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Research Paper

DHX32 Promotes Angiogenesis in Colorectal Cancer Through Augmenting β-catenin Signaling to Induce Expression of VEGFA

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ABSTRACT

We previously reported that overexpression of DHX32 contributes to the growth and metastasis of colorectal cancer (CRC). However, the underlying mechanism is not largely characterized. Herein, we reported that DHX32 in CRC cells upregulated expression of vascular endothelial growth factor A (VEGFA) at the transcription level through interacting with and stabilizing β-catenin. This promoted the recruitment of host endothelial cells to the tumor, and therefore, formation of microvessel in the tumor. Xenograft model revealed that depletion of DHX32 in CRC cells significantly reduced the microvessel density in the grafts and suppressed the growth of grafts. Furthermore, the expression level of DHX32 was positively associated with microvessel density in human CRC and poor outcome of CRC patients. Therefore, the report demonstrates that DHX32 is a pro-angiogenic factor, that inhibition of DHX32-β-catenin pathway can provide a strategy for CRC treatment, and that the expression level of DHX32 has the potential to serve as a biomarker for CRC diagnosis and prognosis.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and accounts for 14% of all new cancer diagnoses(Fitzmaurice et al., 2015). Tumor angiogenesis that is required for cancer growth and metastasis by nourishing cancer cells and helping spread of metastatic cells to distant tissues has been considered as a potential target for CRC treatment (Hanahan and Weinberg, 2011). Understanding the molecular mechanism by which CRC cells promote angiogenesis is required to develop effective antiangiogenesis treatment for CRC.

Angiogenesis is a tightly regulated multistep process that includes endothelial cells breaking through the basement membrane, migrating toward angiogenic stimuli released from tumor cells, proliferating to provide sufficient cells for making a new vessel, and forming tubular structures (Bergers and Benjamin, 2003). The angiogenic switch depends on the balance of pro- and anti-angiogenic factors. Vascular endothelial growth factor (VEGF) is one of the key pro-angiogenic stimuli produced within the tumor microenvironment in pathological states (Carmeliet and Jain, 2011; Conway et al., 2001; Yancopoulos et al., 2000). In CRC, the level of VEGF is elevated and correlate with a poor clinical outcome (Takahashi et al., 1995; Ellis et al., 2000). VEGF has, therefore, been a major focus for the development of anti-cancer drug and is considered a negative prognostic indicator for CRC. Many signaling pathways are interconnected to activate VEGF expression during tumorigenesis and progression (Des Guetz et al., 2006; Kerbel, 2008; Grothey and Galanis, 2009). Transcription of *VEGF* is regulated by multiple external factors. The *VEGFA* promoter contains binding sites for numerous transcription factors, including SP1, AP2, c-JUN, EGR-1, HIF-1, and TCF (Pages and Pouyssegur, 2005; Liu et al., 2016). Recent work reveals that activated β-catenin translocates to the nucleus and complexes with TCF to promote transcription of *VEGFA* (Easwaran et al., 2003; Clifford et al., 2008), indicating that Wnt/β-catenin signaling regulates vessel formation. Therefore, targeting Wnt/β-catenin mediated VEGFA expression can be a new strategy for inhibiting angiogenesis.

RNA helicases are members of the DEAD/H-box family, which are characterized by the presence of a helicase domain and are involved in RNA posttranscriptional procession. In addition to their roles in RNA procession, multiple members of RNA helicases are also implicated in transcription regulations. Aberrant expression of these proteins have been reported in various solid and hematologic malignancies (Wilson and Giguere, 2007; Causevic et al., 2001; Schlegel et al., 2003;

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Abdelhaleem, 2004a). We reported previously that DHX32, a novel member of the DEAH family, is up-regulated in CRC and contributes to CRC proliferation, apoptosis, migration, and invasion. Array analyses revealed that depleting DHX32 in CRC cells suppressed expression of *VEGFA*, indicating that DHX32 is involved in tumor angiogenesis (Huang et al., 2009; Lin et al., 2015). However, the mechanism by which DHX32 upregulates expression of VEGFA remains unknown.

In this study, we showed that DHX32 promoted expression of VEGFA through augmenting β-catenin signaling. Ablation of DHX32 in CRC cells compromised their tumorigenicity and angiogenesis when they were grafted in mice. Furthermore, overexpression of DHX32 was associated with angiogenesis in CRC and poor outcomes of human CRC patients. These results reveal a mechanism by which DHX32 enhances angiogenesis and growth of CRC. It also uncovers the potential of DHX32 as a target for CRC treatments and as a biomarker for CRC diagnosis and prognosis.

2. Materials and Methods

2.1. Cell Culture and Reagents

Human CRC SW480, HCT-8, SW620, and HCT116 cells were grown in the RPMI-1640 medium (Gibco), human CRC HT-29 cells were grown in the Myco5A medium (Gibco), and HUVEC cells were grown in the DMEM medium (Gibco), with supplements of 10% (v/v) fetal bovine serum (Gibco) and 100 units/ml streptomycin and penicillin (Millipore), at 37 °C. Stable strains of SW480 cell with DHX32-overexpression or depletion were generated, characterized, and cultured as described in our previous publication (Lin et al., 2015). The conditioned medium was collected from confluent SW480 cells 24 h after changing the medium to the 0.2% FBS-DMEM medium. The medium was filtered with 0.22 μm filters prior to being used.

The anti-DHX32 antibody, anti-VEGFA antibody and anti-CD31 antibody were purchased from Abcam; anti-β-catenin antibody, anti-phospho-β-catenin (Ser33/37/Thr41) antibody, anti-β-TrCP antibody and anti-ubiquitin antibody from Cell Signaling; anti-β-Actin antibody and anti-HA antibody from Santa Cruz; anti-Flag antibody from Sigma.

2.2. DNA constructs and transfection

Expression vectors carrying the Flag- and His-tagged DHX32 cDNAs were constructed by sub-cloning the PCR product of human DHX32 cDNA into pCMV5 vector and were confirmed by sequencing. Human β-catenin cDNA, β-catenin truncated mutants, pTOPFLASH, and pFOPFLASH were generous gifts from Prof. B. Li. (Xiamen University, China). The renilla luciferase pRL-TK reporter was kindly provided by Prof. H-R Wang. (Xiamen University, China). The β-catenin cDNA was subcloned into pGEX4T-1 vector for bacterial expression. siRNA targeting β-catenin and the control siRNA were purchased from Santa Cruz. siRNA targeting DHX32 and the control siRNA were purchased from QIAGEN.

Transfections of siRNA and plasmid were performed using the HiPerFect Transfection Reagent (QIAGEN) and ViaFect Transfection Reagent (Promega) according to the manufacturer's protocol, respectively.

2.3. Quantitative real-time RT-PCR

Total RNA was extracted from cells using Trizol (TIANGEN) according to the manufacturer's protocol and the cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kits (Fermentas). Quantitative real-time RT-PCR was performed using gene-specific primers as described in our previous study (Huang et al., 2009). The expression of target transcripts was normalized to the β-actin internal control, and relative changes of gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. The primers for DHX32 are 5′-GTCTT TCCATCCACTACCAGCAC-3′(forward) and 5′-ATGATGACCCCATAGCTA CCCAA-3′(reverse), β-actin are 5′-TCACCCACACTGTGCCCATCTACGA-3′(forward) and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′(reverse), VEGFA are 5′-AGGGCAGAATCATCACGAAGT-3′(forward) and 5′-AGGG TCTCGATTGGATGGCA-3′(reverse), and β-catenin are 5′-CATCTAC ACAGTTTGATGCTGCT-3′ (forward) and 5′-GCAGTTTTGTCAGTTCAGG GA-3′(reverse).

2.4. Matrigel Invasion Assay

Cells were seeded in 24-well plates at a density of 2.5×10^4 per well. After attaching to the surface, the cells were washed with PBS and the culture media were replaced with 700 μl 0.2% FBS-DMEM Medium. After being cultured for 48 h, each well was then inserted a Matrigel invasion chamber with an 8.0 μm pore size membrane (BD) containing 0.75×10^5 serum-starved HUVECs in 0.5 ml 0.2% FBS-DMEM medium. After co-cultured for 24 h, non-migrating cells were removed from the upper chamber with a cotton swab and cells migrated through the membrane were fixed with 4% formaldehyde and stained with crystal violet staining solutions. Cell numbers in three randomly selected fields were photographed and counted under a microscope (Leica) with 200× magnification.

2.5. Scrap-wound Healing Assay

Cells were seeded in 6-well plates at a density of 10^5 cells per well and incubated for 24 h. Then a 20 μl pipette tip was used to scratch a linear wound in the cell monolayer. Photographs were taken at 0 and 24 h after scrapping.

2.6. Cell Proliferation Assays

The Cell Counting Kit-8 (CCK-8) (Dojindo) was used to measure cell density. 3×10^3 cells per well were seeded in 96-well plates and incubated for 48 h. The cells were then incubated with the CCK-8 reagent (10 μl per well) for 2 h prior to measure the absorbance of 450 nm with an ELISA plate reader (Thermo).

2.7. In vitro Angiogenesis Assay

HUVECs (2×10^4) were seeded in 24-well plates containing 0.5 ml solidified Matrigel (10 mg/ml) and cultured in the conditioned medium for 12 h. Images were acquired with a phase-contrast microscope. Average numbers of microtubes were counted in three individual wells and presented as mean ± s.d.

2.8. Affinity Purification of DHX32 Binding Proteins

DHX32 transiently expressed in SW480 cells was purified with anti-DHX32 antibody and protein A/G beads (Santa Cruz) in lysis buffer TNPE 0.2% (0.2% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1 mM EDTA). The bead-binding proteins were eluted by boiling in the loading buffer and then subjected to SDS-PAGE. The proteins were detected with silver staining, and specific bands were excised and analyzed by mass spectrometry.

2.9. Immunoprecipitation, Immunoblotting, and Ubiquitination Assays

Immunoprecipitation (IP), immunoblotting (IB), and ubiquitination assays were performed as previously described (Lin et al., 2014). Briefly, cell lysates prepared using ice-cold lysis buffer TNTE 0.5% (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100, containing 10 mg/ml pepstatin A, 10 mg/ml leupeptin, and 1 mM PMSF) were applied to IP or immunoblotting assays with appropriate antibodies. For ubiquitination assay, cell lysates were subjected to anti-β-catenin IP and the ubiquitin-conjugated proteins were detected by immunoblotting and Chemiluminescent HRP substrates (Millipore).