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Decrease in paracellular permeability and chemosensitivity to doxorubicin by claudin-1 in spheroid culture models of human lung adenocarcinoma A549 cells

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ABSTRACT

Chemotherapy resistance is a major problem in the treatment of cancer, but the underlying mechanisms are not fully understood. We found that the expression levels of claudin-1 (CLDN1) and 3, tight junctional proteins, are upregulated in cisplatin (CDDP)-resistant human lung adenocarcinoma A549 (A549R) cells. A549R cells showed cross-resistance to doxorubicin (DXR). Here, the expression mechanism and function of CLDN1 and 3 were examined. CLDN1 and 3 were mainly localized at tight junctions concomitant with zonula occludens (ZO)-1, a scaffolding protein, in A549 and A549R cells. The phosphorylation levels of Src, MEK, ERK, c-Fos, and Akt in A549R cells were higher than those in A549 cells. The expression levels of CLDN1 and 3 were decreased by LY-294002, a phosphoinositide 3-kinase (PI3K) inhibitor, and BAY 11-7082, an NF-κB inhibitor. The overexpression of CLDN1 and 3 decreased the paracellular permeability of DXR in A549 cells. Hypoxia levels in A549R and CLDN1-overexpressing cells (CLDN1/A549) were greater than those in A549, mock/A549, and CLDN3/A549 cells in a spheroid culture model. In contrast, accumulation in the region inside the spheroids and the toxicity of DXR in A549R and CLDN1/A549 cells were lower than those in other cells. Furthermore, the accumulation and toxicity of DXR were rescued by CLDN1 siRNA in A549R cells. We suggest that CLDN1 is upregulated by CDDP resistance through activation of a PI3K/Akt/NF-κB pathway, resulting in the inhibition of penetration of anticancer drugs into the inner area of spheroids.

1. Introduction

Lung cancer is one of the most frequent cancers in the world and is divided into two major subtypes: small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC accounts for 83% of lung cancers diagnosed and is associated with poor survival, with a 5-year survival rate of 17% [1]. Adenocarcinoma, one type of NSCLC, is the most frequent onset type and typically has lower sensitivity to chemotherapy and radiation therapy. An acquired resistance to an anticancer drug could additionally result in cross-resistance to multiple drugs. Approximately 50–70% of patients with lung adenocarcinoma acquire a chemoresistant phenotype after clinical surgery [2]. The development of drug resistance is caused by various mechanisms including increased drug efflux, enhanced detoxification, modification of drug targets, and inhibition of apoptosis [3]. Overexpression of the ATP binding cassette (ABC) transporter ABCC2 in cancer cells confers higher resistance to various anticancer drugs including cisplatin (CDDP), anthracyclines, and vinca alkaloids [4].

The tumor microenvironments of inner cells of spheroids, which are formed *in vivo*, are also a cause of drug resistance. Because of their location far from blood vessels, the inner cells of spheroids experience hypoxia and oxidative stress, nutrients starvation, high intestinal fluid pressure, and altered structure of the extracellular matrix, and cell-cell adhesion [5]. Hypoxia and oxidative stress induce the expression of genes involved in chemoresistance. In addition, tumor microenvironments prevent the delivery of anticancer agents to the inner cells of spheroids. Cancer cells grown in three-dimensional (3D) environments often show more resistance to anticancer agents than those grown in two-dimensional (2D) monolayer cultures [6]. However, the mechanisms underlying chemoresistance are complex and not fully understood in 3D environments.

Epithelial cells form junctional complexes including, tight junctions

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Table 1
Primers for PCR amplification.

Name	Direction	Sequence
CLDN1	Forward	5′-ATGAGGATGGCTGTCATTGG-3′
CLDN1	Reverse	5′-ATTGACTGGGGTCATAGGGT-3′
CLDN2	Forward	5′-ATTGTGACAGCAGTTGGCTT-3′
CLDN2	Reverse	5′-CTATAGATGTCACACTGGGTGATG-3′
CLDN3	Forward	5′-GGATGAACTGCGTGGTGCAGA-3′
CLDN3	Reverse	5′-AGGATGGCCACCACGATGAG-3′
CLDN4	Forward	5′-TTGTCACCTCGCAGACCATC-3′
CLDN4	Reverse	5′-GCAGCGAGTCGTACACCTTG-3′
CLDN5	Forward	5′-AACATCGTGACGGCGCAGACCA-3′
CLDN5	Reverse	5′-TCAGAGCCAGCACCGAGTCGTACA-3′
CLDN7	Forward	5′-TTTTCATCGTGGCAGGTCTT-3′
CLDN7	Reverse	5′-GGCCAAACTCATACTTAATGTTGG-3′
CLDN18	Forward	5′-CTCCGTGTTCCAGTACGAAG-3′
CLDN18	Reverse	5′-CCCAGGATGGTGAAATAGGG-3′
HO-1	Forward	5′-AAGATTGCCCAGAAAGCCCTGGAC-3′
HO-1	Reverse	5′-AACTGTCGCCACCAGAAAGCTGAG-3′
NQO-1	Forward	5′-GAAGAGCACTGATCGTACTGGC-3′
NQO-1	Reverse	5′-GGATACTGAAAGTTCGCAGGG-3′
GCLM	Forward	5′-TGTCTTGGAATGCACTGTATCTC-3′
GCLM	Reverse	5′-CCCAGTAAGGCTGTAAATGCTC-3′
β-Actin	Forward	5′-CCTGAGGCACTCTTCCAGCCTT-3′
β-Actin	Reverse	5′-TGCGGATGTCCACGTCACACTTC-3′

(TJs) and adherens junctions, between neighboring cells. TJs are located in the most apical region of the lateral membrane and control paracellular permeability to ions, other small solutes, and water [7–9]. TJs consist of transmembrane proteins including, claudin (CLDN) and occludin, adaptor proteins, such as zonula occludens (ZO)-1 and ZO-2, and signaling proteins [10,11]. CLDNs constitute a family of over 20 members in mammals and subtypes form homo- and heterophilic associations with each other [12,13]. CLDN1, 3, 4, 5, 7, and 18 are expressed in normal lung epithelia. In contrast, CLDN2 is highly expressed in human lung adenocarcinoma tissues and A549 cells derived from human lung adenocarcinoma [14]. Different combinations of CLDN subtypes can confer distinct properties to epithelial cells in terms of physiological and pathophysiological functions.

Abnormal expression of CLDN subtypes has been reported in a variety of solid cancers. Patients with positive CLDN1 expression had a poorer prognosis than patients with negative CLDN1 expression [15]. Conversely, high miR-375 expression, which reduces CLDN1 expression, has been correlated with a shorter survival time in clinical NSCLC samples. Knockdown of CLDN1 increases invasion and metastasis in lung adenocarcinoma cells [16]. In addition, the overexpression of CLDN1 inhibits cell migration, invasion, and metastatic colonization in CL1–5 lung adenocarcinoma cells, which lack endogenous CLDN1 expression [17]. Therefore, it is unclear whether CLDN1 is involved in the development of lung cancer and its prognosis.

In the present study, we found that the expression of CLDN1 is increased by CDDP resistance in A549 cells. The upregulatory mechanism of CLDN1 was examined using specific inhibitors of intracellular signaling pathways. In addition, the effect of CLDN1 expression on cell damage caused by anticancer drugs was investigated in 2D monolayer and 3D spheroid cell cultures.

2. Material and methods

2.1. Materials

Rabbit anti-CLDN1, anti-CLDN3, anti-CLDN7, and anti-CLDN18 antibodies and mouse anti-CLDN2, anti-CLDN4, anti-CLDN5, and anti-ZO-1 antibodies were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Goat anti-β-actin, rabbit anti-p-ERK1/2 antibodies, and mouse anti-p-c-Fos and anti-Sp1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p-Akt, anti-Akt, anti-ERK1/2, anti-c-Fos, anti-p-MEK and anti-Src antibodies were from Cell

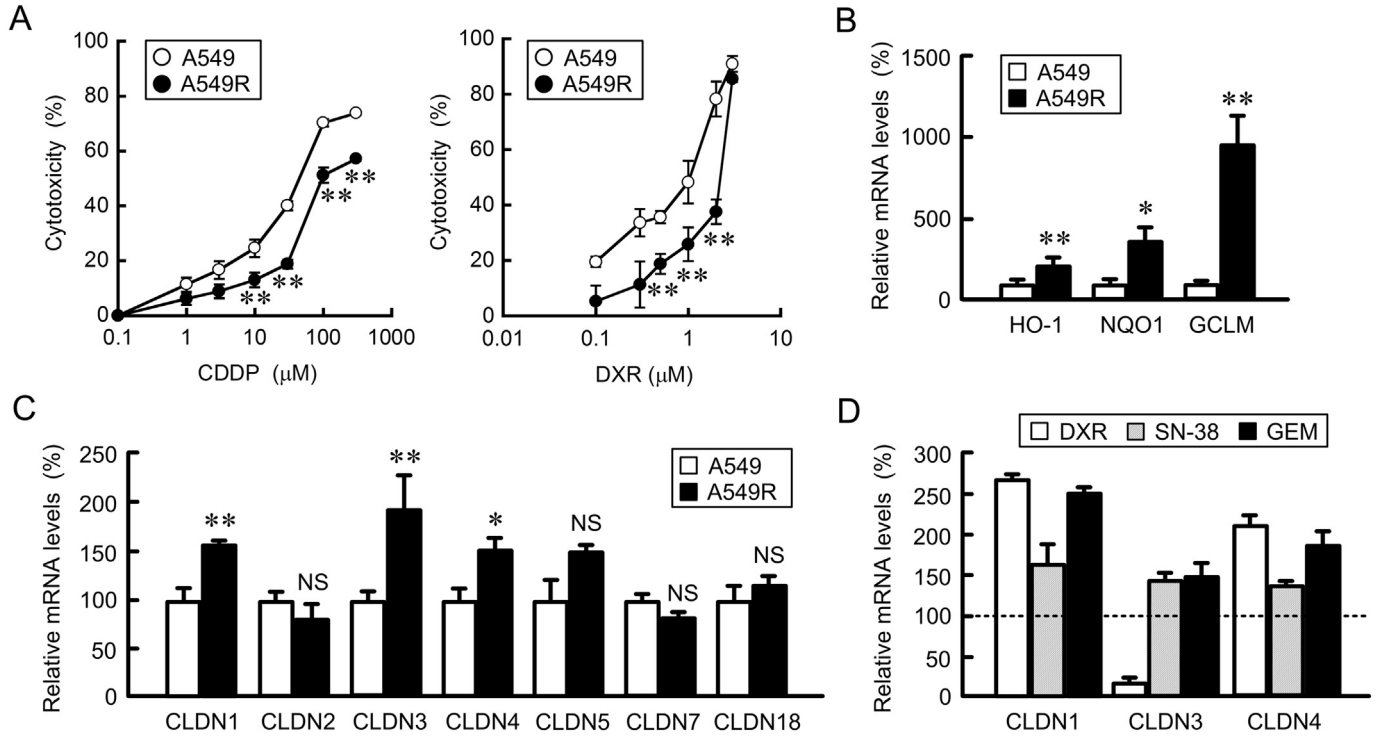

Fig. 1. Effects of chemoresistance on the mRNA levels of anti-oxidant enzymes and CLDNs in A549 cells. (A) A549 and A549R cells were cultured in a 96 well plate for 48 h. CDDP and DXR were treated for 48 h at the concentrations indicated. Cytotoxicity was assessed using WST-1 assays. (B and C) Total RNA was isolated from A549 and A549R cells. After reverse-transcription into cDNA, quantitative real-time PCR was performed using primer pairs for HO-1, NQO1, GCLM, and CLDNs. The expression level of mRNA is represented as a percentage of the values in A549 cells. (D) Total RNA was isolated from DXR-, SN-38-, and GEM-resistant A549 cells. The mRNA levels of CLDN1, 3, and 4 are represented as a percentage of the values in A549 cells. n = 3–4. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with A549 cells. NS, $P > 0.05$.