Original paper

Prognostic value of tumour tissue ANG-1 expression and Ang-1 concentration in patients with non-small-cell lung cancer

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Tumor cells stimulate local angiogenesis, resulting in their further multiplication and spread. Angiogenesis is a multifaceted process in which angiopoietins participate. Angiopoietin-1 (Ang-1) through its receptor Tie2 stimulates endothelial cell survival and the maintenance of the endothelial barrier. These phenomena can support tumour growth by promoting angiogenesis. On the other hand, overproduction of Ang-1 triggers endothelium stability and can lead to angiogenesis inhibition. Because of the ambiguous role of Ang-1, we decided to determine its clinical significance in patients with resectable NSCLC.

In a group of 47 patients, tumours and the adjacent non-cancerous tissues were assessed for *ANG-1* mRNA expression (using Q-RT-PCR analysis) and Ang-1 concentration (by enzyme-linked immunosorbent assay) together with clinical parameters and the five-year survival rate. *ANG-1* expression and Ang-1 concentration were higher in tumour-free tissue, showing no differences between histological types of NSCLC, clinical stage or grading and seemed not to determine the five-year survival. *ANG-1* expression and Ang-1 concentration in tumour and tumour-free tissues in patients with NSCLC seem not to be useful as factors supporting either diagnostics or prognosis.

Key words: angiogenesis, non-small cell lung cancer, angiopoietin-1, five-year survival.

Introduction

Every year, lung cancer causes nearly 1.7 million deaths worldwide [1, 2, 3]. Non-small cell lung cancer (NSCLC) accounts for almost 85% of cases, including large-cell carcinoma, squamous cell carcinoma (20-30%) and adenocarcinoma (40-50%). Because of the initially asymptomatic clinical course, over 60% of newly diagnosed patients are in locally advanced or metastatic stage of the disease [4]. Despite the multimodal care based treatment, the prognosis for patients with lung cancer remains poor and the 5-year overall survival rate is only around 15-18% [5, 6, 7].

Proliferating tumour cells stimulate local angiogenesis which increases their growth and spread into adjacent tissue and organs [8, 9, 10]. Angiogenesis is a multistage process of creating new blood vessels

based on existing ones. It begins with stimulation of endothelial cells by proangiogenic factors, mainly vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Degradation of the vascular basal membrane and extracellular matrix, involving proteolytic enzymes, enables endothelial cell migration. New vascular structures are formed under specific conditions – endothelial cells proliferate alongside adhesive molecules, pericytes and smooth muscle cells. The final stage of vessel formation is their maturation and stabilization. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), among others, participate at this stage of the process [11, 12, 13].

Ang-1 and Ang-2 are ligands for the receptor tyrosine kinase Tie2, expressed in endothelial cells. The Ang-1 receptor is mainly present in pericytes, fibroblasts and smooth muscle cells, whereas on endothelial cells it acts in a paracrine manner. Ang-1 promotes angiogenesis during embryonic development. It stabilises the vascular system by promoting interaction between endothelial cells and the extracellular matrix, but in malignant tissues it inhibits the expansion of vessels [14, 15, 16, 17, 18]. Ang-2, on the other hand, is produced by endothelial cells and acts as an autocrine antagonist of Tie2 activation and a vascular destabiliser [19].

Whilst the role of Ang-2 in angiogenesis during embryogenesis and tumour development has been the subject of detailed study, much less is known about Ang-1 function [19]. The role of Ang-1 in cancer progression is more controversial than that of Ang-2. Although Ang-1 is proven to be at a higher level in many cancer tissues such as breast cancer [20, 21], glioblastoma [22], and gastric cancer [23], its expression is lower in some other cancer types like human hepatocellular carcinoma or lung cancer, compared with normal tissues [18, 24].

We evaluated *ANG-1* mRNA expression and Ang-1 concentration in patients with resectable lung cancer in various clinical stages and analyzed results according to five-year survival in these group.

The aim of our study was to determine the clinical significance of tumour tissue *ANG-1* mRNA expression and Ang-1 concentration and their significance as a prognostic factors in patients with non-small cell lung cancer.

Material and methods

The study group consisted of 47 patients (12 females and 35 males) aged 49 to 75 years (average age: 63.1 ±7.69) with primary NSCLC undergoing surgery in the Thoracic Surgery Ward of Specialist Hospital of Lung Diseases and Tuberculosis in Bystra Slaska, Poland, between 2009 and 2010. During surgery, the specimens of the tumour tissue and the lung parenchyma free from neoplastic infiltration (taken no less than 5 cm from the visible edge of the tumour) were obtained. The specimens were about several mm with a diameter. Each of them was divided into two pieces. One was immediately snap-frozen in a separate tube with liquid nitrogen and stored at –80°C until molecular procedures. The other one was immediately frozen at –20°C for determination of *ANG-1* concentration by enzyme-linked immunosorbent assay (ELISA). Subsequently, the samples were transported on dry ice to the Molecular Research Laboratory of the Department of Medical and Molecular Biology in Zabrze for further examination.

Tumour and the adjacent non-cancerous tissues were excised and evaluated for clinical parameters, such as histopathological type (adenocarcinoma/squamous cell carcinoma), pathological staging of the tumor (pTNM) and the grade of differentiation (G) independently by two pathomorphologists. The absence of tumour infiltration in the parenchyma of the lung that was deemed healthy, was each time confirmed with histopathological examination.

Clinical and pathological characteristics of the patients are presented in Table I.

RNA extraction, reverse transcription and quantitative Real-Time PCR (qRT-PCR) for *ANG-1* and a housekeeping gene *GAPDH*

Total RNA was obtained from ca. 80 mg of frozen tissue samples. Before RNA extraction, samples were thawed slowly and then homogenised in FastPrep®-24 homogeniser (MP Biomedicals, USA) using Lysing Matrix D ceramic beads (MP Biomedicals, USA).

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. In addition to the standard procedure, DNase I (Qiagen, Germany) was used to remove trace amounts of genomic DNA.

RNA was quantified by measuring the absorbance at 260 and 280 nm (NanoDrop ND – 1000 Spectrophotometer, Thermo Fisher Scientific, USA) and the integrity was assessed by electrophoresis in 1.2 % agarose gel ethidium bromide stained (Serva, Germany). RNA isolates were used to cDNA synthesis by RT reaction. 200 ng of total RNA was reverse transcribed into cDNA in a total volume 20 μl using the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) according to manufacturer's instructions. Received cDNA was used to determine *ANG-1* and *GAPDH* genes expression by quantitative real-time PCR assay (TaqMan® system). TaqMan® primers and probe for *ANG-1* were bought as ready to use TagMan® Gene Expression Assay (Hs00375822_m1) and for housekeeping gene

Table I. The clinical and pathological features of NSCLC patients [25, 26]

Variable	Number of patients (%)
Total number	**47 (100)**
Gender	
F	
M	35 (74.5)
Age	
≤ 65	25 (53.2)
> 65	22 (46.8)
Type of histological pattern	
squamous cell carcinoma	34 (72.3)
adenocarcinoma	13 (27.7)
Grading	
G1	1 (2.1)
G2	19 (40.4)
G3	27 (57.5)
Clinical stage	
IA+IB	14 (29.8)
IIA+IIB	21 (44.7)
IIIa+IIIB	11 (23.4)
undefined	1 (2.1)
T: Primary tumour	
T1	11 (23.4)
T2	30 (63.8)
T3	5 (10.7)
T4	1 (2.1)
N: Lymph nodes	
N0	21(44.7)
N1	17 (36.2)
N2	9 (19.1)
Five-year survival	
yes	28 (59.60)
no	19 (40.40)

G – histopathological cancer grading (G1: well differentiated; G2: moderately differentiated; G3: poorly differentiated; G4: undifferentiated); T – tumour size (T1: size ≤ 3 cm; T2: size > 3 cm to ≤ 5 cm; T3: size > 5 cm to 7 cm); N – metastatic lymph nodes (N0: no regional lymph node metastasis; N1: metastasis in ipsilateral peribronchial and/or hilar lymph node and intrapulmonary node; N2: metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes)

GAPDH Endogenous Control (Hs02758991_g1) (Applied Biosystems, USA). Q-PCR for all genes was performed in a volume 20 μl on the ABI PRISM 7300 Real Time PCR Detection System (Applied Biosystems, USA). For each run, a Q-PCR mix was prepared on ice containing 10 μl of Applied Biosystems Universal PCR Master Mix, 1 μl of primers and probe mix and 8 μl of H_2O (Qiagen, Germany). To each well of a 96-well plate, 19 μl of Q-PCR mix and 1 μl of cDNA samples were added. All PCRs were performed in triplicate. In all amplification reactions, negative control was also included. Thermal cycling for *ANG-1* and *GAPDH* genes was initiated with an incubation step at 50°C for 2 minutes, followed by a first denaturation step at 95°C for 10 minutes, and continued with 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Gene expression was considered negligible when the Ct (threshold cycle) value was greater than 40. In view of heterogeneity of the investigated tissue and the lack of ability to use of a commercially available standard, to determine *ANG-1* expression the ΔC_T method was applied [27].

Measurement of tissue concentration of Ang-1

Tissues were rinsed in ice-cold PBS to remove thoroughly excess blood and were consequently cut into small pieces, homogenized in PBS (0.25 g of tissue in 2.25 ml PBS) in short cycles of a few seconds on ice. After this procedure, the samples were frozen at –80°C and next day sonicated on ice to cause the cell membranes to disintegrate, using an UP50H ultrasonic processor (Hielscher Ultrasonics, Germany). Tissue homogenates were centrifuged at 13000 rpm for 10 minutes at 4°C and the supernatants were frozen at –80°C until analysis.

The concentration of Ang-1 in tissue homogenates was determined by Enzyme-linked Immunosorbent Assay (ELISA) using the ANGPT1 SEA008Hu (Cloud-Clone Corp., USA) according to the manufacturer's instructions. The concentration of angiopoietin-1 was expressed in ng per 1 mg of total protein determined by the Lowry method [28].

Statistical analyses

Qualitative variables are presented in absolute terms (number of patients showing the presence of a variable) and as a percentage of the total analyzed the study group, whilst quantitative variables were expressed as mean and standard deviation (mean ±SD).

Statistical analysis was performed using licensed STATISTICA 12.0 software (StatSoft. Inc., Tulsa, OK, USA). Descriptive statistics were initially calculated. The groups were compared using parametric tests, after verification of their assumptions (t-test, analysis of variance with post-hoc tests). In all the other cases, their non-parametric equivalents were applied.

For all the analyses, statistical significance was determined for values of $p < 0.05$. Lack of statistical significance was presented as NS (non-significant).

Table II. Comparison of *ANG-1* expression and Ang-1 concentration in tumour and tumour-free tissue in NSCLC patients

Tissue	*ANG-1* expression	p	Ang-1 concentration (ng/mg)	p
Tumour	0.0004 ±0.0004		0.42 ±0.16	
Tumour-free	0.053 ±0.03	0.001	0.55 ±0.17	0.009

Table III. *ANG-1* expression and Ang-1 concentration in NSCLC patients depending on the histological type of NSCLC, the degree of differentiation, clinical stage and five-year survival

NSCLC	N (%) 47 (100)	*ANG-1* expression		Ang-1 concentration (ng/mg)	
		Tumour	Tumour-free	Tumour	Tumour-free
Histological type					
Squamous cell carcinoma	34 (72.3)	0.0003 ±0.0004	0.06 ±0.04	0.44 ±0.17	0.58 ±0.19
		p = 0.001		p = 0.048	
Adenocarcinoma	13 (27.7)	0.0005 ±0.0004	0.04 ±0.03	0.36 ±0.1	0.48 ±0.07
		p = 0.002		p = 0.031	
Grading					
G2	19 (40.4)	0.0004 ±0.0004	0.06 ±0.04	0.33 ±0.16	0.62 ±0.19
		p = 0.001		p = 0.003	
G3	27 (57.5)	0.0005 ±0.0004	0.05 ±0.03	0.46 ±0.15	0.51 ±0.15
		p = 0.001		NS	
Clinical stage					
IA+IB	14 (29.8)	0.0004 ±0.0004	0.08 ±0.03	0.43 ±0.12	0.53 ±0.07
		p = 0.001		NS	
IIA+IIB	21 (44.7)	0.0004 ±0.0005	0.04 ±0.03	0.37 ±0.09	0.56 ±0.23
		p = 0.001		p = 0.03	
IIIA+IIIB	11 (23.4)	0.0004 ±0.0004	0.06 ±0.03	0.49 ±0.22	0.56 ±0.14
		p = 0.001		NS	
Five-year survival					
Yes	19 (40.40)	0.0005 ±0.0004	0.05 ±0.04	0.43 ±0.17	0.58 ±0.20
		p = 0.001		NS	
No	28 (59.60)	0.0003 ±0.0004	0.04 ±0.03	0.41 ±0.14	0.52 ±0.10
		p = 0.001		NS	

NS – not significant

The Document Personalisation Centre of the Ministry of Internal Affairs and Administration in Warsaw, Poland, kindly granted the access to information about the patients' date of death, which was essential for precise calculation of the length of patients' survival. Based on these data, 5-year survival rates were proven to be correlated with Ang-1 concentration and *ANG-1* expression in cancerous and non-cancerous tissues.

The study protocol was approved by the Ethical Committee of the Medical University of Silesia in Katowice, Poland (KNW/0022/KB1/119/I/09), and conformed to the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained in respect of all study participants.

Results

The analysis encompassed 47 NSCLC tumour specimens and 47 corresponding specimens of pulmonary parenchyma deemed free from tumor infiltration. *ANG-1* expression and Ang-1 concentration in both types of samples were detected. The study group characteristics are presented in Table I.

The mean *ANG-1* expression in tumor-free tissues was significantly higher than in neoplastic tissues as well as the mean Ang-1 concentration in tissues free from tumour infiltration was significantly higher than in tumours (Table II).

Table IV. *ANG-1* expression and Ang-1 concentration in tumour tissues depending on the histological type of NSCLC, the degree of differentiation, clinical stage and five-year survival

NSCLC	*ANG-1* expression	p	Ang-1 concentration (ng/mg)	p
Histological type				
Squamous cell carcinoma	0.0004 ±0.0004	NS	0.45 ±0.17	NS
Adenocarcinoma	0.0005 ±0.0004		0.4 ±0.09	
Grading				
G2	0.0003 ±0.0004	NS	0.42 ±0.17	NS
G3	0.0004 ±0.0004		0.45 ±0.15	
Clinical stage				
I	0.0004 ±0.0003	NS	0.44 ±0.15	NS
II	0.0004 ±0.0005		0.39 ±0.10	
III	0.0004 ±0.0004		0.49 ±0.20	
Five-year survival				
Yes	0.0005 ±0.0004	NS	0.42 ±0.17	NS
No	0.0003 ±0.0004		0.43 ±0.14	

Table V. *ANG-1* expression and Ang-1 concentration in non-tumour tissues depending on the histological type of NSCLC, the degree of differentiation, clinical stage and five-year survival

NSCLC	ANG-1 expression	p	Ang-1 concentration (ng/mg)	p
Histological type				
Squamous cell carcinoma	0.05 ±0.03	NS	0.56±0.18	NS
Adenocarcinoma	0.04 ±0.03		0.49 ±0.07	
Grading				
G2	0.06 ±0.04	NS	0.57 ±0.19	NS
G3	0.09 ±0.03		0.51±0.14	
Clinical stage				
I	0.08 ±0.03	I vs. II:	0.53 ±0.06	NS
II	0.04 ±0.03	p = 0.01	0.56 ±0.14	
III	0.06 ±0.02		0.49 ±0.11	
Five-year survival				
Yes	0.06±0.04	NS	0.56 ±0.18	NS
No	0.05 ±0.03		0.49 ±0.11	

The study compared the expression and concentration of angiopoietin-1 between tumour and tumour-free tissues depending on histological type, grading, clinical stage and five-year survival. Regardless of histological type, grading, clinical stage and five-year survival, both the expression and concentration of Ang-1 have always been higher in tumour-free tissues than in tumours as shown in Table III. These results were significant for *ANG-1* expression in every aspect, but concentration of Ang-1 was not significantly (NS) higher in tumour- free tissues in the case of G3 and in patients in clinical stages IA+B and IIIA+B. Similarly, these parameters seem to be unrelated to five-year survival.

Analysis of both parameters in NCSCL tissues showed no significant differences (Table IV), but in tumour-free tissues a significant difference in *ANG-1* expression was found between patients with clinical stages I and II (Table V). No significant differences were identified in Ang-1 concentration in non-tumour tissues between squamous cell carcinoma and adenocarcinoma, between G2 and G3, between 5-year survivors and non-survivors, and between clinical stages I, II and III.

In summary, *ANG-1* expression was found to be higher in cancer-free tissue than in tumours, irrespectively of histological or clinical criteria and seems to have no impact on five-year survival. Ang-1 concentration was generally higher in margin tissues of both histological types of NSCLC, but it did not show significant differences in G3 patients and those in I and III clinical stages. No significant differences were found between patients who died or survived for over five years following surgery.

Discussion

The vast improvements in our understanding of NSCLC biology and mechanisms of tumour progression have changed the treatment paradigm. Targeted therapy and immunotherapy have led to unprecedented survival benefits, but only in selected patients [7, 29]. Surgical resection is the most frequent and preferred treatment in early stages of disease. Survival rate will depend mainly on the tumour's stage, with a 5-year survival that ranges from 67% to 38% for pathologic tumor stage IA to IIB [30, 31]. In our study group only 40% of patients survived five years following surgery.

Tumour growth, invasion and metastasis strongly depend on oxygen as well as nutrients supply therefore for each of these processes angiogenesis is essential. New vessels development is a complex process which depends on activator and inhibitor molecules interactions. Angiopoietins constitute a family of endothelial growth factors which are ligands for Tie2, type I kinase tyrosine receptor. Ang-1 acts paracrine, agonist, inducing Tie2 phosphorylation and dimerisation/multimerisation. Activated Tie2 binds several other ligands, creating downstream pathways. That results in endothelial cell survival signals and the maintenance of the endothelial barrier [32, 33].

Numerous studies investigated the role of angiopoietins in advanced NSCLC development and their impact on patients' survival, however, the results of these studies remain inconclusive. Takahama *et al.* as early as in 1999 reported that angiopoietin-1, vascular endothelial growth factor, and CD31 mRNAs were higher in cancers than in adjacent noncancerous tissues [34]. Our results, however, indicate the opposite: *ANG-1* expression was higher in cancer-free tissues regardless of NSCLC histological type, grading or clinical stage. Moreover, it has no significant impact on five-year survival rate, while Reinmuth *et al.* found that high tumor cell *ANG-1* expression, combined with the intensity and percentage of positive tumor cells, was statistically associated with a poor survival [35]. Andersen *et al.* [36] underlined the role of another angiopoietins family members: Ang-2 and Ang-4. Low tumour cell expression of Ang-4 and low stromal expressions of both Ang-2 and Ang-4 were individually associated with a poor rate of survival. These factors were analyzed together with VEGF-A expression, which was a powerful adverse prognostic factor in patients with high tumour cell Ang-2 expression, but not in those with low expression [36].

It has been documented that members of the vascular endothelial growth factor and angiopoietin families, mainly secreted by tumour cells, induce tumor angiogenesis. Altogether, VEGF/VEGFRs and angiopoietins/Ties co-regulate the tumour vessels' regression, growth and maturation [37]. In the tumour angiogenesis, angiopoietins are secreted by tumour cells and/or tumour-infiltrating cells [8, 9]. In our study Ang-1 concentration was higher in tumour-free tissue, showing no difference between two histological types of NSCLC, clinical stage or grading. It did not seem to influence the five-year survival rate. Our observation supports the concept, that in NSCLS Ang-1 is more likely to be involved in restricting the process of tumor invasion. We believe it may be connected with its vasculature stabilising properties.

Angiogenesis is regulated by both activator and inhibitor molecules. The transition to the angiogenic phenotype is associated with a change in local balance between positive and negative angiogenesis regulators. Ang-1 can act as a pro-angiogenic or anti-angiogenic factor. It can induce tumor growth by promoting tumor angiogenesis, but overproduction of Ang-1 can lead to inhibition of tumor growth [11]. The intriguing question is whether higher *ANG-1* expression and its product concentration (found in tissues as far as up to 5 cm from visible edge of tumour) could be an attempt at blocking pro-angiogenic signals spreading in normal tissue surrounding the neoplasm.

It was observed that Ang-2 competitively binds Tie-2, destabilising the action of Ang-1 and preparing the tumour vasculature for subsequent action of proangiogenic factors such as VEGF. It is tempting to speculate, that Ang-1 production is an indication of balance reached in tumour-free tissue upon the influence of cancer-derived pro-angiogenic factors. On the other hand, if Ang-1 in NSCLC shows mainly anti-angiogenic activity, its lower expression in tumour tissue (as opposed to normal tissue) will reflect the higher potency of pro-angiogenic molecules.

Angiogenesis inhibitors were extensively tested in different settings and have produced some promising outcomes [38]. Despite the rapid research progress in the field of NSCLS biology, many questions about the molecular mechanisms blocking or promoting angiogenesis remain. According to our observations, Ang-1 seems not to be a good target for future therapy focused on neoangiogenesis/metastasis blockade.

ANG-1 expression and Ang-1 concentration in tumour and tumour-free tissues in patients with NSCLC seem not to be useful either as factors supporting diagnostics or prognosis. Their role in cancer-induced angiogenesis, especially in normal tissue adjacent to tumours, still requires further research.

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The authors declare no conflict of interest.

References

1. https://www.who.int/en/news-room/fact-sheets/detail/cancer
2. Torre LA, Bray F, Siegel RL, et al. Global cancer statistics, 2012. CA Cancer J Clin 2015; 265: 87-108.
3. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136: E359-386.
4. Osmani L, Askin F, Gabrielson E, et al. Current WHO guidelines and the critical role of immunohistochemical markers in the subclassification of non-small cell lung carcinoma (NSCLC): Moving from targeted therapy to immunotherapy. Semin Cancer Biol 2018; 52: 103-109.
5. Xie FJ, Lu HY, Zheng QQ, et al. The clinical pathological characteristics and prognosis of FGFR1 gene amplification in non-small-cell lung cancer: a meta-analysis. Onco Targets Ther 2016; 9: 171-181.
6. Howlader N, Noone AM, Krapcho M, et al. SEER cancer statistics review, 1975–2012. National Cancer Institute, Bethesda, Maryland, 2015.
7. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. Nature 2018; 553: 446-454.
8. Ferrara N. VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2002; 2: 795-803.
9. Holopainen T, Huang H, Chen C, et al. Angiopoietin-1 overexpression modulates vascular endothelium to facilitate tumor cell dissemination and metastasis establishment. Cancer Res 2009; 69: 4656-4664.
10. He Y, Karpanen T, Alitalo K. Role of lymphangiogenic factors in tumor metastasis. Biochim Biophys Acta 2004; 1654: 3-12.
11. Nishida N, Yano H, Nishida T, et al. Angiogenesis in cancer. Vasc Health Risk Manag 2006; 2: 213-219.
12. Park JH, Choi H, Kim YB, et al. Serum angiopoietin-1 as a prognostic marker in resected early stage lung cancer. Lung Cancer 2009; 66: 359-364.
13. Dong Z, Chen J, Yang X, et al. Ang-2 promotes lung cancer metastasis by increasing epithelial-mesenchymal transition. Oncotarget 2018; 27: 12705-12717.
14. Tait CR, Jones PF. Angiopoietins in tumours: the angiogenic switch. J Pathol 2004; 204: 1-10.
15. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996; 87: 1171-1180.
16. Hayes AJ, Huang WQ, Mallah J, et al. Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. Microvasc Res 1999; 58: 224–237.
17. Hawighorst T, Skobe M, Streit M, et al. Activation of the tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. Am J Pathol 2002; 160: 1381-1392.
18. Wong MP, Chan SY, Fu KH, et al. The angiopoietins, tie2 and vascular endothelial growth factor are differentially expressed in the transformation of normal lung to non-small cell lung carcinomas. Lung Cancer 2000; 29: 11-22.
19. Thomas M, Augustin HG. The role of the Angiopoietins in vascular morphogenesis. Angiogenesis 2009; 12: 125-137.
20. Caine GJ, Blann AD, Stonelake PS, et al. Plasma angiopoietin-1, angiopoietin-2 and Tie-2 in breast and prostate cancer: a comparison with VEGF and Flt-1. Eur J Clin Invest 2003; 33: 883-890.
21. Shirakawa K, Tsuda H, Heike Y, et al. Absence of endothelial cells, central necrosis, and fibrosis are associated with aggressive inflammatory breast cancer. Cancer Res 2001; 61: 445-451
22. Stratmann A, Risau W, Plate KH. Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. Am J Pathol 1998; 153: 1459-1466.
23. Wang J, Wu K, Zhang D, et al. Expressions and clinical significances of angiopoietin-1, -2 and Tie2 in human gastric cancer. Biochem Biophys Res Commun 2005; 337: 386-393.
24. Mitsuhashi N, Shimizu H, Ohtsuka M, et al. Angiopoietins and Tie-2 expression in angiogenesis and proliferation of human hepatocellular carcinoma. Hepatology 2003; 37: 1105-1113.
25. Lim W, Ridge CA, Nicholson AG, et al. The 8th lung cancer TNM classification and clinical staging system: review of the changes and clinical implications. Quant Imaging Med Surg 2018; 8: 709-718.
26. Wang J, Wu N, Lv C. et al. Recommended changes for the 8th edition of the TNM classification for lung cancer-the findings of a single-institution evaluation. Ann Transl Med 2020; 8: 123.
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001; 25: 402-408.
28. Lowry OH, Rosebrough NJ, Farr L, et al. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-275
29. Naylor EC, Desani JK, Chung PK. Targeted therapy and immunotherapy for lung cancer. Surg Oncol Clin N Am 2016; 25: 601-609.
30. Birim O, Kappetein AP, Waleboer M, et al. Long-term survival after non-small cell lung cancer surgery: development and validation of a prognostic model with a preoperative and postoperative mode. J Thorac Cardiovasc Surg 2006; 132: 491-498.
31. Fujimoto T, Cassivi SD, Yang P, et al. Completely resected N1 non–small cell lung cancer: Factors affecting recurrence and long-term survival. J Thorac Cardiovasc Surg 2006; 132: 499-506.
32. Fukuhara S, Sako K, Noda K, et al. Tie2 is tied at the cell-cell contacts and to extracellular matrix by Angiopoietin-1. Exp Mol Med 2009; 41: 133-139.
33. Huang H, Bhat A, Woodnutt G, et al. Targeting the ANGPT–TIe2 pathway in malignancy. Nat Rev Cancer 2010; 10: 575-585.
34. Takahama M, Tsutsumi M, Tsujiuchi T, et al. Enhanced expression of Tie2, its ligand angiopoietin-1, vascular endothelial growth factor, and CD31 in human non-small cell lung carcinomas. Clin Cancer Res 1999; 5: 2506-2510.
35. Reinmuth N, Piegelbrock E, Raedel M, et al. Prognostic significance of vessel architecture and vascular stability in non-small cell lung cancer. Lung Cancer 2007; 55: 53-60.
36. Andersen S, Donnem T, Al-Shibli K, et al. Prognostic impacts of angiopoietins in NSCLC tumor cells and stroma: VEGF-A impact is strongly associated with Ang-2. PLoS One 2011; 6(5): e19773.
37. Huang Z, Bao SD. Roles of main pro- and anti-angiogenic factors in tumor angiogenesis. World J Gastroenterol 2004; 10: 463-470.
38. Tabchi S, Blais N. Antiangiogenesis for advanced non-small-cell lung cancer in the era of immunotherapy and personalized medicine. Front Oncol 2017; 29: 7-52.

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