

A novel definition of microvessel density in renal cell carcinoma: Angiogenesis plus vasculogenic mimicry

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Abstract. The present study proposed the novel concept of total microvessel density (TMVD), which is the combination of the MVD and the vasculogenic mimicry (VM) status, and evaluated its clinical significance in patients with renal cell carcinoma (RCC). For that purpose, tumor samples from 183 patients with primary RCC were examined by CD34 single or periodic acid Schiff (PAS)/CD34 dual histology staining. MVD and VM were determined according to previous literature. Clinical information (tumor stage and grade, and duration of survival) was retrieved and analyzed. Survival information and VM-associated gene expression data of patients with RCC were also retrieved from The Cancer Genome Atlas (TCGA) database and the clinical significance of each individual gene was analyzed. The results indicated that MVD exhibited obvious differences among patients with RCC; however, it was not correlated with the stage/grade or length of survival in patients with RCC. In total, 81 patients (44.3%) were CD34(-)/PAS(+) and defined as VM(+), and they had a significantly shorter survival compared with that of VM(-) patients (P=0.0002). VM was not associated with MVD. TMVD was able to distinguish between patients with high and low MVD in terms of survival, thus TMVD was

better compared with MVD alone at distinguishing between patients with different survival prognoses. TCGA data analysis revealed that among the VM-associated genes, nodal growth differentiation factor, caspase-3, matrix metalloproteinase-9 and galectin-3 had a statistically significant impact on the overall/disease-free survival of patients with RCC. In conclusion, the TMVD concept may be more appropriate and sensitive compared with the MVD or VM alone in predicting tumor aggressiveness and patient survival, particularly in RCC, which is a highly vascularized, VM-rich neoplasm, and certain VM formation-associated genes are negatively associated with the survival of patients with RCC.

Introduction

Angiogenesis, which is the development of new blood vessels from existing vasculature, is a major driving force in numerous types of malignancy by delivering oxygen and nutrients for the growth of tumors (1), while facilitating fast metastasis (2). First introduced by Folkman as a potential target for cancer treatment (3), angiogenesis was thereafter considered an essential pathologic feature and sustaining element of cancer, which has a key role in tumor dissemination/metastasis (4). Therefore, it appears reasonable to predict that the extent of tumor vascularity, measured by the pathological microvessel density (MVD), may be closely associated with the aggressiveness of a tumor (5), including its invasive and metastatic potential. MVD is usually defined by the following equation:

$$MVD \text{ (hotspot)} = \text{Individual microvessels (number)} / \text{area}$$

The endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels, tumor cells and other connective tissue elements was considered a single, countable microvessel (6). An inverse association between MVD and patient survival has been reported for several malignancies, including breast cancer (7) and melanoma (8), as well as prostate (9) and bladder (10) cancer. Previous studies have indicated that the MVD was correlated with vascular endothelial growth factor (VEGF) expression, which is also a crucial factor in the vascular biology of multiple tumors as a mediator of angiogenesis. In the field of metastatic renal cell carcinoma

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(RCC), which is a highly vascularized solid tumor type (11), anti-angiogenic agents targeting VEGF/VEGF receptor, such as sunitinib, pazopanib and bevacizumab, have been the standard first-line therapy for years; however, they provide a limited benefit and metastatic RCC remains a challenge (12), which suggests that there may be an alternative blood supply besides angiogenesis. Of note, intra-tumoral MVD has been a controversial prognostic predictor for RCC. Nativ *et al* (13) and Fukata *et al* (14) reported that higher MVD is associated with shorter survival in RCC. Similarly, other studies have demonstrated this association in patients with ccRCC (15-17). Some of the studies found other associations. For example, Paradis *et al* (18) and Zhang *et al* (19) reported a positive association between MVD and VEGF expression levels, and Tuna *et al* (20) reported positive association between MVD and mast cell infiltration. Notably, Slaton *et al* (21) reported no significant correlation between MVD and VEGF, Mohseni *et al* (22) reported lack of correlation between MVD and mast cell infiltration, while others reported a lack of correlation between MVD and survival (23-26). On the contrary, numerous studies (27-32) have reported higher MVD associated with longer survival, and Yoshino *et al* (33) and Sabo *et al* (34) also reported this association in patients with low-stage RCC. Delanunt *et al* (35) reported this association in ccRCC, and Sharaml *et al* (36) reported this tendency yet the P-value was 0.1. Sandlund *et al* (37) reported this trend in 2006, but one year later they switched the marker from CD105 to CD31 and found the association disappeared (38). As for the association with stage or grade, Köhler *et al* (39) reported a negative association between MVD and stage, Hemmerlein *et al* (40) and Baldewijns *et al* (41) reported a negative association between MVD and Fuhrman grade and Kavantzias *et al* (42) reported positive association between MVD and grade, while Sharma *et al* (43) reported no association. Therefore, plethora of literature makes the current understanding of MVD in the setting of RCC controversial (Table I).

Microvessel or microvasculature is defined as ‘the smallest system of blood vessels in a body, including those responsible for microcirculation, that distribute blood within tissues’ (44). Besides angiogenesis, there is an alternative perfusion source termed ‘vasculogenic mimicry’ (VM), also referred to as ‘vascular mimicry’. The initial study and molecular characterization of VM was conducted in melanoma (45). Later, VM was also assessed in breast cancer (46) and hepatic carcinoma (47). Of note, the results of these studies agreed with those of earlier studies suggesting the perfusion of tumors via non-endothelial-lined channels. Since VM may also serve as a supply system of blood including nutrients, the concept of MVD may require to be modified, as the current understanding of the complexity of vasculature, either endothelium- or tumor cell-derived, improves over the years. Therefore, the present study proposed a modified version of MVD, referred to as total MVD (TMVD), which incorporates the number of MVD and the status of VM, and was defined as follows:

$$TMVD = \text{Individual microvessel (number)/area} + VM$$

In the present study, the capability of MVD, VM and TMVD in predicting prognosis of patients with RCC was

evaluated and compared, and a bioinformatics analysis of the possible genes underlying the clinical significance of VM was performed.

Materials and methods

Patients and clinical data. A retrospective study was performed involving 183 patients with histopathologically verified RCC who underwent nephrectomy between January 2006 and December 2016 at Xinhua Hospital Affiliated to Shanghai Jiao Tong University, School of Medicine (Shanghai, China). The cohort had a median age of 59.3±7.0 years (range, 44-73 years) and comprised 104 males and 79 females. The pre-operative radiological evaluation consisted of chest X-ray, abdominal ultrasonography and contrast-enhanced CT. None of the patients received irradiation or chemotherapy prior to surgery. The follow-up comprised of chest X-ray, abdominal ultrasonography or CT scan. The macroscopic and histological features of RCC were assessed, including tumor stage and Fuhrman nuclear grade (26). The tumor stage was defined according to the 2010 TNM classification (48). At presentation, the tumor stage was pT1 in 73, pT2 in 80 and pT3 in 30 cases, and the Fuhrman grade was I in 58, II in 90, III in 29 and IV in 6 tumors. The follow-up program included clinical and radiological examinations. The median follow-up time from diagnosis was 53.9±19.0 months (range, 11-94 months) for surviving patients. The survival time was calculated from the date of surgery to the date of death or latest follow-up. The study was approved by the Ethics Committee of Xinhua Hospital (Shanghai, China; approval no. XHEC-D-2016-061). The requirement for informed consent was waived by the Ethics Committee due to the retrospective nature of this study. The overall/disease-free survival time and gene sequencing data of another 537 patients with RCC were retrieved from The Cancer Genome Atlas (TCGA) database (<https://cancergenome.nih.gov/>), the Kidney RCC cohort (TCGA, provisional) using cBioPortal (<https://www.cbioportal.org/>). Survival time was evaluated based on individual gene expression levels.

Immunohistochemistry (IHC). IHC was performed on conventional 5- μ m-thick histological paraffin-embedded tissue serial RCC sections on poly-L-lysine-coated glass slides. After heat-drying, the sections were deparaffinized in xylene and sequentially rehydrated in gradients of ethanol, and next incubated overnight at 4°C with anti-CD34 antibody (cat. no. ab81289; 1:100 dilution; Abcam). Signals were amplified with the VECTASTAIN® ABC kit (Vector Laboratories, Inc.). At x200 magnification, most of the slides had CD34-positive stain and those without any CD34 signal were considered invalid and restained. Periodic acid Schiff (PAS) staining was performed using a PAS kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol on one of the CD34-stained slides. Sections were counterstained with Mayer's hematoxylin, coverslips were mounted with PermMount Mounting Medium and samples were observed using an Olympus IX73 microscope (Olympus, Corp.). For the negative control, the primary antibody was replaced with non-immune human serum (cat. no. 31876; Thermo Fisher Scientific, Inc.).

Table I. Review of previously published literature on the clinical significance of MVD in patients with RCC.

First author, year	Patients (n)	Marker	Stage/grade association	Clinical significance of higher MVD	(Refs.)
Yoshino, 1995	84	FVIII RAg	/	Longer survival for patients with T1-2 and M0 tumors	(33)
MacLennan, 1995	97	FVIII RAg	No association	Lack of clinical significance	(23)
Köhler, 1996	79	UEAI	Negative association with stage	/	(39)
Anastassiou, 1996	23	CD31	/	Longer survival	(27)
Delahunt, 1997	150	FVIII RAg	Negative association with stage/grade	Longer survival in ccRCC	(35)
Gelb 1997	52	FVIII Rag/CD31	/	Lack of clinical significance	(24)
Nativ, 1998	36	FVIII RAg	Negative association with grade	Shorter survival	(13)
Paradis, 2000	74	CD34	Negative association with grade (in ccRCC)	Positive correlation with VEGF	(18)
Hemmerlein, 2001	58	CD31	Negative association with grade	/	(40)
Sabo, 2001	49	CD34	/	Longer survival in low-stage ccRCC	(34)
Suzuki, 2001	56	CD34	No association	Lack of clinical significance	(25)
Slaton, 2001	46	CD34	No association	Lack of clinical significance/no correlation with VEGF	(21)
Rioux-Leclercq, 2001	73	CD34	Negative association with stage/grade	Longer survival	(28)
Zhang, 2002	70	CD31	No association	Positive correlation with VEGF	(19)
Schraml, 2002	113	CD34	/	Longer survival tendency (P=0.1)	(36)
Yagasaki, 2003	84	CD105	Negative association with stage/grade	Longer survival/negative correlation with VEGF	(29)
Imao, 2004	70	CD34	Negative association with stage/grade	Longer survival	(30)
Joo, 2004	67	CD34	Positive association with stage/negative with grade	Shorter survival in ccRCC	(15)
Fukata, 2005	54	CD34	/	Shorter survival/negative correlation with M/E ratio	(14)
Minardi, 2005	48	CD34	/	Lack of clinical significance	(26)
Tuna, 2006	71	CD31	/	Positive correlation with mast cell infiltration	(20)
Sandlund, 2006	168	CD105	Negative association with stage/grade	Longer survival	(37)
Sandlund, 2007	167	CD31	Negative association with stage/grade	Lack of clinical significance	(38)
Kavantzaz, 2007	53	FVIII RAg	Positive association with grade	/	(42)
Mertz, 2007	284	CD34	/	Longer survival	(31)
Baldewijn 2007	150	CD34	Negative association with grade	/	(41)
Yildiz, 2008	54	CD34	Negative association with stage/grade	Longer survival	(32)
Minardi, 2008	50	CD34	No association	Shorter survival in ccRCC	(16)
Mohseni, 2010	40	CD34	/	No correlation with mast cell infiltration	(22)
Sharma, 2011	41	CD34	No association	/	(43)
Iakovlev, 2012	57	CD34	No association	Shorter survival in ccRCC	(17)

ccRCC, clear-cell renal cell carcinoma; MVD, microvessel density; VEGF, vascular endothelial growth factor.

MVD quantification and VM identification. MVD was assessed according to consensus guidelines (49) independently by two pathologists by counting individual microvessels in 5 fields at a magnification of x200 in a highly vascular tumor area (hot spot), excluding areas with prominent hyalinization and necrosis. Microvessels were defined as any CD34-positive endothelial or endothelial cell cluster with or without a viable lumen. In tumors exhibiting a dense microvasculature network, each branch was interpreted as a single vessel. Large anastomosing sinusoidal vessels were counted as single vessels. Only vessels distinct from one another were counted separately. Large vessels with thick muscular walls were excluded from counting. For each tumor, the mean number of microvessels counted in five fields at x200 magnification was considered as the MVD value, which is a number without unit (50). For CD34/PAS dual-stained slides, VM was defined as any CD34-negative/PAS-positive closed area.

Statistical analysis. Values were expressed as the mean \pm standard error of the mean, while in figures MVD were shown in box and whisker plots as minimum to maximum using GraphPad Prism 6 (GraphPad Software, Inc.). Statistical analyses involved Student's t-test, one-way analysis of variance with Bonferroni's post hoc test, the χ^2 test and the log-rank (Mantel-Cox) test. The analyses were conducted with SPSS 22 (IBM Corp.) or GraphPad Prism 6 (GraphPad Software, Inc.). In the survival analysis, when two Kaplan-Meier curves crossed, Cox time-dependent covariate analysis was used for adjustment of the P-value. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MVD is not associated with the stage or grade of RCC. IHC staining for CD34 was performed on the RCC samples. By microscopic observation under x200 magnification, MVD in a hotspot area was able to be classified into low (between 20 and 30; Fig. 1A), moderate (between 40 and 50; Fig. 1B) and high (between 60 and 80; Fig. 1C). The mean MVD was calculated to be 44.9 ± 12.4 . Regarding different stages, the mean MVD was 43.5 ± 10.0 for stage 1, 46.3 ± 13.6 for stage 2 and 44.8 ± 14.2 for stage 3 (Fig. 1D). The mean MVD for different grades was 42.6 ± 10.9 for grade 1, 46.2 ± 12.4 for grade 2 and 45.5 ± 14.3 for grades 3/4 (Fig. 1E). There was no significant difference in MVD between the different stages or grades, and no increasing or decreasing tendency was observed either. The results of Fig. 1 suggested a weak association between MVD and the stage/grade.

VM exhibits a tendency to increase in patients with advanced-stage/grade RCC. CD34/PAS dual staining was performed on serial RCC sections in order to identify the VM structure. Based on CD34 expression, the slides were classified into VM(-), which corresponded to a CD34(+)/PAS(+) status (Fig. 2A), and VM(+), which was defined by the presence of a CD34(-)/PAS(+) enclosed channel that was lined by tumor cells rather than endothelial cells (Fig. 2B). Patients were stratified based on their VM(+) or VM(-) status. By further

stratifying the patients based on their stage/grade information, it was observed that, although there was a higher proportion of VM(+) patients in stage 3 compared with those in stage 1 ($P=0.0292$; Fig. 2C), the differences between stage 1 and 2 or stage 2 and 3 were not statistically significant. Similarly, a higher proportion of VM(+) patients was present in the grade 3/4 group than in the grade 1 group ($P=0.0325$; Fig. 2D). There was no difference in MVD between patients with VM(+) and VM(-) according to Student's t-test ($P=0.4785$; Fig. 2E). The patients were then stratified into high or low MVD groups and it was observed that there was no difference in the VM(+) ratio between patients with high or low MVD in their tumor according to the χ^2 test ($P=0.2625$; Fig. 2F).

Survival analysis of genes closely associated with the formation of VM. To clarify why the phenotype of VM was reported to be closely associated with the survival of patients with RCC (51,52), the present study attempted to identify the potentially associated genes using TCGA database via cBioPortal. Previous studies reported several genes closely associated with the formation of VM, including vascular endothelial (VE)-cadherin (also known as CDH5), vimentin (VIM) and matrix metalloproteinases (MMPs) (53-55). The clinical data from a large sample were retrieved from TCGA database and the survival length of patients with RCC was analyzed based on the expression levels of those VM-associated genes. Among them, certain genes had a significant negative impact on overall/disease-free survival, including nodal growth differentiation factor (NODAL), caspase-3 (CASP3), MMP9 and galectin-3 (GAL3) (Fig. 3A-H, respectively). Of the two genes that are known to be closely linked to VM, high VE-cadherin was unexpectedly associated with a longer overall survival ($P=0.018$; Fig. 3I), but not disease-free survival ($P=0.494$; Fig. 3J). VIM, a well-known oncogene (56,57), had a significant negative effect on overall survival ($P=0.0092$; Fig. 3K) and disease-free survival ($P=3.92 \times 10^{-7}$; Fig. 3L).

VM rather than MVD is able to distinguish patients with different survival prognoses, while TMVD demonstrates superior discriminating capability. Upon dividing the patients into two groups based on their MVD levels, there was no significant difference between the survival time of patients with high or low MVD ($P=0.348$; Fig. 4A), although the survival time had a tendency to be shorter in patients with higher MVD. Stratification of the patients based on their VM status indicated that VM(+) patients had a significantly shorter survival time ($P=0.0002$; Fig. 4B), demonstrating an inverse association between VM and survival. By applying the TMVD concept, those patients were further stratified into four subgroups. Comparison of the survival curves of these four subgroups indicated that this stratification was able to distinguish patients with different survival prognoses (Fig. 4C). Among patients with a lower MVD, VM(-) patients exhibited significant longer survival than VM(+) patients ($P=0.0076$); and among patients with a higher MVD, VM(-) patients also had a significantly longer survival time than VM(+) patients ($P=0.0093$). Of note, patients with a lower MVD combined with a VM(+) status had an even poorer prognosis than those with a higher MVD combined with a VM(-) status ($P=0.039$).

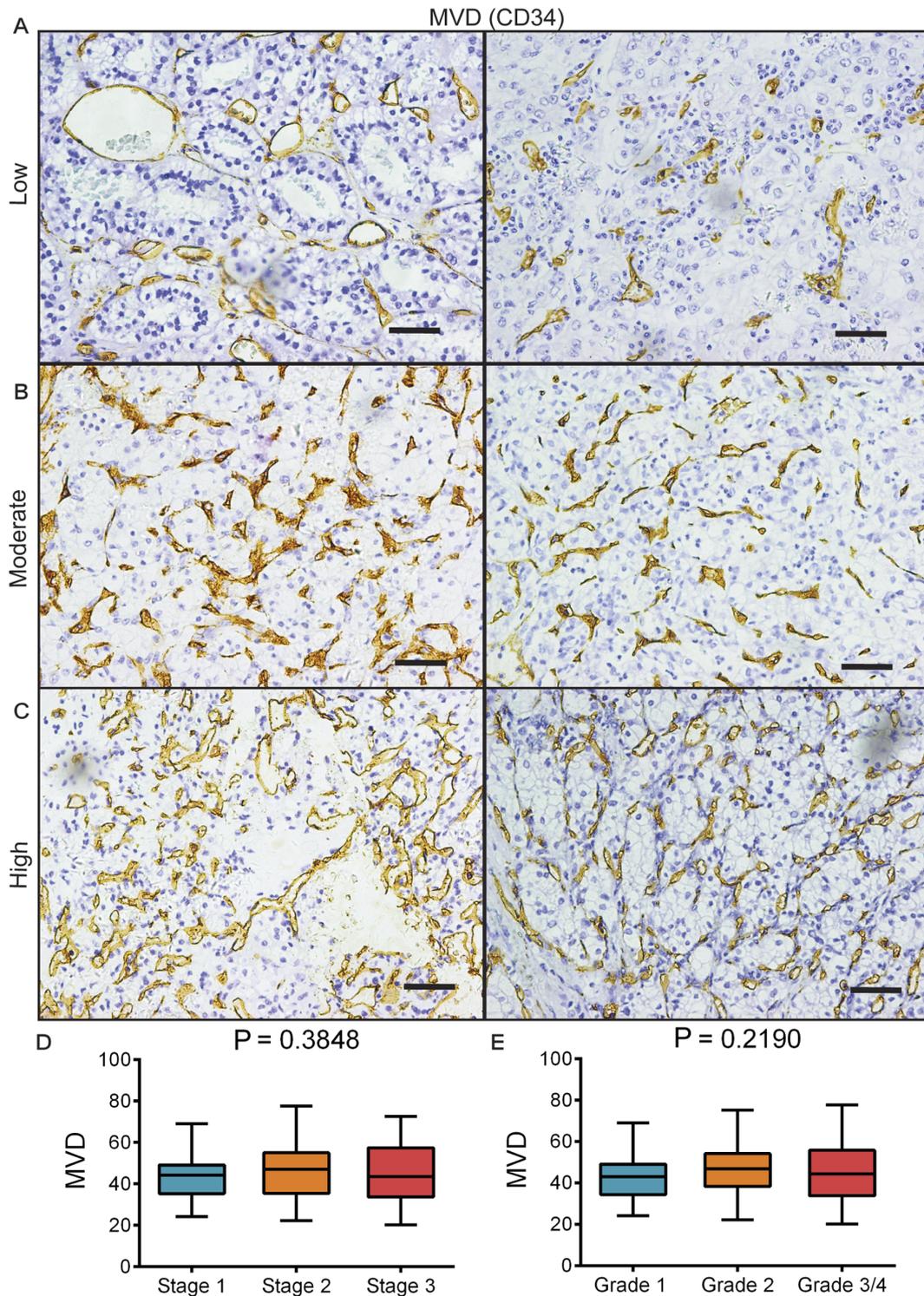


Figure 1. MVD is not associated with the stage or grade in patients with RCC. (A-C) CD34 immunohistochemical staining of clear-cell RCC samples. MVD within hotspots was classified as (A) low (20-30), (B) moderate (40-50) and (C) high (60-80) (scale bar, 10 μ m). Each condition is demonstrated with two representative images. (D and E) Comparison of the mean MVD between different (D) stages and (E) grades. RCC, renal cell carcinoma; MVD, microvessel density.

Discussion

MVD assessment is the most commonly used technique to quantify intratumoral angiogenesis in cancer. It was first developed by Weidner *et al* (58) in 1991, who used panendothelial IHC staining of blood microvessels. The first step was the identification of the area with the highest neovessel density (the so-called

'hot spot'). Individual microvessels were then counted at higher power (magnification, x200) in an adequate area (e.g., 0.74 mm² per field using a 20x objective lens and a 10x ocular lens). Any stained endothelial cells or clusters separated from adjacent vessels were counted as single microvessels. Despite numerous reports of the clinical prognostic significance of MVD in various types of tumor, its predictive value regarding outcomes

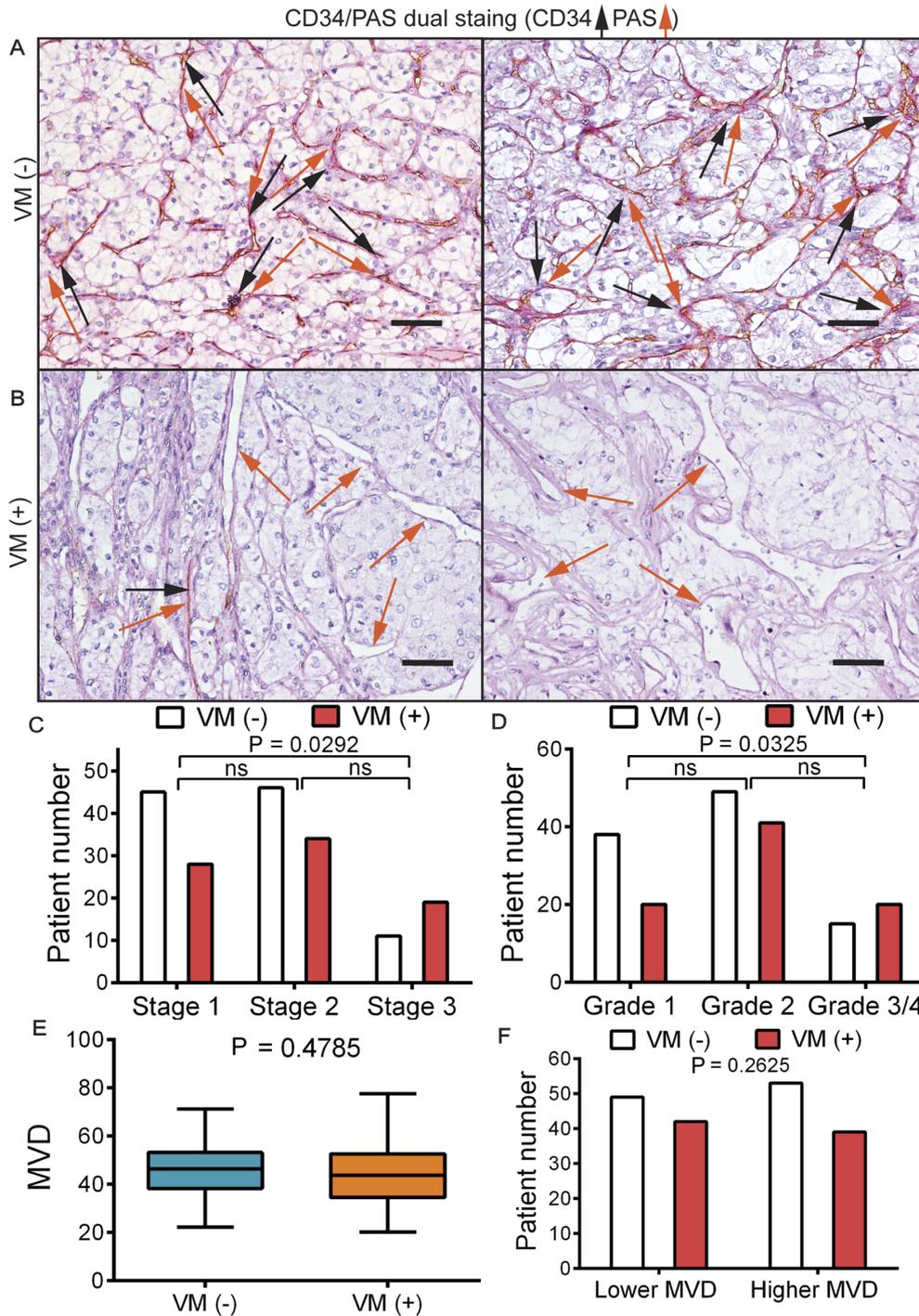


Figure 2. VM tends to increase in patients with high stage or grade. (A-C) CD34/PAS dual staining in serial ccRCC sections. (A and B) As indicated in representative histology images, (A) VM(-) was defined as CD34(+)/PAS(+) and (B) VM(+) was defined as CD34(-)/PAS(+) (scale bar, 10 μ m). Each condition is demonstrated with two representative images. (C) Comparison of VM status between patients with early and advanced stage. (D) Comparison of VM status between patients with low and high grade (E) Comparison of MVD between VM(-) and VM(+) cases. (F) Comparison of VM status between patients with low and high MVD. Black arrows indicate CD34(+) and red arrows PAS(+). VM, vasculogenic mimicry; MVD, microvessel density; PAS, periodic acid Schiff; ns, no significance.

in RCC remains controversial, as summarized in Table I. Some of them reported negative correlation between MVD and prognosis (higher MVD correlated with shorter survival) (13-17), some reported positive correlation (27-32) and others reported no significance (21,23-26,38). This may be associated with several non-mechanistic factors, including sample size, sampling bias, different blood vessel markers (such as the

more commonly used CD34 or CD31, or the less frequently used FVIII Rag or CD105), the quality of IHC staining, the methods of vasculature quantification and the methods of interpretation. For instance, Sandlund *et al* (59) reported in 2006 that a higher MVD was associated with longer survival; however, when CD31 was used as the vessel marker instead of CD105, no association with survival was observed (60). Due to

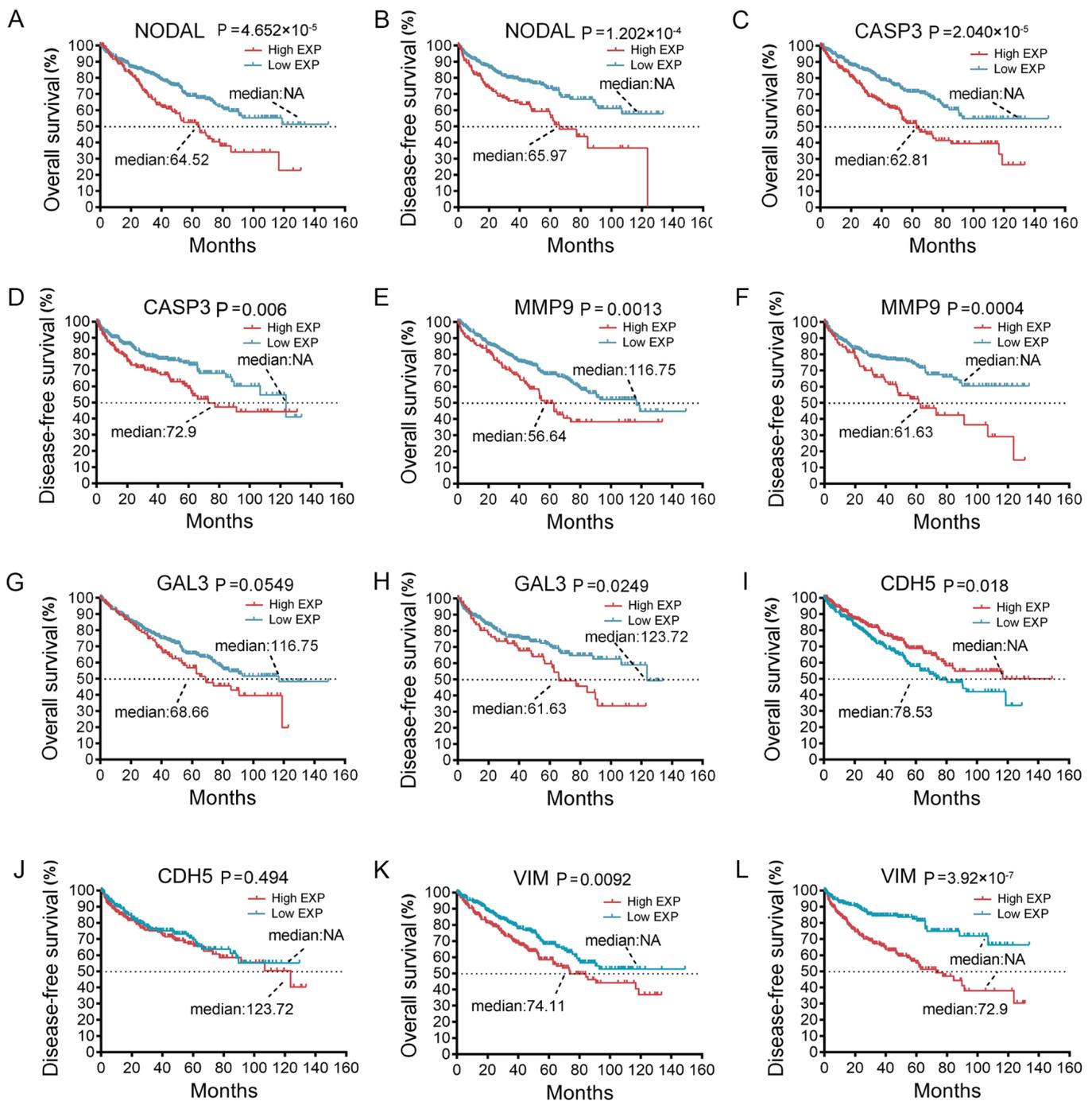


Figure 3. Survival analysis of genes closely associated with the formation of vasculogenic mimicry, which also shorten the overall survival and disease-free survival of patients with renal cell carcinoma, from The Cancer Genome Atlas database. The survival rate was expressed as the percentage. (A) Overall survival and (B) disease-free survival of patients with relatively high or low NODAL expression. Overall survival (C) Overall survival and (D) disease-free survival of patients with relatively high or low CASP3 expression. (E) Overall survival and (F) disease-free survival of patients with relatively high or low MMP9 expression. (G) Overall survival and (H) disease-free survival of patients with relatively high or low GAL3 expression. (I) Overall survival and (J) disease-free survival of patients with relatively high or low VE-cadherin expression. (K) Overall survival and (L) disease-free survival of patients with relatively high or low vimentin expression. EXP, expression; NODAL, nodal growth differentiation factor; CASP3, caspase 3; MMP9, matrix metalloproteinase 9; GAL3, galectin-3; VE-cadherin/CDH5, vascular endothelial cadherin; VIM, vimentin.

the heterogeneity in methodology among these studies, a forest plot may be unpractical and unreasonable. Another possible reason is the different categories of blood vessels. Yao *et al* (61) proposed that, within clear-cell RCC, there are at least two major categories of blood vessels with contrasting prognostic implications, namely undifferentiated vessels (expressing CD31 but not CD34) and differentiated vessels (expressing both CD31

and CD34), with a higher undifferentiated vessel density indicating poorer prognosis and higher differentiated vessel density correlating with better prognosis. Qian *et al* (62) also discussed the complexity of tumor vasculature in RCC and recent studies on the concept of vessel co-option (a non-angiogenic process through which tumor cells utilize pre-existing tissue blood vessels to support tumor growth, survival and metastasis) have

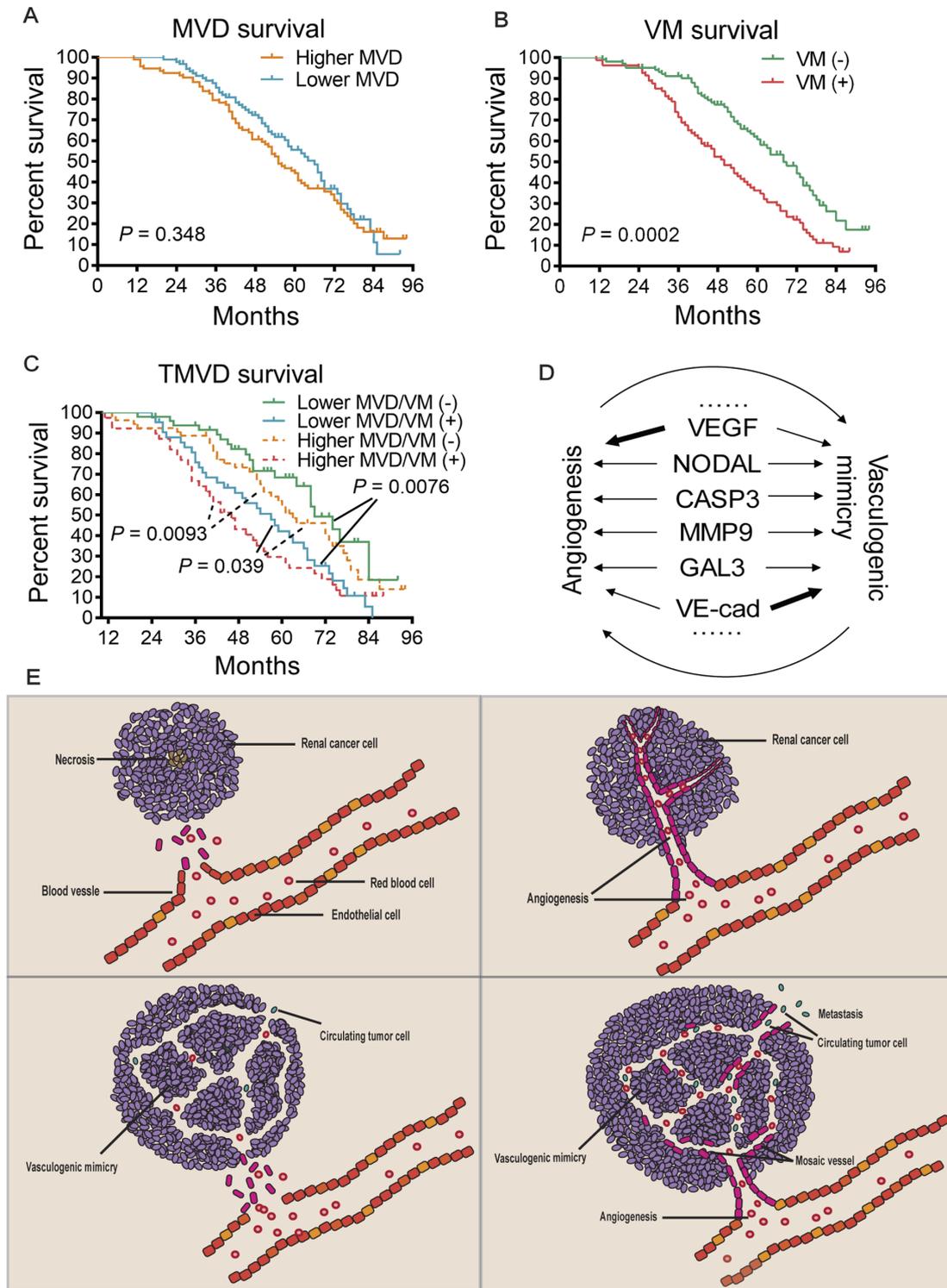


Figure 4. VM rather than the MVD is able to distinguish patients with different survival prognoses, while the TMVD demonstrates superior discriminating capability compared with MVD. (A) Comparison of overall survival between patients with low and high MVD. The survival rate was expressed as the percentage. (B) Comparison of survival between VM(-) and VM(+) patients. (C) Comparison of survival between four different TMVD subgroups of patients. (D) Mechanistic diagram indicating the possible association between angiogenesis and VM via numerous associated genes. (E) Mechanistic scheme illustrating the function of angiogenesis and VM in supplying blood and promoting metastasis. VM, vasculogenic mimicry; TMVD, total microvessel density; VEGF, vascular endothelial growth factor; NODAL, nodal growth differentiation factor; CASP3, caspase 3; MMP9, matrix metalloproteinase 9; GAL3, galectin-3; VE-cadherin, vascular endothelial cadherin.

been published (63-65), thus obscuring whether MVD is a sufficient prognostic factor.

VM is the formation of fluid-conducting channels by highly invasive and genetically dysregulated tumor cells and

acts as a complementary source of blood supply. In the present study, TMVD (i.e., MVD plus VM status) demonstrated a better prognosis-predicting capability compared with that of the MVD or VM alone (Fig. 4C), which may be explained by

the fact that endothelium-lined blood vessels as well as VM are able to transfer blood, nutrients and oxygen, and theoretically, both may facilitate cancer progression. It is reasonable to assume that during treatment with an anti-angiogenic regimen, when neo-angiogenesis is suppressed, tumor growth may be more dependent on the supply from VM. A comprehensive meta-analysis review by Yang *et al* (66) revealed that VM is associated with unfavorable prognosis in >10 different types of tumor, and with cancer differentiation, lymph node metastasis and distant metastasis. In other words, VM is not only functional as a delivering channel, but is in itself a hallmark of potent proliferation and metastasizing capability. Survival analysis of VM-associated genes, including NODAL, CASP3, MMP9 and GAL3, revealed that these genes had a negative impact on overall and disease-free survival in the setting of RCC based on TCGA database. In addition, several studies have been published demonstrating that the above genes also contribute to angiogenesis (67-70). The single most important factor in VM, VE-cadherin, has been indicated to regulate angiogenesis (71) and the single most important factor in angiogenesis, VEGF, has also been reported to promote VM (72). Taken together, angiogenesis and VM may promote tumor progression independently and probably interdependently (Fig. 4D and E). One of the limitations of the present study is that the association between the above-mentioned genes, VM formation and patient survival was not assessed in the present cohort, and therefore, it was not possible to experimentally clarify certain paradoxical results of the bioinformatics analysis, including higher VE-cadherin being associated with longer overall survival.

When the concept of TMVD was proposed, it was expected to be the sum of MVD and VM density, but in reality, the quantification of VM density, if it is able to be quantitated, is rather difficult. The identification process relies greatly on visual observation. If red blood cells (RBCs) are present inside a CD34(-)/PAS(+) area, it is easier to confirm, while the absence of RBCs inside such an area complicates the identification, since PAS staining may not be well demarked. Instead of calculating its density, the status of VM (positive or negative) was incorporated into the formula of TMVD in the present study. Generally speaking, among the four groups classified according to TMVD, the prognosis of patients with low MVD(≤ 45)/VM(+) was the best, that of patients with high MVD(> 45)/VM(-) and low MVD(≤ 45)/VM(+) was intermediate and that of patients with high MVD(> 45)/VM(+) was the worst. The clinical significance and cost-effectiveness of this novel concept of TMVD require to be further investigated, not only in the setting of RCC, but also in other cancer types in which VM may have a critical role. Recently, novel combinational therapy targeting other molecules, including programmed cell death 1 (PDI)/programmed cell death 1 ligand 1 (PDL1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), has demonstrated promising efficiency (73-75). With more clinical trials ongoing, it is possible that checkpoint immunotherapy combined with anti-angiogenesis therapy may be adopted as the first-line treatment for metastatic RCC, and PDI/PDL1/CTLA-4 expression levels, and perhaps other gene expression levels (76-79), combined with TMVD may provide higher accuracy in predicting patient prognosis.

In conclusion, the present study examined the novel concept of TMVD, which is a combination of MVD and VM status, and evaluated its capability in predicting prognosis in patients with RCC compared to that of MVD or VM alone. TMVD demonstrated superior predictive capability, and together with the results of the TCGA data analysis, the present results suggested that angiogenesis and VM promote tumor progression independently and probably interdependently.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

JQ, ZG, JY and JD designed the study. JY and JD supervised the whole process. YW, KD, WG, DW, HT and NW performed the research, among which WG and JY conducted the IHC staining. YW and KD analyzed the data. YW and JD wrote the manuscript. ZG and JD revised the statistics and the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Xinhua Hospital (Shanghai, China; approval no. XHEC-D-2016-061). The requirement of informed consent was waived by the Ethics Committee due to the retrospective nature of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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