Why some tumours trigger neovascularisation and others don’t: the story thus far

Omanma Adighibe¹, Russell D. Leek¹, Marta Fernandez-Mercado²,³, Jiangting Hu⁴, Cameron Snell¹, Kevin C. Gatter¹, Adrian L. Harris⁴ and Francesco Pezzella*¹

Abstract

Background: Angiogenesis is not essential for tumours to develop and expand, as cancer can also grow in a non-angiogenic fashion, but why this type of growth occurs is unknown. Surprisingly, our data from mRNA transcription profiling did not show any differences in the classical angiogenic pathways, but differences were observed in mitochondrial metabolic pathways, suggesting a key role for metabolic reprogramming. We then validated these results with mRNA profiling by investigating differential protein expression via immunohistochemistry in angiogenic and non-angiogenic non-small cell lung cancers (NSCLCs).

Methods: Immunohistochemical staining for 35 angiogenesis- and hypoxia-related biomarkers were performed on a collection of 194 angiogenic and 73 non-angiogenic NSCLCs arranged on tissue microarrays. Sequencing of P53 was performed with frozen tissue samples of NSCLCs.

Results: The non-angiogenic tumours were distinguished from the angiogenic ones by having higher levels of proteins associated with ephrin pathways, mitochondria, cell biogenesis, and hypoxia-inducible factor 1 (HIF1) regulation by oxygen and transcription of HIF-controlled genes but lower levels of proteins involved in the stroma, cell–cell signaling and adhesion, integrins, and Delta-Notch and epidermal growth factor (EGF)-related signaling. However, proteins classically associated with angiogenesis were present in both types of tumours at very comparable levels. Cytoplasmic expression of P53 was strongly associated with non-angiogenic tumours. A pilot investigation showed that P53 mutations were observed in 32.0% of angiogenic cases but in 71.4% of non-angiogenic tumours.

Conclusions: Our observations thus far indicate that both angiogenic and non-angiogenic tumours experience hypoxia/HIF and vascular endothelial growth factor (VEGF) pathway protein expression in a comparable fashion. However, angiogenesis does not ensue in the non-angiogenic tumours. Surprisingly, metabolic reprogramming seems to distinguish these two types of neoplastic growth. On the basis of these results, we raise the hypothesis that in some, but not in all cases, initial tissue remodeling and/or inflammation could be one of the secondary steps necessary to trigger angiogenesis. In the non-angiogenic tumours, in which neovascularisation fails to occur, HIF pathway activation could be the driving force toward metabolic reprogramming.

Keywords: Cancer, Angiogenesis, Hypoxia, Blood vessels

*Correspondence: francesco.pezzella@ndcls.ox.ac.uk
¹ Radcliffe Department of Medicine, Nuffield Division of Laboratory Science, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK
Full list of author information is available at the end of the article

© 2016 Adighibe et al. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background

In 1927, Otto Warburg described what would be called the “Warburg effect,” in which tumour cells exhibited characteristic changes in metabolism, particularly the use of glycolysis rather than oxidative phosphorylation, despite the presence of adequate amounts of oxygen [1, 2]. Warburg believed that this process was the actual cause of neoplastic transformation [3].

Tumour development is now known to be driven by genetic damage. However, mutations in some metabolic enzymes, such as succinate dehydrogenase (SDH) and fumarate hydratase (FH), both parts of the tricarboxylic acid (TCA) cycle, can drive neoplastic transformation, and intermediate products of metabolism can also promote neoplastic progression [4].

Proliferating cancer cells have a high energy requirement to maintain homeostatic cellular processes. The shift in energy production to aerobic glycolysis, while allowing for more rapid production of adenosine triphosphate (ATP), yields far less energy than oxidative phosphorylation: there are two net molecules of ATP per glucose molecule in glycolysis versus 36 molecules of ATP via oxidative phosphorylation [5].

The reasons for the glycolytic energy dependence of proliferating tumour cells are still being debated. Initially, it was believed that the mitochondria in tumours were intrinsically defective. However, it was determined that tumour mitochondria are actually functional, retaining the capacity for oxidative phosphorylation and consuming oxygen at similar rates to normal tissues [6], although it should be appreciated that a degree of variability in mitochondrial activities exists across different neoplasms. Alternatively, high rates of glycolysis might be co-selected with factors that promote the expression of hypoxia-related genes (such as those required for angiogenesis) as an oxygen-independent energy source. Finally, increased intermediate products of glycolysis can easily be shunted into the biosynthetic pathways required for serine and nucleotide synthesis [7].

According to Folkman’s original theory [8], the onset of hypoxia in tumour triggers angiogenesis, which in turn is essential for supplying neoplastic cells with nutrients and oxygen and evacuating metabolic waste and carbon dioxide. The best understood hypoxia signaling mechanism is the stabilization and post-transcriptional activation of the hypoxia-inducible factor (HIF) proteins, which lead to the activation of many different pathways, including the vascular endothelial growth factor (VEGF) pathway. The VEGF pathway prompts and supports neoangiogenesis and glycolysis. Hypoxia-inducible pathway activation also has other effects, which include reducing the activity of mammalian target of rapamycin (mTOR), which in turn can reignite autophagy and promote survival under stress [9].

HIF is a heterodimer of an alpha subunit that is unstable in normoxia and a constitutively present and stable beta subunit. The hypoxia activation of HIF causes the heterodimer to bind to DNA at specific locations, called hypoxic response elements (HREs), eliciting the transcriptional up-regulation of genes required to respond appropriately to hypoxia [9].

In addition to triggering the VEGF pathway, the ubiquitously expressed HIF1 isoform promotes the transcription of glucose transporter 1 (GLUT1), which activates glucose transport inside the cell, lactate dehydrogenase A (LDH-A), which is involved in the glycolytic pathway, erythropoietin (EPO), which enhances erythropoiesis, and nitric oxide synthase (NOS), which promotes angiogenesis and vasodilatation [9].

HIF1 also prevents the entry of pyruvate into the TCA cycle by inducing the expression of pyruvate dehydrogenase kinase 1 (PDK1), thus altering the expressed isoform of cytochrome c and inhibiting mitochondrial biogenesis. This process causes reduced levels of oxygen consumption and a shift away from oxidative phosphorylation. Interestingly, HIF1 can also be activated under normoxic conditions by a variety of oncogenic pathways, such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), and by mutations in von Hippel-Lindau tumour suppressor (VHL), SDH, and FH [10].

In the classic angiogenic pathway, VEGF binds to VEGF receptor 2 (VEGFR2) on endothelial cells, increasing the expression of the Notch ligand Delta-like 4 (DLL4) on the same cells. DLL4 then binds to its receptor Notch on the adjacent endothelium. Further expression of VEGF2 and VEGFR1, as well as a smaller amount of VEGFR3, then follows, leading to triggering/amplification of the downstream phospholipase C family γ (PLCγ)–protein kinase C (PKC)–Raf kinase–MAP kinase–ERK kinase (MEK)–mitogen-activated protein kinase (MAPK) pathway, concomitantly prompting cell proliferation and cell survival throughout the phosphoinositide 3-kinase (PI3 K)/protein kinase B (AKT) pathway [11].

The switch to glycolysis in neoplasia was, according to Warburg, irreversible [3], yet a more complex picture has emerged over the last decade. There have been observed instances in which oxidative phosphorylation predominates during neoplastic transformation [12]. This variation between OxPhos and glycolysin cancer cells has been increasingly linked to specific disturbances in cell signaling pathways [13].

Additionally, tumours of the same genetic lineage can develop different metabolic adaptations depending on the host tissue from which they arise, suggesting that the stromal environment might play a crucial role in shaping the
metabolic profile [14]. The different molecular mechanisms 
being postulated to explain this variability of the Warburg 
effect include the following: inhibition of pyruvate dehy-
drogenase (PDH) by PDK1, reduction of mitochondrial 
biogenesis and inhibition of oxidative phosphorylation, 
both are caused by P53 inactivation and mutations [15].

Warburg raised two important issues: first, how tumour 
cells are supplied with glucose; and second, how they are 
supplied with oxygen [1]. Folkman’s work addressed the 
latter question with the hypothesis that tumour growth 
is strictly angiogenesis-dependent [16]. The work under-
taken to test this hypothesis led to the inclusion of “angi-
ogenesis” as one of the hallmarks of cancer [8].

Although there is strong evidence that angiogenesis fre-
cently occurs in cancer, we also now know that this event 
does not always occur. Indeed, some tumours, called “non-
angiogenic tumours,” can grow without triggering new 
vessel formation by co-opting preexisting vessels [17, 18].

Non-angiogenic growth was first identified by histol-
ysis in primary and metastatic lung carcinomas because 
neoplastic cells filled the alveolar spaces, co-opting the 
pre-existing capillary network and exhibiting a character-
istic “chicken-wire” appearance [17]. A gene expression 
signature for non-angiogenic non-small cell lung cancer 
(NSCLC) was published in 2005 [19]. Surprisingly, rather 
than the classic angiogenesis-related genes, the differ-
entially expressed genes were involved in mitochondrial 
metabolism, transcription, protein synthesis, and the cell 
cycle. Lack of differential mRNA expression between 
tumour phenotypes was noted for genes classically associ-
ated with hypoxia and angiogenesis. This result suggested 
that the response to hypoxia does not necessarily trigger 
neovascularisation, as would be observed in angiogenic 
tumours, but could actually be dependent on the genetic 
background of neoplastic cells, and in some instances, it 
could lead to metabolic reprogramming [19]. We there-
fore postulated that the degree to which a tumour will rely 
on angiogenic or non-angiogenic growth could be associ-
ated with a variety of events, including hypoxia, pseudo-
hypoxia, and metabolic re-programming.

In the first part of the present study, we investigated 
whether there were truly no differences in the expression 
of hypoxia-and angiogenesis-related proteins between 
angiogenic and non-angiogenic tumours, as suggested by 
mRNA profiling. We also investigated the degree of these 
proteins expression and the expression of some mito-
chondrial biogenesis proteins via immunohistochemistry. 
Notably high cytoplasmic P53 expression in non-angio-
genetic tumours, compared to angiogenic tumours, was 
found after completing the first part of this study. We 
therefore performed a second investigation, in which we 
examined and sequenced the p53 gene in these tumours.

Methods
Tissue specimens
Clinical specimens of NSCLCs were obtained from a 
series of consecutive patients who underwent surgical 
treatment at the John Radcliffe Hospital, Oxford, UK. 
This collection had ethical committee approval (study 
number C02.216—The pathophysiology of human neo-
plasia). The tissues were formalin-fixed and paraffin-
embedded. Tissue microarrays were constructed using 
the Beecher Instrument MTA-1 manual arrayer (Beecher 
Instruments, Inc., Sun Prairie, WI, USA). Up to four suit-
able areas of appropriate tumour were chosen from a 
slide stained with hematoxylin and eosin (H&E), avoiding 
as areas of necrosis.

Immunohistochemical staining of tissue sections
The 4-μm sections were cut from paraffin blocks and 
mounted on glass histology slides. Non-specific pro-
tein binding was blocked by incubating with 2.5% nor-
mal horse serum (Vector Laboratories, Burlingame, CA, 
USA), and the primary antibody was then applied. Details 
of all of the antibodies used are presented in Table 1. Sub-
stitution of the primary antibody with phosphate-buff-
ered saline (PBS) served as a negative control. The slides 
were then counterstained with hematoxylin for 20 s.

Scoring
The immunohistochemical staining was scored for cyto-
plasmic and nuclear localization and, when present, for 
membrane staining. Two observers scored the slides. 
Intensity was scored on a scale of 0–3 (0 = no staining, 
1 = weak, 2 = moderate, 3 = strong staining). The per-
centage of positive cells was recorded on a scale from 0 to 
4 (1 = 1%–10%, 2 = 11%–50%, 3 = 51%–80%, 4 = 81%–
100%) or alternatively on a continuous scale from 0% 
to 100%. The intensity and percentage values were then 
multiplied to provide a score called the “intensity per-
centage score” (IPS), the maximum score of which ranged 
from 12 to 300 [20].

Disputed scores were discussed and a consensus 
reached. For tissue microarrays, cores that did not con-
tain tumour tissue or that were more than 50% incom-
plete were excluded. The number of cases scored for each 
marker was between a minimum of 73 and a maximum 
of 194 cases for angiogenic tumours and between a mini-
mum of 48 and a maximum of 73 cases for non-angi-
genetic tumours.

Statistical analysis
To evaluate the association between protein biomarker 
expression and angiogenic status, the Mann–Whitney 
two-tailed non-parametric test was performed using
Pathway visualization

Three lists were made: two of proteins more highly expressed in angiogenic or non-angiogenic tumours at least in one subcellular location and the third of proteins always equally expressed in all of their subcellular localizations (Table 2). To visualize the pathways associated with these proteins, each list was imported into the Web-based Enricher facility (http://amp.pharm.mssm.edu/Enrichr/) [21]. The data were visualized from the online databases for gene ontologies (GO biological process and GO cellular component) and for pathways [Kyoto
Encyclopedia of Genes and Genomes (KEGG) 2015, WikiPathways 2015, Reactome 2015, and Panther. The results were visualized by bar graph sorted by combined score.

P53 sequencing

Genomic DNA was isolated from 33 specimens of NSCLC (25 angiogenic cases, seven non-angiogenic cases, and one undetermined case) and five specimens of peri-tumour lung tissues. The coding regions corresponding to exons 3–9 (amino acids 25–331) were sequenced. DNA was whole-genome amplified using GenomiPhi (GE Healthcare, Piscataway, NJ, USA). The primers for polymerase chain reaction (PCR) amplification and subsequent sequencing reactions are described in Table 3. PCR was performed using ThermoStart PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. PCR products were purified and bidirectionally sequenced using the BigDye Terminator cycle sequencing kit, version 1.1 (Applied Biosystems, Foster City, CA, USA), and an ABI 3100 Genetic Analyzer (Applied Biosystem, Paisley, UK). Sequence data were analyzed using Mutation Surveyor, version 3.25 (Softgenetics, State College, PA, USA). Predicted effects on protein were assessed in silico using PolyPhen2 software (http://genetics.bwh.harvard.edu/phyt/). Fisher's two-sided exact test was performed to compare mutation frequencies in angiogenic versus non-angiogenic cases. All sequencing experiments were performed in duplicate.

Results

Immunohistochemical staining

The complete results for the cytoplasmic and membranous expression are reported in Table 4, and those for nuclear expression are reported in Table 5, while a summary of these results appears in Table 6. The complete original results of ontology and pathway visualization appear in Additional file 1.

In Table 7, a selection of pathways shows that proteins expressed in both types of tumours are associated with angiogenesis-, VEGF-, and oxidative stress-related pathways. The non-angiogenic tumours are distinguished from the angiogenic tumours by having higher levels of proteins related to ephrin pathways, response to hypoxia, HIF1 regulation by oxygen, and transcription

---

### Table 2 Lists of proteins used for pathway visualization using the EnrichrWebd facility [21]

| Expression status | Proteins |
|-------------------|----------|
| Equal expression in both tumour types | HIF2, VEGFA, TMYP, KDR, KDRp34, FIH, PHD1, SOD1, EPHB4, BCL2, EGFR, FGF, SP1, LON, MEF2D, RPSA, CHGA, SYPA |
| Higher expression in angiogenic tumours in at least one subcellular location | HIF1, PHD2, PHD3 (cytoplasmic), CXCRRN, TSP, DLL4, BNIP3, P3B, EGF, FOS, STAT3, ITG8A, ITGAV |
| Higher expression in non-angiogenic tumours in at least one subcellular location | CA9, PHD3 (nuclear), EPHB2, EPHB3, NCAM, P53, TRAP1, JMY, GST |

---

### Table 3 Primers for polymerase chain reaction (PCR) amplification and subsequent P53 sequencing reactions

| Primer | Sequence | Annealing temperature (°C) | Amplicon length (bp) |
|--------|----------|---------------------------|----------------------|
| Exon 3–4 Forward: GTGGAAAGCGAAAATCTCCAT Reverse: GCCAGGCTTGGTGGTCAT | 60 | 506 |
| Exon 5–6 Forward: TGTTCACTTGTGCCCTGACT Reverse: TTAACCCCTCCTCCCAGAGA | 60 | 467 |
| Exon 7 Forward: GAGCTTGCAGTGAGCTGAGA Reverse: GGGATGTGATGAGAGGTGGA EX7F_seq CCTGCTTGCCACAGGTCT (to be used for sequencing instead of Exon 7 forward primer) | 61.5 | 390 |
| Exon 8–9 Forward: GACAAGGGTGGTGCGGAAATAA Reverse: GCCCAATTCGAGTAAAC | 60 | 500 |
Table 4 Cytoplasmic and membranous expression of the proteins in angiogenic versus non-angiogenic non-small cell lung cancers (NSCLCs)

| Protein          | Intensity percentage score (IPS) of protein expression | P value | Tumour type with higher expression |
|------------------|-------------------------------------------------------|---------|-----------------------------------|
|                  | Angiogenic tumours | Non-angiogenic tumours |                  |                                   |
| HIF1             | 6.22 ± 0.33        | 6.00 ± 0.48            | 0.87              | –                                 |
| HIF2             | 0.00 ± 0.00        | 0.00 ± 0.00            | Not applicable    | –                                 |
| CA9 cytoplasm    | 2.38 ± 0.16        | 3.50 ± 0.23            | <0.001            | Non-angiogenic                   |
| CA9 membrane     | 4.00 ± 0.40        | 3.30 ± 0.88            | 0.62              | –                                 |
| VEGFA            | 8.83 ± 0.35        | 7.38 ± 0.78            | 0.07              | –                                 |
| TYMP             | 3.20 ± 0.54        | 2.21 ± 0.32            | 0.28              | –                                 |
| KDR              | 94.56 ± 1.64       | 97.80 ± 1.54           | >0.05             | –                                 |
| KDRp34           | 10.40 ± 0.30       | 10.55 ± 0.62           | 0.71              | –                                 |
| FIH              | 9.10 ± 0.32        | 10.00 ± 0.47           | 0.22              | –                                 |
| PHD1             | 3.74 ± 0.27        | 4.20 ± 0.48            | 0.47              | –                                 |
| PHD2             | 3.50 ± 0.30        | 2.46 ± 0.53            | 0.02              | –                                 |
| PHD3             | 2.70 ± 0.09        | 3.38 ± 0.17            | <0.001            | Non-angiogenic                   |
| DLL4vessels      | 2.34 ± 0.10        | 1.53 ± 0.09            | <0.001            | Angiogenic                        |
| TSP stroma       | 16.98 ± 2.75       | 2.00 ± 1.74            | <0.001            | Angiogenic                        |
| CXCR4            | 2.47 ± 0.20        | 2.34 ± 0.31            | 0.92              | –                                 |
| EP HB2           | 7.78 ± 0.27        | 9.06 ± 0.33            | <0.01             | Non-angiogenic                   |
| EP HB3 cytoplasm | 159.3 ± 8.84       | 202.20 ± 9.95          | <0.01             | Non-angiogenic                   |
| EP HB3 membrane  | 23.83 ± 7.23       | 86.51 ± 12.78          | <0.001            | Non-angiogenic                   |
| EP HB4           | 7.82 ± 0.26        | 7.40 ± 0.38            | 0.57              | –                                 |
| SOD1             | 4.36 ± 0.45        | 9.39 ± 4.22            | 0.27              | –                                 |
| BCL2             | 1.05 ± 0.25        | 0.68 ± 0.35            | 0.54              | –                                 |
| FOS              | 8.05 ± 0.37        | 5.31 ± 0.55            | <0.001            | Angiogenic                        |
| EGF              | 203.40 ± 10.79     | 166.30 ± 25.74         | 0.33              | –                                 |
| EGF              | 18.90 ± 3.03       | 14.44 ± 4.15           | 0.72              | –                                 |
| FGF              | 9.20 ± 0.42        | 9.72 ± 0.71            | 0.74              | –                                 |
| BNP3             | 6.08 ± 0.36        | 5.92 ± 0.82            | 0.88              | –                                 |
| PS3              | 0.28 ± 0.12        | 2.28 ± 0.56            | <0.001            | Non-angiogenic                   |
| PI3              | 4.62 ± 0.36        | 4.92 ± 0.61            | 0.51              | –                                 |
| SP1              | 3.70 ± 0.37        | 3.38 ± 0.55            | 0.86              | –                                 |
| STAT3            | 8.78 ± 0.39        | 7.11 ± 0.79            | 0.03              | Angiogenic                        |
| LON              | 102.50 ± 11.20     | 134.20 ± 17.10         | 0.18              | –                                 |
| MEF2D cytoplasm  | 50.11 ± 4.16       | 65.67 ± 9.66           | 0.25              | –                                 |
| MEF2D membrane   | 60.00 ± 7.12       | 68.50 ± 12.67          | 0.47              | –                                 |
| JMY              | 1.84 ± 0.18        | 2.42 ± 0.31            | 0.05              | Non-angiogenic                   |
| TRAP1            | 4.22 ± 0.28        | 5.48 ± 0.31            | <0.01             | Non-angiogenic                   |
| GST cytoplasm    | 236.20 ± 7.16      | 249.80 ± 11.40         | 0.23              | –                                 |
| GST membrane     | 28.44 ± 5.52       | 69.38 ± 14.03          | <0.01             | Non-angiogenic                   |
| NCAM             | 0.40 ± 0.09        | 3.92 ± 0.07            | <0.001            | Non-angiogenic                   |
| CHGA             | 0.08 ± 0.04        | 0.27 ± 0.14            | >0.05             | –                                 |
| SYP              | 0.12 ± 0.05        | 0.44 ± 0.15            | >0.05             | –                                 |

All data are presented as mean ± standard error

HIF hypoxia-inducible factor; CA9 carbonic anhydrase 9; VEGFA vascular endothelial growth factor A; TYMP thymidine phosphorylase; KDR vascular endothelial growth factor receptor 2; KDRp34 vascular endothelial growth factor p34; FIM factor-inhibiting HIF; PHD prolyl hydroxylase dehydrogenase; DLL4 Delta-like 4; TSP1 thymidine phosphorylase 1; CXCR4 chemokine (C-X-C motif) receptor 4; EP HB ephrin; SOD1 superoxide dismutase 1; BCL2 B cell lymphoma 2; FOS FBJ murine osteosarcoma viral oncogene homolog; EGF epidermal growth factor; EGF R epidermal growth factor receptor; BNIP3 BCL2 adenovirus E1B 19 kDa interacting protein 3; PI3 peptidase inhibitor 3; SP1 Sp1 transcription factor; STAT3 signal transducer and activator of transcription 3; LON Lon protease; MEF2D myocyte enhancer factor 3D; JMY junction-mediating and regulatory protein, p53 cofactor; TRAP1 TNF receptor-associated protein 1; GST glutathione S-transferase; NCAM neural cell adhesion molecule; CHGA chromogranin A; SYP synaptophysin

– Indicates equal expression
of HIF-controlled genes but lower levels of proteins involved in stromal cell–cell signaling and adhesion, integrins, and Delta-Notch- and EGF-related signaling.

Gene ontology analysis confirmed that proteins usually associated with angiogenesis were present in both types of tumours, whereas higher levels of proteins associated with Notch, extracellular matrix, and cell adhesion were present in the truly angiogenesis-producing tumours. However, in the tumours that grow in a non-angiogenic fashion, proteins associated with mitochondria, cell biogenesis, carbonic dehydratase, ephrins, and axon guidance-related functions were more commonly detected (Table 8).

**Sequencing**

The higher level of p53 expression in the cytoplasm of the non-angiogenic tumours, as compared with the angiogenic tumours, was one of the most striking results (Table 9) and raised the question of whether non-angiogenic cells had wild-type p53 or a different set of mutations from the angiogenic tumours. Our results showed that p53 mutations were observed in eight of 25 angiogenic cases (32.0%) but

### Table 5 Nuclear expression of the proteins in angiogenic versus non-angiogenic NSCLCs

| Protein | Intensity percentage score (IPS) of protein expression | P value | Tumour type with higher expression |
|---------|------------------------------------------------------|---------|----------------------------------|
|         | Angiogenic tumours | Non-angiogenic tumours |
| HIF1    | 5.49 ± 0.31       | 3.79 ± 0.39       | 0.01 | Angiogenic |
| VEGFA   | 6.09 ± 0.52       | 5.62 ± 0.32       | 0.74 | – |
| TYMP    | 1.94 ± 0.22       | 1.16 ± 0.21       | 0.42 | – |
| KDR     | 231.20 ± 8.55     | 229.80 ± 9.88     | >0.05 | – |
| KDRp34  | 11.69 ± 0.16      | 11.45 ± 0.39      | 0.77 | – |
| FHI     | 7.50 ± 0.35       | 8.15 ± 0.72       | 0.50 | – |
| PHD1    | 3.10 ± 0.34       | 2.50 ± 0.35       | 0.99 | – |
| PHD2    | 2.56 ± 0.27       | 1.41 ± 0.35       | 0.02 | Angiogenic |
| PHD3    | 2.18 ± 0.13       | 0.65 ± 0.20       | <0.001 | Angiogenic |
| CXCR4   | 5.13 ± 0.23       | 3.72 ± 0.37       | <0.01 | Angiogenic |
| EPHB2   | 3.26 ± 0.26       | 4.91 ± 0.35       | <0.001 | Non-angiogenic |
| EPHB3   | 99.27 ± 7.41      | 147.30 ± 9.23     | <0.001 | Non-angiogenic |
| EPHB4   | 5.56 ± 0.30       | 5.73 ± 0.34       | 0.81 | – |
| SOD1    | 4.21 ± 0.45       | 5.00 ± 0.91       | 0.44 | – |
| C-FOS   | 7.50 ± 0.52       | 5.53 ± 0.75       | 0.05 | Angiogenic |
| EGF     | 235.20 ± 5.60     | 214.8 ± 8.68      | 0.01 | Angiogenic |
| FGF     | 7.51 ± 0.38       | 8.68 ± 0.76       | 0.11 | – |
| BNP3    | 3.96 ± 0.40       | 0.00 ± 0.00       | <0.001 | Angiogenic |
| P53     | 2.66 ± 0.36       | 2.32 ± 0.53       | 0.91 | – |
| p13     | 0.53 ± 0.13       | 0.00 ± 0.00       | <0.02 | Angiogenic |
| SP1     | 5.31 ± 0.46       | 4.04 ± 0.60       | 0.24 | – |
| STAT3   | 7.80 ± 0.39       | 6.31 ± 0.81       | 0.09 | – |
| MEF2D   | 251 ± 4.62        | 243.10 ± 8.04     | 0.38 | – |
| JMY     | 2.01 ± 0.17       | 2.60 ± 0.27       | 0.07 | – |
| TRAP1   | 1.57 ± 0.18       | 4.58 ± 0.32       | <0.001 | Non-angiogenic |
| GST     | 243.30 ± 6.15     | 244.10 ± 10.91    | 0.55 | – |
| CHGA    | 0.00 ± 0.00       | 0.34 ± 0.12       | >0.05 | – |

All data are presented as mean ± standard error

HIF hypoxia-inducible factor; C99 carbonic anhydrase 9; VEGFA vascular endothelial growth factor A; TYMP thymidine phosphorylase; KDR vascular endothelial growth factor receptor 2; KDRp34 vascular endothelial growth factor p34; FHI factor inhibiting HIF; PHD prolyl hydroxylase dehydrogenase; DLL4 Delta-like 4; TSP1 thymidine phosphorlyase 1; CXCR4 chemokine (C-X-C motif) receptor 4; EPH ephrin; SOD1 superoxide dismutase 1; BCL2 B cell lymphoma 2; FOS FBJ murine osteosarcoma viral oncogene homolog; EGF epidermal growth factor; EGFRT epidermal growth factor receptor; BNP3P3 BCL2 adenovirus E18 19 kDa interacting protein 3; PI3 peptidase inhibitor 3; SP1 Sp1 transcription factor; STAT2 signal transducer and activator of transcription 3; LON Lon protease; MEF2D myocyte enhancer factor 2D; JMY junction-mediating and regulatory protein, p53 cofactor; TRAP1 TNF receptor-associated protein 1; GST glutathione S-transferase; NCAM neural cell adhesion molecule; CHGA chromogranin A; SYT synaptophysin

– Indicates equal expression
in five of seven non-angiogenic cases (71.4%) \( (P = 0.091 \) by Fisher’s two-tailed exact test) (Table 9).

All of the detected mutations were heterozygous, and almost all of them corresponded to hot spots previously reported in different tumour types (http://genetics.bwh.harvard.edu/ggi/pph2/c2e64e1d60f39a55ca76a2a364ae4f2392360/1121012.html). The locations of the mutations were randomly distributed across the sequenced region. No specific pattern of mutation location seemed to be related to tumour subtype, angiogenic or non-angiogenic (Fig. 1 and Table 9).

### Discussion

As initially suggested by our mRNA profiling work [19] and also confirmed by the immunohistochemical data presented here, we failed to reveal significant differences...
between angiogenic and non-angiogenic NSCLCs as far as the expression of proteins associated with the classic hypoxia/angiogenesis pathway is concerned. Because neovascularisation is found in some tumours but not in others, we suggest that activation of the classical angiogenic pathways is necessary, but not sufficient, to induce the sprouting of new vessels in cancer.

The higher levels of expression of proteins associated with extracellular matrix, cell adhesion, and inflammation were in agreement with the observed presence of tumour-associated stroma and chronic inflammation in many angiogenic tumours [18, 22]. mRNA profiling of these tumours has equally shown that stromal remodeling, cell adhesion, and inflammation are enhanced in angiogenesis [19]. Both mRNA and immunohistochemical data suggest a crucial role in angiogenic cancers for FBJ murine osteosarcoma viral oncogene homolog (FOS), a protein involved in cell proliferation, remodeling, and inflammation. The question remains of whether tissue remodeling is a consequence or a cause of the triggering of angiogenesis. Because non-angiogenic tumours usually preserve the pre-existing architecture, we hypothesize that the triggering of tissue destruction could be a secondary step necessary for the activation of angiogenesis.

Table 8 Selection of visualized ontologies

| Proteins equally expressed in angiogenic and non-angiogenic tumours | Proteins up-regulated in angiogenic tumours | Proteins up-regulated in non-angiogenic tumours |
|---------------------------------------------------------------|------------------------------------------|-----------------------------------------------|
| **Ontology**                                                  | **GO-ontology database**                 | **Ontology**                                   | **GO-ontology database**                      | **Ontology**                                   | **GO-ontology database**                      |
| Cell migration/sprouting angiogenesis GO0002042                | Biological process                       | Immune response-activating signals GO0002757   | Biological process                            | Regulation of synapses GO0051365               | Biological process                            |
| Endothelial cell migration GO0043534                          | Biological process                       | Immune response-regulating cell signaling GO0002768 | Biological process                            | Axon guidance and neuronal regulation GO0031290 | Biological process                            |
| VEGF signaling pathways GO0038084                             | Biological process                       | Mesodermal cell differentiation GO0048333     | Biological process                            | –                                               | Biological process                            |
| –                                                             | –                                        | Activation of immune response GO0002253       | –                                               | Positive regulation cell biogenesis GO0044089   | Biological process                            |
| –                                                             | –                                        | Basement membrane GO0005604                   | –                                               | –                                               | –                                             |
| –                                                             | –                                        | Extracellular matrix part GO0044420           | –                                               | Mitochondrial intermembrane space and matrix GO0005758 | Cell component                               |
| –                                                             | –                                        | Complex involve in cell adhesion GO0098636    | –                                               | –                                               | –                                             |
| PDGF receptor-binding GO0005161                               | Molecular function                       | Fibronectin- and extracellular matrix-binding GO0001968 | Molecular function                            | Ephin receptor activity GO0005003              | Molecular function                            |
| Extracellular matrix-binding GO0005840                        | Molecular function                       | Notch-binding GO0005112                       | Molecular function                            | Carbonate dehydratase activity GO0004089      | Molecular function                            |
| –                                                             | –                                        | –                                               | –                                               | Axon guidance GO0008046                        | Molecular function                            |


Nonetheless, this process would be far from a general rule because we have observed, in a subset of NSCLC, that angiogenesis can occur in the absence of tissue destruction [23]. Clearly, other mechanisms must exist.

If classical angiogenesis pathways are similarly active in both angiogenic and non-angiogenic tumours, how does their biology differ? Our previous transcriptional profiling work demonstrated higher levels of mRNA coding for molecules associated with oxidative phosphorylation and mitochondrial biogenesis in non-angiogenic tumours [19]. In the present study, we did not examine components of the oxidative phosphorylation pathway, although we did investigate the expression of some proteins involved in mitochondrial functions. Our results showed that proteins related to mitochondria and to cell biogenesis-promoting processes were more highly expressed in non-angiogenic tumours than in angiogenic tumours. This feature was consistent with higher mitochondrial regulatory activity and a possible metabolic switch in non-angiogenic tumours. Again, whether this switch is a cause or an effect of HIF activation remains unclear.

Interestingly, our data suggested increased involvement of response to hypoxia and to HIF regulation by oxygen in non-angiogenic tumours. We hence speculate that, in these tumours, although the HIF pathway failed to induce new vessel formation, it could well be involved in metabolic reprogramming.

Finally, we noted markedly higher levels of cytoplasmic P53 expression in non-angiogenic tumours than in angiogenic tumours via immunohistochemistry. In a pilot study, we sequenced \( p53 \) in a limited number of angiogenic and non-angiogenic NSCLCs. Non-angiogenic tumours had a higher incidence of mutations, which were all missense mutations, whereas angiogenic tumours had an amalgam of frameshifts and missense and nonsense mutations. Because \( p53 \) also affects mitochondrial respiration [24], it will be necessary to investigate further how the observed \( p53 \) mutations could functionally affect its ability to regulate respiration and/or angiogenesis.

**Conclusions**

On the basis of our observations collected so far, from the mRNA profiling, immunohistochemical and histopathologic data, we conclude that all tumours, angiogenic and non-angiogenic, experience hypoxia/HIF and VEGF pathway activation. However, angiogenesis does not always ensue. Based on these findings, we suggest that in non-angiogenic tumours, HIF pathway activation could be the driving force toward metabolic reprogramming.

### Table 9: Summary of \( p53 \) mutations detected in non-angiogenic and angiogenic NSCLC cases

| Sample ID | Sample type       | Mutation location | Mutation type         | Domain       | Predicted effect on protein activity |
|-----------|-------------------|-------------------|-----------------------|--------------|-------------------------------------|
| 104       | Non-angiogenic    | c.761T>TA; p.I254S| Missense              | HCD IV       | Damaging                            |
| 105       | Non-angiogenic    | c.734G>GA; p.G245D| Missense              | HCD IV       | Damaging                            |
| 121       | Non-angiogenic    | c.488A>AG; p.Y163C| Missense              | DNA binding  | Damaging                            |
| 152       | Non-angiogenic    | c.634T>TG; p.F212V| Missense              | DNA binding  | Damaging                            |
| 249       | Non-angiogenic    | c.314G>GA; p.G105D| Missense              | DNA binding  | Damaging                            |
| 133       | Angiogenic        | c.511G>GT; p.E171X| Nonsense (truncated   | HCD III      | Truncating                          |
| 138       | Angiogenic        | c.824G>GA; p.C275Y| Missense              | HCD IV       | Damaging                            |
| 139       | Angiogenic        | c.het_del216C,p.V73Wfs48X| Frameshift (truncated | HCD II       | Damage                             |
| 141       | Angiogenic        | c.407A>AC; p.Q136P| Missense              | DNA binding  | Potentially damage                  |
| 147       | Angiogenic        | c.524G>GA; p.R175H| Missense              | DNA binding  | Damage                              |
| 133       | Angiogenic        | c.407A>AC; p.Q136P| Missense              | DNA binding  | Damage                              |
| 147       | Angiogenic        | c.524G>GA; p.R175H| Missense              | DNA binding  | Damage                              |
| 121       | Non-angiogenic    | c.488A>AG; p.Y163C| Missense              | DNA binding  | Damage                              |

**Fig. 1** Localization of the mutations detected on the \( p53 \) gene. The sequenced region is indicated with an orange line in the figure above. Mutation locations are indicated with arrowheads (purple: mutations found in angiogenic samples; gray: mutations found in non-angiogenic samples). Human P53 protein (HP53) can be divided into five domains, each corresponding to specific functions: yellow is the highly conserved domain I (HCD I)/transactivation domain; red is the second transactivation domain, which is proline-rich; blue is the DNA-binding domain essential for \( p53 \)-DNA interactions that also contains HCD II-V and is the target of 90% of the \( p53 \) mutations found in human cancers; green is the nuclear export signal (NES) localized in the oligomerization domain of \( p53 \).
8. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74. doi:10.1016/j.cell.2011.02.013.

9. Harris AL. Hypoxia—a key regulatory factor in tumour growth. Nat Rev Cancer. 2002;2(1):38–47. doi:10.1038/nrc704.

10. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer. 2008;8(9):705–13. doi:10.1038/nrc2468.

11. Kerbel RS. Tumor angiogenesis. N Engl J Med. 2008;358(19):2039–49. doi:10.1056/NEJMra0706596.

12. Jose C, Bellance N, Rossignol R. Choosing between glycolysis and oxidative phosphorylation: a tumor’s dilemma? Biochim Biophys Acta. 2011;1807(8):532–61. doi:10.1016/j.bjbaio.2010.10.012.

13. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. Nature. 2012;491(7424):364–73. doi:10.1038/nature11706.

14. Yuneva MO, Fan TW, Allen TD, Higashi RM, Ferraris DV, Tsukamoto T, et al. The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type. Cell Metab. 2012;15(2):157–70. doi:10.1016/j.cmet.2011.12.015.

15. Obre E, Rossignol R. Emerging concepts in bioenergetics and cancer research: metabolic flexibility, coupling, symbiosis, switch, oxidative tumors, metabolic remodeling, signaling and bioenergetic therapy. Int J Biochem Cell Biol. 2015;59:167–81. doi:10.1016/j.biocel.2014.12.008.

16. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285(21):1182–6. doi:10.1056/NEJM197111282852108.

17. Pezzella F, Di Bacco A, Andreola S, Nicholson AG, Pastorino U, Harris AL. Angiogenesis in primary lung cancer and lung secondarys. Eur J Cancer. 1996;32A(14):2494–500.

18. Pezzella F, Pastorino U, Tagliabue E, Andreola S, Sozzi G, Gasparini G, et al. Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. Ann J Pathol. 1997;151(3):1417–23.

19. Hu J, Bianchi F, Fergusson M, Cesario A, Margarotta S, Granci P, et al. Gene expression signature for angiogenic and nonangiogenic non-small-cell lung cancer. Oncogene. 2005;24(7):1212–9. doi:10.1038/sj.onc.1208242.

20. Bates GJ, Fox SB, Han C, Launchbury R, Leek RD, Harris AL, et al. Expression of the forkhead transcription factor FOXP1 is associated with that of estrogen receptor-beta in primary invasive breast carcinomas. Breast Cancer Res Treat. 2008;111(3):453–9. doi:10.1007/s10549-007-9812-4.

21. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics. 2013;14:128. doi:10.1186/1471-2105-14-128.

22. Ferguson M. Angiogenesis in human lung tumours. Oxford: Oxford University Press, 2008.

23. Passalidou E, Trivella M, Singh N, Fergusson M, Hu J, Cesario A, et al. Vascular phenotype in angiogenic and non-angiogenic lung non-small cell carcinomas. Br J Cancer. 2002;86(2):244–9. doi:10.1053/bjoc.6600015.

24. Matoba S, Kang J, Patino W, Wragg A, Boehm M, Gavrilova O, et al. p53 regulates mitochondrial respiration. Science. 2006;315(5870):1650–3.