Original Article

Modified microvessel density based on perfusion distance: a preferable NSCLC prognostic factor

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Background: Despite the vital role of blood perfusion in tumor progression, the prognostic value of typical blood perfusion markers, such as microvessel density (MVD) or microvessel area (MVA), in patients with non-small cell lung cancer (NSCLC) is still unclear. This study established a modified MVD (mMVD) measurement based on perfusion distance and determined its prognostic value in patients with NSCLC.

Methods: A total of 100 patients with NSCLC were enrolled in this retrospective study. The intratumor microvessels of NSCLC patients were visualized using immunohistochemical staining for CD31. The blood perfusion distance was evaluated as the distance from each vessel to its nearest cancer cell (D_{mvcc}), and the cutoff value for prognosis was determined. Apart from the total MVD (tMVD), microvessels near cancer cells within the cutoff-D_{mvcc} were counted as mMVD. Predictive values for mortality and recurrence were evaluated and compared.

Results: The D_{mvcc} ranged from 1.6 to 269.8 μm (median, 13.1 μm). The mMVD (range: 2–70; median 23) was counted from tMVD according to the cutoff-D_{mvcc} (~20 μm). Compared with tMVD, a larger fraction of mMVD (80% *vs.* 2.9%) played a significant role in overall survival, with an improved area under the receiver operating characteristic (ROC) curve (AUC) (0.74 *vs.* 0.56). A high mMVD was an independent positive indicator of overall survival (OS) and progression-free survival (PFS). In contrast, tMVD was only related to PFS at the optimal cutoff.

Conclusions: Perfusion-distance-based mMVD is a promising prognostic factor for NSCLC patients with superior sensitivity, specificity, and clinical applicability compared to tMVD. This study provides novel insights into the prognostic role of tumor vessel perfusion in patients with NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); vascular perfusion distance; microvessel density; microvessel distance; prognosis marker

Submitted Nov 10, 2021. Accepted for publication Jan 05, 2022.
doi: 10.21037/atm-21-6566
View this article at: https://dx.doi.org/10.21037/atm-21-6566

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Introduction

Blood perfusion is crucial for tumorigenesis and progression (1-4). Furthermore, vascular remodeling can improve drug delivery (5). Thus, blood perfusion profile-based biomarkers, including microvessel density (MVD) and microvessel area (MVA), have emerged as mainstream markers of patient survival and outcomes, and may be predictive of patient prognosis (6-11). However, their prognostic value in terms of patient survival in various types of solid tumors (12,13), including non-small cell lung cancer (NSCLC), has been controversial (7,9,14-16) . This is not unexpected because the quantity of microvessels is only one of many decisive parameters reflecting perfusion efficiency. In addition to MVD and MVA, blood perfusion efficiency is based on vessel diffusion distance, blood flow rate, and resistance to blood perfusion in the extracellular matrix (ECM) (17), especially in desmoplastic tumors (18,19).

In most solid tumors, cancer cell niches are surrounded by a rich desmoplastic stroma, which separates cancer cells from microvessels (3,18,20). The diffusion and convection of nutrients and oxygen crossing the stroma to the cancer cells was found to be impaired by matrix-fibro and high interstitial fluid pressure caused by the dense extracellular matrix (18,20). Elongated distances can result in significant deficiencies in perfusion of the tumor (17,21,22). Therefore, microvessels near cancer niches likely allow superior perfusion compared to those far away from cancer cells. Our previous study determined the diffusion distances between microvessels and their nearest cancer cells, defined as microvessels near cancer cells, D_{mvcc}. Long distances were shown to be strongly associated with poor survival (23). However, D_{mvcc} cannot accurately reflect the MVD. Herein, we hypothesized that the density of microvessels near cancer cells may be more representative of the real perfusion system and a more powerful prognostic factor compared with MVD.

In this study, a modified MVD (mMVD) value was defined as the density of microvessels within the optimal cutoff of D_{mvcc} (23). The mMVD was shown to be a preferable prognostic prediction marker for NSCLC patients compared to total MVD. The following article is presented in accordance with the REMARK reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6566/rc).

Methods

Patients and samples

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by institutional ethics committee of Zhejiang Cancer Hospital (No. IRB-2017-67). Individual patient consent for this study was waived due to the retrospective nature of this investigation.

This retrospective study included a total of 100 patients who underwent surgical resection for the treatment of NSCLC at Zhejiang Cancer Hospital (Hangzhou, China) between July 2011 and October 2012. The inclusion criteria were as follows: (I) the patients were histologically diagnosed with primary NSCLC; (II) the patients underwent surgical resection and had available tumor tissue of the primary lesion; (III) the patients with full clinicopathologic information. All clinicopathological characteristics and survival outcome data were available. Tumor stage, histology, and differentiation classifications were performed in accordance with the tumor, node, and metastasis (TNM) system or the World Health Organization (WHO) criteria (24). The overall survival (OS) was calculated from the day of diagnosis until death or the last follow-up. Progression-free survival (PFS) was recorded from the day of diagnosis until evidence of recurrence was observed. Tumor recurrence was monitored using abdominal computed tomography (CT), abdominal ultrasonography, magnetic resonance imaging (MRI), and chest X-ray examinations. The last data of follow-up was July 20th, 2016.

Immunohistochemistry

Paraffin-embedded tumor tissues were consecutively cut into 5 μm thick sections and processed for immunohistochemical staining using the endothelial cell marker CD31, as described previously (23). Briefly, Antigen retrieval was carried out using the EnVisionTM FLEX Target Retrieval Solution (pH =9.0, Dako), followed by incubation with a rabbit polyclonal CD31 antibody (dilution: 1:300, Proteintech, Rosemont, USA) at 4 ℃ overnight. Sections were then incubated with the secondary antibody (PV-9000, Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1 hour, followed by color development with

3,3'-diaminobenzamine in Tris-HCl (50 mmol/L, pH =7.5) containing 0.005% hydrogen peroxide, and counterstaining with hematoxylin.

Assessment of D_{mvcc}, tMVD, and mMVD

The immunohistochemically stained tumor tissue sections were observed under a microscope at 100× magnification. According to a previously described method (25,26), the field showing the most intense vascularization was selected as the "hotspot". Two experienced investigators measured the D_{mvcc} as the distance from each microvessel to its nearest cancer cell within the hotspot, at a 200× magnification. The optimal D_{mvcc} cutoff value for prognostic prediction was calculated using Cutoff Finder (27), an online software developed by the Translational Tumor Research Team at the Institute of Pathology, Charité-Universitätsmedizin Berlin (version 2.1, January 8, 2013, available at https://molpathoheidelberg.shinyapps.io/CutoffFinder_v1/). Subsequently, the number of total microvessels or microvessels within the cutoff-D_{mvcc} from the cancer niche was determined as tMVD or mMVD, respectively, as previously described (25,26).

Statistical analysis

Statistical analyses were conducted using SPSS software (Version 23.0, IBM Inc., New York, USA), Cutoff Finder (27) (Version 2.1, Institute of Pathology, Berlin, Germany) and GraphPad Prism software (version 7.00, GraphPad Software, La Jolla, CA, USA). Categorical data are expressed as counts and percentages, while continuous data are expressed as median values and ranges. Where suitable, Student's *t*-test, and linear regression analysis were performed. To evaluate the strength of the prognostic prediction of D_{mvcc}, tMVD, and mMVD, each possible cutoff was investigated separately and a Cox proportional hazard model was fitted to each of the corresponding groups of patients. The corresponding hazard ratios (HRs) were plotted for all cutoff points. The optimal cutoff point was defined as the point with the most significant split (log-rank test). Hazard ratios (HRs), including 95% confidence intervals (CIs), were calculated. Patients were divided into groups based on tMVD and mMVD, and the survival curves were computed using the Kaplan-Meier method and compared using the log-rank test. Multivariate analysis was performed using the Cox regression model to assess the predictive potential of each factor independent of other clinical characteristics. A two-tailed P value <0.05 was considered statistically significant.

Results

Patients

A total of 100 patients, including 74 males and 26 females, with a median age of 59 years (range: 40–79 years) were included in this study (*Table 1*). The majority of patients (71/100) presented with early-stage NSCLC (stages I and II). The differentiation status was classified into 3 categories, namely, poorly differentiated (n=49), moderately differentiated (n=47), and well-differentiated (n=1). There were 54 cases of adenocarcinomas and 43 cases of squamous carcinomas. The median follow-up time was 51.1 months (range: 45.5–60.0 months). At the end of the follow-up period, 29 (29%) patients died. Eight patients were lost to follow-up and 52.2% of the remaining patients (48/92) experienced recurrence.

D_{mvcc} profile

The value of D_{mvcc} in the tumor samples varied from 1.61–269.75 μm, with a median value of 13.10 μm. No significant differences in D_{mvcc} values were observed between subpopulations stratified by age (P=0.768), gender (P=0.133), smoking history (P=0.168), disease stage (P=0.834), histology (P=0.052), nor differentiation status (P=0.887). The optimal D_{mvcc} cutoff value for prognosis was determined to be 20 μm.

tMVD and mMVD profiles

Representative images of tMVD are presented in *Figure 1A*. The median values for tMVD and mMVD were 38 (range: 17–81) and 23 (range: 2–70), respectively (*Table 1* and *Figure 1B*). Patients presenting with different disease stages showed significantly different tMVD (P=0.003) and mMVD values (P=0.019) (*Table 1* and *Figure 1C*). However, when subpopulations were stratification by age, gender, smoking history, histology, or differentiation status, there were no significant differences among the tMVD (P=0.134–0.775) nor mMVD values (P=0.455–0.891). The mMVD values were 2.6–98.6% of the tMVD values, with a median ratio of 72.5%, which was remarkably lower than that of tMVD, and was independent of clinicopathological characteristics.

Prognostic strength and significance of the mMVD and tMVD values

The strength and significance of the tMVD and the mMVD for prognostic stratification were demonstrated

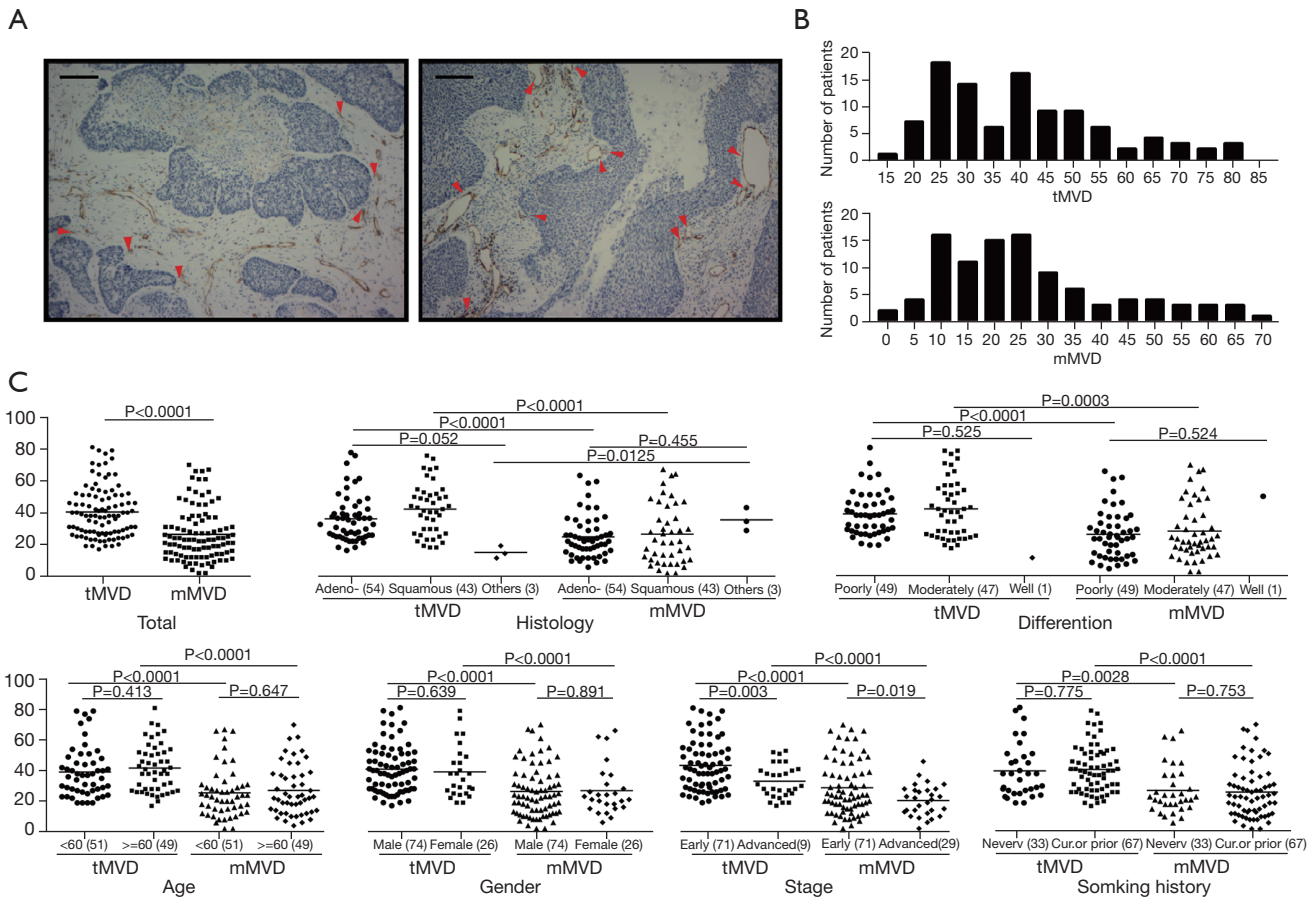

Figure 1 The total and modified microvessel densities in non-small cell lung cancer patients. (A) Tumor tissues were immunohistochemically stained with the endothelial cell marker CD31. Microvessels within a 20 μm distance from cancer cells are marked with a red triangle (bar, 50 μm). (B) A histogram depicting the tMVD and mMVD. (C) Scatter plots showing the tMVD and mMVD values in patients classified according to various clinicopathological characteristics, such as age, gender, smoking history, tumor histology, tumor differentiation, and disease stage. tMVD, total microvessel density; mMVD, modified MVD; NSCLC, non-small cell lung cancer.

using both receiver operating characteristic (ROC) curves and significance test plots (*Figure 2A*). The area under the ROC curve (AUC) for OS analysis was 0.56 for tMVD (with sensitivity of 81.2% and specificity of 34.5%) and 0.74 for mMVD (sensitivity of 87.3% and specificity of 51.7%). In contrast, the AUC values for PFS analysis were comparable (0.63 and 0.66 for tMVD and mMVD, respectively). Only 2.9% (1 out of 34) and 27.3% (9 out of 33) of the investigated tMVD cutoff points were significantly correlated with OS and PFS, respectively. In contrast, 80% and 58.8% of the mMVD cutoff points were significantly correlated with OS and PFS, respectively. The optimal cutoff values of mMVD for OS and PFS were determined as 13 and 34, respectively, while those of tMVD were 26.5, for both OS and PFS (*Figure 2B*).

Univariate survival analysis

To further evaluate the prognostic value of MVD, the population was stratified based on the optimal MVD cutoff or median MVD. As shown in *Figure 3A*, the tMVD was not a satisfactory prognostic predictor for OS, either dichotomized by the optimal cutoff value (HR =0.45, 95% CI: 0.28 to 1.14, P=0.269) or the median value (HR =0.80, 95% CI: 0.39 to 1.66, P=0.409). Instead, the mMVD was a promising prognostic predictor of OS, either dichotomized by the optimal cutoff value (HR =0.21, 95% CI: 0.081 to 0.53, P<0.0001) or the median value (HR =0.22, 95 % CI: 0.11 to 0.46, P=0.0003). The PFS was significantly correlated with both the optimal cutoff value of mMVD (HR =0.25, 95% CI: 0.14 to 0.46, P=0.0005) and the tMVD

Table 1 Patient clinicopathologic characteristics and microvessel parameters

Clinicopathologic variables	Number (%)	D_{mvcc} (μm)†		MVD				
		D_{mvcc}	P value‡	tMVD	P value‡	mMVD	P value‡	P value§
Age (years)								
<60	51 (51.0)	25.3±5.5	0.768	39.1±2.3	0.413	25.6±2.3	0.647	<0.0001***
≥60	49 (49.0)	23.4±3.8		41.7±2.3		27.1±2.4		<0.0001***
Gender								
Male	74 (74.0)	27.4±4.3	0.133	40.8±1.8	0.639	26.2±1.9	0.891	<0.0001***
Female	26 (26.0)	15.9±3.4		39.1±3.4		26.7±3.2		0.0098
Smoking history								
Never	33 (33.0)	17.8±3.4	0.168	39.7±3.0	0.775	27.1±2.7	0.753	0.0028
Prior or current	67 (67.0)	27.6±4.7		40.7±1.9		26.0±2.1		<0.0001***
Disease stage								
Early (stage I & II)	71 (71.0)	23.9±4. 5	0.834	43.4±2.0	0.003**	28.8±2.116	0.019*	<0.0001***
Advanced (stage III & IV)	29 (29.0)	25.5±3.6		33.0±2.0		20.34±1.895		<0.0001***
Tumor histology								
Adenocarcinoma	54 (54.0)	17.2±2.7	0.052	37.7±2.1	0.134	25.89±1.879	0.455	<0.0001***
Squamous	43 (43.0)	33.7±6.8		44.1±2.5		27.67±2.983		<0.0001***
Others	3 (3.0)	20.2±9.3		37.0±4.4		15.67±2.333		0.0125
Tumor differentiation								
Poorly	49 (49.0)	23.6±3.0	0.887	38.8±2.0	0.525	25.8±2.187	0.524	<0.0001***
Moderately	47 (47.0)	22.7±5.8		42.0±2.7		27.94±2.603		0.0003***
Well	1 (1.0)	153.0		50.0		11.0		
		24.4±3.3		40.4±1.59		26.4±1.64		<0.0001***

†, D_{mvcc} represents the distance from microvessels to the nearest cancer cell; ‡, intra-group difference; §, difference between tMVD and mMVD; *, P<0.05; **, P<0.01; ***, P<0.001. tMVD, total microvessel density; mMVD, modified microvessel density based on perfusion distance.

(HR =0.42, 95% CI: 0.19 to 0.93, P=0.0037). However, no significant correlations with either the median value of mMVD (HR =0.66, 95% CI: 0.36 to 1.22, P=0.151) nor the tMVD value (HR =0.68, 95% CI: 0.38 to 1.19, P=0.172) were observed. Moreover, the strength of the mMVD in survival prediction was verified in subgroup survival analysis with most clinicopathological characteristics (*Figure 3B*).

Multivariate survival analysis

Cox's proportional hazard estimation was conducted to assess the independent prognostic values of tMVD and mMVD for survival outcomes. As shown in *Figure 4*, higher mMVD, regardless of either cut-off or median setting, robustly predicted both longer OS (cutoff value: HR =0.16, 95% CI: 0.07 to 0.37, P<0.001; median value: HR =0.26, 95% CI: 0.10 to 0.67, P=0.005) and PFS (cutoff value: HR =0.31, 95% CI: 0.12 to 0.80, P=0.013; median value: HR =0.40, 95% CI: 0.21 to 0.76, P=0.005), and this was independent of other clinicopathologic characteristics. In contrast, tMVD was not an independent predictor of disease-related death (cutoff value: HR =0.47, 95% CI: 0.21 to 1.05, P=0.066; median value: HR =1.30, 95% CI: 0.58 to 2.92, P=0.530) nor recurrence (median value: HR =1.02, 95% CI: 0.55 to1.91, P=0.946), Interestingly, patients with higher tMVD stratified by optimized cutoff

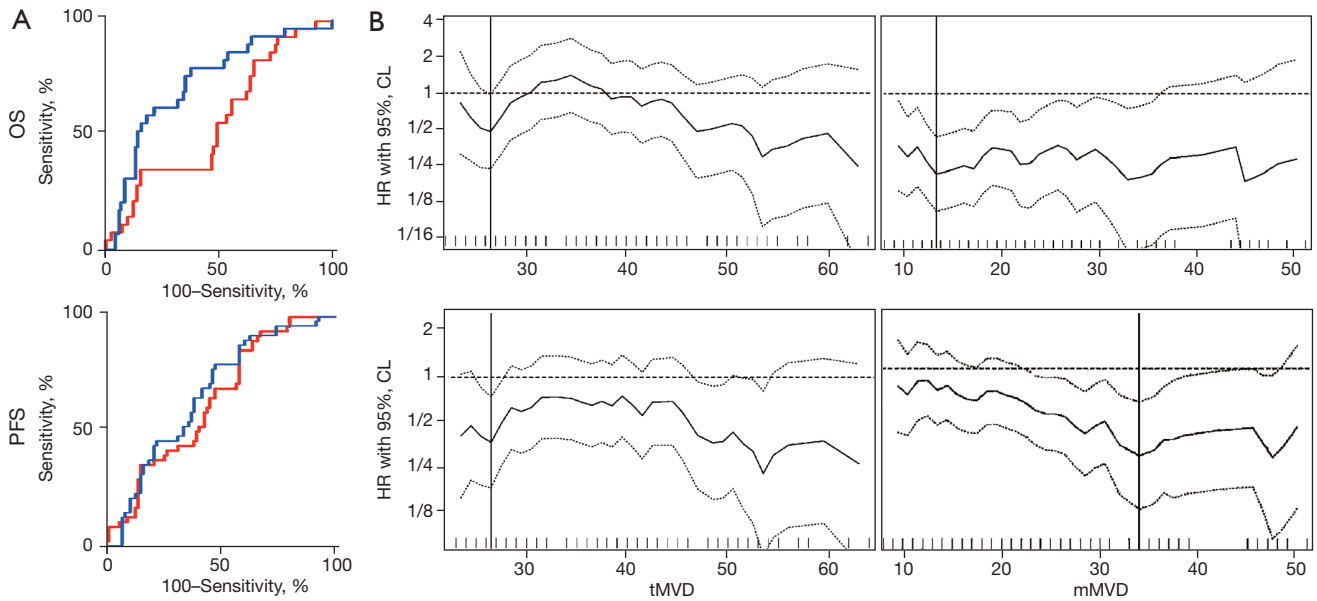

Figure 2 The strength, significance, and cutoff-value optimization of the tMVD and mMVD for prognostic stratification. (A) Sensitivity test: ROC curves predicting mortality (upper panel) and recurrence (lower panel) show improved strength with mMVD (blue line), especially for mortality, The mMVD curve shows a higher AUC (0.74 *vs.* 0.56) and improved specificity (51.7% *vs.* 34.5%) compared with tMVD curve (red line). (B) Significance test: The hazard ratio (HR, middle line, high- *vs.* low-level MVD) of OS and PFS at various cutoff values of tMVD or mMVD (bottom of the figures) is demonstrated with its upper (upper line) and lower limits (lower line) of 95% CI. A significant role of mMVD in prognosis indicates that it is robust over a larger range of cutoff point choices compared with tMVD (80% *vs.* 2.9%). The vertical line designates the dichotomization showing the most significant correlation with survival. tMVD, total microvessel density; mMVD, modified MVD; ROC, receiver operating characteristic; AUC, area under the ROC curve; OS, overall survival; PFS, progression-free survival; CI, confidence interval.

were associated with longer PFS (HR =0.29, 95% CI: 0.14 to 0.59, P=0.001). In addition, advanced stage of disease independently predicted a higher risk of disease-related mortality and recurrence.

Discussion

Blood perfusion, as a major contributor to tumor progression, has a profound influence on nutrient supply, oxygen diffusion, and drug delivery in solid tumors. Therefore, biological factors based on the intratumor blood perfusion system, including MVD and MVA, have been theoretically proposed as potential prognostic markers (17). Nevertheless, to date, none have been proven to be a reliable and intrinsic prognostic marker across different types of solid tumors (17). This may be partly attributed to the fact that they are not absolute determinant factors of intratumor blood perfusion, and at least the distance from microvessels to cancer niches plays a crucial role in perfusion deficiency, but was is not considered in evaluation of MVD or MVA. The present study identified complex contributors to perfusion deficiency, including vascular density and perfusion distance. The results revealed that the mMVD of a perfusion distance-confined sub-cluster of microvessels, which is in close proximity to cancer cells (within 20 μm), was inversely correlated with OS and PFS. Moreover, it was shown to have greater prognostic prediction power compared to tMVD.

Blood perfusion efficiency is determined by multiple factors, including microvessel density, vessel diffusion distance, blood flow rate, and resistance to blood perfusion in the ECM (17). In particular, in tumor tissue desmoplastic stroma between cancer-niches and microvessels, the delivery of oxygen, nutrients, and drugs can be significantly hampered by a thick ECM and high-levels of interstitial fluid pressure (IFP) (18,19). Therefore, a short D_{mvcc} is considered fundamental for efficient perfusion (17). For instance, the oxygen partial pressure (pO_2) is inversely

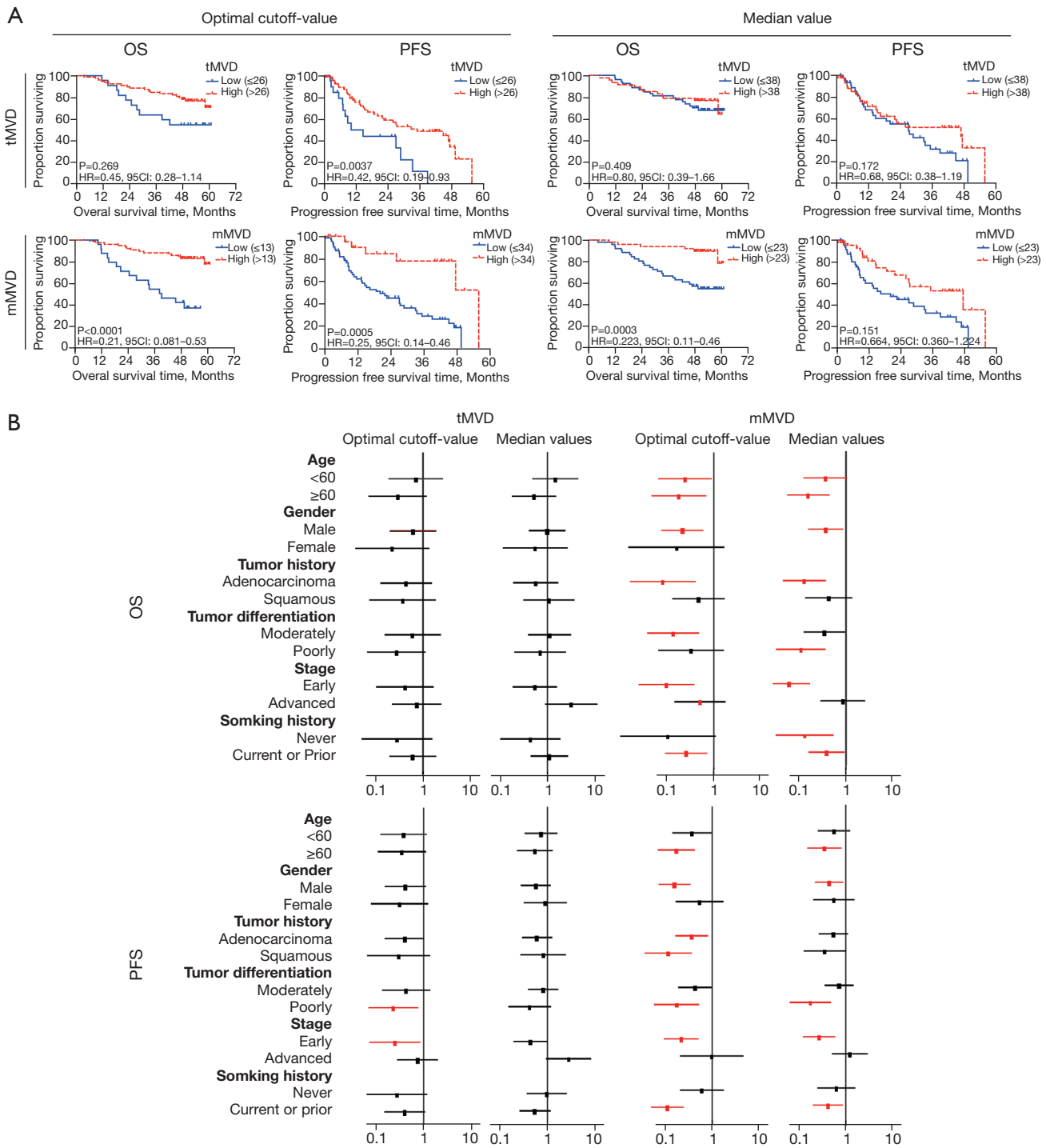

Figure 3 Univariate survival analysis of OS and PFS for subpopulations stratified by tMVD, mMVD, and clinicopathological characteristics. (A) Kaplan-Meier survival curves of patients stratified by tMVD (upper panels) and mMVD (lower panels). (B) Forest plots of HRs for high-MVD and low-MVD (tMVD, left panels; mMVD, right panels) in terms of OS (upper panels) and PFS (lower panels) in various subpopulations stratified by age, gender, tumor histology, tumor differentiation, stage, and smoking history. The subpopulation in which MVD has a significant prognostic role is highlighted in red. OS, overall survival; PFS, progression-free survival; tMVD, total microvessel density; mMVD, modified MVD; HR, hazard ratio.

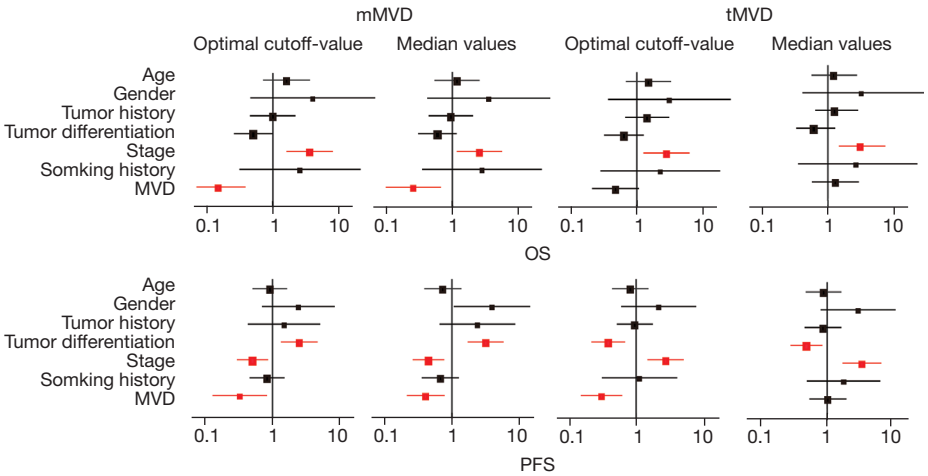

Figure 4 Forest plots of HRs for clinicopathological characteristics evaluated using multivariate analysis (COX) of OS and PFS for NSCLC patients. The factors with significant correlation to survival are highlighted in red. HR, hazard ratio; OS, overall survival; PFS, progression-free survival; NSCLC, non-small cell lung cancer.

proportional to the square of the perfusion distance (28). Data from breast cancer xenografts showed that pO_2 was decreased by approximately 40% and 100% at distances of 50 and 70 μm from the vessels, respectively (29,30). Furthermore, hypoxia (pO_2 <50% of vascular oxygen pressure) was observed at a distance of less than 50 μm in NSCLC specimens (31). Glucose concentration has been reported to decrease by 40% at a diffusion distance of 100 μm (29). Despite the meaningful impact of delivery distance on the perfusion efficiency of tumors, the perfusion profile data of patients are difficult to evaluate. Therefore, delivery distance was introduced as a surrogate marker. In this current study, the D_{mvcc} of NSCLC patients varied from 1.6 to 270 μm, and this was significantly correlated with the survival outcome. A D_{mvcc} of 20 μm was determined to be the cutoff distance for prognosis. For the rich desmoplastic stroma in patient tumors, the cutoff distance was shorter than that obtained from xenograft data. Thus, 20 μm was used as the cutoff value to distinguish microvessels near cancer cells from those far away from cancer cells. The microvessels near the cancer niches (within 20 μm) were counted and defined as mMVD. As a result, approximately 75% of the total microvessels were close to the cancer niche.

The prognostic values of mMVD and tMVD were evaluated and compared in three aspects: strength of prediction determined using ROC analysis and significance test with continuous data, univariate survival analysis using the log-rank test, and multivariate survival analysis using Cox regression with variously dichotomized data. As a more comprehensive perfusion-based factor to tMVD, mMVD represented not only the quantity but also the perfusion distance profiles of microvessels and was presented as a more powerful prognostic factor. Compared with tMVD, mMVD was a superior prognostic factor, especially for OS, with a higher AUC value (0.74 *vs.* 0.56), a larger fraction of OS-predictable-MVD (80% *vs.* 2.9%), and a meaningful role in univariate, subgroup, or multivariate analysis with either an optimal cutoff value or median value.

Interestingly, in this cohort of NSCLC patients, low-level mMVD and tMVD was associated with poor survival. To date, the prognostic value of tMVD remains controversial. Although tMVD was shown to be a detrimental prognostic factor in a pooled meta-analysis of 35 studies (7), its prognostic value was dismissed in a 17-center study for NSCLC (16). Moreover, positive correlations between low-tMVD and poor survival have been reported in other solid tumors, including esophageal (14) and ovarian cancers (15). This may be attributed to the fact that lower mMVD induces poorer blood perfusion and results in a higher numbers of hypoxia-susceptible tumor cells, which is a prime motivator of tumor progression via numerous pathways including apoptosis-resistance, immune escape, and chemoresistance (31-33). In line with this, substantial evidence indicates that poorly vascularized solid cancer regions are hypoxic and more likely to be resistant to chemotherapy and radiotherapy (14,34).

These results demonstrated that a complex factor

involving microvessel quantity and perfusion distance may be a favorable prognostic marker for NSCLC patients. However, there were certain limitations to this study. In the absence of data on perfusion distance and perfusion efficiency in patients, the cutoff distance was determined according to patient survival. Therefore, the cutoff value of D_{mvcc} should be examined in other tumors to verified the results. Further investigations regarding the reliability and applicability of the cutoff value in various types of solid tumors should be conducted. In addition, there are several other potential factors may influence perfusion, such as microenvironment. For example, specific subtype of perivascular like cells in microenvironment of NSCLC can promote vascular leakage (35). The macrophages expressing tie2, which regulated by hypoxia inducible factor α subunits, affect tumor perfusion in rat breast cancer (36). These factors were not included in analyses of this study. Finally, several factors may be associated with the prognosis of NSCLC, such as the tumor differentiation and TNM stage, *et al.* In our study, the mMVD was found significantly different in different group of TNM stage. Also, we analyzed tMVD and mMVD values in patients classified according to various other clinicopathological characteristics. The mutation status of driver genes and related targeted therapy may also affect the outcome of NSCLC. However, all patients analyzed in this study were hospitalized in from 2011 to 2012, the detection of driver genes was not prevalent at that time. We failed to collect the signature mutations of these patients retrospectively and the relationship with mMVD. Further research may be carried out in the future.

In summary, this report demonstrated that mMVD can act as a prognostic marker of OS and PFS in patients with NSCLC. It presented superior sensitivity, specificity, and clinical applicability compared to tMVD. A complex consideration of vascular intensity and the diffusion distance from vessels to cancer cells by introducing the definition of a modified MVD might provide novel insights into neovascularization-based prognostic predictions.

Acknowledgments

Funding: This work was supported by the Natural Science Foundation of China (Grant numbers: 81773819 and 81973396 to Luo Fang, and 81803585 to Like Zhong); the Natural Science Foundation of Zhejiang Province (Grant number: Y16H160129 to Luo Fang, LYY18H310006 to Like Zhong, and LQ17H300001 to Yu Song); the Science and Technology in Zhejiang Province Chinese Medicine Program (Grant number: 2015ZA036 to Luo Fang); the Medical Science Research Foundation of Zhejiang Province (Grant number: 2019KY473 to Zheng Shi, and 2013KYA027 to Luo Fang); and Key Laboratory of Clinical Cancer Pharmacology and Toxicology Research of Zhejiang Province (2020E10021).

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6566/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6566/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6566/coif). YS reports that this work was supported by the Natural Science Foundation of Zhejiang Province (Grant number: LQ17H300001). LZ reports that this work was supported by the Natural Science Foundation of China (Grant numbers: 81803585) and the Natural Science Foundation of Zhejiang Province (Grant number: LYY18H310006). ZS reports that this work was supported by the Medical Science Research Foundation of Zhejiang Province (Grant number: 2019KY473). LF reports that this work was supported by the Natural Science Foundation of China (Grant numbers: 81773819 and 81973396), the Natural Science Foundation of Zhejiang Province (Grant number: Y16H160129), the Science and Technology in Zhejiang Province Chinese Medicine Program (Grant number: 2015ZA036), and the Medical Science Research Foundation of Zhejiang Province (Grant number: 2013KYA027). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by institutional ethics committee of Zhejiang Cancer Hospital (No. IRB-2017-67). Individual patient consent for this study was waived due to the retrospective nature of this study.

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Cite this article as: Tong Y, Yang D, Mi X, Song Y, Xin W, Zhong L, Shi Z, Xu G, Ding H, Fang L. Modified microvessel density based on perfusion distance: a preferable NSCLC prognostic factor. Ann Transl Med 2022;10(2):43. doi: 10.21037/atm-21-6566

