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Nuclear distribution of claudin-2 increases cell proliferation in human lung adenocarcinoma cells

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ABSTRACT

Claudin-2 is expressed in human lung adenocarcinoma tissue and cell lines, although it is absent in normal lung tissue. However, the role of claudin-2 in cell proliferation and the regulatory mechanism of intracellular distribution remain undefined. Proliferation of human adenocarcinoma A549 cells was decreased by claudin-2 knockdown together with a decrease in the percentage of S phase cells. This knockdown decreased the expression levels of ZONAB and cell cycle regulators. Claudin-2 was distributed in the nucleus in human adenocarcinoma tissues and proliferating A549 cells. The nuclear distribution of ZONAB and percentage of S phase cells were higher in cells exogenously expressing claudin-2 with a nuclear localization signal than in cells expressing claudin-2 with a nuclear export signal. Nuclear claudin-2 formed a complex with ZO-1, ZONAB, and cyclin D1. Nuclear distribution of S208A mutant, a dephosphorylated form of claudin-2, was higher than that of wild type. We suggest that nuclear distribution of claudin-2 is up-regulated by dephosphorylation and claudin-2 serves to retain ZONAB and cyclin D1 in the nucleus, resulting in the enhancement of cell proliferation in lung adenocarcinoma cells.

1. Introduction

Lung cancer is one of the leading causes of cancer-related deaths, and its incidence is increasing all over the world. Non-small cell lung cancer (NSCLC) comprises over 80% of lung cancers diagnosed and has poor survival with a 5-year survival rate of 15% [1]. The long-term survival rate of patients with NSCLC remains unsatisfactory due to an unclear pathological mechanism. NSCLC includes two major histological subtypes: lung adenocarcinomas and squamous cell carcinomas. Adenocarcinomas are the most common type in the United States and Asia [2]. Tumor cells have the basic characteristics of unlimited proliferation, invasion, and metastasis. Cell–cell adhesion is important in maintaining epithelial morphology, cell proliferation, and migration [3].

At the apical pole of the intercellular junction of the lateral membrane, epithelial cells form the tight junctions (TJs), which compose a large complex of proteins including integral membrane proteins and cytoplasmic plaque proteins [4]. The TJs separate apical and basolateral epithelial compartments and limit the free diffusion of solutes across these compartments. Furthermore, some tight junctional proteins were shown to be involved in the regulation of cell proliferation [5,6]. Claudins and occludin are tetraspanning proteins that extend their extracellular loops across neighboring cells. The carboxy terminus of most claudins has a PDZ-binding motif that can interact with the PDZ domains of scaffolding proteins including zonula occludens (ZO)-1, -2, and -3 [7]. Claudins comprise a large family of 27 members that form homo- and heterotypic associations with each other [8,9]. Different combinations of claudins can confer different properties to epithelial cells in terms of physiological and pathophysiological functions.

Several claudins have been reported to exhibit abnormal expression in human cancers. For example, claudin-1 is down-regulated in breast cancer [10], whereas it is up-regulated in colon carcinoma [11] and melanoma [12]. Claudin-4 is up-regulated in pancreas [13] and biliary tract [14] cancers. Normal lung epithelia express claudin-1, -3, -4, -5, -7, and -18 [15,16]. In contrast, the expression of claudin-2 is high in adenocarcinomas [17]. Claudin-2 expression may be useful in a differential diagnosis and as a target of chemotherapy against lung adenocarcinoma. Recently, we revealed that claudin-2 is up-regulated by activation of a MEK/ERK/c-Fos pathway in human lung adenocarcinoma A549 cells [17]. However, the pathophysiological function of claudin-2 has not been fully elucidated in A549 cells.

ZO-1 associated nucleic acid binding protein (ZONAB) is a Y-box transcription factor that suppresses cell proliferation mediated by its interaction with the SH3 domain of ZO-1 in highly confluent epithelial

Abbreviations: ZONAB, ZO-1 associated nucleic acid binding protein; NSCLC, non-small cell lung cancer; TJs, tight junctions

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cells [18]. In contrast, ZONAB is accumulated in the nucleus and up-regulates the expression of cell cycle regulators such as cyclin D1 and proliferating cell nuclear antigen in proliferating cells. ZONAB is overexpressed in several cancer tissues including the liver [19], pancreas [20], and colon [21]. The nuclear accumulation of ZONAB may be involved in the abnormal proliferation of cancer cells.

In the present study, we found that claudin-2 is distributed in the nucleus of human lung adenocarcinoma tissue and in both the nucleus and TJs of proliferating A549 cells. The introduction of claudin-2 siRNA decreased nuclear levels of ZONAB and cell cycle regulators, resulting in the suppression of G1/S cell cycle progression. The exogenous expression of claudin-2 with nuclear localization signal increased nuclear ZONAB levels and G1/S cell cycle progression in the endogenous claudin-2 knockdown cells. Claudin-2 formed a complex with ZO-1, ZONAB, and cyclin D1 in the nucleus. Nuclear distribution of claudin-2 was increased by dephosphorylation of Ser208. Our results indicate that claudin-2 may be abnormally distributed in the nucleus mediated by its dephosphorylation and enhances cell proliferation in lung adenocarcinoma.

2. Materials and methods

2.1. Materials

Rabbit anti-claudin-1, claudin-2, and ZO-1, goat anti-occludin, and mouse anti-claudin-2, and ZO-1 antibodies were obtained from Zymed Laboratories (South San Francisco, CA, USA). Unless otherwise indicated, rabbit anti-claudin-2 and mouse anti-ZO-1 antibodies were used in immunoblotting and immunofluorescence staining. Mouse anti-E-cadherin and nucleoporin p62 antibodies were from Becton Dickinson Biosciences (San Jose, CA, USA), rabbit anti-cyclin E1, mouse anti-Na^+/K^+-ATPase α subunit, and goat anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse anti-cyclin D1 antibody was from Lab Vision (Fremont, CA, USA), rabbit anti-ZONAB antibody was from Upstate Cell Signaling Solutions (Lake Placid, NY, USA), and rabbit anti-Rb and phosphorylated Rb (phosphor-Ser795) antibodies were from Signalway antibody (College Park, MD, USA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and 3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (PI) were from Dojindo laboratories (Kumamoto, Japan). All other reagents were of the highest grade of purity available.

2.2. Plasmid constructs

Human claudin-2 cDNA was amplified by RT-PCR and subcloned into the vector pCMV-Tag2, containing the FLAG epitope (BD Biosciences Clontech, Mountain view, CA, USA). The oligonucleotides encoding the nuclear localization signal (NLS) or nuclear export signal (NES) were fused to the amino-terminus of FLAG-claudin-2. The primers used to generate NLS-claudin-2 and NES-claudin-2 are listed in Supplementary Table 1. PCR products were digested by both Nhe I and Sal I, and were then cloned into the pTRE2/hyg vector (BD Biosciences Clontech). The S208A mutant of claudin-2 was generated using QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Sequence analysis was consigned to Bio Matrix Research (Chiba, Japan).

2.3. Cell culture and transfection

The human lung adenocarcinoma A549 cell line was obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 5% fetal calf serum (HyClone, Logan, UT, USA), 0.07 mg/ml penicillin-G potassium, and 0.14 mg/ml streptomycin sulfate in a 5% CO_2 atmosphere at 37 °C. Cells were transiently transfected with wild type claudin-2/pCMV-Tag2, S208A claudin-2/pCMV-Tag2, NLS-claudin-2/pTRE2, or NEL-claudin-2/pTRE2 vector using Lipofectamine 2000. Cells stably expressing the claudin-2/pSingle-tTS-shRNA vector were cultured in the presence of 1 μg/ml doxycycline for the inducible knockdown of endogenous claudin-2 expression [22]. Cell growth was assessed by counting the number of viable cells using a Neubauer chamber.

2.4. RNA isolation and RT-PCR

Total RNA was isolated from A549 cells using TRI reagent (Sigma-Aldrich). Reverse transcription was carried out with M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random primers. Single strand cDNA was amplified by PCR using GoTaq DNA polymerase under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 0.5 min; these steps were repeated for 30 cycles. The primers used to PCR are listed in Supplementary Table 1. Quantitative real time PCR was performed using FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany). The threshold cycle (ct) for each PCR product was calculated with the instrument's software, and ct values obtained for claudin-1, claudin-2, E-cadherin, and ZO-1 were normalized by subtracting the ct values obtained for β-actin. The resulting Δct values were then used to calculate the relative change in mRNA expression as a ratio (R) according to the equation $R = 2^{-(\Delta ct(\text{each time}) - \Delta ct(24\ h))}$.

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The preparation of cell lysates was performed as described previously [22]. Nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Samples were applied to SDS-PAGE and blotted onto a PVDF membrane. Phos-tag SDS-PAGE was used to analyze phosphorylation states of claudin-2. The membrane was then incubated with each primary antibody (1:1000 dilution) at 4 °C for 16 h, followed by a peroxidase-conjugated secondary antibody (1:5000 dilution) at room temperature for 1 h. Finally, the blots were incubated in Pierce immunoblotting Substrate (Thermo Fisher Scientific) and exposed to film, or incubated in ECL Prime Western Blotting Detection System (GE healthcare UK Ltd., Buckinghamshire, England) and scanned with a C-DiGit Blot Scanner (LI-COR Biotechnology, Lincoln, NE, USA). Band density was quantified with Doc-It LS image analysis software (UVP, Upland, CA, USA).

2.6. Immunoprecipitation

Cells were lysed in a immunoprecipitation buffer containing 150 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4), a protease inhibitor cocktail (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were incubated with anti-claudin-2 or ZONAB antibody and Protein G-sepharose (GE Healthcare UK Ltd.) or ExactaCruz (Santa Cruz Biotechnology) for 16 h at 4 °C with gentle rocking. A negative control was immunoprecipitated with rabbit IgG. After centrifugation at 6,000 ×g for 1 min, the pellet was washed three times with the immunoprecipitation buffer. The immune pellets were solubilized in a sample buffer for SDS-PAGE.

2.7. Immunofluorescence staining

Lung tumor tissue array was obtained from BioChain Institute (Newark, CA, USA). Tissue was fixed in formalin followed by embedded in paraffin. Tissue arrays are 6 μm in thickness and mounted on positively charged glass slides followed by stained with H & E to ensure the quality. The slide includes 15 sections prepared from patients with lung adenocarcinoma. After blocking with 5% bovine serum albumin for 1 h, the

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