

Potential implication of IL-24 in lymphangiogenesis of human breast cancer

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Abstract. Lymphangiogenesis is involved in the dissemination of malignant cells from solid tumours to regional lymph nodes and possibly to various distant sites. Lymphangiogenesis is regulated by vascular endothelial growth factor (VEGF)-C and VEGF-D. Interleukin (IL)-24 is known as a cytokine with potent antitumour and tumour-suppressive activity which functions through its receptor (IL-22R). Expression of IL-24 has been shown to be reduced in breast cancer, and the reduced expression is associated with lymphatic metastases and a poor prognosis. However, the involvement of IL-24 in lymphangiogenesis during lymphatic metastasis remains unclear. The aim of the present study was to determine whether there is an association between IL-24, IL-22R and lymphangiogenic factors and markers in breast cancer. Analysis of IL-24, IL-22R and lymphangiogenic factors in malignant breast tissue samples (n=127) revealed a correlation between increased expression of lymphangiogenic markers (podoplanin, Prox-1 and LYVE-1) and reduced levels of IL-24 and IL-22R. Samples stained with a high degree of positivity for lymphangiogenic factors and markers whereas staining for IL-24 was weak. *In vitro* assays showed that the average perimeter length of microtubules formed by endothelial cells treated with IL-24 was significantly reduced compared to the control. The growth of endothelial cells was significantly reduced when exposed to a high concentration of IL-24 (250 ng/ml). Treatment of HECV cells with IL-24 resulted in significantly reduced expression of VEGF-C (P<0.05) and VEGF-D (P<0.001). In conclusion, reduced expression of IL-24 and IL-22R in breast cancer is correlated with increased expression of specific lymphangiogenic markers. IL-24 suppressed *in vitro* growth and microtubule formation of endothelial cells. IL-24 may downregulate the expression of lymphangiogenic markers and

factors although further research is required. This suggests that IL-24 plays a profound role in suppressing tumour lymphangiogenesis, thereby, reducing the likelihood of cancer metastasis via the lymphatic route.

Introduction

Lymphangiogenesis is the process by which new lymphatic vessels form from pre-existing lymphatic vessels or lymphatic endothelial progenitor cells (1). The process is important in the normal development of the lymphatic system and is also implicated in pathological processes such as inflammation, lymphedema and importantly cancer metastasis. The lymphatic vasculature serves as a major route for the dissemination of malignant cells from solid tumours, particularly to regional lymph nodes (2). The extent of lymph node metastasis is a major determinant of both staging and prognosis in breast cancer.

The recent discovery of a number of molecular and cellular markers thought to be specific to lymphatic endothelial cells has enabled more detailed research into lymphangiogenesis. The most important lymphangiogenic markers include podoplanin (a glomerular podocyte membrane mucoprotein) (3), Prox-1 (a homeobox gene involved in embryonic development of the lymphatic system) (4) and LYVE-1 (a marker shown to be found exclusively on lymphatic vessels in normal tissues and tumours) (5). Several studies have reported that high expression levels of lymphangiogenic markers (particularly podoplanin and LYVE-1) in breast tumours are correlated with increased lymphatic vessel density, increased likelihood of tumour metastasis to regional lymph nodes and therefore a poor prognosis (6-9).

The process of lymphangiogenesis is thought to be regulated by the interaction of vascular endothelial growth factor (VEGF)-C or VEGF-D with the cell surface receptor VEGFR-3 (2). Research suggests that VEGF-C expression in breast cancer may correlate with increased lymph node metastases (10) and significantly poorer disease-free survival (assessed at five years) (11). In contrast, other studies have indicated that a high VEGF-C to VEGF-D ratio in breast tumours may be a more accurate predictor of increased likelihood of lymph node metastasis (12-14). High expression levels of lymphangiogenic factors or markers may be important in indicating an increased potential for lymphangiogenesis and cancer metastasis.

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Interleukin (IL)-24 is a member of the IL-10 cytokine family. The secreted protein is 206 amino acids in length and has a predicted size of 35-40 kDa (15). Previous studies suggest that IL-24 operates through interaction with IL-20R1/IL-20R2 or IL-22R1/IL-20R2 heterodimers (15,16). IL-24 was originally detected in human melanoma cells. However, it appears to be lowly expressed in cells of the immune system, including spleen cells, thymus cells and normal melanocytes (16).

IL-24 is thought to possess antitumour characteristics. By suppressing signals generated by tumour cells and through interference with tumour vasculature, IL-24 may act to inhibit tumour growth and metastasis (17). Forced expression of IL-24 in breast cancer cells was found to cause potent growth suppression (18). Results from *in vitro* assays suggest that IL-24 significantly reduces migration and motility of breast cancer cells (19).

Immunohistochemical staining has demonstrated that normal breast tissue contains substantially higher levels of IL-24 compared to malignant breast tissue. Lower transcript levels of IL-24 were found to be correlated with node-positive breast tumours, increased likelihood of distant metastasis, a poor prognosis and shorter disease-free survival. Patients with higher levels of IL-24 were more likely to have a better prognosis and remain alive and disease-free (19). The correlation between loss of IL-24 expression and increased tumour invasion may indicate that IL-24 has an important role in tumour suppression. However, how IL-24 is involved in lymphatic metastasis remains unclear.

The aim of the present study was to assess whether there is an association between levels of IL-24, IL-22R and the expression of lymphangiogenic factors and markers in breast cancer tissue samples obtained from a cohort of 127 women. *In vitro* studies were then carried out to investigate how IL-24 alters the expression of lymphangiogenic factors and markers. The effect of IL-24 on specific functions (growth and microtubule formation) of endothelial cells was also examined.

Materials and methods

Cohort of tissue samples. Breast cancer tissue samples (n=127) were collected from patients at the University Hospital of Wales, Cardiff. Ethical approval and informed consent was obtained. RNA extraction of the samples was performed using an RNA extraction kit (AbGene Ltd., Surrey, UK) along with reverse transcription. Pairs of PCR primers were designed using the Beacon Designer™ software and synthesised by Sigma-Aldrich. IcyClerIQ™ (Bio-Rad, Hemel Hempstead, UK) was used to carry out real-time quantitative PCR [based on previously described methods (19,20)] to detect transcript levels of IL-24, IL-22R and lymphangiogenic factors and markers in the breast cancer samples. The Amplifluor system (Intergen Inc., New York, NY, USA) was utilized under the following conditions: an initial period of 15 min at 95°C followed by 60 cycles of 95°C for 15 sec, 55°C for 60 sec and 72°C for 20 sec together with QPCR Master Mix (ABgene, Surrey, UK).

Materials and cell lines. HECV cells purchased from Interlab (Milan, Italy) were maintained in Dulbecco's modified Eagle's

medium (DMEM) (Sigma-Aldrich, Poole, UK) supplemented with benzylpenicillin, amphotericin B, streptomycin and 10% fetal bovine serum (Sigma-Aldrich). The cells were incubated at 37°C in 5% CO₂ and 95% humidity until confluent. Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Products (Bedford, MA, USA).

Treatment of cells with IL-24 and conventional and quantitative polymerase chain reaction. Six 12.5-cm² tissue culture flasks were seeded with 1x10⁶ HECV cells and incubated at 37°C for 24 h. Cells were treated with 25 ng/ml human recombinant IL-24 (R&D Systems Europe) and incubated at 37°C for 1, 2, 4 and 24 h. Total RNA reagent (TRI) was used to extract the RNA from IL-24-treated cells using the provided protocol (Sigma-Aldrich). RNA was quantified using a spectrophotometer (WPA UV 1101; Biotech Photometer, Cambridge, UK) and standardised to a concentration of 500 ng. Reverse transcription was performed using the iScript cDNA synthesis kit (PrimerDesign Ltd., Southampton, UK).

Conventional PCR was carried out in a T-Cy Thermocycler (Creacon Technologies Ltd., The Netherlands) using REDTaq® ReadyMix™ PCR reaction mix (Sigma-Aldrich), cDNA from cells and the following primers: GAPDH (which served as a control), VEGF-C and VEGF-D (Table I). The reaction conditions used were: 5 min at 94°C, 40 sec at 94°C, 40 sec at 55°C, 1 min at 72°C for 35 cycles and 72°C for 10 min. PCR products were then stained with SYBR Safe™ (Invitrogen) separated on a 1% agarose gel, visualised under UV light and photographed. ImageJ software was used to semi-quantify the results.

Real-time quantitative PCR was carried out using the iCycler iQ™ Real-Time detection system (Bio-Rad) to determine transcript levels of VEGF-C, VEGF-D and Prox-1 in IL-24-treated HECV cells. QPCR was carried out in a 96-well plate using a previously described method (21) with 10 pmol sense primer, 1 pmol antisense Z primer (Table I) and 10 pmol FAM-probe, using a custom Hot-Start QPCR master mix, under the following conditions: 95°C for 15 min, followed by 50 cycles at 95°C for 15 sec, 55°C for 4 sec and 72°C for 15 sec. The levels of transcripts were calculated as lymphangiogenic factor or lymphangiogenic marker/GAPDH ratio.

Western blotting. HECV cells (1x10⁶) contained in 25 cm² flasks were treated with IL-24 (25 ng/ml) for 0, 1, 2 or 4 h. HMCSF buffer containing 1% Triton X-100, 2 mM CaCl₂, 100 µg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 10 mM sodium orthovanadate was used to detach and lyse the cells. Insoluble components were then removed by rotating samples on a wheel for 1 h and centrifuging at 13,000 x g. The protein samples were blotted onto Hybond-C Extra nitrocellulose membranes (Amersham Biosciences UK Ltd., Bucks, UK). Protein expression of GAPDH and lymphangiogenic factors and markers in the samples were assessed. Antibodies specific to GAPDH, VEGF-C, LYVE-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and VEGF-D (R&D Systems Europe) were used. Visualisation of the protein bands was carried out using SuperSignal West Dura Extended Duration substrate chemiluminescent system (Perbio Science UK Ltd., Cramlington,

Table I. Primer sequences.

| Method | Name | Sequences |
|--------|-------------|--|
| PCR | VEGF-C F9 | TTTGCCAATCACACTTCCTG |
| | VEGF-C R9 | CAGGCACATTTTCCAGGATT |
| | VEGF-D F9 | CAGGGCTGCTTCTAGTTTGG |
| | VEGF-D R9 | TTCTTCAGGGATCTGGATGG |
| | GAPDH F8 | ATGATATCGCCGCGCTCGTC |
| | GAPDH R8 | GCTCGGTCAGGATCTTCA |
| QPCR | VEGF-C F12 | CTACAGATGTGGGGGTTGCT |
| | VEGF-C ZR12 | ACTGAACCTGACCGTACAGTAGCTCGTGCTGGTGTTC |
| | VEGF-D F12 | TCCACATTGGAACGATCTGA |
| | VEGF-D ZR12 | ACTGAACCTGACCGTACACTCCACAGCTTCCAGTCCTC |
| | Prox-1 F11 | AGAGCAGGAAATGGCTGAAA |
| | Prox-1 ZR11 | ACTGAACCTGACCGTACATCCGGTTGTAAGGAGTTTGG |
| | GAPDH F | CTGAGTACGTCGTGGAGTC |
| | GAPDH ZR | ACTGAACCTGACCGTACAGAGATGATGACCCTTTTG |

F, forward; R, reverse; VEGF, vascular endothelial growth factor.

UK) and images were captured using a UViprochem camera system (UVItec, Cambridge, UK).

The values shown graphically for QPCR, conventional PCR with semi-quantitative analysis and western blotting are expressed as the percentage of decrease in lymphangiogenic transcript expression compared to control cells (the expression in the cells not treated with IL-24 was regarded as baseline, i.e. having 100% lymphangiogenic factor/marker expression). The change in expression was shown as a percentage (%) in comparison with the control.

In vitro microtubule formation assay. The processes used were modified from previously published methods (22,23). Briefly, 96-well plates were coated with 100 μ l/well of Matrigel (diluted in a 1 to 1 ratio with serum-free medium) and incubated for 60 min to allow the thin gel layer to set. HECV cells (3×10^4 /well) were seeded onto the Matrigel layer. The cells were treated with IL-24 (2.5, 25 and 250 ng/ml) or a preparation of serum-free medium or normal medium and incubated for 4-6 h to allow tubule formation to occur. Subsequently, the microtubule lengths were visualized under bright light microscopy and quantified (total perimeter length per field) using ImageJ.

In vitro cell growth assay. The processes used were based on a previously published method (24). HECV cells (3,000/well) were plated into 96-well plates followed by treatment with IL-24 (2.5, 25 and 250 ng/ml) and a period of incubation (either overnight or 3 days). The cells were fixed in 4% formaldehyde and stained with 0.5% crystal violet. After washing, 10% acetic acid was added, and the absorbance was determined using a Bio-Tek ELx800 multiplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA) at a wavelength of 540 nm. Absorbance was used to represent the cell number.

Results

IL-24 and the expression of lymphangiogenic factors/markers in breast cancer. Following quantitative determination of the transcripts of IL-24, IL-22R, lymphangiogenic factors and markers in the breast cancer tissues (12,19), correlations among these factors were analysed.

Table II depicts the results where IL-24 and IL-22R are found in the presence of CK19 (a specific epithelial cell marker). Levels of IL-24 and IL-22R were therefore normalised values representative of those found within epithelial-derived malignant breast tissue. High levels of IL-22R were associated with significantly high levels of IL-24 expression ($P < 0.01$). A significant correlation was evident between increased expression of LYVE-1 and reduced expression of IL-24 (correlation coefficient -0.288) ($P < 0.05$) in the cohort of breast cancer tissue samples. Similarly, the expression of another lymphangiogenic marker, podoplanin, was also significantly inversely correlated with the expression of IL-24 ($P < 0.01$) and IL-22R ($P < 0.05$). Although the expression of other examined lymphangiogenic markers and factors (Prox-1, VEGFR-3 and VEGF-C) also appeared to increase in the tumours with lower expression of IL-24, no statistically significant correlation was noted. VEGF-D, however, a promoter of lymphangiogenesis tended to be positively associated with the expression of IL-24 ($P = 0.03$).

Both epithelial-derived malignant breast tissue and surrounding stromal cells (including endothelial cells) are important factors in lymphangiogenesis of tumours. Therefore levels of IL-24 and IL-22R (non-normalised) in the cohort of breast cancer tissue samples are also provided in Table II. The expression of all lymphangiogenic factors (VEGF-C and VEGF-D) and lymphangiogenic markers (podoplanin, Prox-1, VEGFR-3 and LYVE-1) was inversely correlated with the expression of IL-24. In contrast VEGF-D, VEGF-C and

Table II. Correlations between IL-24/IL-22R and lymphangiogenic factors/markers at their transcript levels in breast cancer tissues.

| Transcript name | IL-24 | IL-22R |
|-----------------|-----------------------------------|-----------------------------------|
| VEGF-C | -0.115 (P=0.358)/-0.047 (P=0.684) | 0.108 (P=0.384)/0.120 (P=0.293) |
| VEGF-D | 0.324 (P=0.03)/-0.068 (P=0.624) | 0.289 (P=0.054)/0.105 (P=0.449) |
| Podoplanin | -0.332 (P=0.001)/-0.183 (P=0.059) | -0.260 (P=0.011)/-0.094 (P=0.33) |
| Prox-1 | -0.199 (P=0.053)/-0.067 (P=0.486) | -0.057 (P=0.578)/0.102 (P=0.291) |
| VEGR3 | -0.156 (P=0.131)/-0.046 (P=0.637) | -0.134 (P=0.192)/0.025 (P=0.792) |
| LYVE-1 | -0.288 (P=0.017)/-0.161 (P=0.145) | -0.039 (P=0.749)/-0.042 (P=0.707) |
| IL-22R | 0.268 (P=0.008)/0.305 (P=0.001) | |

Correlation coefficient data comparing expression of lymphangiogenic factors/markers with expression of IL-24 and IL-22R in samples of breast cancer tissue. The correlation coefficient (P-value) of normalised transcript levels/the correlation coefficient (P-value) of non-normalised transcript levels are shown. Transcripts of each gene normalised against the corresponding CK19 levels, respectively, are shown as a ratio against CK19. VEGF, vascular endothelial growth factor.

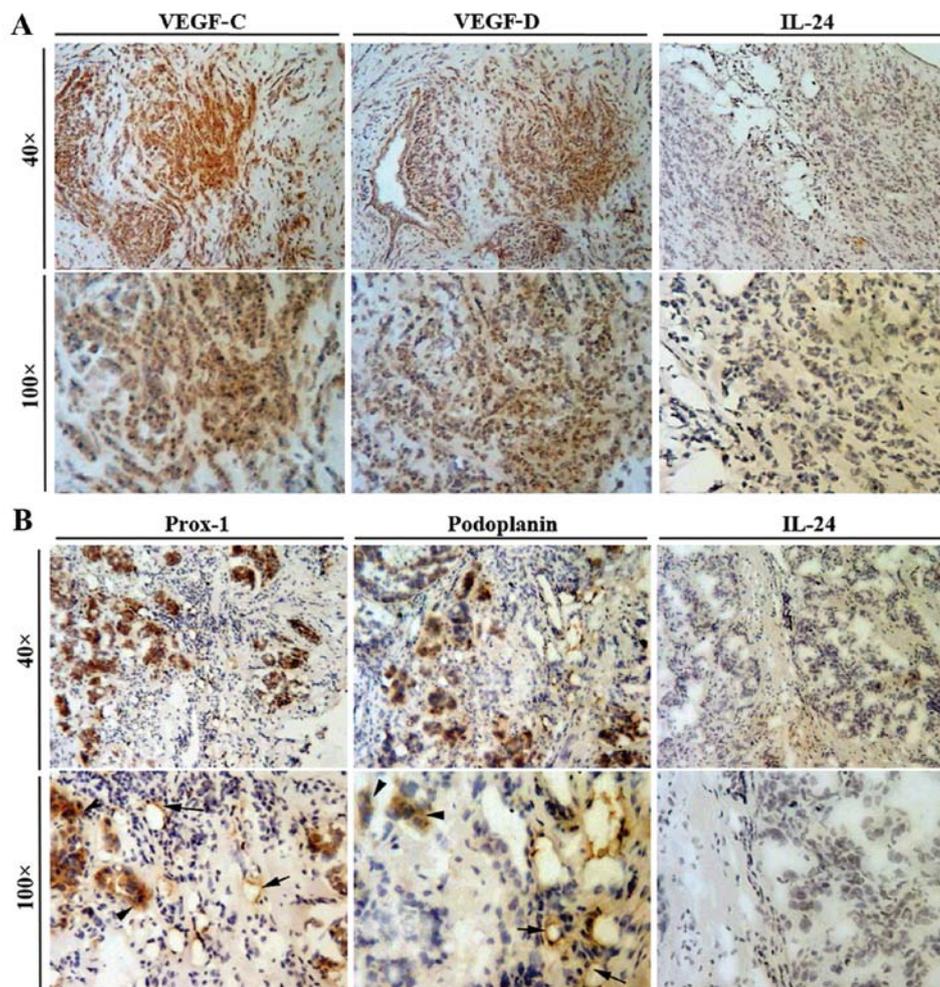


Figure 1. Immunohistochemical staining of IL-24 and lymphangiogenic factors/markers in breast cancer specimens. (A) Breast cancer tissue samples were immunohistochemically stained for the presence of VEGF-C, VEGF-D and IL-24. Breast cancer cells stained positively for VEGF-C and VEGF-D but were virtually negative for the presence of IL-24. (B) Breast cancer tissue samples were immunohistochemically stained for the presence of Prox-1, podoplanin and IL-24. Breast cancer cells (arrowheads) and endothelial cells (arrows) stained positively for Prox-1 and podoplanin but were virtually negative for the presence of IL-24. Shown are the staining of different molecules in the matched specimens.

VEGFR-3 (promoters of lymphangiogenesis) were positively associated with expression of IL-22R. None of the non-normalised results was found to be significant.

Breast cancer tissue samples were immunohistochemically stained for levels of IL-24 and lymphangiogenic factors (VEGF-C and VEGF-D), IL-24 and lymphangiogenic markers

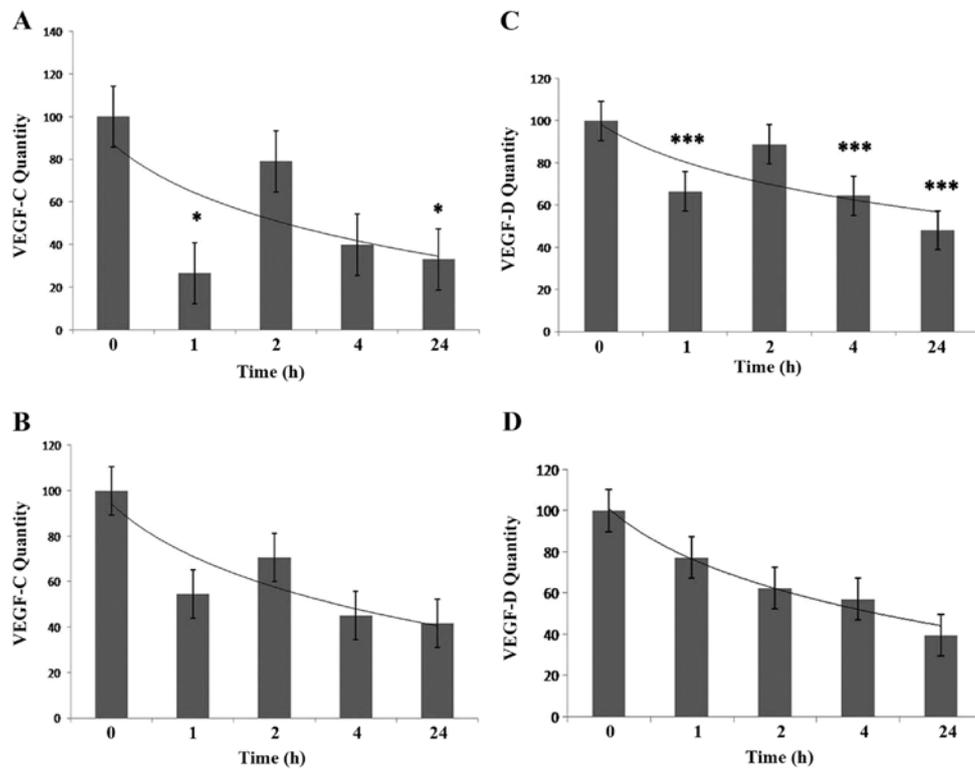


Figure 2. Effect of IL-24 on expression of VEGF-C and VEGF-D in endothelial cells. (A) Semi-quantitative analysis of conventional PCR demonstrating the effect of IL-24 on the expression of VEGF-C in HECV endothelial cells. Treatment of HECV cells with IL-24 for 24 h resulted in a significantly reduced expression of VEGF-C compared to the control; * $P \leq 0.05$. (B) Quantitative PCR demonstrating the effect of IL-24 on the expression of VEGF-C in HECV endothelial cells. (C) Semi-quantitative analysis of conventional PCR demonstrating the effect of IL-24 on the expression of VEGF-D in HECV endothelial cells. Treatment of HECV cells with IL-24 for 1, 4 and 24 h resulted in significantly reduced expression of VEGF-D compared to the control; *** $P \leq 0.001$. (D) Quantitative PCR demonstrating the effect of IL-24 on the expression of VEGF-D by HECV endothelial cells.

(Prox-1 and podoplanin). A high degree of VEGF-C and VEGF-D positivity was found to be present in breast cancer cells (Fig. 1). In comparison, endothelial cells contained within the tumour tissue did not stain positively for the lymphangiogenic factors VEGF-C and VEGF-D. Fig. 1 also shows that both cancer cells and endothelial cells stained with a high degree of positivity for the lymphangiogenic markers Prox-1 and podoplanin. Breast cancer cells appeared to contain low levels of IL-24 and therefore the staining for this cytokine was very weak or absent from the cancer cells.

Regulation of lymphangiogenic factors/markers in endothelial cells by IL-24. Transcript levels of lymphangiogenic factors and markers in the IL-24-treated HECV cells were detected using conventional PCR with semi-quantitative analysis and QPCR. Conventional PCR with semi-quantitative analysis and QPCR demonstrated that treatment of HECV endothelial cells with IL-24 (at a concentration of 25 ng/ml) resulted in time-dependent downregulation of VEGF-C and VEGF-D over 24 h. Results obtained using both methods showed the highest levels of mRNA expression of VEGF-C and VEGF-D in the control cells (0 h). There was rapid and significant downregulation of VEGF-C after 1 h as detected using semi-quantitative PCR ($P < 0.05$). This was followed by increased expression of VEGF-C after 2 h to levels almost equivalent to those detected in the control. A gradual decrease in expression of VEGF-C was then observed after 4 and 24 h of treatment. At 24 h significantly reduced levels of VEGF-C

were detected using semi-quantitative PCR compared to the control ($P < 0.05$) (Fig. 2A). QPCR results showed the same trend as those observed using semi-quantitative analysis. No QPCR results, however, were found to be significant, (Fig. 3B). In a same way, semi-quantitative analysis showed levels of expression of VEGF-D to be significantly reduced after 1 h of treatment with IL-24 ($P < 0.001$). Significant downregulation of VEGF-D (when compared to control) was then observed at 2 h ($P < 0.001$), 4 h ($P < 0.001$) and 24 h ($P < 0.001$) (Fig. 2C). Semi-quantitative analysis and QPCR results showed a trend of gradually decreased expression of VEGF-D over the 24 h period (Fig. 2C and D).

Protein expression of lymphangiogenic factors and markers in the IL-24-treated HECV cells was detected using western blot analysis. The results indicate a time-dependent downregulation of VEGF-C protein expression by IL-24. Levels of VEGF-C protein expression were highest at 0 h with decreased expression being detected at 1 and 2 h. Significantly low levels of VEGF-C expression (compared to control) were detected after 4 h of treatment with IL-24 ($P < 0.05$) (Fig. 3). In contrast, levels of VEGF-D and LYVE-1 protein expression appeared to decrease in a time-dependent manner (at 1 and 2 h). After 4 h, however, VEGF-D and LYVE-1 expression increased to levels originally detected at time 0 (Fig. 3). Further research is required to elucidate whether downregulation of VEGF-D and LYVE-1 protein expression occurs when HECV cells are treated with IL-24 for a longer period of time (>4 h).

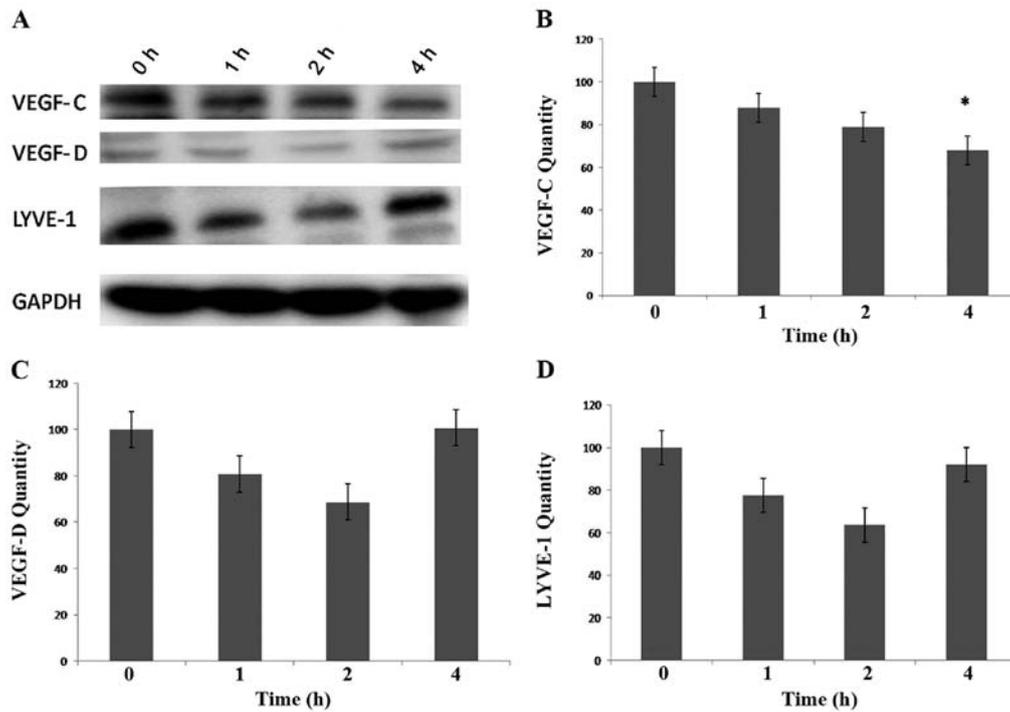


Figure 3. Western blotting demonstrating the effect of IL-24 on protein expression of VEGF-C, VEGF-D and LYVE-1 in HECV endothelial cells. (A) Treatment of HECV cells with IL-24 caused significant downregulation of VEGF-C expression after 4 h compared to the control; * $P \leq 0.05$. (B-D) Shown are quantification of the protein bands normalised against the corresponding loading control.

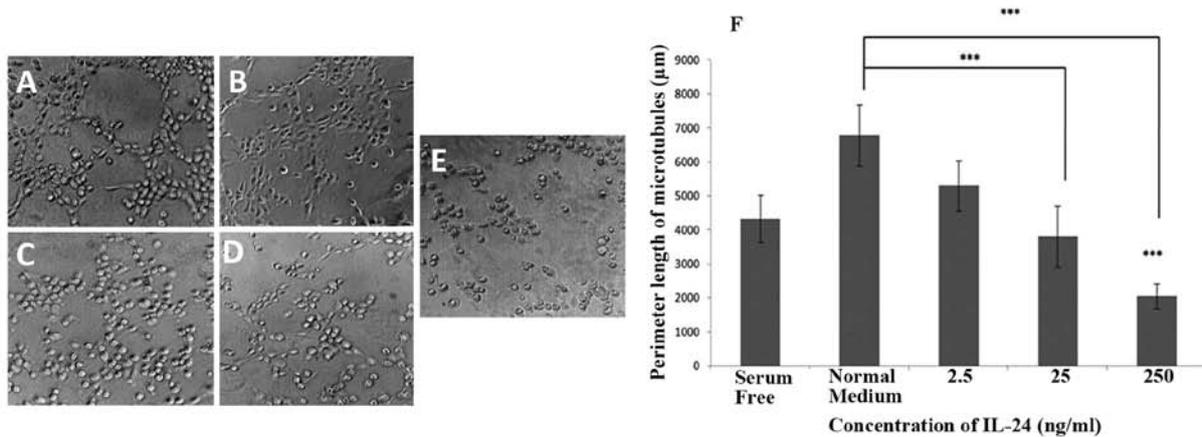


Figure 4. Effect of IL-24 on the ability of HECV endothelial cells to form microtubules. HECV cells were treated with either (A) normal medium as control, (B) serum-free medium as control, (C) 2.5 ng/ml IL-24, (D) 25 ng/ml IL-24 or (E) 250 ng/ml IL-24. IL-24 (25 and 250 ng/ml) significantly inhibited tubule formation. (F) Quantitative analysis of the perimeter length of the microtubules formed by HECV endothelial cells treated with IL-24. IL-24 (at a concentration of 250 ng/ml) significantly inhibited the formation of microtubules compared to serum-free medium and normal medium; *** $P \leq 0.001$. IL-24 (at a concentration of 25 ng/ml) significantly inhibited the formation of microtubules compared to the normal medium; *** $P \leq 0.001$.

IL-24 affects in vitro tubule formation and growth of endothelial cells. IL-24 exhibited a concentration-dependent inhibition on microtubule formation of HECV endothelial cells (Fig. 4). HECV cells that were incubated with normal medium showed the longest perimeter length of microtubules. The most profound reduction in tubule formation was observed in cells treated with the highest concentrations of IL-24 (25 and 250 ng/ml). In the presence of IL-24 at a concentration of 250 ng/ml, microtubule formation of HECV cells was significantly impaired compared to microtubule

formation observed when normal medium was used ($P < 0.001$) and when serum-free medium was used ($P < 0.001$). In the presence of IL-24 at a concentration of 25 ng/ml, microtubule formation of HECV cells was significantly impaired compared to microtubule formation in the presence of normal medium ($P < 0.001$).

HECV cells incubated with normal medium showed relatively high rates of growth between day 1 and day 3. In comparison, HECV endothelial cells exposed to IL-24 at concentrations of 2.5 and 25 ng/ml showed reduced growth.

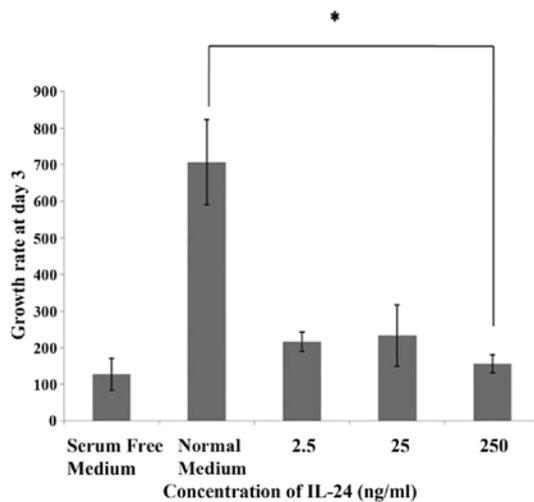


Figure 5. Absorbance results of the growth assay demonstrating the effect of IL-24 on the growth of HECV endothelial cells. IL-24 (at a concentration of 250 ng/ml) significantly inhibited the growth of HECV endothelial cells compared to normal medium; * $P \leq 0.05$.

The growth rate observed at day 3 was significantly reduced ($P < 0.05$) compared to the control when HECV endothelial cells were incubated with a high concentration of IL-24 (250 ng/ml) (Fig. 5).

Discussion

Results obtained in previous studies suggest a link between loss of IL-24 and increased likelihood of cancer invasion and metastasis particularly in breast cancer (14). IL-24 exerts its effects via IL-22R, a cell surface tyrosine kinase receptor. The present study attempted to ascertain whether loss of IL-24 or IL-22R is associated with increased levels of expression of lymphangiogenic markers and factors in a large cohort of women with breast cancer.

Low expression levels of IL-24 have been found to be associated with significantly higher levels of expression of the lymphangiogenic markers podoplanin ($P < 0.05$) and LYVE-1 ($P < 0.05$), and low levels of expression of IL-22R were associated with significantly increased levels of expression of LYVE-1 ($P < 0.05$). In addition, breast cancer tissue samples were immunohistochemically stained for lymphangiogenic factors and markers and IL-24. Within the tissue samples, cancer cells and endothelial cells stained strongly for VEGF-C and VEGF-D, whereas endothelial cells displayed a strong positivity for Prox-1 and podoplanin. The tissue samples that stained strongly for lymphangiogenic factors and markers stained very weakly for IL-24. High levels of expression of lymphangiogenic markers and factors found within the tissue samples may indicate a high density of lymphatic capillaries draining the tumour and this may be linked to reduced levels of expression of IL-24. What remains unclear is whether IL-24 has a role in inhibiting the process of lymphangiogenesis of tumours thereby reducing the density of lymphatic capillaries draining the primary cancer and preventing the dissemination of malignant cells to regional lymph nodes.

A number of *in vitro* assays were carried out to investigate the role of IL-24 in reducing lymphangiogenesis. Results

obtained during the present study suggest that IL-24 acts in a time-dependent manner to reduce mRNA expression of lymphangiogenic factors VEGF-C and VEGF-D and the lymphangiogenic marker Prox-1. Within 24 h of treatment of cells with IL-24, downregulation in the mRNA levels of lymphangiogenic factor and marker were detected. Downregulation of protein expression of VEGF-C was detected after cells were treated with IL-24 for 4 h. In addition, IL-24 had a significant effect on microtubule formation and growth of HECV cells.

The mechanism by which IL-24 exerts anti-lymphangiogenic activity is yet to be fully elucidated. Previous studies suggest that IL-24 operates through interaction with IL-20R1/IL-20R2 or IL-22R1/IL-20R2 heterodimers (15,16). Receptor activation is associated with activation of Janus activated kinase (JAK)/signal transducers and activators of transcription (STAT) signalling. Ramesh *et al* (17) report that inhibition of microtubule formation is mediated by the interaction of IL-24 with IL-22R1 resulting in the activation of STAT-3. STAT proteins are important in cytokine signalling pathways. Al-Rawi and Jiang (2) suggested that stimulation of VEGFR-3 results in strong activation of STAT-3. It is possible therefore that the regulation of VEGFR-3 signalling (and lymphangiogenesis) can be controlled by other cytokines such as IL-24, although this requires further investigation.

In conclusion, IL-24 significantly inhibits growth and microtubule formation of endothelial cells *in vitro* and downregulates the expression of some lymphangiogenic factors and markers. By modifying the ability of endothelial cells to function normally, IL-24 may act to impair lymphangiogenesis. In addition to the reduced expression of IL-24 and IL-22R in breast cancer, this is also correlated with increased expression of specific lymphangiogenic factors and markers. This suggests that IL-24 plays a role *in vivo* to suppress tumour lymphangiogenesis thereby reducing the likelihood of cancer metastasis via the lymphatic route. Further research is however required to fully elucidate the mechanisms by which IL-24 inhibits lymphangiogenesis.

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