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Original Article

Vascular mimicry formation is promoted by paracrine TGF-β and SDF1 of cancer-associated fibroblasts and inhibited by miR-101 in hepatocellular carcinoma

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A B S T R A C T

Vascular mimicry (VM) describes the phenomenon that tumor cells but not endothelial cells form vascular-like channels, which provide blood perfusion for tumor tissues. VM is associated with tumor growth, metastasis and worse survival of different cancers. The mechanisms of VM formation remain largely unknown. We showed that the conditioned medium of cancer-associated fibroblast (CM-CAF) promoted tumor cells to form capillary-like structure *in vitro*. Consistently, co-implantation of CAFs with tumor cells significantly enhanced VM formation in mouse xenografts, and higher amount of CAFs was found in VM^+ human HCC tissues compared to VM^- ones. However, the CM-CAF-promoted VM formation was attenuated when TGF-β or SDF1 signaling was abrogated. Similar to CM-CAF, recombinant TGF-β1 and SDF1 induced VM formation. We further disclosed that the CAF-secreted TGF-β and SDF1 enhanced the expression of VE-cadherin, MMP2 and laminin5γ2 via TGF-βR1 and CXCR4 in tumor cells, thereby promoted VM formation. Moreover, tumor cells with high activity of self-sustaining TGF-β signaling displayed strong capability of VM formation. Subsequent investigations showed that miR-101, which was down-regulated in both tumor cells and CAFs, suppressed the CAF-promoted VM formation *in vitro* and *in vivo*. Gain- and loss-of-function analyses revealed that miR-101 attenuated TGF-β signaling transduction by targeting TGF-βR1 and Smad2 in tumor cells, and simultaneously abrogated SDF1 signaling by suppressing SDF1 expression in CAFs and inhibiting VE-cadherin expression in tumor cells. Our findings suggest that the miR-101-TGF-β/SDF1-VE-cadherin/MMP2/LAMC2 networks regulate VM formation and represent the potential targets for cancer therapy.

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Introduction

Development of adequate microcirculation is essential for overgrowth and metastasis of tumors. It has long been believed that microcirculation in cancers was dependent on angiogenesis, the formation of new capillaries from pre-existing vessels [1]. Recent studies have revealed that vascular mimicry (VM) is an important complement for tumor microcirculation [2,3]. VM refers to the vascular channel-like structure that is consisted of tumor cells but not endothelial cells, which is similar to embryonic vasculogenesis [4,5]. VM is correlated with short survival of malignant tumors [6–8]. Studies in the xenografts of breast cancer and melanoma reveal the perfusion ability of the VM channels [9,10], suggesting that VM provides an alternative pathway to supply oxygen and nutrition for rapidly growing tumors, as well as an escape route for metastasis. A few molecules, including VE-cadherin, matrix metallopeptidases (MMPs) and laminin have been shown to be critical for VM formation [5,11]. Expression of VE-cadherin in tumor cells can upregulate MMP14 expression and then activates MMP2, in turn cleaves laminin5γ2 (LAMC2) chain and results in the formation of γ2′ and γ2x fragments, which stimulates migration, invasion and VM formation of tumor cells [8,12]. Moreover, knockdown of VE-cadherin, LAMC2 or neutralizing antibody of MMP2/MMP14 inhibits melanoma cells to form VM structure

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in vitro, suggesting that activation of VE-cadherin/MMPs/LAMC2 pathway is essential for VM formation [13]. However, the extracellular signals that trigger the VM formation of tumor cells remain largely unknown.

The interaction between stromal cells and tumor cells plays a major role in tumor progression. Cancer-associated fibroblasts (CAFs) constitute the majority of stromal cells in cancer tissues [14]. The presence of CAFs is correlated with tumor development and worse survival of cancer patients [15,16]. CAFs secrete a wide spectrum of chemokines and cytokines to tumor microenvironment, thus promoting growth, invasion and angiogenesis of cancers [14,17]. To date, the role of CAFs in VM formation is not reported yet.

In this study, we found that the CAF-secreted TGF-β and SDF1 enhanced the expression of VE-cadherin, MMP2 and LAMC2 in tumor cells, consequently promoted VM formation. We further showed that miR-101, a microRNA (miRNA) that was downregulated in both tumor cells and CAFs, could suppress the CAF-promoted VM formation by abrogating both TGF-β and SDF1 signaling. These findings disclose a novel regulatory network of VM formation and provide potential targets for anti-cancer therapy.

Materials and methods

Human tissue specimens

Normal liver tissues were obtained from patients underwent resection of hepatic hemangiomas. Tumor tissues were obtained from pathologically confirmed HCC patients who underwent tumor resection at the Cancer Center of Sun Yat-sen University. No local or systemic anticancer treatment had been conducted before operation and no post-operative anticancer therapies were administered prior to relapse. Informed consent was obtained from each patient and the study was approved by the Institute Research Ethics Committee at Cancer Center, Sun Yat-sen University.

Reagents

The sources of reagents are described in the Supporting Materials and Methods.

Tumor cell lines

Human HCC cell lines QGY-7703, MHCC-97L and SMMC-7721 were maintained in DMEM supplemented with 10% FBS. Primary HCC cells, VETC-1 and VETC-2, were isolated from two HCC patients previously [18] and cultured in DMEM supplemented with 10% FBS. The primary fibroblasts including normal skin fibroblasts (NSF), normal liver fibroblasts (NLF) and CAFs were prepared as described in the Supporting Materials and Methods.

Preparing the conditioned medium of CAF (CM-CAF)

CAFs (2.5×10^5) were seeded in a 30-mm dish for 24 h, and then refreshed with 10% FBS-containing DMEM. The conditioned medium (CM) was collected 3 days later by centrifugation at 500*g* to remove the detached cells and then at 14,000*g* to discard cell debris (4 °C, 10 min each). Aliquots of CM were stored at −80 °C until used. CM-CAF was inactivated by heating at 100 °C for 10 min if needed.

Three-dimensional tube formation assays

Three-dimensional (3-D) culture was used to evaluate the ability of tumor cells to form VM structure. Cells (1.8×10^4) were resuspended in 200-μl mixture of equal volume of serum-free DMEM and 10% FBS-containing RPMI or CM-CAF, then added to a Matrigel-coated 48-well plate. The concentration of Matrigel used was 60%, unless otherwise indicated. After incubation at 37 °C for 4 h, the branch points of the formed tubes, which represented the degree of VM *in vitro*, were quantitated in 5 random low-power fields (150×) under a light microscope.

RNA oligoribonucleotides

All small interference RNA (siRNA) duplexes or single strand miRNA inhibitors (Supplementary Table 1) were purchased from GenePharma (Shanghai, PR China). siRNAs targeting human *TGF-β1* (NM_000660.5), *TGF-βR1* (NM_004612.3), *Smad2* (NM_001003652.3), *CXCR4* (NM_001008540.1) and *VE-cadherin* (XM_011522801.1), which were designated as siTGF-β1, siTGF-βR1, siSmad2, siCXCR4 and siVE-cadherin respectively, were designed using online tool siDESIGN (Dharmacon, IL, USA). The negative controls for RNA duplex (NC) or miRNA inhibitor (anti-NC) were non-homologous to any human genome sequence.

Vectors and luciferase reporter assay

The p-SBE reporter plasmid which contains twelve tandem Smad2/3 binding elements (SBEs) (generously provided by Dr. Ten Dijke, Leiden University Medical Center, the Netherlands) [19] was used to examine TGF-β signaling activity.

The luciferase reporter plasmids used to verify the target genes of miR-101 and the lentiviral vector that expressed human miR-101 were constructed as described in Supporting Materials and Methods. Primers used for PCR are provided in Supplementary Table 1.

Analysis of gene expression

Gene expression was analysed by real-time quantitative RT-PCR (qPCR), Western blotting or immunohistochemical staining as explained in Supporting Materials and Methods.

Double-staining for CD34 and Periodic Acid-Schiff (PAS)

To determine the VM pattern in human tissues and mouse xenografts, tissue sections were immunohistochemically stained for CD34, then stained with PAS (cat. BA4080A, BASO, Zhuhai, China) and counterstained with hematoxylin.

Mouse xenograft models

All experimental procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications Nos. 80-23, revised 1996), and according to the institutional ethical guidelines for animal experiments.

For subcutaneous xenograft model, female BALB/c athymic nude mice at 5–6 weeks of age were used. QGY-7703 cells (1×10^6) alone or mixed with 1×10^6 CAFs were injected subcutaneously into either side of the posterior flank of the same mice. Tumor growth was examined every 5 days over a course of 20 days. Tumor volume (V) was monitored by measuring the length (L) and width (W) of the tumor with calipers and was calculated with the formula $V = (L \times W^2) \times 0.5$.

To examine the effect of miR-101 on VM formation *in vivo*, the miR-101-expressing lentiviruses (3×10^8 vg) were intratumorally injected into tumor-bearing mice at the 15th day post-implantation of tumor cells. Five days later, xenografts were dissected and analysed for VM and tumor volume.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. The differences between the groups were analysed by Student's *t* test when two groups were compared or by one-way ANOVA followed by Tukey's test when more than two groups were compared. Analyses were performed with GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA, USA). All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

Results

CAFs promote VM formation in vitro and in vivo

To elucidate the effect of CAFs on VM formation, CAFs were isolated from primary human HCC tissues and then characterized by positive staining for the activated fibroblast marker (α-SMA) and mesenchymal marker (vimentin) and negative staining for epithelial marker (E-cadherin) and macrophage marker (CD68, Supplementary Figure S1). We first evaluated the effect of CM-CAF on the VM formation of tumor cells, using the *in vitro* 3-D culture model. As shown, QGY-7703, MHCC-97L and two cell lines isolated from primary HCC tissues, VETC-1 and VETC-2, hardly formed capillary-like structures, whereas treatment with CM-CAF significantly promoted these tumor cells to form VM (Fig. 1A and Supplementary Figure S2). On the other hand, SMMC-7721 cells exhibited strong tube formation ability even in the absence of CM-CAF, although the number of branch points decreased when the concentration of matrigel was reduced from 60% to 40%. Notably, CM-CAF also enhanced the *in vitro* VM formation of SMMC-7721 cells (Fig. 1B). Moreover, the promoting function of CM-CAF in VM formation was abrogated when CM-CAF was inactivated by heating (Fig. 1A, right panel). These results suggest that CAFs may promote tumor cells to form VM via secreted molecules.

To confirm the contribution of CAFs to VM formation *in vivo*, QGY-7703 cells alone or mixed with CAFs were injected

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