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Original Article

Connexin 43 affects intrinsic gefitinib resistance of NSCLC cells by GJIC

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Abstract: Gefitinib efficiency in non-small-cell lung cancer (NSCLC) therapy is limited as a majority of NSCLC cells with no epidermal growth factor receptor (EGFR) mutations are intrinsic gefitinib resistance. The molecular mechanisms of intrinsic gefitinib resistance remain unclear. In this study, we found that wild type EGFR (WT EGFR) cell lines A549 and H1299 were intrinsic gefitinib resistance, while WT EGFR cell line Calu3 was gefitinib sensitive. The results showed that Connexin (Cx) 43, the predominant Cx isoform expressed in various NSCLC cell lines, was highly expressed in the gefitinib-resistant (GR) cells lines and the up-regulation of Cx43 at the plasma membrane and their derived gap junctional intercellular communication (GJIC) in A549 and H1299 cells may contribute to the intrinsic resistance to gefitinib by activation of PI3K/Akt signaling pathway.

Keywords: NSCLC, Cx43, gefitinib resistance, GJIC

Introduction

Lung cancer, especially the non-small-cell lung cancer (NSCLC), is the leading cause of cancer death in many countries [1]. Chemotherapy, the mainstay of treatment in advanced disease, shows only marginally effective [2, 3]. Gefitinib, a kind of tyrosine kinase inhibitors (TKIs), which inhibits the EGFR pathway, shows promise in the treatment of metastatic NSCLC [4]. Although those TKIs have an impressive responses in a subset of treated cancer patients, recent study shows that only tumors with EGFR mutations are highly sensitive to gefitinib while tumors with no EGFR mutations show resistance to gefitinib [5-7]. Therefore, it is critical to establish the mechanisms of this drug resistance for the application of this knowledge to the development of strategies to combat the resistance [8-10].

Connexins are a group of homologous proteins that form the inter-membrane channels of gap junctions [11]. Cx43 is one of the most common of the connexins and the major Cx homolog expressed in lung tissue [12-14]. Studies have shown that Cx43 plays important roles in cancer development, cell proliferation, apoptosis, invasion and metastasis in lung cancer [15-17]. Most importantly, Cx43 is able to sensitize NSCLC cells to CDDP and ionizing radiation [18, 19]. Many human tumors, including lung cancer, have been reported to be deficient in the expression of Cx43 mRNA and protein levels [20-24]. And it has been reported that Cx43 is closely related with drug resistance [25].

In the present study, three wild type EGFR cell lines (A549/H1299/Calu3) was chosen to detect the IC_{50} of gefitinib. We found that Cx43 is involved in the intrinsic resistance to gefitinib in these cell lines. And the up-regulation of Cx43 at the plasma membrane and their derived GJIC in A549 and H1299 cells may contribute to the intrinsic resistance to gefitinib by activation of PI3K/Akt signaling pathway.

Materials and methods

Reagents and antibodies

Gefitinib was provided by AstraZeneca (London, UK) and dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 10 mM (stored at

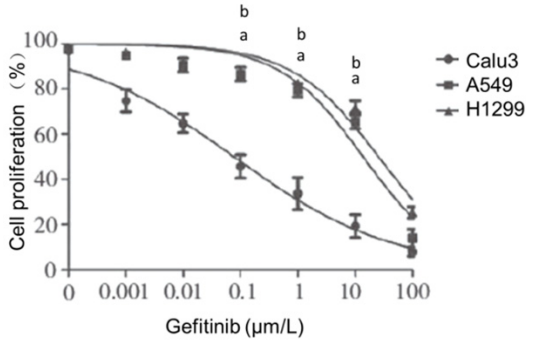

Figure 1. Comparison of gefitinib sensitivity between Calu3/A549, and H1299 cells. Cell proliferation was measured by MTT assay. Data are presented as mean ± S.D. from four independent experiments. a: $P<0.01$ for A549 cells versus Calu3 cells. b: $P<0.01$ for H1299 versus Calu3 cells.

-20°C) and then diluted in a culture medium before use. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Polyclonal antibodies against Cx43 and anti-β-actin, HRP-labeled Goat anti-rabbit secondary antibody and H&L-labeled donkey anti-rabbit secondary antibody were purchased from Abcam company. Monoclonal antibody against Cx43 was purchased from Santa Cruz company (American). Monoclonal antibodies against p-Akt (Ser473) and Akt were purchased from CST company.

Cell lines and cell culture

The human lung cancer cells Calu3, A549 and H1299 were obtained from the American Type Culture Collection (Manassas, USA) and routinely cultured in RPMI medium 1640 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified 37°C incubator containing 5% CO_2.

MTT assay

The three kinds of NSCLC cells were cultured in 96-well culture plates 100 μL per well at an initial density of 1×10^5 cells/mL. Following adherence overnight, the cells were devided into 3 groups: gefitinib group (0, 0.001, 0.01, 0.1, 1.0, 10, 100 μmol/L of gefitinib), blank group (solvent only), control group (medium only). After 96 h's culturing, 20 μL Thiazolyl Blue was added to each well of the 96-well culture plates. Then the medium was removed and 150 μL DMSO was added to per well after 4 h's incubation. Cell viability was detected by an automatic microplate reader (TEAN, Swiss) at a 570 nm wavelength. The inhibitory rate and IC_{50} of cell proliferation was calculated.

Western blot detect the expression of Cx43 and p-Akt

Cells were harvested using lysis buffer (Tris–HCl pH 7.4 20 mM, NaCl 150 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, ethylene glycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 1 mM, Triton 1%, sodium pyrophosphate 2.5 mM, Na3V04 1 mM, β-glycerophosphate 1 mM, protease inhibitors 1:1000). Cell lysate was sonicated and centrifuged at 12000 rpm for 30 min at 4°C. DC protein assay kit was used to determine the total protein concentration (Bio-Rad Co., Hercules, CA, U.S.A.). Twenty-five micrograms of protein from each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. The blotted membrane was incubated with primary antibodies at final dilutions ranging between 1/1000 and 1/2000 and then probed with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody. All Western blotting exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by Quantity One software on a GS-800 densitometer (Bio-Rad Laboratories).

"Parachute" Dye-coupling assay

This assay for gap junction function was performed as described by Goldberg *et al.* [26] and Koreen *et al.* [27]. Cells were grown to confluence in 12-well plates. Donor and receiver cells were grown to confluence. Donor cells from one well were incubated with a freshly made solution of 5 μM calcein-AM in growth medium for 30 min, at 37°C. Calcein-AM was converted into the gap junction-permeable dye calcein intracellularly. The donor cells were then trypsinized and seeded onto the A549, H1299 and Calu3 receiver cells at a 1:150 donor/receiver ratio. They were allowed to attach to the monolayer of receiver cells and form gap junctions for 4 h at 37°C and then examined with a fluorescence microscope. The average number of receiver cells containing calcein per donor cell was considered as a measure of the degree of

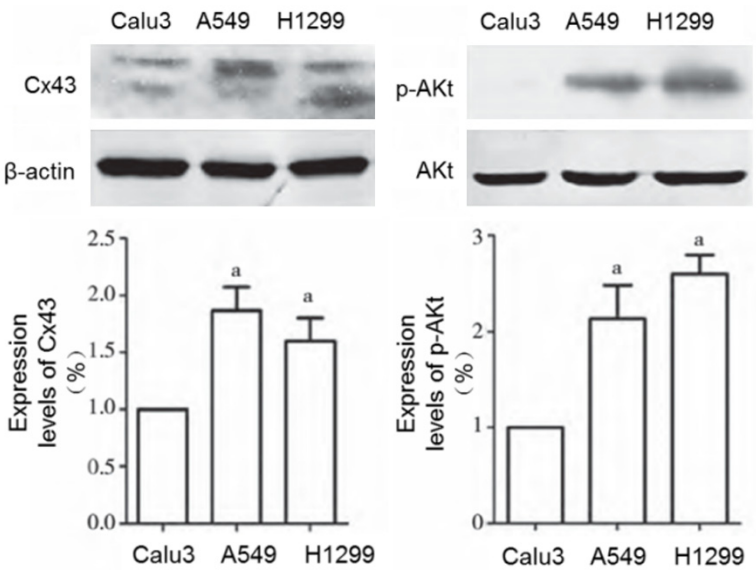

Figure 2. Western blot analysis of Cx43 and p-Akt proteins. Bar graphs are derived from densitometric scanning of the blots. Error bars are mean ± S.D. from three independent experiments. a, $P<0.01$ for A549 and H1299 cells versus Calu3 cells.

GJIC. Drugs tested by this way were added to cell simultaneously with donor cell to receiver cell except GJIC enhancer retinoic acid (RA), which was added to receiver cells 24 h before testing.

Immunofluorescence detect the location of Cx43 in cell

The cells were grown on coverslips for 24 h and then fixed in cold methanol for 10 min. Cells were blocked in 2% bovine serum albumin for 30 min at room temperature and incubated with rabbit anti-Cx43 primary antibody (diluted 1:50, Santa Cruz, Dallas, TX, USA) overnight at 4°C. The cells were then incubated with H&L-labeled Donkey Anti-rabbit secondary antibody (diluted 1:200) for 1 h at room temperature followed by counterstaining with 4', 6-diamidino-2-phenylindole (DAPI). Images were acquired on a confocal laser scanning fluorescence microscope (Nikon A1) and analyzed using the NIS-Elements software (Nikon Corporation, Tokyo, Japan).

Statistical analysis

Data were presented as means ± S.D. One-way ANOVA and LSD were used to analyze all experimental data by using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA)

Results

Effects of gefitinib on the NSCLC cells proliferation in vitro

To explore the gefitinib sensitivity of these NSCLC cells, cell proliferation was measured by MTT assay. By continuous exposure to gefitinib starting at 0.001 μM and increasing in a stepwise manner to 100 μM, we found that Calu3 was sensitive to gefitinib with IC_{50} of 0.064±0.011 μM, while A549 and H1299 were much less sensitive with IC_{50} of 13.64±0.18 μM and 20.054±0.11 μM, respectively (**Figure 1**). So we concluded that NSCLC cells A549 and H1299 were gefitinib intrinsic resistance cells [28].

Cx43 and p-Akt are highly expressed in A549 and H1299 cells

To study the relationship between the expression level of Cx43/p-Akt and the sensitivity of different NSCLC cells to gefitinib, we detected the expression level of Cx43 and p-Akt. As described in **Figure 2**, the expression levels of Cx43 and p-Akt in A549 and H1299 cells were much higher than those in Calu3 cells ($p<0.01$). It indicated that the higher expression level of Cx43 and p-Akt in intrinsic GR A549 and H1299 cells may be related to the activation of PI3K/Akt signal pathway.

Cx43 acts on the intrinsic GR of NSCLC cells by GJIC

As it has been reported that Cx43 plays important roles in lung cancer development, cell proliferation, apoptosis, invasion and metastasis by GJIC. We next explored whether the intrinsic GR of A549 and H1299 cells was related to the GJIC by "Parachute" Dye-coupling assay. We found that there were detectable fluorescence transfers in A549 and H1299 cells while fluorescence transfer in Calu3 cells was undetectable. In order to exclude the involvement of undetectable GJIC in Calu3 cells, GJIC was further measured in the presence of 10 μM of RA for 24 h. The result still showed no GJIC in

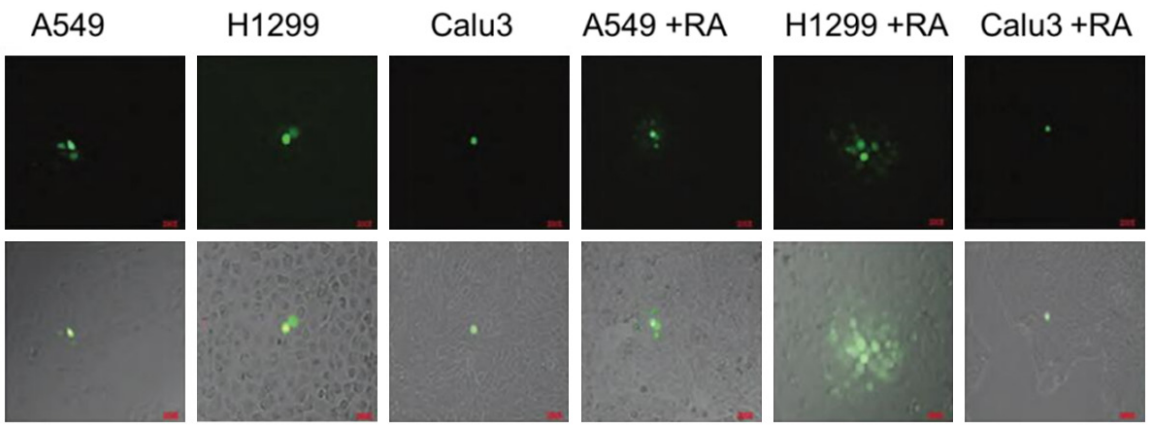

Figure 3. Functional GJIC was detected by parachute assay. No detectable GJIC was found in Calu3, and there were detectable GJIC in A549 and H1299 cells. No enhancement of GJIC in these cells incubated with RA. Top: fluorescence images. Bottom: overlaid the corresponding phase-contrast images. Original magnification, ×200.

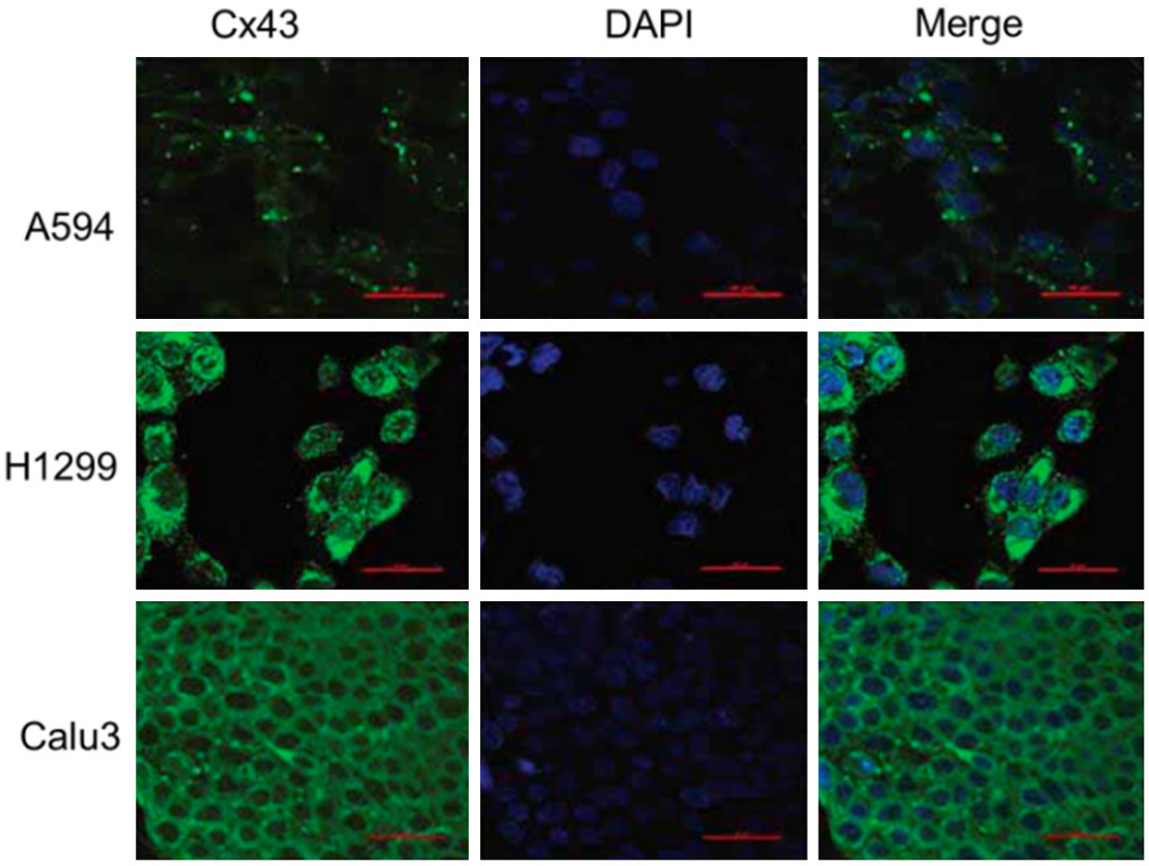

Figure 4. Immunofluorescence staining of the cellular localization of Cx43 with or without RA treatment.

Calu3 cells (**Figure 3**). Then we detected the location of Cx43 in cells by Immunofluorescence. As shown in **Figure 4**, Cx43 was mainly expressed in plasma membrane in A549 cells and expressed in both plasma membrane and cytoplasm in H1299 cells. But Cx43 was mainly expressed in cytoplasm in Calu3. From all these results we can conclude that there was no GJIC

in Calu3 cells while GJIC was existed in A549 and H1299 cells, Cx43 participated in the intrinsic GR of NSCLC cells by exerting GJIC.

Discussion

Despite the clinical success of several TKI drugs, the intrinsic resistance to TKIs in cancer therapy remains a critical limitation to the efficacy of these agents, therefore, it is crucial that we find out the thorough understanding of the underlying drug resistance mechanisms to develop effective strategies to manage or prevent drug resistance. Up to now, researches on the intrinsic EGFR-TKIs resistance are seldom, and most of the researches refer to mutations, including EGFR 20 exon insertion mutation [29], KRAS mutation [30] and EML4-AKT gene rearrangement [31]. As it has been reported that an abnormal expression level and distribution of connexins are closely related to tumor formation [32, 33]. And it has also been verified that the upregulation of GJIC and connexin 43 expression is associated with chemosensitivity in neuroblastoma cells [34]. It's also reported that Connexin 43 enhances the temozolomide resistance of human glioma cells by a GJIC independent pathway [35]. In this research, we found that the expression levels of Cx43 were significantly improved in the intrinsic GR cell lines A549 and H1299, and the GJIC was enhanced in both of the two cell lines. While, Cx43 in the gefitinib-sensitive cell line Calu3 was down-regulated, and GJIC was deficient. Cx43 in A549 and H1299 cells were mainly expressed in plasma membrane, but Cx43 was mainly expressed in cytoplasm in Calu3. This means Cx43 participate in the intrinsic GR of NSCLC cells by exerting GJIC. As PI3K/Akt pathway is known to play a prominent role in drug resistance in cancers and Cx43 could contribute to activation of PI3K/Akt signaling [36, 37]. We also found that the expression levels of protein p-Akt were significantly enhanced in the GR A549 and H1299 cell lines. Despite further studies are needed to explore the exact regulatory relationship between Cx43 expression and PI3K/Akt pathway. It can be concluded that up-regulation of Cx43 at the plasma membrane and their derived GJIC may contribute to the intrinsic GR by activation of PI3K/Akt signaling pathway in A549 and H1299 cells.

Disclosure of conflict of interest

None.

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