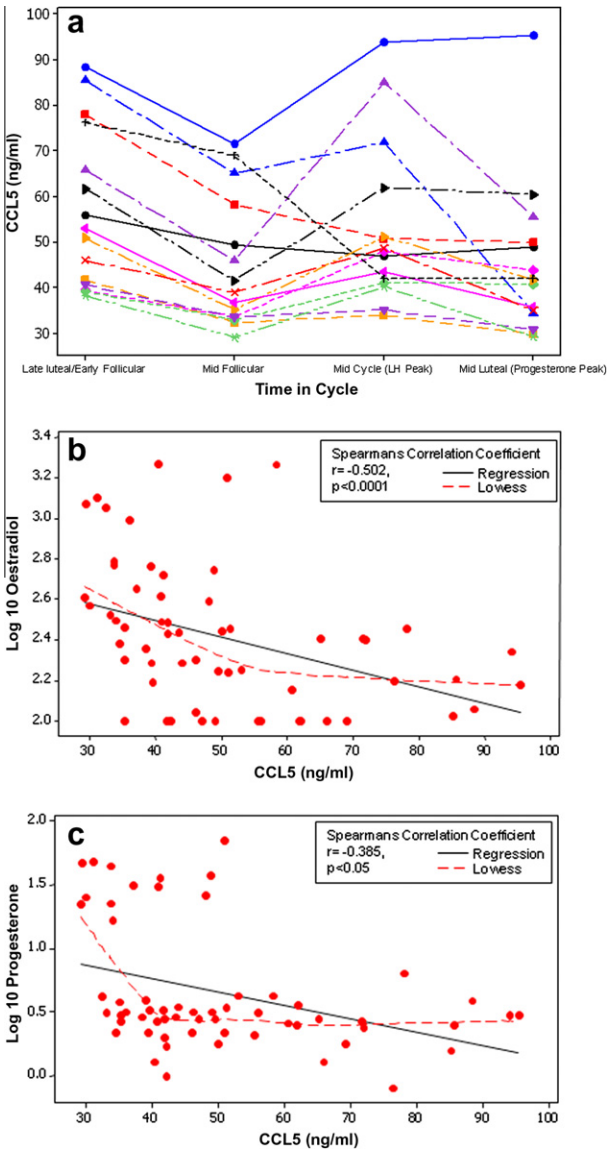

Fig. 3 – (a) Variation of systemic CCL5 across phases of menstrual cycle ($n = 15$ samples over four consecutive weeks, total $n = 60$). The levels of CCL5 were significantly decreased in the transition from late luteal/early follicular to mid follicular phase ($p < 0.05$). (b) Scatterplot depicting significant negative correlation between CCL5 and Oestradiol ($n = 60$, $r = -0.502$, $p < 0.0001$). (c) Scatterplot showing significant negative correlation between CCL5 and Progesterone ($n = 60$, $r = -0.385$, $p < 0.05$).

of the stromal populations examined (Fig. 5). A significant negative correlation was found between CCL5 and TGFβ1 gene expression in primary stromal cells ($n = 32$, $r = -0.38$, $p < 0.05$).

4. Discussion

In contrast to previous studies,[6,7] the results presented show no significant difference between circulating CCL5 levels in breast cancer patients and healthy controls. The contradicting findings may be as a result of the fact that this study contains the largest cohort of breast cancer patients ($n = 102$) to date. Niwa et al. compared plasma samples from breast cancer patients ($n = 43$) to healthy controls ($n = 12$), whilst Eissa et al.[7] detected elevation of CCL5 in serum samples from 60 breast cancer patients and 30 healthy controls. At initial stages of the current study, CCL5 levels were also found to be elevated in breast cancer patients, however when the number of samples was increased, this pattern was lost. The control group in the current study also contained an equal proportion of pre- and post-menopausal subjects as the breast cancer group, which may impact the outcome, particularly considering the observed relationship between CCL5 and circulating hormones.

Previous studies have shown that Estradiol replacement therapy decreases serum CCL5.[15] The significant negative correlation between serum CCL5, Oestradiol and Progesterone observed in this study in healthy premenopausal volunteers provides direct evidence of a cyclical variation of the chemokine. A previous study reported cyclical variation of vascular endothelial growth factor (VEGF), and its importance in terms of choosing the optimal time point of surgery in the menstrual cycle of premenopausal breast cancer patients.[25] The results presented here may warrant consideration in determining the optimal time point in the menstrual cycle for surgical intervention in premenopausal breast cancer patients.

Previous evidence suggests that circulating CCL5 is elevated in late stage breast cancer.[6,8] In this study, a trend towards increased CCL5 levels was observed as the number of positive lymph nodes increased. This pattern has previously been associated with TGFβ1,[19] and further investigation revealed a significant positive correlation between circulating CCL5 and TGFβ1. Further investigation of expression of CCL5 and TGFβ1 in tumour tissue from a subset of the same patients also revealed a correlation between the two factors, adding further significance to this finding.

Although no difference in circulating CCL5 levels was observed, in agreement with previous reports based on immunohistochemistry,[6] a significant increase in CCL5 expression in tumour tissue compared to normal tissue was detected. Also, expression of CCL5 and its principle receptor CCR5, displayed a significant positive correlation, indicating a strong affinity between the ligand and receptor. TGFβ1 gene expression was also significantly higher in tumour tissue compared to normal tissue, whilst expression of TGFβII remained unchanged.

Whilst a positive correlation between CCL5 and TGFβ1 was observed at the systemic level, and also in whole tumour tissues, upon isolation of primary tumour stromal cells, an inverse relationship between the two factors was observed. CCL5 was found to be significantly elevated in tumour, compared to normal stromal cells, whilst TGFβ1 was decreased. Interestingly, previous studies have shown that reduced TGFβ signalling in tumour stroma supports breast cancer metastasis,[26] whereas increased CCL5 secretion from adjacent stromal cells has been shown to stimulate metastasis of breast cancer cells.[27] Cheng et al.[26] reported that TGFβRII deletion in an *in vivo* model led to blockage of the stromal autocrine TGFβ loop and increased proliferation in fibroblasts. This group also found that TGFβRII deletion in stromal cells altered the paracrine stromal–epithelial crosstalk and led to increased proliferation of the mammary epithelium.[26] Forrester

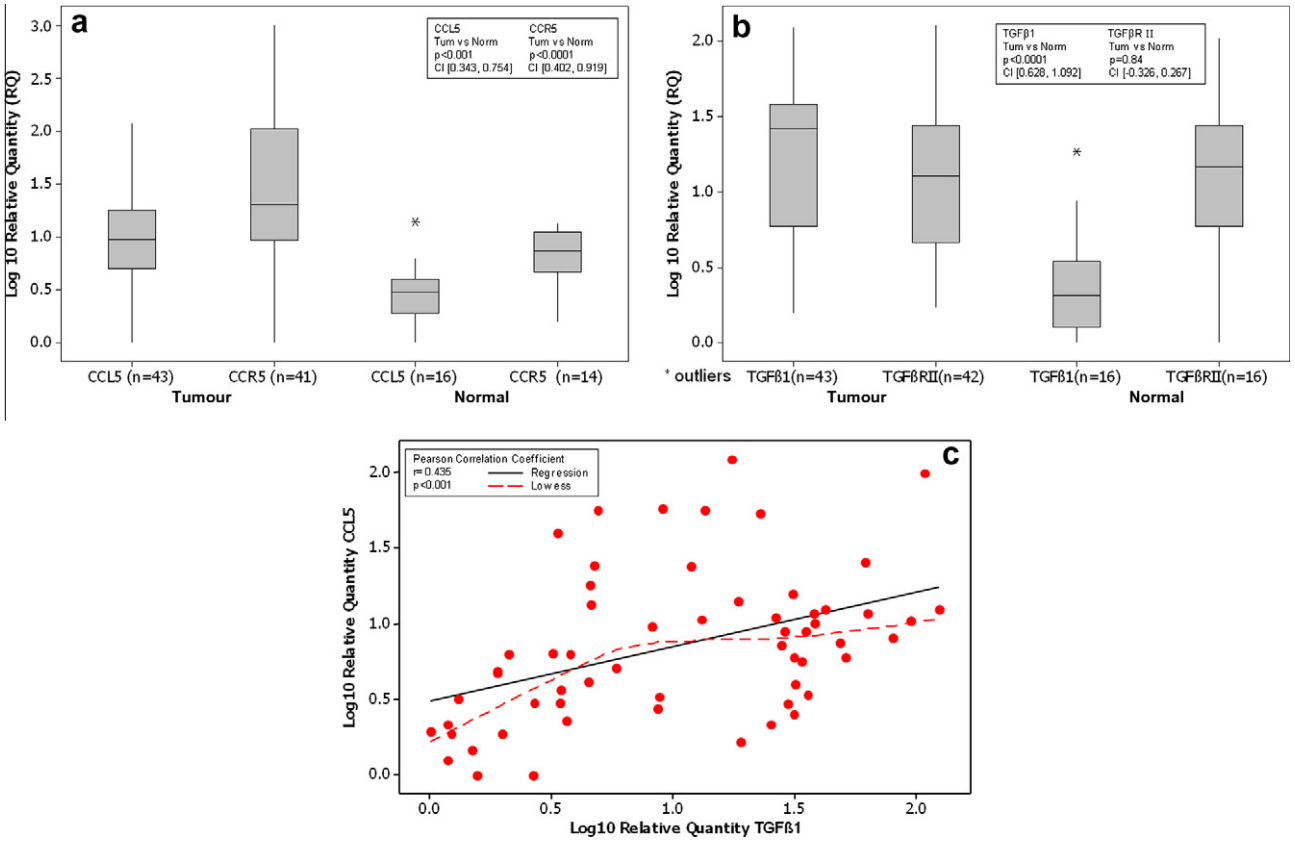

Fig. 4 – (a) Gene expression analysis targeting CCL5 and CCR5 in breast cancer tissue ($n = 43$) compared to normal tissue ($n = 16$). Significantly higher expression of CCL5 and CCR5 was found in tumour compared to normal breast tissue ($p < 0.0001$). The data are presented as boxplots showing the range of Log_{10} Relative Quantity gene expression of CCL5 and CCR5. (b) Gene expression analysis targeting TGFβ1 and TGFβRII in tumour and normal breast tissue. A significant increase in TGFβ1 expression was found in breast cancer tissue compared to normal controls ($p < 0.0001$) whereas no significant difference in gene expression of its receptor TGFβRII was found. (c) Pearson correlation between CCL5 and TGFβ1 gene expression in all breast tissue samples examined ($n = 59$, $r = 0.44$, $p < 0.001$).

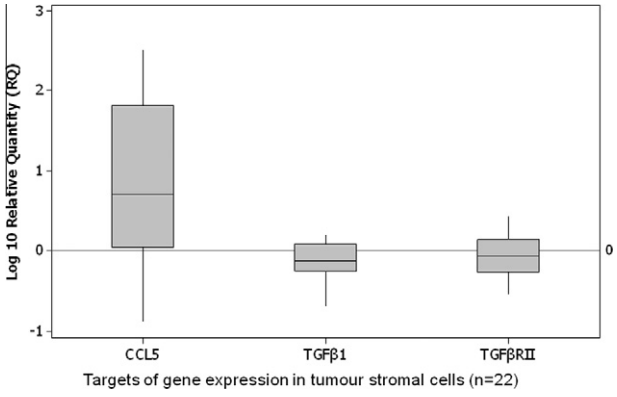

Fig. 5 – Boxplots depicting CCL5, TGFβ1 and TGFβRII expression in primary tumour stromal compared to normal stromal cells harvested at reduction mammoplasty ($n = 3$). Normal stromal cell expression is represented by the reference line.

et al.[28] also showed in an *in vivo* model of breast tumourigenesis, that loss of TGFβRII in tumour cells lead to increased pulmonary metastases and decreased time to tumour formation.

In a study incorporating an admix of Mesenchymal Stem Cells and epithelial breast cancer cells, MSC-secreted CCL5 was shown to exert its action in a paracrine manner and stimulate increased breast cancer metastasis *in vivo*.[27] Pinilla et al.[29] found that co-culture with tissue resident stem cells stimulated increased invasion of breast cancer cell lines, an effect which was blocked in the presence of an antibody to CCL5. The adipose-derived stem cells were identified as the source of CCL5 secretion, which was found to be induced through the influence of tumour derived humoral factors.

It is worth noting that CCR5 expression was not detected in any of the tumour stromal cells examined in the current study, supporting paracrine action of the chemokine in the tumour microenvironment. This may also have impacted a previous report showing that CCL5 on its own has little or no effect in breast cancer growth and metastasis.[12] The investigation employed a mouse model of breast cancer established using epithelial cells alone. The current study, along with previous reports, suggests that *in vivo* models using mixed stromal–epithelial xenografts may be more appropriate to elucidate the true role of CCL5 in breast cancer.

Although unchanged at a systemic level, CCL5 expression in the tumour microenvironment is significantly increased

compared to healthy tissue, with the stromal compartment partly responsible for this. The cyclical variation in CCL5 detected supports a role for hormonal control of the chemokine. It appears that CCL5 may play an important role in the primary tumour microenvironment, most likely through paracrine effects on tumour epithelial cells. This study has also identified a potentially important relationship between CCL5 and TGFβ1 in breast cancer which warrants further investigation.

Grant support

This study was supported by funding from the National Breast Cancer Research Institute (NBCRI), a Breast Cancer Ireland Research Fellowship Award (M.C. Hartmann), and the Health Research Board of Ireland (R.M. Dwyer, J. Newell).

Conflict of interest statement

None declared.

REFERENCES

1. Balkwill F, Mantovani A. Inflammation and Cancer: back to Virchow? *Lancet* 2001;**357**(9255):539–45.
2. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;**420**(6917):860–7.
3. Maghazachi AA, Al-Aoukaty A, Schall TJ. CC chemokines induce the generation of killer cells from CD56+ cells. *Eur J Immunol* 1996;**26**(2):315–9.
4. Soria G, Ben-Baruch A. The inflammatory chemokines CCL2 and CCL5 in breast cancer. *Cancer Lett* 2008;**267**(2):271–85.
5. Samson M, Stordeur P, Labbe O, et al. Molecular cloning and chromosomal mapping of a novel human gene, ChemR1, expressed in T lymphocytes and polymorphonuclear cells and encoding a putative chemokine receptor. *Eur J Immunol* 1996;**26**(12):3021–8.
6. Niwa Y, Akamatsu H, Niwa H, et al. Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer. *Clin Cancer Res* 2001;**7**(2):285–9.
7. Eissa SA, Zaki SA, El-Maghraby SM, Kadry DY. Importance of serum IL-18 and RANTES as markers for breast carcinoma progression. *J Egypt Natl Canc Inst* 2005;**17**(1):51–5.
8. Luboshits G, Shina S, Kaplan O, et al. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. *Cancer Res* 1999;**59**(18):4681–7.
9. Soria G, Yaal-Hahoshen N, Azenshtein E, et al. Concomitant expression of the chemokines RANTES and MCP-1 in human breast cancer: a basis for tumor-promoting interactions. *Cytokine* 2008;**44**(1):191–200.
10. Stormes KA, Lemken CA, Lepre JV, Marinucci MN, Kurt RA. Inhibition of metastasis by inhibition of tumor-derived CCL5. *Breast Cancer Res Treat* 2005;**89**(2):209–12.
11. Adler EP, Lemken CA, Katchen NS, Kurt RA. A dual role for tumor-derived chemokine RANTES (CCL5). *Immunol Lett* 2003;**90**(2–3):187–94.
12. Jayasinghe MM, Golden JM, Nair P, et al. Tumor-derived CCL5 does not contribute to breast cancer progression. *Breast Cancer Res Treat* 2008;**111**(3):511–21.
13. Yaal-Hahoshen N, Shina S, Leider-Trejo L, et al. The chemokine CCL5 as a potential prognostic factor predicting disease progression in stage II breast cancer patients. *Clin Cancer Res* 2006;**12**(15):4474–80.
14. Christodoulakos GE, Lambrinoudaki IV, Economou EV, et al. Circulating chemoattractants RANTES, negatively related to endogenous androgens, and MCP-1 are differentially suppressed by hormone therapy and raloxifene. *Atherosclerosis* 2007;**193**(1):142–50.
15. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of raloxifene evaluation. *Jama* 1999;**281**(23):2189–97.
16. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Jama* 2006;**295**(23):2727–41.
17. Rao VS, Dyer CE, Jameel JK, Drew PJ, Greenman J. Potential prognostic and therapeutic roles for cytokines in breast cancer (Review). *Oncol Rep* 2006;**15**(1):179–85.
18. Padua D, Massague J. Roles of TGFbeta in metastasis. *Cell Res* 2009;**19**(1):89–102.
19. Lebrecht A, Grimm C, Euller G, et al. Transforming growth factor beta 1 serum levels in patients with preinvasive and invasive lesions of the breast. *Int J Biol Markers* 2004;**19**(3):236–9.
20. Barcellos-Hoff MH, Akhurst RJ. Transforming growth factor-beta in breast cancer: too much, too late. *Breast Cancer Res* 2009;**11**(1):202.
21. Kong FM, Anscher MS, Murase T, et al. Elevated plasma transforming growth factor-beta 1 levels in breast cancer patients decrease after surgical removal of the tumor. *Ann Surg* 1995;**222**(2):155–62.
22. Grau AM, Wen W, Ramroopsingh DS, et al. Circulating transforming growth factor-beta-1 and breast cancer prognosis: results from the Shanghai Breast Cancer Study. *Breast Cancer Res Treat* 2008;**112**(2):335–41.
23. McNeill RE, Miller N, Kerin MJ. Evaluation and validation of candidate endogenous control genes for real-time quantitative PCR studies of breast cancer. *BMC Mol Biol* 2007;**8**:107.
24. Potter SM, Dwyer RM, Curran CE, et al. Systemic chemokine levels in breast cancer patients and their relationship with circulating menstrual hormones. *Breast Cancer Res Treat* 2009;**115**(2):279–87.
25. Heer K, Kumar H, Speirs V, et al. Vascular endothelial growth factor in premenopausal women – indicator of the best time for breast cancer surgery? *Br J Cancer* 1998;**78**(9):1203–7.
26. Cheng N, Bhowmick NA, Chytil A, et al. Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* 2005;**24**(32):5053–68.
27. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;**449**(7162):557–63.
28. Forrester E, Chytil A, Bierie B, et al. Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. *Cancer Res* 2005;**65**(6):2296–302.
29. Pinilla S, Alt E, Abdul Khalek FJ, et al. Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion. *Cancer Lett* 2009;**284**(1):80–5.