BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

RACK1 Suppresses Gastric Tumorigenesis by Stabilizing the β-Catenin Destruction Complex

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BACKGROUND & AIMS: Dysregulation of Wnt signaling has been involved in gastric tumorigenesis by mechanisms that are not fully understood. The receptor for activated protein kinase C (RACK1, GNB2L1) is involved in development of different tumor types, but its expression and function have not been investigated in gastric tumors. **METHODS:** We analyzed expression of RACK1 in gastric tumor samples and their matched normal tissues from 116 patients using immunohistochemistry. Effects of knockdown with small interfering RNAs or overexpression of RACK1 in gastric cancer cell lines were evaluated in cell growth and tumor xenograft. RACK1 signaling pathways were investigated in cells and zebrafish embryos using immunoblot, immunoprecipitation, microinjection, and in situ hybridization assays. **RESULTS:** Expression of RACK1 was reduced in gastric tumor samples and correlated with depth of tumor infiltration and poor differentiation. Knockdown of RACK1 in gastric cancer cells accelerated their anchorage-independent proliferation in soft agar, whereas overexpression of RACK1 reduced their tumorigenicity in nude mice. RACK1 formed a complex with glycogen synthase kinase Gsk3β and Axin to promote the interaction between Gsk3β and β-catenin and thereby stabilized the β-catenin destruction complex. On stimulation of Wnt3a, RACK1 repressed Wnt signaling by inhibiting recruitment of Axin by Dishevelled 2 (Dvl2). Moreover, there was an inverse correlation between expression of RACK1 and localization of β-catenin to the cytoplasm/nucleus in human gastric tumor samples. **CONCLUSIONS: RACK1 negatively regulates Wnt signaling pathway by stabilizing the β-catenin destruction complex and act as a tumor suppressor in gastric cancer cells.**

Keywords: Dvl2; Tumor Suppressor; *GNB2L1*; Stomach Cancer.

Gastric cancer is one of the most common malignancies and the second leading cause of cancer-related death worldwide.[1] Although *Helicobacter pylori* infection plays a role in gastric neoplastic transformation by causing chronic gastritis at an early stage of gastric cancer pathogenesis, the progression into an invasive cancer is a multistep process with numerous alterations in cell proliferation, differentiation, and/or survival; the underlying mechanism is largely unclear.[2]

Wnt/β-catenin signaling plays a pivotal role in embryogenesis as well as tumorigenesis.[3] In the absence of Wnt ligands, Ser/Thr residues in the N-terminus of β-catenin undergo constitutive phosphorylation by the cytoplasmic destruction complex containing adenomatous polyposis coli (APC), Axin, CK1α, and GSK3β, which in turn facilitates ubiquitination of β-catenin by β-TrCP E3 ligase.[4] The binding of Frizzled/low-density lipoprotein receptor-related protein (LRP) coreceptor with Wnt ligands initiates a signaling cascade, resulting in the inactivation of the cytoplasmic destruction complex. Consequently, β-catenin accumulates in cytoplasm and subsequently translocates into nucleus, where it acts as a transcription coactivator. Although the general framework for Wnt signaling has been well established, components of the signaling pathway have not been completely identified. For instance, although the recruitment of Axin by Dishevelled (Dvl) is very crucial in relaying Wnt/β-catenin signaling from cell membrane to the cytoplasmic β-catenin destruction complex,[5] the mechanism underlying the regulation of the interaction between Dvl and Axin is not fully understood.

It has been reported that Wnt/β-catenin signaling is one of the major causes of gastric cancer development. Nuclear accumulation of β-catenin, a hallmark of Wnt signaling activation, is found in more than 50% of gastric cancers.[6,7] However, unlike colon cancer, APC gene muta-

Abbreviations used in this paper: Dvl, Dishevelled; LRP, low-density lipoprotein receptor-related protein; MO, morpholino nucleotides; PKC, protein kinase C; RACK1, receptor for activated protein kinase C.

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tions are not common in gastric cancer, and β-catenin mutations are present in less than 30% of the Wnt-activated gastric cancers.[8] Thus, other mechanism(s) may contribute to the hyperactivation of Wnt signaling in gastric cancer.

Receptor of activated protein kinase C (RACK1, GNB2L1) is a 36-kilodalton cytosolic protein with a propeller-like structure of 7 WD40 repeats.[9] Because of its association with a large number of signaling proteins such as protein kinase C (PKC),[10] Src,[11] and PDE4D5,[12] RACK1 has been identified as an adaptor protein in multiple intracellular signal transduction pathways. As to the role of RACK1 in carcinogenesis, it was involved in the coordination of cell growth and movement.[13,14] However, some contradictory results exist in its oncogenic property.[9,13] The function of RACK1 in gastric cancer has not been explored.

In the present study, we show that the expression of RACK1 is decreased in gastric cancer, which is associated with tumor infiltration depth and poor differentiation status. In gastric cancer cells, knockdown of RACK1 promotes cell growth in liquid culture as well as in soft agar, whereas overexpression of RACK1 attenuates tumorigenicity of gastric cancer cells. Mechanistically, we identified RACK1 as a novel component of the β-catenin destruction complex, which negatively regulates Wnt/β-catenin signaling. RACK1 stabilizes β-catenin destruction complex in the "Wnt-off" state and suppresses Wnt-induced recruitment of Axin by Dvl in the "Wnt-on" state, both of which promote the phosphorylation, ubiquitination, and subsequent degradation of β-catenin. Our results suggest that RACK1 is a negative regulator in gastric cancer.

Materials and Methods

Cell Culture, Transfection, and Reporter Gene Assay

Details of cell culture, transfection, and reporter gene assay are available in Supplementary Materials and Methods.

Plasmid Construction

The open reading frame of human RACK1, β-catenin, and Dvl2 complementary DNA was cloned into the eukaryotic expression vector pCMV-myc, pCS2+MT, and pCMV-Tag2B (Invitrogen, Carlsbad, CA), respectively. The enhanced green fluorescent protein plasmid was purchased from BD Bioscience Clontech (Palo Alto, CA)(PT3027-5).

RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

RAN extraction and real-time polymerase chain reaction analysis were performed as described previously.[15] The procedures are detailed in Supplementary Materials and Methods.

Nuclear Protein Extraction

Nuclear extracts were prepared by the mini-extraction method as described previously.[16] Details are available in Supplementary Materials and Methods.

RNA Interference–Mediated Knockdown of RACK1

In our experiments, FG12 lentiviral vector was used to produce small, double-stranded RNA (siRNA) as described previously.[16] Two sequences (6# and 31#) targeting the messenger RNA (mRNA) of RACK1 are shown in Supplementary Table 1. Details are available in Supplementary Materials and Methods.

Immunofluorescence

Cells (HEK293, HEK293/con, or HEK293/RACK1) plated on cover slides were stimulated with Wnt3a conditioned medium or control medium and then washed thrice with phosphate-buffered saline at room temperature, fixed in methanol at −20°C for 10 minutes, and blocked with 2% bovine serum albumin at room temperature for 1 hour. Primary antibody incubations were performed overnight at 4°C (RACK1, 1:300; Axin, 1:100; Dvl2, 1:150; GSK3β, 1:150). After extensive washing with phosphate-buffered saline, secondary antibody (1:1000) incubations were performed at room temperature for 1 hour. Samples were washed again with phosphate-buffered saline and then mounted in Mowiol 4-88 (Sigma-Aldrich Inc, St Louis, MO). Fluorescence was monitored by inverted confocal laser microscopy (Carl Zeiss Thornwood, NY).

Immunohistochemistry

A total of 116 gastric tumor (26 gastric dysplasia) tissues and paired normal tissues were obtained from Cancer Hospital (Fudan University, Shanghai, China) from 2006 to 2009 after obtaining written informed consent. Immunohistochemistry was performed as described previously using anti-RACK1 (1:150) and anti–β-catenin (1:100) antibodies.[15] Details are available in Supplementary Materials and Methods.

Western Blot Analysis, Immunoprecipitation, and 2-Step Coimmunoprecipitation

Western blot analysis, immunoprecipitation, and 2-step coimmunoprecipitation were performed as described previously.[15,17] Details are available in Supplementary Materials and Methods. Antibodies to Flag, HA, myc, nucleporin62, c-Jun, β-catenin, β-actin, ubiquitin, Axin (sc-8567), and tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to GSK3β (used for Western blot and immunoprecipitation) and RACK1 were purchased from BD Transduction Laboratories (San Diego, CA); antibodies to phosphor-β-catenin (Ser33/37/Thr41) and Dvl2 antibody (used for immunoprecipitation) were purchased from Cell Signaling Technology Inc (Danvers, MA) (#3216); and Dvl2 antibody (sc-13974) and GSK3β antibody (sc-9166) used for immunostaining were purchased from Santa Cruz Biotechnology.

Crystal Violet Assay, Soft Agar, Boyden Chamber Assay Tumorigenicity Assay, and In Vivo Metastasis Assay

The details of crystal violet assay, soft agar, Boyden chamber assay, tumorigenesis assay, and in vivo metastasis assay are available in Supplementary Materials and Methods.

Zebrafish Experiments and Microscopy

Zebrafish experiments were performed as described.[18] Details are available in Supplementary Materials and Methods.