Identification of novel vascular targets in lung cancer

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Background: Lung cancer remains the leading cause of cancer-related death, largely owing to the lack of effective treatments. A tumour vascular targeting strategy presents an attractive alternative; however, the molecular signature of the vasculature in lung cancer is poorly explored. This work aimed to identify novel tumour vascular targets in lung cancer.

Methods: Enzymatic digestion of fresh tissue followed by endothelial capture with Ulex lectin-coated magnetic beads was used to isolate the endothelium from fresh tumour specimens of lung cancer patients. Endothelial isolates from the healthy and tumour lung tissue were subjected to whole human genome expression profiling using microarray technology.

Results: Bioinformatics analysis identified tumour endothelial expression of angiogenic factors, matrix metalloproteases and cell-surface transmembrane proteins. Predicted novel tumour vascular targets were verified by RNA-seq, quantitative real-time PCR analysis and immunohistochemistry. Further detailed expression profiling of STEAP1 on 82 lung cancer patients confirmed STEAP1 as a novel target in the tumour vasculature. Functional analysis of STEAP1 using siRNA silencing implicates a role in endothelial cell migration and tube formation.

Conclusions: The identification of cell-surface tumour endothelial markers in lung is of interest in therapeutic antibody and vaccine development.
Minced tissue was digested in DMEM containing 2 mg ml⁻¹ of DNAse I (Qiagen, Hilden, Germany). The tissue was digested with collagenase type V (Sigma, Gillingham, UK), 7.4 mg ml⁻¹ of DNAse I (Qiagen, Hilden, Germany), and 50 U ml⁻¹ of actinomycin (Sigma) at 37°C. Endothelial cells were isolated from the digested cell suspension by positive selection using Ulex europaeus lectin-coated magnetic beads (Invitrogen, Paisley, UK). All images were acquired using a Leica DM IL microscope (Leica, Milton Keynes, UK) and USB 2.0 2M Xli camera (XL Imaging LLC, Carrolls, TX, USA).

MATERIALS AND METHODS

**Ulex-bead isolation.** Healthy and tumour lung tissue was obtained immediately following surgery with full patient consent and ethics approval (Heartlands Hospital, REC, 07/MRE08/42). Minced tissue was digested in DMEM containing 2 mg ml⁻¹ of collagenase type V (Sigma, Gillingham, UK), 7.4 mg ml⁻¹ of actinomycin (Sigma) and 30 kU ml⁻¹ of DNase I (Qiagen, Crawley, UK) at 37°C. Endothelial cells were isolated from the digested cell suspension by positive selection using *Ulex europaeus* lectin-coated magnetic beads (Invitrogen, Paisley, UK).

**Microarray.** cRNA extracted from Ulex-bead isolated samples was converted to cRNA, then subjected to amplification and labelling. Labelled cRNA samples were then hybridised to an Agilent 4 x 44k whole human gene expression microarray (Agilent, Wokingham, UK). The Bioconductor packages preprocessor Core and Limma were used to subtract background and quantile-normalise probe signal intensities prior to performing differential gene expression analyses. Principle component analysis (PCA) was performed in R.

**RNA-seq.** Seventy-nine and 84 million paired end reads (50 bp + 35 bp) were sequenced on the SOLiD4 2nd generation sequencer (Applied Biosystems, Foster City, CA, USA) for endothelium from fresh tumour and healthy tissue, respectively. Reads were mapped to the Human genome (University California Santa Cruz, version hg19) with Tophat 1.3.3 (Trapnell et al., 2009). Default parameters for colour space mapping were used with the exception of the following: 1 g⁻¹-max-multihits was set to 1 to identify the single best mapped read; 2 library-type was set to fr-secondstrand to reflect the sequencing library preparation; 3 G provided Tophat with a model set of gene annotation genome positions from the Refseq hg19 transcriptome. The Tophat output bam files were sorted using samtools (Version: 0.1.8). And (Li et al., 2009)), and ‘HTSeq-count’ version 0.4.7p4 (Anders, 2010) was used, in conjunction with the Human transcriptome GTF Refseq version 19, to assign gene counts and produce a tab delimited file of transcript/gene counts. Differential gene expression analysis and P-value generation on the count data was carried out using the R Bioconductor package DESeq v1.5 (Anders and Huber, 2010).

Quantitative real-time PCR. RNA extraction, complementary DNA preparation and quantitative real-time PCR (qPCR) were performed using LightCycler real-time quantitative PCR (Roche, Burgess Hill, UK) by following previously described methods (Armstrong et al., 2008). The Primer sequences are provided in Supplementary Table 1. The double delta Ct method was used to compare the expression levels in tumour relative to healthy endothelial isolates.

**Immunohistochemistry.** Immunohistochemistry of placental tissue or lung tumour sections were immunostained with antisera to the targets (all antisera from Abcam, Cambridge, UK). The sections were then visualised using ImmPRESS universal antibody kit and NovaRed chromagen (Vector labs, Burlingame, CA, USA). Finally, the sections were counterstained with hematoxylin, dehydrated and mounted in diystyrene-plasticizer–xylene resin.

**Functional assay with siRNA knockdown.** Transfection with siRNA and functional assays were performed as previously described (Armstrong et al., 2008). STEAP1 siRNA duplexes were:

- D1: sense: 5'-CUAUUUAUCAGAGCGAUAUATT-3'; anti-sense: 5'-UAGCUUGCUAGCUUUAGTG-3';
- D2: sense: 5'-GAAUAUGUGGAAGUAUUAATT-3'; anti-sense: 5'-UAUUCAUGCUUACUUUCCAC-3' (Ambion, Chipping Norton, UK).

The open area of the wound was quantified using a cell intelligence quotient analyzer or Image J software (Image J website, rsweb.nih.gov). The effect of STEAP1 knockdown on Matrigel assays was analysed by Angiogenesis Analyzer for ImageJ. All images were acquired using a Leica DM IL microscope (Leica, Milton Keynes, UK) and USB 2.0 2M Xli camera (XL Imaging LLC, Carrollton, TX, USA).

**RESULTS**

Isolation of lung endothelium from fresh tissue. Previous studies have shown that a high purity of endothelial isolates can be achieved using *Ulex*-conjugated beads (Jackson et al., 1990) but has not yet applied to human lung tissue. *Ulex* agglutinin I is a lectin that specifically binds to L-fucose residues present in glycoproteins on the human endothelial surface (Holthofer et al., 1982). Here we examine this approach for the isolation of endothelium from fresh lung specimens. Fresh healthy or tumour lung tissue samples (1–3 g) were processed within 3 h post surgery. The tumour tissue was dissected from the viable region of the
tumour core and the patient-matched healthy tissue was resected >10 cm away from the tumour core. Endothelial cells were positively isolated using magnetic beads coupled to Ulex lectin (workflow illustrated in Figure 1A). To verify endothelial enrichment, expression of the universal endothelial marker CD31 was examined by qPCR in the endothelial isolates and compared with that in the bulk tissue. A 15-fold enrichment of endothelium was achieved in the bead isolated samples when compared with whole tumour extracts. Afour-fold enrichment was seen in endothelial cells isolated from healthy lung (Figure 1B). The differing fold increase in CD31 seen in healthy and tumour samples is likely owing to the proportion of endothelial cells being higher in healthy lung (30%) than in tumours (3%–5%). RNA integrity analysis of a typical RNA isolate is shown in Figure 1C. The data confirm that the Ulex-bead isolation approach can effectively isolate the endothelial population from lung.

Microarray of endothelial isolates from lung cancer patients. For expression profiling, a microarray analysis was performed on four pairs of NSCLC patient-matched healthy and tumour lung endothelial isolates. Clinical and pathologic data was obtained from Birmingham Heartlands Hospital (Table 1, patients 1–4). A PCA plot shows variation in both tumour and healthy lung samples and between the samples of each group. This was to be expected as samples were collected and extracted from different patients and statistically significant genes are those that are consistent across replicate samples. Despite this the tumour and healthy isolates fall into two discrete groups (Figure 2).

To better understand the role of known angiogenesis-associated genes in NSCLC, a differential expression analysis was performed using the programme Limma. The analysis revealed a panel of known angiogenesis-associated genes including COL1A1, VEGF-A, TEM7, TNC, EPHB2, IL8, FGF1, ANGPTL2 and TEM8 to be elevated in lung tumour endothelium (Table 2). As tumour angiogenesis proceeds by proteolysis of the extracellular matrix (Sottile, 2004), elevated matrix metalloproteinase (MMP) activity is associated with active angiogenesis and tumour progression. The analysis also identified a number of MMPs that are upregulated in lung tumour compared with healthy lung endothelium (Table 3).

Identification and validation of putative tumour vascular targets in NSCLC. For target identification, differentially expressed genes from the microarray data were filtered through several selection criteria: Log2 fold change magnitude > 1, a P-value < 0.5 and containing a transmembrane or signal peptide domain, which generated a list comprised of 584 genes. Twelve target candidates were chosen for further validation based on additional criteria including the level of association with endothelial cells, previously published work, sites of expression and relation to known genes with interesting functional properties (Table 4). To validate putative targets, qPCR was performed on the four pairs of endothelial isolates used in the microarray. Figure 3 shows that all candidates had elevated expression in tumour compared with that in healthy endothelium ranging from a 3-to 35-fold increase in expression.

Table 1. Clinical-pathological data of lung cancer patients used in the genomic analysis

| ID | Age | Gender | Pack years smoked | Histology of tumour | Tumour stage | Application |
|----|-----|--------|-------------------|---------------------|--------------|-------------|
| 1  | 65  | M      | 13                | Squamous           | T1N0M0       | Microarray  |
| 2  | 71  | M      | 40                | Squamous           | T1N0M0       | Microarray  |
| 3  | 63  | F      | 17                | Squamous           | T1N1M0       | Microarray  |
| 4  | 67  | M      | 50                | Squamous           | T3N0M0       | Microarray  |
| 5  | 73  | F      | 25                | Adeno              | T2N0M0       | RNA-Seq    |
| 6  | 83  | M      | 35                | Adeno              | T2N1M0       | RNA-Seq    |
| 7  | 52  | M      | 25                | Adeno              | T2N1M0       | RNA-Seq    |

Figure 1. A Ulex-coated magnetic bead isolation achieved substantial endothelial enrichment and gave good quality RNA. (A) The workflow of the main steps in Ulex-bead isolation of endothelial cells from healthy/tumor lung tissue. (B) Confirmation of endothelial enrichment using the Ulex-beads approach. Real-time PCR using a primer set for the endothelial marker CD31 was performed on the bead isolated endothelial cells and bulk tissue. Expression of CD31 in the bead isolated sample was normalised to that in the bulk tissue (n = 3). (C) Good quality RNA (RIN > 7) was obtained from Ulex-bead isolated endothelial cells from healthy and tumour lung tissue. ECs = Endothelial cells.

Figure 2. Principle component analysis plot of microarray of the four pairs of healthy and tumour lung endothelial isolates. A PCA plot showing that the endothelial transcriptomes of healthy and tumour lung show a clear difference. The separation between healthy and tumour lung endothelium was highlighted by dotted lines.

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Expression profiling of lung endothelial isolates by RNA-seq. RNA-seq using deep sequencing technology provides an in-depth resolution of RNA snapshots by generating millions of reads that can be assembled and mapped to a known transcriptome, allowing the measurement of differential gene expression. RNA-seq has the advantage of querying novel transcripts and does not rely on prior knowledge and annotation. Here we used RNA-seq to verify the genes that had been identified through the microarray analysis. We note that a lower yield of RNA was obtained from healthy lung tissue compared with that from tumour. This was possibly owing to the endothelial cells in healthy lung tissue being in a quiescent state compared with the active endothelium in tumours. For this reason, endothelial RNA isolated from three healthy lungs samples (pooled; Table 1, patients 5–7) and one tumour lung tissue (Table 1, patient 6) were sequenced as one healthy and one tumour sample on a SOLiD4 sequencer.

### Table 2. Upregulated angiogenesis-associated genes in lung cancer

| Gene ID | Gene Symbol | GenBank accession no. | LogFC | P-value |
|---------|-------------|-----------------------|-------|---------|
| Collagen, type I, alpha 1 | COL1A1 | NM_000088 | 5.11 | 0.00 |
| Vascular endothelial growth factor A | VEGF-A | NM_001025366 | 2.59 | 0.00 |
| Plexin domain containing 1 | PLXDC1(TEM7) | NM_020405 | 2.04 | 0.01 |
| Tenascin C | TNC | NM_002160 | 1.95 | 0.01 |
| Eph receptor B2 | EPHB2 | NM_004442 | 1.76 | 0.00 |
| Interleukin 8 | IL8 | ENST00000401931 | 1.36 | 0.16 |
| Fibroblast growth factor 1 (Acidic) | FGF1 | NM_000800 | 0.82 | 0.08 |
| Angiopoietin-like 2 | ANGPTL2 | NM_012098 | 0.69 | 0.40 |
| Anthrax toxin receptor 1 | ANTXR1(TEM8) | NM_032208 | 0.65 | 0.15 |

**Abbreviation:** LogFC = log2 fold change; TEM = tumour endothelial marker. Differential expression analysis of microarray data for the identification of elevated angiogenesis-associated genes. Listed genes were ranked by LogFC in descending order.

### Table 3. Upregulated matrix metallopeptidases in lung cancer

| Gene ID | Gene Symbol | GenBank accession no. | LogFC | P-value |
|---------|-------------|-----------------------|-------|---------|
| Matrix metallopeptidase 11 | MMP11 | NM_005940 | 4.10 | 0.00 |
| Matrix metallopeptidase 9 | MMP9 | NM_004994 | 4.00 | 0.00 |
| Matrix metallopeptidase 12 | MMP12 | NM_002426 | 3.80 | 0.00 |
| Matrix metallopeptidase 7 | MMP7 | NM_002423 | 3.29 | 0.17 |
| Matrix metallopeptidase 1 | MMP1 | NM_002421 | 2.49 | 0.05 |
| Matrix metallopeptidase 3 | MMP3 | NM_002422 | 1.81 | 0.02 |
| Matrix metallopeptidase 10 | MMP10 | NM_002425 | 1.74 | 0.12 |
| Matrix metallopeptidase 14 | MMP14 | NM_004995 | 1.55 | 0.00 |
| Matrix metallopeptidase 13 | MMP13 | NM_002427 | 1.21 | 0.04 |
| Matrix metallopeptidase 2 | MMP2 | NM_004530 | 1.03 | 0.12 |

**Abbreviations:** Log FC = log fold change; MMP = matrix metalloprotease. Differential expression analysis of microarray data for the identification of elevated matrix metallopeptidases. Listed genes were ranked by logFC in descending order.

### Table 4. Putative vascular targets in lung cancer

| Gene ID | Gene Symbol | GenBank accession no. | LogFC | P-value | TM |
|---------|-------------|-----------------------|-------|---------|----|
| Six transmembrane epithelial antigen of the prostate 1 | STEAP1 | NM_012449 | 4.19 | 0.00 | 6 |
| Synaptotagmin Xi | SYT12 | NM_177963 | 4.16 | 0.00 | 1 |
| Gap junction protein, beta 2, 26kDa | GJB2 | NM_004004 | 4.13 | 0.00 | 4 |
| Solute Carrier organic anion transporter family, member 1B3 | SLCO1B3 | NM_019844 | 3.65 | 0.00 | 11 |
| Basal acinar cell repeat containing 5 | BIRC5 | NM_001012271 | 3.45 | 0.00 | 0 |
| Protocadherin 7 | PCDH7 | NM_002589 | 2.29 | 0.00 | 1 |
| Prominin 2 | PROM2 | NM_001165978 | 2.05 | 0.00 | 6 |
| Plexin Domain Containing 1 | PLXDC1(TEM7) | NM_020405 | 2.04 | 0.01 | 1 |
| Bmp and activin membrane-bound inhibitor homologue | BAMBI | NM_012342 | 1.99 | 0.00 | 1 |
| Lemur tyrosine kinase 3 | LMTK3 | NM_001080434 | 1.94 | 0.07 | 2 |
| Trophoblast glycoprotein | TPBG | NM_006670 | 1.48 | 0.03 | 1 |
| C-Ros oncogene 1, receptor tyrosine kinase | ROS1 | ENST00000403284 | 1.39 | 0.22 | 1 |

**Abbreviations:** Log FC, log fold change; TEM, tumour endothelial marker. Differential expression analysis of microarray data for the identification of putative vascular targets for lung cancer. Listed genes were ranked by logFC in descending order.
expression analysis of the RNA-seq data was performed using the DESeq v1.5 package (Anders and Huber, 2010). The analysis confirmed most of the unregulated angiogenesis-associated genes, MMPs and putative targets identified through the microarray analysis (Figure 4). Analysis of the RNA-seq data alone generated a list of 477 genes with the same criterion used in the microarray analysis for target identification. The intersection of the microarray and RNA-seq gene pools comprises a list of 122 genes, which provides a rich source for target identification (Supplementary Table 2). The discrepancy between the two analyses is likely owing to the cancer type (squamous vs. adenoc). We next used siRNA knockdown to seek a function of STEAP1 in the endothelial cells.

Expression of TEM candidates in angiogenic tissue and lung cancer. To further validate the candidate targets, we investigated protein expression by immunohistochemistry. We have previously shown that placental vasculature is a rich source of endothelial gene expression. Thus, immunohistochemical staining was performed first on the human placental tissue using antibodies to the lung TEM targets. Amongst the twelve targets, six genes: ROS1, PCDH7, BIRC5, STEAP1, GJB2 and PROM2 showed expression in human placental vessels (Supplementary Figure 1). The tumour and healthy lung tissue was then immunostained and these six candidates are indeed overexpressed in the lung tumour vessels, whereas absent or at a low level in the healthy lung tissue (Figure 5). Some of the targets were not restricted to the tumour endothelium; for example, ROS1 and STEAP1 also showed positive expression on some tumour cells or macrophages and this may be beneficial for developing drugs targeting the tumour and its vasculature simultaneously. It is also worth mentioning that other target candidates should not be completely eliminated for further investigation simply owing to the lack of antibody reactivity in immunohistochemistry.

Expression of STEAP1 in lung cancer. We then focused on the top-ranked target STEAP1. To confirm whether STEAP1 is differentially expressed in the endothelium within the healthy and tumour lung tissue, an expression profiling was carried out on human lung cancer tissues by immunohistochemistry. Eighty-two patients were examined (Table 5). The intensity of the signal was classified as absent, low, medium or high. Representative images of STEAP1 staining in lung cancer are shown in Figure 6A. From the 82 cases examined, a clear overexpression of STEAP1 in tumour vessels was observed; for example 45% of the vessels highly expressed STEAP1 in lung cancer vs only 5% in matching healthy lung. The proportion of vessels that are ‘low’ and ‘no expression’ of STEAP1 was 77% in healthy lung, but only 14% in lung tumours (Figure 6B). These data confirm that STEAP1 is differentially expressed between the tumour and healthy lung vasculature and presents a possible vascular target for lung cancers.

Function of STEAP1 in endothelial cells. We next used siRNA knockdown to seek a function of STEAP1 in the endothelial cells.

Figure 3. Validation of putative lung vascular targets by qPCR. Quantitative real-time PCR validation of tumour vascular target candidates in the endothelial cells isolated from the healthy and tumour lung tissue. Flotillin 2 was used as the house keeping gene to which the data was normalised. The double delta Ct method was used to compare the expression levels in tumour relative to healthy endothelial isolates.

Figure 4. Confirmation of upregulated angiogenesis-associated genes, MMP and putative vascular targets in lung cancer by RNA-seq. Differential gene expression analysis of RNA-seq data confirmed a panel of elevated angiogenesis-associated genes, MMP genes and lung cancer vascular target candidates identified through microarray analysis.
STEAP1 protein expression was efficiently knocked down by two independent siRNA duplexes (Figure 7A). Migration of HUVEC after STEAP1 knockdown was compared with that of mock and negative siRNA-transfected controls in a scratch-wound assay. At 24 h, control wounds showed 60% closure, whereas in STEAP1 knockdown cells the wound had only closed by 35%–40% (Figure 7B and C). STEAP1 knockdown also compromised tube formation on Matrigel. Tubes showed a significant decrease in mesh size compared with controls (Figure 7D and E).

## DISCUSSION

The ineffectiveness of current treatments for NSCLC prompted the search for alternatives. Although TKIs of EGFR initially showed promising outcomes in several trials, resistance developed in all the patients (Lovly and Carbone, 2011). Antiangiogenic/anti-tumour vascular therapy remains a viable alternative (Vasudev and Reynolds, 2014). VEGF (receptor) blockers have been used to treat NSCLC patients in early clinical trials; however, concerns have arisen from the limited efficacy in achieving tumour regression and the tendency to develop resistance (Pallis and Syrigos, 2013). In addition, the lack of effective biomarkers for patient pre-treated selection, emphasises the need for novel targets and biomarkers. Our study describes the first molecular profiling of endothelium from NSCLC patient tissue.

A major obstacle that hinders the expression profiling of in vivo endothelium is the challenge to obtain pure endothelial isolates. Favre et al described attempts for mouse lung endothelial isolates, using the unpurified sample as the control, aimed at identifying endothelial specific genes (Favre et al, 2003). An effort to isolate mouse endothelium from a Lewis lung carcinoma tumour model was reported by Allport and Weissleder, (2003); however, the isolated endothelial cells were subjected to in vitro culture and only the characterised angiogenesis genes were investigated (Allport and Weissleder, 2003). In this study, we demonstrate that the Ulex-bead isolation approach has proven to be an effective approach to obtain a pure endothelial population from the human lung. The purity of the endothelial isolates allows for the first time to precisely document the transcriptome of the lung vasculature using microarray analysis and deep sequencing platforms.

Our microarray analysis of endothelial isolates from cancer patients identified a panel of angiogenesis-associated genes and MMPs elevated in tumour endothelium, which have potential to be biomarkers for NSCLC. The elevated MMP2 expression is in agreement with a reported increased MMP2 level in serum that was shown to be a predictor of metastasis in NSCLC patients (Guo et al, 2007). MMP2 and MMP9 have also been reported to associate with tumour grade in various solid tumours (John and Tuszynski, 2001). The expression of MMP7 and MMP9 were previously found to be significantly upregulated in NSCLC compared with that of healthy lung and benign lung tumours (Safranek et al, 2009). Although clinical trials investigating inhibitors targeting multiple MMPs yielded limited efficacy (Heath et al, 2001; Goffin et al, 2005), possibilities remain to design drugs that are more specific to those highly expressed MMPs in NSCLC.

RNA-seq using deep sequencing technology has been intensively applied to molecular profiling of many tissue types and cell populations from different species (Guduric-Fuchs et al, 2012; Voellenkle et al, 2012). This work is the first description of RNA-seq for profiling the endothelium from human lung cancer. Further, cross referencing the microarray data identified 122 genes

| Patients (n) | % |
|-------------|---|
| Age | 63.8 ± 9.5 |
| Sex | |
| Male | 64 | 78% |
| Female | 18 | 22% |
| Histology | |
| Squamous cell carcinoma | 47 | 57.3% |
| Adenocarcinomas | 19 | 23.2% |
| Large cell carcinoma | 5 | 6.1% |
| Bronchioloalveolar carcinoma | 7 | 8.5% |
| Carcinosarcoma | 2 | 2.4% |
| Clinical stage | |
| I | 34 | 41.5% |
| II | 35 | 42.7% |
| III | 13 | 15.8% |

Table 5. Clinical characteristics of lung cancer patients for STEAP1 profiling

Figure 5. Validation of putative lung vascular targets by immunohistochemistry. Identified putative lung TEMs were validated by immunohistochemistry. Representative immunohistochemistry for lung vascular target candidates on the healthy and tumour lung tissue.

Figure 7. A, Efficiency of STEAP1 knockdown in HUVEC. 

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was detected in a subpopulation of NSCLC patients (Bergethon et al., 2012; Janne and Meyerson, 2012). Despite intensive study of ROS1 in lung cancer, there has been no previous report of its expression in the tumour endothelium. BIRC5 or survivin, belongs to a family of inhibitors of apoptosis. BIRC5 inhibits the caspase activation regulating apoptosis. Disruption of the BIRC5 signalling pathway leads to tumour cell apoptosis and growth delay. BIRC5 protein is often present in tumour cells and fetal tissues but has rarely been described in the healthy tissue (Sah et al., 1989). ROS1 belongs to a transmembrane protein that belongs to the connexin family. Connexins have a role in many physiological processes and embryonic development including the microvasculature. Defects in GJB2 lead to the most common form of congenital deafness (Apps et al., 2007). Thus, most GJB2 studies have centred on this pathology. Nevertheless, a recent study reported the expression of GJB2 in lymphatic endothelium in the mouse embryo, and that the deletion of GJB2 in mice disrupted the development of lymphatic vessels and was embryonic lethal (Dicke et al., 2011). PROM2 is a multi-pass membrane protein and belongs to the prominin family of pentaspan membrane glycoproteins. PROM2 is at present comparatively uncharacterised.

Low shear stress and turbulent flow are mechanical factors that regulate endothelial gene expression (Wasserman and Topper, 2004). Around 600 genes are regulated by shear stress in the endothelial cell (Ando and Yamamoto, 2009; Mura et al., 2012). Like the previously described TEMs CLEC14A and Robo4, STEAP1, PCDH7 and BIRC5 are all upregulated in the endothelium exposed to reduced shear stress (Bicknell et al., unpublished data). Reduced blood flow and shear stress may account for their expression on tumour vessels.

To conclude, our work not only enhances our knowledge of proteins that are differentially expressed on the lung tumour endothelium but has also identified several promising biomarkers/targets for future investigation. Cell-surface expression of some of

Figure 6. Expression profiling of STEAP1 expression in clinical lung cancer samples. (A) Representative images of STEAP1 in healthy (i–ii) and tumour (iii–vi) lung tissue; expression level classified as no expression (i), low (ii–iii), medium (iv) and high (v–vi). Images were acquired using an optical microscope at a magnification of × 20. (B) Expression profiling of STEAP1 in clinical samples by immunohistochemistry (n = 82).
these targets, for example STEAP1, will facilitate the pre-clinical validation with antibodies. Further work is needed to characterise their functions and their roles in the endothelial biology and angiogenesis in the lung.

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