JWA inhibits melanoma angiogenesis by suppressing ILK signaling and is an independent prognostic biomarker for melanoma

Jing Lu1,2,8, Yun Tang1, Maham Farshidpour1, Yabin Cheng1, Guohong Zhang1, Seyed Mehdi Jafarnejad1, Alan Yap1, Magdalena Martinka1, Ziming Dong2, Jianwei Zhou3, Jinhua Xu4 and Gang Li5

1Department of Dermatology and Skin Science, Research Pavilion, Vancouver Coastal Health Research Institute, University of British Columbia, 828 West 10th Avenue, Vancouver, British Columbia, V5Z 1L8, Canada, 2Department of Pathophysiology, Basic Medical College, Zhengzhou University, Zhengzhou 450001, Henan, China, 3Department of Pathology, Shantou University Medical College, Shantou, Guangdong 515041, China, 4Department of Pathology, Vancouver Coastal Health Research Institute, University of British Columbia, Vancouver, British Columbia, V5Z 1M9, Canada, 5Department of Translational Oncology, Jiangsu Key Lab of Cancer Biomarkers, Prevention & Treatment, Cancer Center, Nanjing Medical University, Nanjing 210029, China and 6Department of Dermatology, Huashan Hospital, Fudan University, Shanghai 200040, China

*To whom correspondence should be addressed. Tel: +1 604 875 4891; Fax: +1 604 675 2478; Email: lujing966@gmail.com

Melanoma is the deadliest cutaneous malignancy because of its high incidence of metastasis. Melanoma growth and metastasis relies on sustained angiogenesis; therefore, inhibiting angiogenesis is a promising approach to treat metastatic melanoma. JWA is a novel microtubule-associated protein and our previous work revealed that JWA inhibited melanoma cell invasion and metastasis. However, the role of JWA in melanoma angiogenesis and the prognostic value are still unknown. Here, we report that JWA in melanoma cells significantly inhibited the tube formation of endothelial cells. In addition, JWA regulated integrin-linked kinase (ILK) through integrin αvβ3 and such regulation was achieved through the transcription factor Sp1. Notably, both in vitro and in vivo angiogenesis assays revealed that JWA dramatically suppressed melanoma angiogenesis by inhibiting ILK signaling. Furthermore, we examined the expression of JWA protein in a large set of melanocytic lesions (n = 505) at different stages by tissue microarray and found an inverse correlation between JWA expression and melanoma progression (P = 5 × 10^-4). Importantly, reduced JWA expression was correlated with a poorer overall, and disease-specific 5 year survival of patients (P = 0.001 and 0.007, respectively). Multivariate Cox regression analyses indicated that JWA was an independent prognostic marker for melanoma patients. Moreover, we found a significant negative correlation between JWA and ILK in melanoma biopsies, and their concomitant expression was closely correlated with melanoma patient survival (P = 0.004), further indicating the regulation of ILK expression by JWA is critical in melanoma. Taken together, our data highlight the function of JWA in melanoma angiogenesis and reveal the clinical prognostic value of JWA.

Introduction

Melanoma is the deadliest cutaneous malignancy and its incidence frequency has continued to grow for the last 30 years (1). It is estimated that there are nearly 1 000 000 melanoma patients in the USA, and an additional 76 690 individuals might be diagnosed in 2013 (1). Melanoma has a high capacity for metastasis and up to 20% of patients will develop a metastatic tumor (2,3). Once metastasis occurs, the 5 year survival rate drops to <10% (4). As the underlying mechanisms that regulate melanoma progression and metastasis were still poorly understood, the treatment options for metastatic melanoma were scarce and without overall survival benefit (5). Angiogenesis is essential for solid tumor growth and metastasis, and controlling tumor-associated angiogenesis is a promising tactic in limiting cancer progression (6,7). Thus, the factors driving melanoma angiogenesis need to be identified for further therapeutic exploitation.

JWA, also known as ADP-riboseylation-like factor 6 interacting protein 5, was initially cloned from human tracheal bronchial epithelial cells (8). Several JWA homologues (e.g. ARL-6, GTPAP3-18, addicin and JM4) were later identified (9,10). Subsequent studies indicated that JWA was a structurally novel microtubule-associated protein, which regulated cancer cell migration via the mitogen-activated protein kinase signaling pathway, and JWA depletion blocked the inhibitory effect of As2O3 on HeLa cell migration (11). In addition, our recent data have shown that JWA inhibits the metastasis of melanoma cells by suppressing integrin αvβ3 signaling (12). However, the function of JWA in melanoma progression, as well as the prognostic of melanoma patients, has not been elucidated. Also, the potential role of JWA in melanoma angiogenesis still remains unknown.

Integrin-linked kinase (ILK) is a multifunctional intracellular serine/threonine kinase and adapter protein that associates with β1, β2 and β3 integrins (13). When it is dysregulated, ILK induces evasion of apoptosis, cell proliferation, anchorage-independent cell growth, metastasis and angiogenesis (14,15). Furthermore, ILK expression is often elevated in human malignancies and it closely correlates with tumor stage and grade in colon cancer and gastric cancer (16). Importantly, strong ILK expression predicts poor patient survival in non-small-cell lung cancer and prostate cancer (17,18). We have previously confirmed that ILK upregulation closely correlated with melanoma invasion and progression, and inversely correlated with 5 year survival of melanoma patients (19). We have demonstrated that ILK promoted melanoma angiogenesis by upregulating the interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3)/vascular endothelial growth factor (VEGF) signaling pathway (20). However, the molecular mechanism of elevated ILK expression in melanoma is still not clear and the regulation for ILK in melanoma angiogenesis is not well understood.

In this study, we show for the first time that JWA functions as a suppressor of melanoma angiogenesis in vitro and in vivo. In addition, this inhibitory effect of JWA is dependent on regulating ILK signaling. Importantly, reduced JWA expression is significantly associated with melanoma progression and a worse patient survival. Our findings underscore the importance of JWA in melanoma angiogenesis and progression, and suggest that JWA may be used as a promising antiangiogenic therapeutic target and a novel prognostic marker for melanoma patients.

Materials and methods

Study approval

All experiments involving animals were approved by the Animal Care Committee of the University of British Columbia (protocol A10-0193). All human samples were obtained the following receipt of informed consent. The use of human tissues and the waiver of patient consent in this study were approved by the Clinical Research Ethics Board of University of British Columbia (CREB study ID H09-01321). The study was conducted according to the principles expressed in the Declaration of Helsinki.

Abbreviations: AJCC, American Joint Committee on Cancer; CM, conditioned medium; DAPI, 4',6-diamidino-2-phenylindole; HR, hazard ratio; HUVEC, human umbilical vein endothelial cell; IL, interleukin; ILK, integrin-linked kinase; KD, knockdown; NF-κB, nuclear factor-kappaB; siRNA, small interfering RNAs; STAT, signal transducer and activator of transcription; TMA, tissue microarray; VEGF, vascular endothelial growth factor.
Cell lines

The human melanoma cell lines MMRU and MMLH were cultured in Dulbecco’s modified Eagle’s medium (HyClone, South Logan, UT) supplemented with 10% fetal bovine serum (Hyclone). Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell medium (SchenCell, Carlsbad, CA).

Plasmids and transfection

3xFlag-JWA and shJWA vectors were generated as described previously (12). pcDNA3.1-ILK was kindly provided by Dr. Shoukat Dedhar (University of British Columbia). The shILK plasmid and shILK MMRU stable cell line were described previously (20). Human ILK promoter, cloned into the vector pGL2-Basic, was kindly provided by Prof. Nelly Kieffer (Laboratoire Franco-Luxembourgeois de Recherche Biomédicale) (21). ILK promoter mutants carrying disrupted DNA consensus motifs for NF-kB/rel-p53 and p-1 transcription factors were kindly provided by Prof. Ute Reuning (Frauenklinik der Technischen Universität München) (22). For plasmid transfection, MMRU and MMLH cells were grown to ~60% confluency and then transiently transfected with 3xFlag-JWA, pcDNA3.1-ILK, or ILK promoter plasmids, or transfected with shJWA, or shILK plasmids by Effectene reagent (Qiagen, Mississauga, ON), according to the manufacturer’s instructions. After transfection plus 48 h, cells were collected for use.

Quantitative real-time reverse transcription–PCR assay

Total RNA was extracted from cells using the QIAzol lysis reagent (Qiagen). One microgram of RNA was used for the reverse transcription reaction with a complementary DNA Synthesis Kit (Roche, Basel, Switzerland). Real-time PCR was carried out in triplicate with SYBR Green PCR Master Mix (Roche) using a 7900HT real-time PCR system (Applied Biosystems, Foster, CA). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. The Cq value for each sample was normalized to glyceraldehyde 3-phosphate dehydrogenase. Primer sequences are listed in Supplementary Table 1, available at Carcinogenesis Online.

Western blot and ELISA

Protein extracts for western blot were prepared with lysis buffer (10 mM N-2-hydroxyethylpiperezine-N-2-ethanesulfonic acid, pH 8.0, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM diethiothreitol, 0.2 mM ethylenediaminetetraacetic acid), complete protease inhibitor cocktail tablet. Protein concentration was checked by Bio-Rad protein assay (Bio-Rad). Western blot was performed as described previously (23). The following antibodies were used in this study: anti-ILK1, anti-integrin αV, anti-integrin β3, anti-NF-κB p65, anti-Stat3 (Cell signaling, Boston, MA), anti-Sp1 (Millipore, Billerica, MA), anti-p-Ser32-Ser386ERK1/2 (Cell Signaling), and anti-ILK1 (Novus Biologicals, Littleton, CO), anti-p50 and anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), anti-JWA (Abmax, Beijing, China), anti-Flag-tag (Applied Biological Materials, Richmond, British Columbia, Canada) and anti-actin (Sigma, St Louis, MO). Signals were detected with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). The quantification of western blots was analyzed by Image J (National Institutes of Health, Bethesda, MD).

For ELISA, the secreted IL-6 protein level in the conditioned medium (CM) was measured with a human IL-6 ELISA kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

Small interfering RNAs

MMRU cells were transfected at 60% confluency with small interfering RNAs (siRNAs) against integrin αV, integrin β3 and Sp1 (Qiagen ID numbers: SI00034342, SI00034349, SI00004585, SI00004599, SI01550983 and SI01550990) using SiLenTect reagent (Bio-Rad, Mississauga, ON) according to the manufacturer’s instructions.

Luciferase reporter assay

293T cells were seeded in triplicate in 24-well plates (1 x 10⁵ cells per well) and transiently transfected with ILK promoter plasmid and 3xFlag-JWA plasmid. To normalize for varying transfection efficiency, cells were co-transfected with the Renilla plasmid pRL-SV40. Luciferase activities were determined 48 h later using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Tube formation assay of HUVECs

The tube formation assay was performed as described previously (20,24). MMRU and MMLH cells were cultured in six-well plates to ~70% confluency with fresh serum-free medium for 24 h, and 1 ml of CM was collected. Multimicrofluidic BD Biosciences (Mississauga, Ontario, Canada) was coated on 96-well plates and kept at 37°C for 2 h. Then, 2 x 10⁴ HUVECs were suspended in 100 μl CM and added to the precoated 96-well plate. HUVECs tube formation was examined after incubation at 37°C incubator for 10 h. The cells were photographed under microscope, and the tubular structures were counted in randomly selected fields.

Matrigel plug assay in vivo and immunofluorescent staining

The matrigel plug assay was performed as described previously (20,24). MMRU cells with stable knockdown of ILK were transiently transfected with shJWA or shCtrl for 48 h. Four groups of cells (shCtrl/Vector, shJWA/Vector, shCtrl/shILK and shJWA/shILK) were suspended in 100 μl phosphate-buffered saline, mixed with 300 μl matrigel and implanted subcutaneously into the flanks of 5-week-old male severe combined immunodeficiency mice (4 x 10⁶/mouse, three mice per group). The mice were killed after 10 days and the implanted matrigel plugs were excised, photographed immediately and embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, Torrance, CA). After freezing at ~80°C for 1 h, 5 μm sections were cut from the embedded tissues using a CM1850 cryostat (Leica Microsystems, Concord, Ontario, Canada) and applied to glass slides, then stained with anti-mouse CD31, anti-human IL-6 and anti-human VEGF antibodies (Santa Cruz). 4’,6-diamidino-2-phenylindole (DAPI), Sigma, nuclear staining indicated the overall cell density of each matrigel plug. Confocal images were sequentially acquired by Zeiss AIM software on a Zeiss LSM 510 confocal microscope (Zeiss, Toronto, Ontario, Canada) with excitation at 488 nm (fluorescein isothiocyanate) and 340 nm (DAPI). Mean fluorescent intensity of CD31, IL-6 and VEGF was analyzed by Image J.

Tissue microarray construction

We selected 365 melanoma tissues as the ‘training set’ from the 1992 to 2001 archives of the Department of Pathology, Vancouver General Hospital. To validate the findings from the training set, we used an additional 199 melanoma tissue samples from January 2002 to 2009 as the ‘validation set’. In addition, 49 normal nevi and 100 dysplastic nevi samples were also retrieved. Due to loss of biopsy cores or insufficient tumor cells present in the cores, 383 melanoma and 122 cases of nevi were evaluated for JWA staining. The distributions of selected demographic characteristics of melanoma patients in the training set and validation set are listed in Supplementary Table 2, available at Carcinogenesis Online. To further confirm the JWA expression in human samples, we used a second TMA, and also stained ILK to investigate the correlation between JWA and ILK. A total of 237 biopsies, including 66 dysplastic nevi, 118 primary melanomas and 53 metastatic melanomas, were used for this TMA construction. Due to loss of biopsy cores, 45 dysplastic nevi, 81 primary melanomas and 49 metastatic melanomas could be evaluated for JWA or ILK staining.

Immunohistochemistry and evaluation of immunostaining

The immunohistochemistry procedure was performed as described previously (25). Slides were incubated with a primary monoclonal mouse anti-JWA antibody (1:30, Abcam), or a polyclonal rabbit anti-ILK antibody (1:100, Cell signaling) at 4°C overnight. The slides were then incubated with biotin-labeled secondary antibody and streptavidin–peroxidase for 30 min each, followed by development with diaminobenzidine substrate kit (DAKO Diagnostics) and counterstained with hematoxylin. The evaluation of JWA or ILK staining was blindly and independently examined by 2 observers, including one dermatopathologist. In the few cases with discrepancy between the two observers, the immunostained slides were reviewed with a double-viewing microscope so that the discrepancy was settled. JWA or ILK staining intensity was scored as 0, 1, 2+ and 3+. The percentage of JWA or ILK positive cells was also scored into four categories: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%). On the basis of the immunoreactive score, JWA or ILK staining pattern was defined as: negative (0), weak (1), moderate (2–6) or strong (7–12). We grouped negative and weak staining as low expression, and moderate and strong staining as high expression because the optimal value of cutoff points for the JWA scores was identified as 2 based on the predictive value of this cutoff point for patient survival.

Statistical analysis

In cell-based models, the data represents mean ± SD from three independent experiments unless otherwise indicated. Statistical analysis was performed by Student’s t-test. A P-value of <0.05 was considered significant. Differences in the demographic and clinical characteristics and expression levels of JWA and ILK were evaluated by chi-square test between patient subgroups. The Kaplan–Meier method and log-rank test were used to evaluate the correlation of JWA or ILK expression with the survival of patients. Multivariate Cox proportional hazard regression models were performed to estimate the hazard ratios (HRs) or adjusted HRs and their 95% confidential intervals. SPSS version 16 (SPSS Inc, Chicago, IL) software was used for all analyses.
Results

**JWA overexpression in melanoma cells inhibits the tube formation of HUVECs**

We used two melanoma cell lines, MMRU and MMLH, to overexpress or knock down JWA with 3×Flag-JWA or shJWA plasmids. The expression of JWA at protein and mRNA levels was tested by western blot and real-time PCR (Figure 1A and B). To provide evidence for the regulation of JWA on the angiogenic phenotype of melanoma cells, we determined the angiogenic ability of HUVECs by tube formation assay in matrigel *in vitro*. We found that when the CM from JWA-overexpressing cells was applied to HUVECs, the tube-forming network was drastically reduced compared with the control group (*P* < 0.01, *P* < 0.001, respectively; Figure 1C). However, HUVECs had greater angiogenic ability when cultured in CM collected from JWA knockdown (KD) cells (*P* < 0.01, *P* < 0.001, respectively; Figure 1C). Furthermore, we demonstrated that restoration of JWA in the JWA-KD cells can restore the inhibition of tube formation of HUVECs (*P* < 0.001, Figure 1D). These findings indicated that JWA expression in melanoma cells inhibits tube formation of HUVECs.

![Figure 1](https://academic.oup.com/carcin/article-abstract/34/12/2778/2464177/177)

**Fig. 1.** JWA in melanoma cells inhibits the tube formation of HUVECs. MMRU and MMLH cells were transiently transfected with the plasmids expressing JWA or JWA KD or their respective controls (3×Flag vector and sh control vector). (A) Whole-cell lysates were collected to check JWA expression by western blot. (B) RNA was extracted with Trizol and JWA messenger RNA level was determined by real-time PCR. ***P* < 0.001, compared with vector control group by Student’s *t*-test. (C) The conditioned media were prepared from MMRU and MMLH cells transfected with JWA, shJWA and control vector for detection of the tube formation of HUVECs. (D) The conditioned media were prepared from MMRU and MMLH cells transfected with shJWA/Vector, shCtrl/JWA, shJWA/JWA and control vector for detection of the tube formation of HUVECs. Tube formation in each group was photographed using a light microscope (scale bar 40 μm). The number of tubes formed per field was counted in five random fields. All data were presented as means ± SD. **P* < 0.01; ***P* < 0.001, compared with vector control group. ###P* < 0.001, compared with shJWA group by Student’s *t*-test.
JWA regulates ILK expression through integrin αVβ3 and Sp1

Because our previous study showed that ILK overexpression in melanoma cells enhanced the tube-forming ability of endothelial cells in vitro and microvessel formation in vivo (20), we asked whether there is a correlation between ILK and JWA expression in melanoma cells. We found that JWA overexpression inhibited, whereas JWA KD increased, ILK at the mRNA and protein levels (Figure 2A and B). We also examined whether ILK could exert any effect on JWA expression and found that both ILK overexpression and ILK KD did not have an effect on JWA expression (Supplementary Figure 1, available at Carcinogenesis Online). These findings indicated that JWA is the upstream regulator of ILK.

Next, we investigated how JWA regulates ILK expression. Because Lossner et al. (22) showed that integrin αVβ3 upregulated ILK expression in human ovarian cancer cells by enhancing ILK gene transcription, and our previous work demonstrated that JWA expression regulated melanoma metastasis by integrin αVβ3 signaling (12), we hypothesized that JWA may regulate ILK through integrin αVβ3. As expected, JWA overexpression downregulated, and JWA KD upregulated, integrin αVβ3 in MMRU cells (Figure 2C). To confirm that JWA regulates ILK through integrin αVβ3, we used specific siRNAs to silence integrin αV or integrin β3, and found that knocking down integrin αV or β3 abolished the upregulation of ILK in JWA-KD MMRU cells (Figure 2D).

Fig. 2. JWA inhibits ILK expression through integrin αVβ3 and Sp1. MMRU and MMLH cells were transiently transfected with 3×Flag-JWA, shJWA and vector control. (A) ILK mRNA levels in JWA overexpression or KD MMRU and MMLH cells were examined by real-time PCR. *P < 0.05; **P < 0.01, compared with vector control group. Student’s t-test. (B) The protein levels of 3×Flag-JWA and ILK were examined by western blot in JWA overexpression or KD MMRU and MMLH cells. (C) The protein levels of JWA, integrin αV and integrin β3 were examined by western blot in JWA overexpression or KD MMRU cells. (D) After silencing integrin αV or integrin β3 for 72 h using specific siRNA, the protein levels of JWA, integrin αV, integrin β3 and ILK were examined by western blot in JWA-KD MMRU cells. (E) 293T cells overexpressing JWA were transfected with the ILK promoter luciferase reporter plasmid. Whole-cell extracts were prepared after transfection for 48 h, and luciferase levels were measured by luminometry. **P < 0.01, compared with vector control group. Student’s t-test. (F) The protein levels of JWA in cytoplasm, and Sp1 in nuclei was examined by western blot in JWA overexpression or KD MMRU cells. (G) After silencing Sp1 for 72 h using specific siRNA, the protein levels of JWA, integrin αV, integrin β3, ILK and Sp1 were examined by western blot in JWA-KD MMRU cells.
To examine if JW A regulates ILK expression at the transcriptional level, the activity of the ILK promoter reporter was evaluated by dual-luciferase reporter assay. The results demonstrated that JW A-overexpressing cells exhibited a 47% inhibition (P < 0.01) in ILK promoter activity compared with vector control and normalized with Renilla activity (Figure 2E). Several transcription factor-binding sites within ILK promoter regions have been characterized, including regulatory elements for NF-κB, Ap-1, p53, Sp1 and so on (21,22). We used ILK promoter mutants for NF-κB/c-rel, p53 and Ap-1 transcription factors to perform luciferase assays and found that JWA overexpression does not have a significant effect on ILK promoter activity when the DNA consensus motifs for these transcription factors were disrupted (P > 0.05, Supplementary Figure 2, available at Carcinogenesis Online). Our previous study demonstrated that JWA KD could intensify melanoma integrin αvβ3 signaling by regulating nuclear factor Sp1 (12), and major transcription factors binding to the ILK promoter region are proteins of the Sp family (21). Here, we further determined whether JWA regulates ILK expression through Sp1. We found that JWA overexpression inhibited, whereas JWA KD promoted, transcriptional factor Sp1 expression in the nucleus (Figure 2F), and silencing Sp1 partially abrogated the upregulation of integrin αvβ3 and ILK in JW A-KD MMRU cells (Figure 2G). Sp1 is a ubiquitous transcription factor; to prove the decrease in integrin αvβ3 and ILK is a special response to knocking down Sp1, we checked inhibitor of growth 4 protein levels when Sp1 was knocked down. We found that inhibitor of growth 4 protein levels did not change when Sp1 was knocked down 72 h (Supplementary Figure 3, available at Carcinogenesis Online). Taken together, these results indicated that JWA regulates ILK through integrin αvβ3 and Sp1.

JWA inhibits the angiogenic ability of HUVECs through suppressing ILK signaling

Because we have demonstrated that JWA inhibited the tube formation of HUVECs (Figure 1C and D) and JWA is an upstream regulator of ILK (Figure 2A and B), and our previous study also showed that ILK was involved in melanoma angiogenesis (20), we hypothesized that JWA may regulate tube formation of HUVECs via ILK. We found that the CM from JWA-overexpressing cells clearly reduced, whereas CM from ILK-overexpressing cells significantly increased, the tube formation of HUVECs compared with the control group (P < 0.01, P < 0.001, respectively; Figure 3A). Interestingly, the CM from cells overexpressing both JWA and ILK showed significantly enhanced angiogenic activity of HUVECs compared with JWA overexpression alone (P < 0.001; Figure 3A), indicating that restoring ILK expression abolished the inhibition of tube formation by JWA.

To test if suppression of tube formation by endogenous JWA is dependent on ILK expression, we knocked down JWA or ILK alone in combination in MMRU cells, followed by the tube formation assay. We found that JWA KD drastically promoted, whereas ILK KD inhibited, the tube formation, compared with the control group (P < 0.001 for both; Figure 3B). Importantly, JWA/ILK double KD significantly decreased the angiogenic ability of HUVECs compared with JWA KD alone (P < 0.001; Figure 3B), further indicating that JWA-regulated angiogenic ability of HUVECs is ILK dependent.

Based upon our previous study about ILK’s regulation of melanoma angiogenesis through the nuclear factor-kappaB (NF-κB)/IL-6/STAT3/VEGF signaling pathway (20), further investigation of the function of JWA in this signaling pathway is deserved. We found that JWA overexpression significantly decreased, whereas JWA KD increased, IKK, IL-6 and VEGF mRNA levels in MMRU and MMLH cells (Supplementary Figure 4A–C, available at Carcinogenesis Online). We also observed that JWA inhibited the expression of p50, pSTAT3 and VEGF at the protein level in MMRU and MMLH cells (Supplementary Figure 4D, available at Carcinogenesis Online).

On the basis of these findings, we hypothesized that JWA regulated the NF-κB/IL-6/STAT3/VEGF signaling pathway through ILK. We found that JWA overexpression significantly inhibited, whereas ILK overexpression distinctly promoted, IL-6 and VEGF mRNA levels (Figure 3C). Furthermore, ILK overexpression partially abolished the repressive function of JWA (Figure 3C). To test if JWA regulates this signaling via ILK, we performed western blotting and IL-6 ELISA, and found that JWA overexpression clearly inhibited pIKBα, p50, pSTAT3, VEGF and IL-6 protein expression compared with the control group, whereas ILK overexpression dramatically increased the expression of these proteins (Figure 3D and Supplementary Figure 5, available at Carcinogenesis Online). Importantly, ILK overexpression partially abolished JWA-induced inhibition of the expression of these proteins (Figure 3D and Supplementary Figure 5, available at Carcinogenesis Online). This further indicated that JWA was able to regulate the NF-κB/IL-6/STAT3/VEGF signaling pathway through ILK.

JWA-regulated blood vessel formation is dependent on ILK in vivo

We next evaluated whether JWA could regulate angiogenesis through ILK in vivo. We have previously established stable ILK-KD MMRU cells by transfecting ILK-short hairpin RNA or control short hairpin RNA sequences (26). To clarify the role of ILK in JWA-regulated angiogenesis, we knocked down JWA in stable ILK-KD MMRU cells, and subsequently implanted the cells with matrigel into severe combined immunodeficiency mice. Visual examination of the excised matrigel plugs revealed varying levels of vascularization, with more blood vessels observed in JWA-KD group, but fewer blood vessels in ILK-KD group compared with the control group. Excitingly, ILK-KD abolished JWA-KD-induced blood vessel formation (Figure 4A), indicating that JWA-regulated blood vessel formation is dependent on ILK expression in vivo.

We then investigated the CD31 immunofluorescent staining in these matrigel plugs and found that the JWA-KD group contained denser and more extensive microvessel networks. In contrast, ILK-KD group exhibited a scanty pattern of endothelial structures (Figure 4B and C). Furthermore, there was a dramatically reduced blood vessel formation in both the ILA and ILK-KD groups, compared with JWA-KD alone group (Figure 4B and C). These data further confirmed that JWA-regulated blood vessel formation is dependent on ILK expression in vivo. Immunofluorescent staining was also conducted with anti-IL-6 and anti-VEGF antibodies, and expression patterns similar to CD31 were observed, suggesting that JWA regulates neo blood vessel formation by regulating IL-6 and VEGF expression (Figure 4B and C).

Reduced JWA expression closely correlates with melanoma progression

Our previous animal study showed that JWA inhibited melanoma metastasis (12). Furthermore, our present in vitro and in vivo study clarifies that JWA inhibits melanoma angiogenesis. We next asked if JWA expression is associated with melanoma progression and patient survival. We performed JWA staining in the TMA training set and validation set. We found that there was a clearly reduced JWA staining from normal nevi to dysplastic nevi, to primary and metastatic melanoma (P = 0.06, 0.0019 and 5 × 10^−6, respectively, Figure 5), suggesting that reduced JWA expression closely correlates with melanoma progression. From primary melanoma to metastatic melanoma, JWA staining was also dramatically reduced (P = 0.0002, Figure 5). Moreover, JWA staining was significantly decreased from American Joint Committee on Cancer (AJCC) stage II to stage III (P = 5 × 10^−5, Supplementary Table 3, available at Carcinogenesis Online), indicating that reduced JWA expression is crucial for melanoma metastasis. We also used a second TMA to further confirm the JWA expression in melanoma biopsies and obtained similar results (data not shown).

JWA expression significantly correlates with poor patient survival and is an independent prognostic marker for melanoma

A total of 370 melanoma patients had complete follow-up and clinical information. The Kaplan–Meier analyses revealed that reduced JWA expression was associated with poor overall (P = 0.039) and disease-specific 5 year survival (P = 0.028) in the training set (Figure 6A and B). This association was confirmed in the validation set for overall
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When these two sets were combined, the correlation between JWA expression and overall, and disease-specific 5 year survival was more significant ($P = 0.001$ and 0.007, respectively; Figure 6E and F).

Multivariate Cox proportional hazard regression analysis showed that JWA expression was a significantly favorable prognostic factor for overall (HR, 0.68; 95% confidential interval, 0.49–0.69; $P = 0.024$), and disease-specific 5 year survival (HR, 0.60; 95% confidential interval, 0.42–0.85; $P = 0.004$) (Figure 6G). These results indicate

Fig. 3. JWA inhibits the tube formation of HUVECs through suppressing ILK signaling. (A) MMRU cells were transiently transfected with pcDNA3.1/Flag V, pcDNA3.1/JWA, Flag V/ILK and JWA/ILK. The conditioned media were obtained from these MMRU cells and used to evaluate the effect on the tube formation of HUVECs (scale bar 40 μm). Quantification of the number of tubes was performed in five random fields for each group. $**P < 0.01; ***P < 0.001$, comparison with vector control group. $###P < 0.001$, compared with JW A overexpression group by Student’s t-test. (B) MMRU cells were transfected with Vector/shCtrl, Vector/shJWA, shCtrl/shILK and shJWA/shILK. Detection of the tube formation of HUVECs in each group (scale bar 40 μm). The number of tubes was counted in 5 random fields for each group. $***P<0.001$, compared with vector control group. $***P<0.001$, compared with JWA-KD group by Student’s t-test. (C) IL-6 and VEGF messenger RNA levels in JWA or ILK overexpression, and JWA/ILK co-overexpression MMRU cells, compared with respective controls as determined by real-time PCR. $***P < 0.001$, comparison with vector control group. $###P < 0.001$, compared with JWA overexpression group by Student’s t-test. (D) ELISA detection of IL-6 protein levels in the conditioned media of JWA or ILK overexpression, JWA/ILK co-overexpression and control MMRU cells. *$P < 0.05$; **$P < 0.01$, compared with vector control group. $##P<0.01$, compared with JWA overexpression group by Student’s t-test.
that JW A expression is an independent prognostic factor for melanoma patients.

Next, we examined whether JW A expression is correlated with patient survival after chemotherapy. Our results clearly indicated that the patients with high JW A expression who received chemotherapy had better 5 year survival than those with low JW A expression ($P = 0.012$) (Supplementary Figure 6, available at Carcinogenesis Online).

**Correlation between JW A and ILK expression and the influence of their concomitant expression on melanoma patient survival**

In this study, we have demonstrated that JW A-regulated melanoma angiogenesis is ILK dependent. Based on this finding, we next asked whether there is a correlation between JW A and ILK expression in melanoma patient samples, and whether this correlation can influence their concomitant expression on 5 year survival of melanoma patients. After the analysis, we found a negative correlation between JW A and ILK ($P = 0.026$; Supplementary Figure 7A, available at Carcinogenesis Online). Importantly, the patients with high JW A/low ILK expression showed a significantly increased 5 year survival rate, compared with patients with low JW A/high ILK expression using Kaplan–Meier survival curves ($P = 0.004$; Supplementary Figure 7B, available at Carcinogenesis Online). These data demonstrated that the expression pattern between JW A and ILK exerts significant influence on melanoma patient survival.

**Discussion**

High tendency to metastasize to other organs is the leading cause of melanoma patient deaths. Angiogenesis is one of the hallmarks of
tumor development, invasion and metastasis (7,27), and rapid angiogenesis of cutaneous melanomas dramatically enhances the risk of lethality that contributes to the progression of melanoma in young adults (28). Therefore, it is a priority to identify the factors that are involved in the regulation of melanoma angiogenesis. The JWA gene is located on chromosome 3p, a region associated with various cancers, including renal and lung cancer (29). We reported previously that JWA suppressed cell migration of cervical carcinoma, melanoma and liver cancer through mitogen-activated protein kinase cascade activation and F-actin cytoskeleton rearrangement (11). Furthermore, our recent study demonstrated that JWA inhibited melanoma cell metastasis in an animal model (12). However, there is no report on the potential role of JWA in tumor angiogenesis. In this study, we found that JWA overexpression significantly inhibited, whereas JWA KD strongly promoted, the tube formation of HUVECs. Strikingly, in vivo studies also indicated that JWA KD enhanced the melanoma angiogenesis, which prompted us to investigate the mechanisms involved in this process.

ILK is a multifunctional intracellular effector, and targeted ablation of ILK in the mammary gland resulted in delayed tumor growth in vivo (30). Blockade of ILK in prostate cancer has been shown to suppress VEGF-mediated endothelial cell migration, capillary formation and angiogenesis (31). In addition, our recent research showed that ILK overexpression in melanoma cells enhanced the tube formation ability of endothelial cells (20). The opposite properties of ILK and JWA in melanoma angiogenesis led us to explore their correlation in melanoma cells. We found that JWA regulated ILK expression, whereas ILK did not have any effect on JWA expression, suggesting that JWA is an upstream regulator of ILK. Because the molecular mechanism of elevated ILK expression in melanoma is not clear, how JWA regulates ILK expression deserves further investigation.

Some research has shown that integrins αV and β3 are crucial regulators of tumor angiogenesis. Blocking integrin αVβ3 was shown to successfully inhibit angiogenesis and promoted regression of tumors (32,33). The expression of integrin αVβ3 in various tumors was also associated with increased tumor growth and metastasis (34,35). Recent research demonstrated that integrin αVβ3 upregulated ILK expression by enhancing ILK gene transcription in human ovarian cancer cells (22). Combined with our previous result that JWA regulated melanoma metastasis by regulating Sp1 (12), we asked whether JWA regulated ILK via integrin αVβ3. As expected, silencing integrin αVβ3 using specific siRNA partially abolished the regulation of JWA on ILK expression.

Having shown the important role of JWA in regulating ILK expression, we further investigated whether JWA downregulates ILK expression at the transcriptional level. We found that JWA overexpression significantly inhibited ILK promoter activity. Several transcription factor-binding sites within ILK promoter regions have been characterized, including regulatory elements for NF-κB, Ap-1, Sp1 and so on (21,22). Our previous study demonstrated that JWA KD could intensify melanoma integrin αVβ3 signaling by regulating Sp1 (12). Sp1 is a zinc finger transcription factor, which is important.

Fig. 5. Correlation between JWA expression and melanoma progression. (A–H) Representative images of JWA immunohistochemical staining in human melanocytic lesions. (A and E) Strong JWA staining in normal nevi. (B and F) Moderate JWA staining in dysplastic nevi. (C and G) Weak JWA staining in primary melanoma. (D and H) Negative JWA staining in metastatic melanoma. (A–D, scale bar 40 μm; E–H, scale bar 20 μm). (I) JWA expression was reduced from normal nevi to dysplastic nevi, to primary melanoma and metastatic melanoma (n = 505, P = 0.06, 0.0019 and 5 × 10−4, respectively, chi-square test). JWA expression was reduced in metastatic melanoma compared with normal nevi, dysplastic nevi or primary melanoma (n = 505, P = 5 × 10−6, P = 2 × 10−6, P = 0.0002, chi-square test). NN, normal nevi; DN, dysplastic nevi; PM, primary melanoma; MM, metastatic melanoma.
for the transcription of many cellular and viral genes containing GC boxes in their promoters, and plays an important role in tumor growth and metastasis. Abnormal Sp1 expression and activation contribute to human cancer development and progression (36), and high Sp1 expression correlates with tumor aggressiveness and poor clinical outcome of patients (37). Our present data showed that JWA overexpression inhibited Sp1 expression, and silencing Sp1 inhibited both integrin αVβ3 and ILK expression to some extent. These findings
suggest that JWA regulates ILK expression through integrin αvβ3, and Sp1 plays an important role during this process. ILK is also identified as a promoter of vascular development and angiogenesis (38). When ILK is deleted in mice and zebrafish, vasculature formation was markedly decreased (39). VEGF has been shown to be the downstream target of ILK in regulating angiogenesis (31). The two mRNA isoforms of ILK (ILK1 and ILK2) share similar structural and functional properties. ILK1 is ubiquitously expressed in normal tissues and is upregulated in various tumors independent of transforming growth factor-β1. In contrast, ILK2 is expressed in highly invasive fibrosarcoma and melanoma cell lines, but not in normal tissue in a transforming growth factor-β1-dependent manner (40). In this study, we investigated the function of ILK (ILK1) in JWA-mediated effects on melanoma angiogenesis. We found that JWA overexpression inhibited, whereas JWA-KD promoted, ILK-induced angiogenic ability of HUVECs. The function of ILK2 in this process is still unclear and deserves further investigation in the future. Previously, we reported that increased NF-κB p50 expression significantly correlated with melanoma progression and poor patient prognosis (41). Recently, we further reported that the NF-κB p50 subunit promoted melanoma angiogenesis by upregulating IL-6 expression (23). IL-6 is a secreted proangiogenic factor (42) and is known to regulate VEGF through the STAT3 signaling pathway in some types of cancers and thereby promotes angiogenesis (43–45). Our present data further demonstrated that JWA inhibits melanoma angiogenesis through suppressing NF-κB/IL-6/STAT3/VEGF signaling via ILK.

Both our in vitro and in vivo data supported the fact that JWA functioned as a suppressor of melanoma angiogenesis, which inspired us to further investigate the value of JWA expression in melanoma progression. We used TMA and immunohistochemistry and found that JWA expression was significantly decreased from normal nevi to primary and metastatic melanoma (P \leq 0.0019 and 5 \times 10^{-6}, respectively). As a result, a decreased JWA expression was apparently crucial in melanoma progression. Because the main cause of melanoma patient death is tumor metastasis, we further analyzed the correlation between JWA expression and patient survival. We demonstrated that low JWA expression significantly correlated with a poor overall and disease-specific 5 year survival of melanoma patients (P = 0.001 and 0.007, respectively), and JWA expression was an independent factor for disease-specific 5 year survival (P = 0.004). One of the most important prognostic factors for melanoma patient outcome is the AJCC stage at the time of diagnosis. Our findings indicated that JWA expression was significantly decreased from early AJCC stages I and II to advanced stages III and IV (P = 0.0001), and the main difference of JWA staining was between AJCC stages II and III (P \geq 5 \times 10^{-6}), which corresponds to transition from primary melanoma to lymph node metastasis. This suggests a key role for JWA in melanoma metastasis. As metastasis is the leading cause of melanoma patient death (46), and angiogenesis is essential for tumor growth and metastasis (67, 6), it is not surprising to see that reduced JWA can lead to increased melanoma angiogenesis and promotion of tumor invasion and metastasis, and eventually results in worse survival of melanoma patients.

Previous reports showed that ILK expression correlated with tumor stage and grade in colon cancer and gastric cancer (16), and strong ILK expression predicts poor patient survival in non-small-cell lung cancer and prostate cancer (17,18,47). We previously showed that ILK upregulation strongly correlated with melanoma progression, invasion and inversely correlated with 5 year survival of melanoma patients (19). In our study, we compared the staining pattern of JWA and ILK proteins in our TMA result and found that there was a significant inverse correlation between their expressions. This further consolidated our observation that ILK expression was regulated by JWA in melanoma. More importantly, the expression levels of JWA and ILK were closely related to 5 year survival of patients (P = 0.0004). Moreover, patients with high JWA and low ILK expression showed a drastically increased 5 year survival compared with patients with low JWA but high ILK expression. This implies that the regulation of ILK expression by JWA plays a critical role for melanoma patient survival. Several small-molecule inhibitors of ILK have been tested in animal model studies and clinical trials, such as QLT0267, abolishing ILK downstream signaling to play a strong role in the treatment of acute myeloid leukemia (48,49). Therefore, JWA/ILK signaling in melanoma might provide new opportunities for therapeutic intervention.

Taken together, our data for the first time indicate that JWA functions as a suppressor in melanoma angiogenesis and this suppressive effect is through inhibiting ILK signaling. Also, TMA results demonstrate that reduced JWA expression is a critical event during melanoma progression and is associated with a worse melanoma patient survival. Furthermore, there is a significant inverse correlation between JWA and ILK, and their expression is closely associated with 5 year survival rates for melanoma patients. Therefore, these findings highlight the role of JWA as a promising therapeutic target for antiangiogenesis therapy in melanoma and it might be a novel prognostic marker for melanoma.

Supplementary material
Supplementary Tables 1–3 and Figures 1–7 can be found at http://carcin.oxfordjournals.org/

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