

Predictive value of microvascular density for response to anlotinib in advanced NSCLC

Danqing Liu, MS, Guozheng Ding, MS* 

Abstract

Nonsmall cell lung cancer (NSCLC) is the most common type of lung cancer. This study aimed to categorize the microvessels in advanced NSCLC and determine the relationship between intratumoral microvascular density (MVD) and the efficacy of anlotinib for NSCLC.

The clinical data of 68 patients receiving anlotinib as third-line treatment or beyond for advanced NSCLC were retrospectively collected. Microvessels were stained for CD31 and CD34 by using immunohistochemical staining and were classified as undifferentiated (CD31+CD34-) and differentiated vessels (CD31+CD34+). The relationship between MVD and anlotinib efficacy and patient prognosis was analyzed.

Patients were divided into the high or low MVD groups according to the median MVD of differentiated (9.4 vessels/field) and undifferentiated microvessels (6.5 vessels/field). There were significantly more patients with high undifferentiated-vessel MVD in the disease control group than in the disease progression group (72.7% vs 16.7%, $P < .001$). Patients with high undifferentiated-vessel MVD had significantly longer median progression-free survival than those with low undifferentiated-vessel MVD (7.1 vs 3.7 months, $P < .001$).

Anlotinib as third- or beyond line therapy is safe and effective for advanced NSCLC. Patients with a higher density of undifferentiated microvessels have better response to anlotinib and longer progression-free survival.

Abbreviations: CI = confidence interval, MVD = microvascular density, NSCLC = nonsmall cell lung cancer, PFS = progression-free survival, VEGF = vascular endothelial growth factor.

Keywords: anlotinib, efficacy, microvascular density, nonsmall cell lung cancer, prediction, prognosis

1. Introduction

Angiogenesis is essential for development and metastasis of malignant tumors.^[1] Microvascular density (MVD) is a primary biomarker for assessing tumor angiogenesis.^[2] Higher MVD is associated with metastasis in breast cancer and nonsmall cell lung cancer (NSCLC).^[3,4] In addition, MVD can predict patient survival and efficacy of antiangiogenic therapy in NSCLC.^[5,6]

There are differentiated and undifferentiated microvessels in clear cell renal cell carcinoma and NSCLC.^[6,7] The differentiated microvessels have supporting pericytes and are characterized by

the expression of CD34. The undifferentiated microvessels have no supporting pericytes and are characterized by the expression of CD31 but no CD34. A higher density of undifferentiated microvessels was associated with shorter survival in patients with clear cell renal cell carcinoma who were treated by surgery alone,^[7] but was associated with good response to antiangiogenic therapy in NSCLC,^[6] suggesting that undifferentiated microvessels may be more sensitive to antiangiogenic agents and predict better treatment outcomes.

Anlotinib is a novel multi-target antiangiogenic-tyrosine kinase inhibitor and has obtained good outcomes in treating advanced NSCLC.^[8] However, there is still no proved predictive biomarkers of response to anlotinib, which are essential to choose patients who can gain the most from this drug.

The present study aimed to determine the relationship between intratumoral MVD and treatment efficacy of anlotinib in patients with advanced NSCLC.

2. Materials and methods

2.1. Patients

This is a retrospective study analyzing the patients with advanced NSCLC treated between 2018 and 2020 at our hospital. The inclusion criteria were: pathological evidence of NSCLC; disease progression after second-line treatment, with at least one evaluable tumor according to the RECIST (version 1.1); 18 to 80 years of age; Eastern Cooperative Oncology Group performance status of 0 to 1; and received anlotinib as third-line treatment or beyond. Patients with the following conditions were excluded: active bleeding of the primary lesion within the last 3 months; uncontrollable hypertension or diabetes; renal

Editor: Haijun Zhang.

The authors have no funding and conflicts of interest to disclose.

Supplemental Digital Content is available for this article.

The datasets generated and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

Pulmonary and Critical Care Medicine, Anqing Hospital Affiliated to Anhui Medical University, Anhui, China.

* Correspondence: Guozheng Ding, Pulmonary and Critical Care Medicine, Anqing Hospital Affiliated to Anhui Medical University, No. 87, Tianzhu Shandong Road, Yixiu District, Anqing 24600, Anhui, China (e-mail: dgz499@163.com).

Copyright © 2022 the Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial License 4.0 (CCBY-NC), where it is permissible to download, share, remix, transform, and buildup the work provided it is properly cited. The work cannot be used commercially without permission from the journal.

How to cite this article: Liu D, Ding G. Predictive value of microvascular density for response to anlotinib in advanced NSCLC. *Medicine* 2022;101:3(e28647).

Received: 28 August 2021 / Received in final form: 3 December 2021 /

Accepted: 30 December 2021

<http://dx.doi.org/10.1097/MD.00000000000028647>

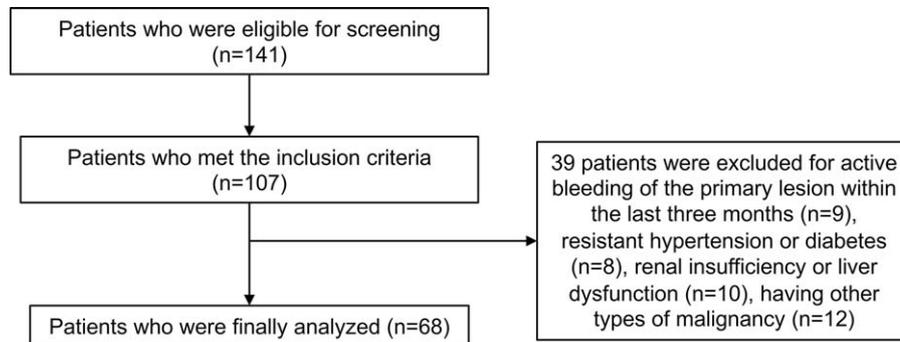


Figure 1. A flow chart of the patient inclusion process.

insufficiency or liver dysfunction; and having other types of malignancy. The patient inclusion flowchart is shown in Figure 1. The study was approved by the Ethical Committee of our hospital (approval number: Ethical review of Medicine [2021] No. 31). Informed consent was waived due to the retrospective nature of the study.

2.2. Treatment protocol

All included patients had disease progression after second- or later-line therapy for NSCLC. Anlotinib (12 mg a day) was orally administered 30 minutes before breakfast for 2 weeks, followed by discontinuation for 1 week. A treatment cycle consisted of 21 days. Doses of anlotinib were reduced to 10, then 8 mg, if severe adverse events occurred, or discontinued if disease progressed. All patients had follow-up.

2.3. Evaluation of treatment response

To assess patient response to anlotinib treatment, chest computed tomography images after 2 cycles of treatment were compared with that before the treatment. According to the RECIST (version 1.1), treatment response was classified into complete remission, partial remission, stable disease, and progressive disease. The objective response rate was the sum of the complete remission rate and partial remission rate. The disease control rate was the sum of the rates of complete remission, partial remission, and stable disease. Progression-free survival (PFS) was the time between the initiation of anlotinib therapy and disease progression or death. Adverse events were graded as I to IV according to the Common Terminology Criteria for Adverse Events (version 4.0).

2.4. Immunohistochemical staining

All tumor specimens were biopsied by using bronchoscopy before the anlotinib therapy. The tissue was fixed in 10% neutral formalin, dehydrated in graded ethanol, embedded in paraffin, and cut into 4- μ m sections. Two continuous sections were stained for CD31 and CD34 separately. Briefly, the section was dewaxed in xylene, rehydrated in graded ethanol, and heated in EDTA for 30 minutes. The section was incubated with the primary antibody (mouse antihuman CD31 or CD34 monoclonal antibody, MXB Biotechnologies, Fuzhou, China) overnight at 4°C. The second antibody was added for 40 minutes and stained with DAB. Nuclei were counter-stained with hematoxylin.

Negative controls were obtained by omitting the primary antibody.

2.5. Quantification of microvessels

The microvessels were counted according to Wang et al.^[9] and Weidner et al.^[10] Any tube-like structure or endothelial cell cluster that was stained as brownish-yellow was considered a microvessel, as long as it had no contact with other cells. Branches of the same vessels were regarded independent microvessels. Three “hotspots” of 400 \times fields with the most CD31+ endothelial cells were selected in a CD31-stained section. The corresponding 3 areas in the continuous CD34-stained section were found. The identical areas in the 2 continuous sections were analyzed to evaluate the differential expression of CD31 and CD34. The CD31+ and CD34+ microvessels in the 3, 400 \times field of each section were counted separately. The MVD of differentiated microvessels was the mean number of CD34+ microvessels of the 3 fields. The MVD of undifferentiated microvessels was calculated by subtracting the mean number of CD34+ microvessels of the 3 fields from that of the CD31+ microvessels. The microvessels were counted by 2 pathologists independently who did not know the diagnosis and treatment protocol. The mean values of the 2 raters were used for analysis.

2.6. Statistical analysis

The relationship between MVD and treatment response to anlotinib was analyzed by using the Chi-square test. Survival was analyzed by using the Kaplan–Meier method, which was compared by using the log-rank test. A two-tailed *P*-value <.05 was considered statistically significant. All analyses were performed by using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Patient general information

A total of 68 patients with advanced NSCLC were included in our study (Fig. 1). The demographic and clinical characteristics of the patients is shown in Table 1.

3.2. Treatment response

The median follow-up time was 5 months (range, 1–16 months). At the last follow-up visit, complete remission was obtained in 0

Table 1
Demographic and clinical characteristics of the patients (n=68).

Characteristics	No. of patients	%
Sex		
Male	59	86.8
Female	9	13.2
Age, yr		
<65	33	48.5
≥65	35	51.5
Smoking status		
Smoker	29	42.6
Nonsmoker	39	57.4
Histology		
Adenocarcinoma	46	67.6
Squamous cell carcinoma	22	32.4
Clinical stage		
IIIb	14	20.6
IV	54	79.4
ECOG performance status		
0	24	35.3
1	44	64.7
EGFR gene mutation		
Yes	7	10.3
No	61	89.7
Brain metastasis		
Yes	27	39.7
No	41	60.3

ECOG=Eastern Cooperative Oncology Group, EGFR=epidermal growth factor receptor.

patient, partial remission in 7, stable disease in 37, and progressive disease in 24. Disease control (complete remission, partial remission, and stable disease) was obtained in 44 patients. The objective response rate (complete remission and partial remission) was 10.3% and the disease control rate was 64.7%. The median PFS was 5.0 months (95% confidence interval [CI]: 3.6–6.4 months) (Fig. 2).

3.3. Adverse effects

All 68 patients were evaluable for adverse effects. No patient died during the follow-up. The most common adverse effects were hypertension (38.2%), fatigue (36.8%), and hand-foot syndrome (36.8%) (Table 2). The dose of anlotinib was reduced to 8 mg a day in 1 patient for resistant hypertension and proteinuria, to 10 mg day in another patient for severe vomiting and fatigue. The drug was discontinued in 2 patients for gastrointestinal bleeding and hematuria, respectively.

3.4. Histological results

There was more CD31+ microvessels than the CD34+ microvessels. All microvessels were stained positive for CD31. The undifferentiated microvessels had small lumens or no lumens and were positive for CD31 but negative for CD34 (Fig. 3). The differentiated microvessels were positive for both CD31 and CD34.

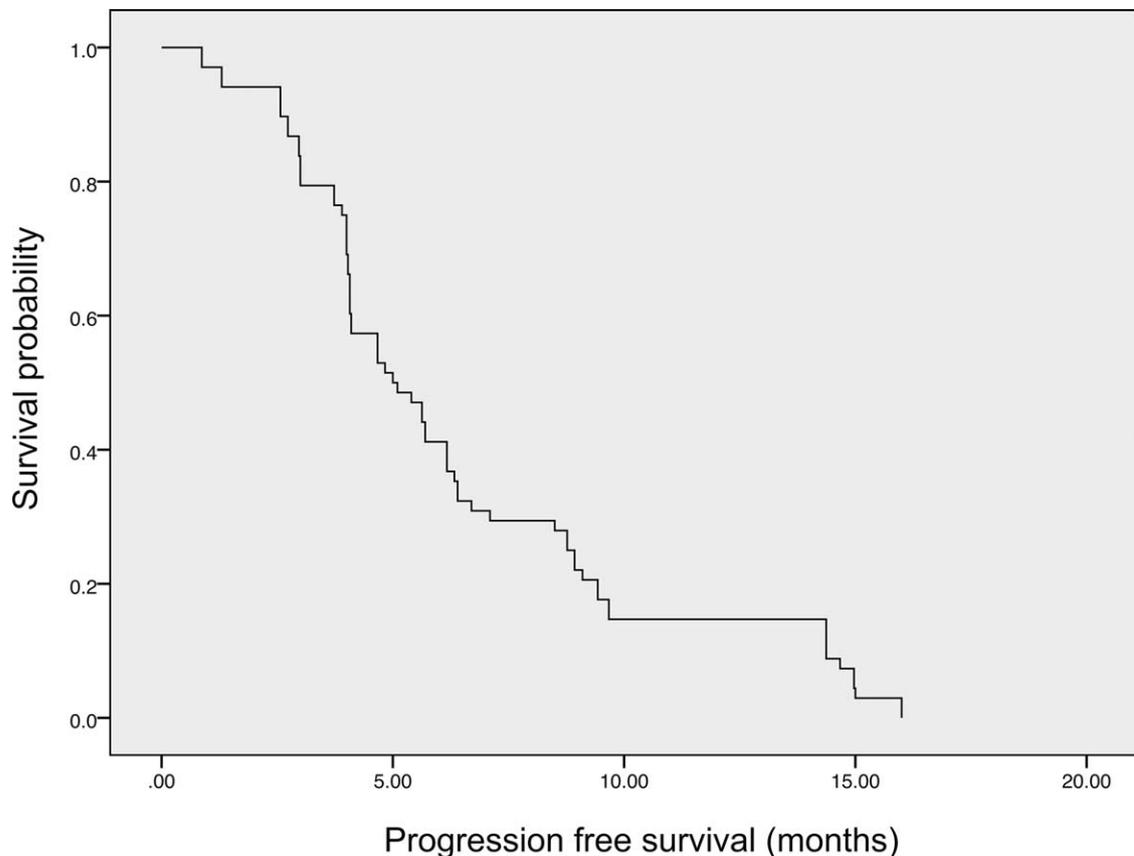


Figure 2. The Kaplan–Meier curve of progression-free survival of the patients.

Table 2
Number of patients who had adverse effects [n (%)].

Adverse effects	≤Grade II	Grade III	Grade IV	Total (n=68)
Hypertension	25 (36.8)	0	1 (1.5)	26 (38.2)
Fatigue	24 (35.3)	1 (1.5)	0	25 (36.8)
Hand-foot syndrome	25 (36.8)	0	0	25 (36.8)
Proteinuria	6 (8.8)	1 (1.5)	0	7 (10.3)
Hypothyroidism	1 (1.5)	0	0	1 (1.5)
Elevated total bilirubin	1 (1.5)	0	0	1 (1.5)
Elevated alanine aminotransferase	1 (1.5)	0	0	1 (1.5)
Elevated aspartate aminotransferase	1 (1.5)	0	0	1 (1.5)
Elevated total cholesterol	1 (1.5)	0	0	1 (1.5)
Oral ulcer	9 (13.2)	0	0	9 (13.2)
Vomiting	4 (5.9)	0	0	4 (5.9)
Loss of appetite	1 (1.5)	0	0	1 (1.5)
Oral mucositis	5 (7.4)	0	0	5 (7.4)
Gastrointestinal bleeding	0	0	1 (1.5)	1 (1.5)
Hematuria	0	1 (1.5)	0	1 (1.5)

3.5. Relationship between MVD and treatment response

Patients were divided into the high or low MVD groups according to the median MVD of differentiated (9.4 vessels/field) and undifferentiated microvessels (6.5 vessels/field). No significant difference in the various demographic and clinical characteristics was found between patients with high or low MVD (Tables S1 and S2, Supplemental Digital Content, <http://links.lww.com/MD2/A845>, <http://links.lww.com/MD2/A846>).

There were significantly more patients with high undifferentiated-vessel MVD in the disease control group than in the disease progression group (72.7% vs 16.7%, $P < .001$). No significant difference was found in the proportion of patients with high differentiated-vessel MVD between the disease control group and the disease progression group (52.3% vs 45.8%, $P = .612$) (Table 3).

3.6. Relationship between MVD and survival

Patients with high undifferentiated-vessel MVD had significantly longer median PFS than those with low undifferentiated-vessel MVD (7.1 months [95% CI: 4.3–9.9] vs 3.7 months [95% CI: 2.9–4.5], $P < .001$; Fig. 4A). However, no significant difference in

median FPS was found between patients with high differentiated-vessel MVD and those with low differentiated-vessel MVD (4.7 months [95% CI: 3.1–6.2]) vs 5.0 months [95% CI: 3.3–6.7], $P = .514$; Fig. 4B).

4. Discussion

Lung cancer now ranks the second most common cancer worldwide, accounting for 11.4% of new cancer cases and 18% of cancer mortality.^[11] Due to the low availability of early screening in developing countries, substantial lung cancer patients are diagnosed at an advanced stage.^[12]

Angiogenesis is essential for the development and progression of lung cancer.^[11] Various factors play critical roles in cancer angiogenesis, including vascular endothelial growth factor (VEGF) and its receptor.^[13] Anlotinib is a multi-target tyrosine kinase inhibitor targeting VEGFR 2/3, fibroblast growth factor receptors 1-4 (FGFR1-4), platelet-derived growth factor receptors (PDGFR) α/β , c-Kit, and Ret.^[14,15] Two clinical trials showed significant extend in PFS and overall survival in patients with advanced NSCLC after treatment with anlotinib as third-line therapy.^[16,17] However, some patients had adverse effects

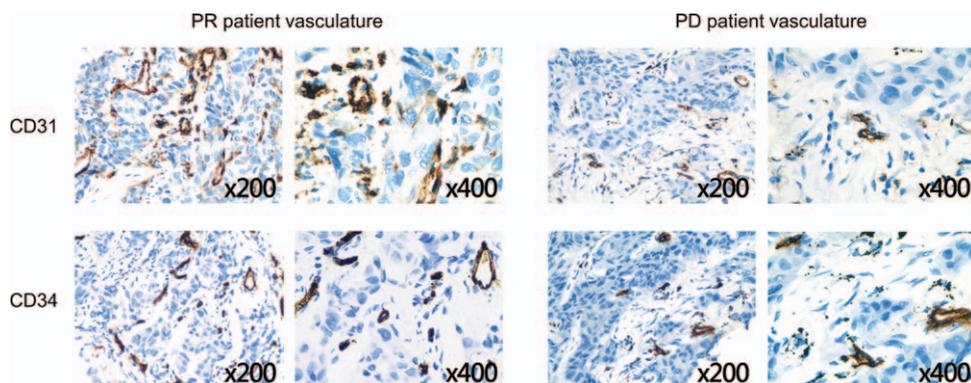


Figure 3. CD31 and CD34 expression in advanced nonsmall cell lung cancer tissue of a patient with PR and a patient with PD. PR = partial remission. PD = progressive disease.

Table 3**Relationship between MVD and treatment response to anlotinib.**

	Disease control group (n=44)	Disease progression group (n=24)	P-value
Undifferentiated vessels (CD31+CD34-)			<.001
High MVD	32	4	
Low MVD	12	20	
Differentiated vessels (CD31+CD34+)			.612
High MVD	23	11	
Low MVD	21	13	

MVD = microvascular density.

and poor response to anlotinib. Therefore, predicting treatment efficacy of anlotinib is greatly needed.

MVD is a widely used parameter for assessing tumoral microvasculature. High MVD is associated with poor prognosis in cancers of the breasts, prostate gland, nasopharynx, and kidneys.^[7,18–20] A 2002 meta-analysis found that high MVD was associated with poor prognosis in NSCLC, regardless of factor VIII, CD31, or CD34 being used to label the vessels.^[21] However, this study only included surgically treated patients who had early-stage NSCLC but no patients with advanced NSCLC who are treated with antiangiogenic therapy.

Tumoral vessels may arise from bone marrow stem cells or tissue stem cells. Different origins of tumor vessels lead to endothelial cell differentiation and vascular variation.^[22] Differentially differentiated vessels have been found in cancers of the prostate gland, kidneys, lungs, and brain by using various vascular biomarkers.^[7,9,19,23] For instance, only the density of undifferentiated microvessels is associated with the grade and survival of patients with clear cell renal cell carcinoma.^[7] These studies all demonstrate that the different types of tumoral microvessels have different prognostic values.

Suppressing VEGF can induce selective ablation of immature vessels but not mature vessels.^[24] Periendothelial cells around the mature vessels may promote the survival of endothelial cells by secreting VEGF, which are not present around the immature vessels.^[25,26] Patients with lung adenocarcinoma of high undifferentiated-vessel MVD were more sensitive to bevacizumab.^[6] Another study found that recombinant human vascular endothelial growth inhibitor significantly reduced the number of

CD31+ but not CD34+ vessels in a mouse model of lung cancer, suggesting that the immature microvessels can be inhibited by endostatin.^[27]

Our study analyzed the clinical data of 30 patients with advanced NSCLC who were treated with anlotinib. The tumoral vasculature was stained for CD31 and CD34. We found that there were more CD31+ vessels than CD34+ vessels. Two types of microvessels were recognized in our study, the differentiated (CD31+/CD34+) and the undifferentiated (CD31+/CD34-). We also found that patients with high undifferentiated-vessel MVD had better response to anlotinib and longer PFS than those with low-undifferentiated vessel MVD. Therefore, the undifferentiated-vessel MVD may be used as a predictive factor for beyond-third-line anlotinib therapy for advanced NSCLC.

The most common adverse effects of anlotinib in our study were hypertension (38.2%), fatigue (36.8%), and hand-foot syndrome (36.8%). Our results were consistent with a previous phase III clinical trial of anlotinib in advanced NSCLC.^[28] Most of the anlotinib-associated adverse effects were manageable, and only 4 patients required dose reduction or drug discontinuation in our study.

Our study has some limitations. First, the objective response rate, disease control rate, and median PFS in our study were all lower than those in the ALTER0303 study.^[17] This may be attributed to the small number of patients and the beyond-third-line anlotinib therapy in some patients. Second, the follow-up time was short and the overall survival was not evaluated. Third, the included patients were heterogenous in terms of baseline status and previous chemotherapy and must had an Eastern

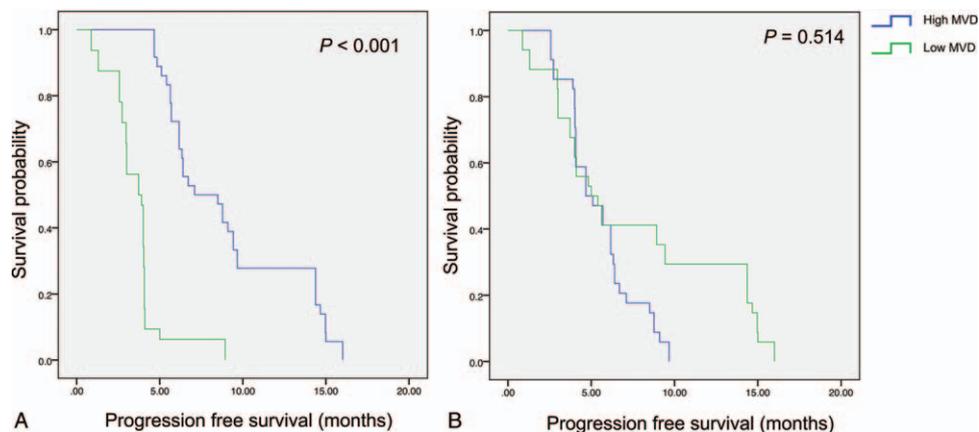


Figure 4. Progression-free survival curves of patients with high or low undifferentiated-vessel (A), and high or low differentiated-vessel (B) microvascular density. MVD = microvascular density.

Cooperative Oncology Group score of 0 to 1. This may introduce bias and limit the representativeness of our patients.

In conclusion, our study confirmed the existence of CD31+ CD34+ differentiated microvessels and CD31+ CD34− undifferentiated microvessels in advanced NSCLC. Patients with high undifferentiated-vessel MVD had better response to third-or later-line anlotinib therapy and longer PFS.

Author contributions

Conceptualization: Guozheng Ding.

Data curation: Danqing Liu.

Formal analysis: Danqing Liu.

Methodology: Guozheng Ding.

Project administration: Guozheng Ding.

Resources: Danqing Liu.

Visualization: Guozheng Ding.

Writing – original draft: Danqing Liu.

Writing – review & editing: Danqing Liu, Guozheng Ding.

References

- [1] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
- [2] Hasan J, Byers R, Jayson GC. Intra-tumoural microvessel density in human solid tumours. *Br J Cancer* 2002;86:1566–77.
- [3] Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA. Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet* 1992;340:145–6.
- [4] Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991;324:1–8.
- [5] Fontanini G, Lucchi M, Vignati S, et al. Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. *J Natl Cancer Inst* 1997;89:881–6.
- [6] Zhao Y-Y, Xue C, Jiang W, et al. Predictive value of intratumoral microvascular density in patients with advanced non-small cell lung cancer receiving chemotherapy plus bevacizumab. *J Thorac Oncol* 2012;7:71–5.
- [7] Yao X, Qian C-N, Zhang Z-F, et al. Two distinct types of blood vessels in clear cell renal cell carcinoma have contrasting prognostic implications. *Clin Cancer Res* 2007;13:161–9.
- [8] Ye H, Li Z, Liu K, Zhang F, Cheng Z. Anlotinib, a novel TKI, as a third-line or further-line treatment in patients with advanced non-small cell lung cancer in China: a systemic review and meta-analysis of its efficacy and safety. *Medicine (Baltimore)* 2021;100:e25709.
- [9] Wang HC, Wang BD, Chen MS, et al. An underlying pathological mechanism of meningiomas with intratumoral hemorrhage: undifferentiated microvessels. *World Neurosurg* 2016;94:319–27.
- [10] Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;84:1875–87.
- [11] Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;71:209–49.
- [12] Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016;66:115–32.
- [13] Ferrara N. Molecular and biological properties of vascular endothelial growth factor. *J Mol Med (Berl)* 1999;77:527–43.
- [14] Lin B, Song X, Yang D, Bai D, Yao Y, Lu N. Anlotinib inhibits angiogenesis via suppressing the activation of VEGFR2, PDGFR β and FGFR1. *Gene* 2018;654:77–86.
- [15] Sun Y, Niu W, Du F, et al. Safety, pharmacokinetics, and antitumor properties of anlotinib, an oral multi-target tyrosine kinase inhibitor, in patients with advanced refractory solid tumors. *J Hematol Oncol* 2016;9:105.
- [16] Han B, Li K, Zhao Y, et al. Anlotinib as a third-line therapy in patients with refractory advanced non-small-cell lung cancer: a multicentre, randomised phase II trial (ALTER0302). *Br J Cancer* 2018;118:654–61.
- [17] Qin T, Liu Z, Wang J, et al. Anlotinib suppresses lymphangiogenesis and lymphatic metastasis in lung adenocarcinoma through a process potentially involving VEGFR-3 signaling. *Cancer Biol Med* 2020;17:753–67.
- [18] Kato T, Kameoka S, Kimura T, Nishikawa T, Kobayashi M. The combination of angiogenesis and blood vessel invasion as a prognostic indicator in primary breast cancer. *Br J Cancer* 2003;88:1900–8.
- [19] de la Taille A, Katz AE, Bagiella E, et al. Microvessel density as a predictor of PSA recurrence after radical prostatectomy. A comparison of CD34 and CD31. *Am J Clin Pathol* 2000;113:555–62.
- [20] Qian CN, Min HQ, Liang XM, Zheng SS, Lin HL. Primary study of neovascularity correlating with metastatic nasopharyngeal carcinoma using computer image analysis. *J Cancer Res Clin Oncol* 1997;123:645–51.
- [21] Meert AP, Paesmans M, Martin B, et al. The role of microvessel density on the survival of patients with lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer* 2002;87:694–701.
- [22] Bussolati B, Grange C, Camussi G. Tumor exploits alternative strategies to achieve vascularization. *FASEB J* 2011;25:2874–82.
- [23] Mineo TC, Ambrogio V, Baldi A, et al. Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB-IIA non-small cell lung cancer. *J Clin Pathol* 2004;57:591–7.
- [24] Benjamin LE, Golijanin D, Itin A, Podes D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 1999;103:159–65.
- [25] Gee MS, Procopio WN, Makonnen S, Feldman MD, Yeilding NM, Lee WMF. Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. *Am J Pathol* 2003;162:183–93.
- [26] Darland DC, Massingham LJ, Smith SR, Piek E, Saint-Geniez M, D'Amore PA. Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Dev Biol* 2003;264:275–88.
- [27] Fu W, Zhuo J, Hu L. Differential effects of recombinant human endostatin treatment on differentiated and undifferentiated blood vessels in Lewis lung cancer. *Oncol Lett* 2017;13:196–200.
- [28] Si X, Zhang L, Wang H, et al. Management of anlotinib-related adverse events in patients with advanced non-small cell lung cancer: Experiences in ALTER-0303. *Thorac Cancer* 2019;10:551–6.