An immunohistochemical pilot investigation into the changes of lymphatic vasculature due to carcinoma and cervical intraepithelial lesions of the uterine cervix

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INTRODUCTION

Carcinoma of the uterine cervix is the third most common malignant neoplasm, and in 2008 more than half a million females globally were diagnosed with this condition.[1] Tumour metastasis is thought to be involved in more than 90% of all cancer deaths.[2] It is, therefore, apparent that the metastatic process is intimately connected with patient prognosis. The preferred route of metastasis for carcinomas is via the lymphatic system.[3] Research into the mechanisms involved in tumour/lymphatic interactions has highlighted that tumours may disseminate through existing lymphatic vessels and induce lymphangiogenesis and metastasise via these newly formed vessels.[4]

Much of the earlier research into the role of lymphangiogenesis in the pathogenesis of cervical carcinoma has focused on the lymphatic vessel density (LVD) of tumour lymphatics. There is little information regarding the distribution of LVD in normal cervix and premalignant conditions and the exact anatomical location of the cervix used to obtain LVD for control purposes is often omitted. As 90% of all cervical lesions occur in the region of the cervix termed the transformation zone, any difference in the LVD of this anatomical region as compared to other regions of the cervix, eg ectocervix, is of particular importance.

Previous studies have made observations describing the morphological appearance of lymphatic vessels in cervical tissue. One study observed that the lymphatics in normal cervical tissue appear open with regular shape whilst those in the peritumoral regions of carcinoma tissue appear large and dilated.[5] Similar findings are observed in two other studies.[6,7] To build on this observational data, the present study aims to utilise quantitative data obtained from image analysis to describe the number, size and shape of lymphatic vessels in the uterine cervix via measurements of LVD, vessel area and circularity. Lymphangiogenisis is thought to occur via the sprouting of endothelial cells from existing lymphatic vessels.[8] If this is the case, a subset of smaller lymphatic vessels may be visible in tissue from carcinoma specimens. The structural arrangement of these newly formed vessels in 3D space will influence how functional they are as compared to those found in normal cervix. This study will address these issues.

Ethical approval was granted for the undertaking of this project by the National Research Ethics Service, North West 11 Research Ethics Committee. (26 February 2010. REC reference number 10/H1016/16.)

MATERIALS AND METHODS

In total, 41 cases that underwent cervical cone biopsy, at least 20 years ago, were taken from the archive of paraffin-embedded tissue stored within the histopathology department of Royal Lancaster Infirmary: cervical intraepithelial neoplasia (CIN) grades 1-3 (n=21) and squamous cell carcinoma (n=20). The control group (n=21) consisted of 20-year-old hysterectomy specimens taken in cases of menorrhagia. A single section was taken from each block and stained with haematoxylin and eosin. The cases were split between six consultant histopathologists and one block from each case was chosen. This block was regarded as the best representation of the diagnosis for each selected case.

A 4µm section was taken from each selected block and placed onto a charged slide (Super Frost Plus, Thermo Scientific). D2-40 antibody workup was conducted to optimise the immunohistochemical staining protocol. Immunohistochemistry was carried on a Ventana Benchwork XT immunostainer (Ventana Medical Systems Inc, Tucson, Arizona). In short, sections were deparaffinised and re-hydrated in EZ Prep (Ventana Medical Systems Inc, Tucson, Arizona). Antigen retrieval was performed using CC1 buffer (Ventana Medical Systems Inc, Tucson, Arizona) heated to 95°C for 30 minutes. The primary monoclonal antibody D2-40 (DAKO Corporation, Carpinteria, California) was applied to the sections for 32 minutes at 37°C at a dilution of 1/100. D2-40 binding was visualised using IView Detection Kit (Ventana Medical Systems Inc, Tucson, Arizona). Slides were counter stained in Haematoxylin II (Ventana Medical Systems Inc, Tucson, Arizona) for 12 minutes, then dehydrated through graded alcohols, cleared in xylene and mounted in DPX (Surgipath, Peterborough, UK).

Sections immunohistochemically stained with the monoclonal antibody D2-40 were analysed using the Coolscope (Nikon UK Ltd, Kingston-upon-Thames, Surrey). Image analysis was performed using NIS Elements software version BR 2.3 (Nikon UK Ltd, Kingston-upon-Thames, Surrey). Images were saved in jpeg 2000 format.

LVD was assessed as described by the authors of a 1991 study,[15] with modifications:

- sections were scanned at low power (x4). Areas of tissue with the greatest density of lymphatic vessels 'hotspots' were selected
- positively stained structures were counted as lymphatic vessels if they resembled lymphatic endothelial cells regardless of whether they had a visible lumen
- for normal ectocervix, only 'hotspots' lying within 2mm of epithelial basement membrane were selected
- for invasive squamous carcinoma and CIN groups, 'hotspots' lying within 2mm of a tumour nest or pre-neoplastic lesion were used

- in control cervix, 'hotspots' located in the region 2mm from the last glandular structure underneath squamous epithelium to the new squamoucolumnar junction were considered to be lying within the transformation zone
- 'hotspots' were examined at higher magnification (x20), this equated to a total visual field area of 137374µm2. Total count of lymphatic vessels seen within this area equated to field LVD. Up to ten fields were counted within each specimen and the mean LVD calculated. In normal uterine cervix samples, up to ten fields were counted for both ectocervix and transformation zone

All results were recorded in Microsoft XL 2003 (Redmond, Washington, USA).

Statistical methods

As LVD is a count, it was modelled as Poisson distributed. Data was analysed using a hierarchical mixed-effects model. Model fitting was carried out using the functions lme and glmmPQL, respectively found in the nlme and MASS libraries in R version 2.12.0 (R Development Core Team 2010).

RESULTS

In total, 62 specimens were analysed within three experimental groups. The control group (n=21) consisted of 20-year-old hysterectomy specimens taken in cases of menorrhagia. From this group, two distinct areas were examined: ectocervix, and/or transformation zone. Due to limitations of the specimens, some specimens did not yield both regions. So out of the 21 specimens, 20 of them provided information of the ectocervix and 16 of them provided information of the transformation zone. The two other groups were cervical CIN (n=21) and invasive carcinoma (n=20). On average, 8.8 fields (range 1 to 19) were counted for each specimen. These fields contained an average of 8.2 vessels (range 1 to 45 vessels). This data is summarised in table 1.

The statistical model also tested whether the within-field standard deviation of the circularity variable was different in the different experimental groups. The within-field standard deviation in the control ectocervix group was only 85% (95% confidence interval (CI) 78% to 93%) of that in the invasive carcinoma group. For the control transformation zone group, this figure was 98% (95% CI 90% to 105%) and for the CIN group it was 91% (95% CI 85% to 97%). These results provide some evidence that lymphatic vessels in squamous carcinomas of the uterine cervix are aligned less regularly in 3D space than those found in control ectocervix or CIN specimens.

Immunohistochemical staining using the antibody D2-40 showed distinct staining of lymphatic endothelial cells. These appear as thin-walled circular structures with clear endothelial nuclei protruding into the lumen. Blood vessels with typical morphology and containing erythrocytes were not demonstrated with D2-40 in any section used in this study. Therefore, the distinction between lymphatic and blood vessels was clearly observable (see figure 1). The staining reaction was not entirely specific to lymphatic endothelium. Positive staining was seen in the basal cells of some stratified squamous epithelium of the uterine ectocervix (see figure 2). D2-40 positive tumour cells were found in 12 of the invasive squamous carcinoma group (see figure 3). In these cases, the D2-40 staining was localised to the periphery of the tumour nests.

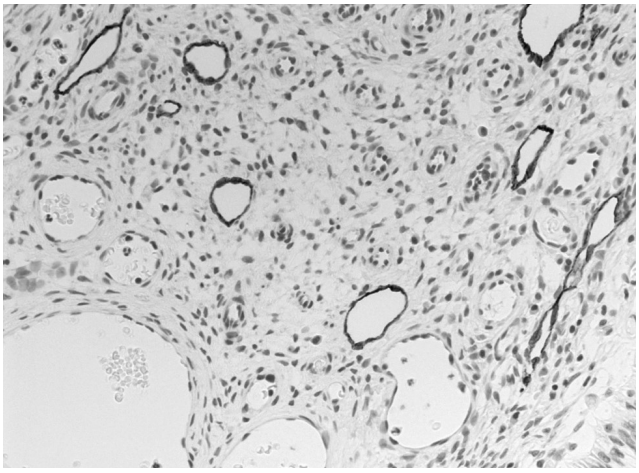

Figure 1 D2-40 staining lymphatic vessels. Blood vessels appear negative (x20)

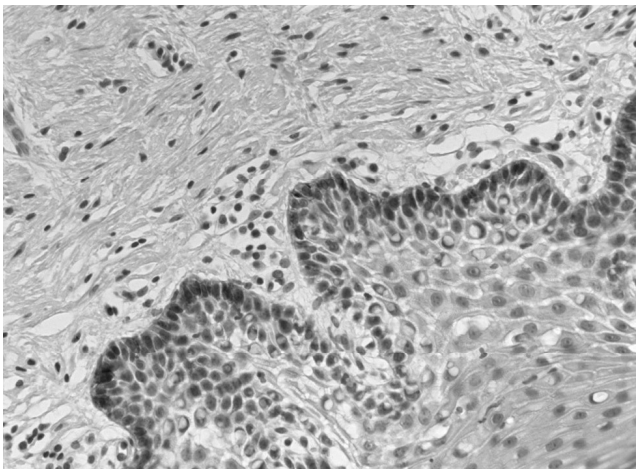

Figure 2 D2-40 staining in basal cells of stratified basal epithelium (x20)

	Number of specimens	Average number of fields per specimen (range)	LVD per 'hotspot' (SD)	Vessel area (SD)	Circularity (SD)
Normal cervix	21	12.7 (5 to 19)	3.38 (2.17)	1633 (2555)	0.57 (0.21)
Ectocervix	20	9.2 (5 to 10)	2.34 (0.90)	2062 (2764)	0.54 (0.21)
Transformation zone	16	5.2 (2 to 9)	5.67 (2.55)	1248 (2285)	0.60 (0.21)
Cervical CIN	21	5.6 (2 to 8)	5.53 (1.75)	912 (1489)	0.61 (0.19)
Invasive carcinoma	20	8.0 (1 to 10)	9.04 (4.55)	523 (934)	0.56 (0.22)

Table 1 Summary statistics for this study

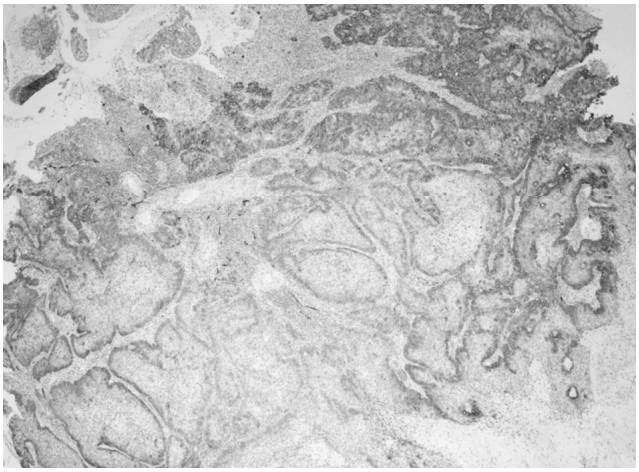

Figure 3 D2-40 staining in periphery of tumour nests (x4)

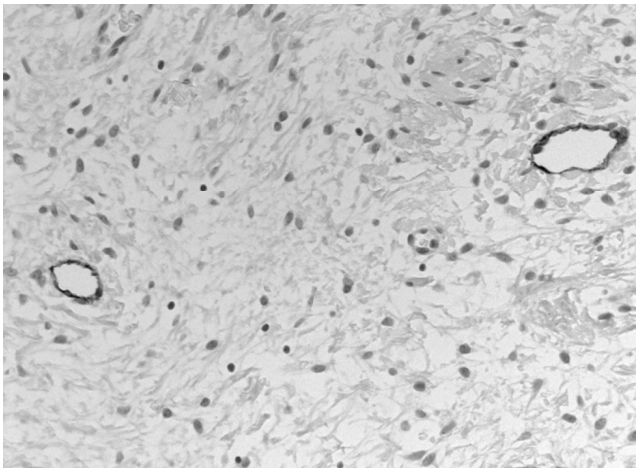

Figure 4 D2-40 staining lymphatic vessels in control ectocervical stroma (x20)

Lymphatic vessels observed in control stroma appeared open and of regular shape (see figure 4). A range of morphologically different vessels were observed in the stroma of the squamous carcinoma experimental group. Large, dilated, and irregularly shaped lumen were observed along with small condensed loci of positivity with no discernible lumen (see figures 5 and 6).

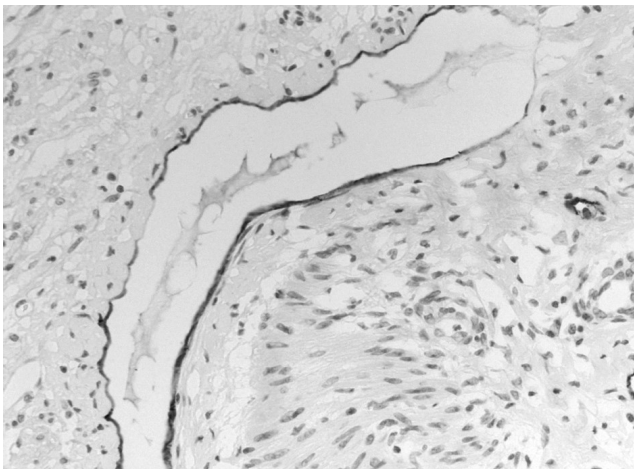

Figure 5 D2-40 staining in cervical carcinoma. Demonstrating large, irregularly shaped lymphatic vessel (x20)

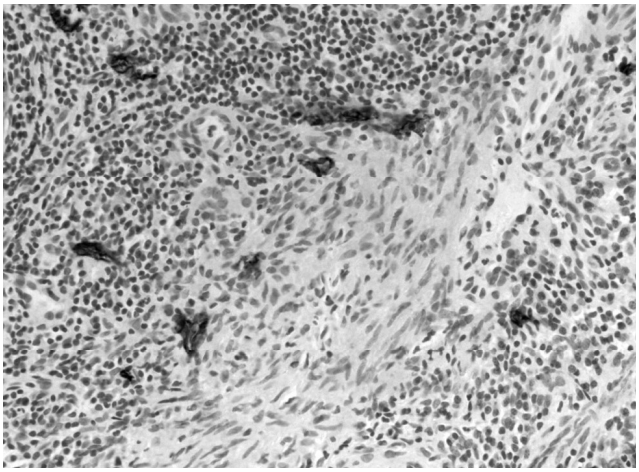

Figure 6 D2-40 staining in cervical carcinoma. Demonstrating small foci of positivity with little or no lumen (x20)

Lymphatic invasion by tumour emboli was clearly demonstrated in 45% of squamous carcinoma cases.

The measure of circularity could potentially lead to some ambiguity. Figure 7 demonstrates two vessels with a circularity of 0.49. One vessel is of irregular shape whilst the other is cut in a more longitudinal plane.

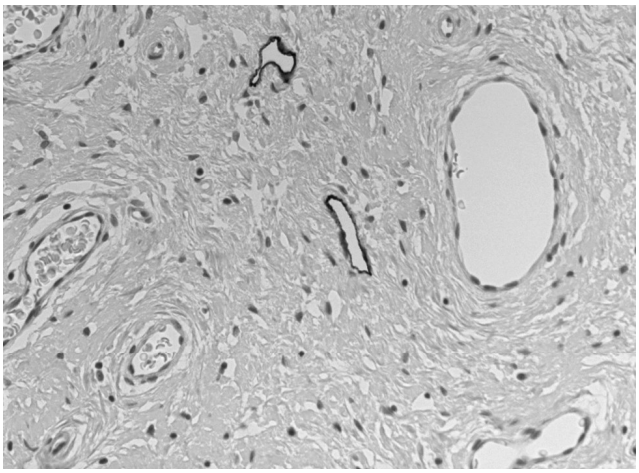

Figure 7 Two lymphatic vessels stained with D2-40. One has irregular shaped lumen whilst the other is cut in a more longitudinal plain. Both have a circularity of 0.49 (x20)

DISCUSSION

This study has demonstrated differences in the properties of the lymphatic vasculature within different regions of the normal cervix and according to the presence of CIN or invasive carcinoma. The LVD was 3.72 times higher in tissue from the invasive carcinoma group than for the control ectocervix group. This is in keeping with the findings of previous studies and provides further evidence that a lymphangiogenic effect is exerted over the adjacent cervical stroma by squamous cell carcinomas.[5,7,9,10] Molecular pathways to explain this behaviour have been reported. Traditionally, tumours have been regarded as anaplastic aggregates of neoplastic cells that lack tissue-like organisation.[16] Recent evidence suggests that at the very least the interior and periphery of tumours differ in their molecular expression. After looking at the expression of VEGF-C and

VEGF-D (both associated with lymphangiogenesis)[5] in carcinomas of the uterine cervix, a 2007 study concluded that tumour cells at the invasive front have biological properties that distinguish them from those in the central portions of the tumour, and that these may play a significant role in tumour progression.[11] Our study in part helps corroborate this statement. In 12 specimens of the squamous carcinoma of the uterine cervix used within this study, D2-40 was found to stain the periphery of the tumour nests but not the interior (see figure 3). This would seem to indicate different molecular expression between the periphery and interior of the tumour mass.

The area of the lymphatic vessels within the stoma of carcinoma tissue (see figures 4 and 6) was found to be smaller than those of control tissue. Within the literature, two models of lymphangiogenesis have been described. One model sees lymphangiogenesis occurring from the sprouting of pre-existing vessels,[12] the second model proposes that new vessels are formed by the convergence of lymphatic endothelial cells that have slewed off from pre-existing lymphatic vessels and migrated through the stroma.[13] Regardless of which model of lymphangiogenesis actually occurs during lymphangiogenesis in the uterine cervix due to the presence of squamous carcinoma, one would expect these newly formed vessels to be smaller than existing vessels.

We examined the circularity of lymphatic vessels to try to gain evidence regarding the morphological and organisation of vessels undergoing lymphangiogenesis. This is a difficult measurement to interpret. There have been some observational reports within the literature that lymphatic vessels in squamous cell carcinoma of the uterine cervix are of a more irregular shape than those vessels found in normal cervical tissues. Based upon these observations it may be expected that the measure of lymphatic circularity might decrease in these specimens. The problem with this measurement is that it is a product of two parameters. The first is the regularity of the lumen shape. The second is the plane in which the vessel is sectioned. If a vessel is sectioned longitudinally, it will have a low measure of circularity regardless of the regularity of its lumen (see figure 7). To offset this difficulty in interpretation, we calculated the standard deviation of circularity between the vessels within each 'hotspot' field; the premise being that if the lymphatic system is less organised, as may be the case in lymphangiogenesis due to a neoplastic process, the standard deviation of circularity would be greater. There is some evidence that this standard deviation is higher in invasive carcinoma specimens than in CIN or control specimens, suggesting that lymphatic vessels in squamous carcinomas of the uterine cervix are aligned less regularly in 3D space than those found in control or CIN specimens. The statistical methods used to obtain this result are discussed separately in a forthcoming publication [confirmation pending].

The 2007 study measured the LVD in CIN lesions and found that they were raised when compared to control ectocervix.[10] They concluded that this raised LVD was due to a lymphangiogenesis occurring in the progression of CIN lesions. We also found that the LVD count was raised in CIN lesions when compared to the LVD measured in control ectocervix, 5.53 and 2.34 respectively. This would then seem to support these previous findings. However, when the LVD from the transformation zone and CIN lesions are compared, a different picture presents itself. Our study found the LVD of the transformation zone and CIN lesions to be very similar: 5.67 and 5.53 respectively. As the majority of CIN lesions occur in the transformation zone this would suggest that contrary to the conclusions of the 2007 study,[10] the increase of LVD in CIN lesions is not due to lymphangiogenesis occurring in pre-neoplastic disease, but is connected to an earlier event shared by the transformation zone, possibly during puberty and the formation of the transformation zone.

During the process of cervical eversion, the epithelium of the lateral endocervix and underlying tissues are pushed outward to lie in the region formally occupied by the ectocervix. If the LVD of the endocervix is higher than the ectocervix as this tissue is everted to form the transformation zone, the LVD will appear raised in relation to the normal ectocervix.

Alternatively, the increased LVD measured in the transformation zone could be attributed to lymphangiogenesis occurring during cervical eversion and the subsequent squamous metaplastic process. The acidic environment of the vagina can cause tissue damage in the everted endocervical epithelium and underlying stroma due to the relatively sparse protection offered by a single layer of columnar epithelium. The response to this tissue damage is an inflammatory reaction. The lymphangiogenic ligands VEGF-C and VEGF-D can be released by inflammatory cells and may account for the raised LVD in the transformation zone and similar LVD in the CIN specimens.

The 'hotspot' methodology, as it has been applied in this and previous studies, only examines the local effects of lymphangiogenesis, those immediately adjacent to cervical lesions. This produces a very limited view, may miss many morphological features of lymphangiogenesis that may be of importance and give misleading interpretations of the data. The methodology dictates that the researcher looks for 'hotspots' fields demonstrating a high number of lymphatic vessels. By definition, this will then exclude fields containing fewer larger vessels as observed by a 2009 study.[7] We also observed these dilated lymphatic vessels (see figure 5). If such fields were included in the data, then it would be reasonable to conclude that the LVD would reduce and the mean vessel area would increase. It could, therefore, be argued that although the 'hotspot' methodology may be useful in detecting the presence of lymphangiogenesis, it will not be the most appropriate method to gain a more holistic view of the effect of lymphangiogenesis and its progenitors on the morphology of the lymphatic system in its entirety. 3D reconstruction techniques, the measurement of random fields or measurement of total lymphatic density within a section as used by the authors of a 2010 study,[14] may be more appropriate.

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