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Research paper

CD44 variant–dependent regulation of redox balance in *EGFR* mutation–positive non–small cell lung cancer: A target for treatment

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ABSTRACT

Objectives: The regulation of redox balance in cancer cells is an important factor in tumor development and chemoresistance, with oncogene activation having been shown to induce the generation of reactive oxygen species (ROS). Activating mutations of the epidermal growth factor receptor gene (*EGFR*) are oncogenic drivers in non–small cell lung cancer (NSCLC), but it has remained unknown whether ligand-independent EGFR signaling conferred by *EGFR* mutation triggers ROS generation in NSCLC cells.

Materials and Methods: HEK293T cells were transfected with an expression vector for mutant EGFR. The expression of CD44 variant (CD44v) isoforms in NSCLC cell lines was evaluated by flow cytometry. Cells were depleted of CD44v by RNA interference and assayed for ROS and glutathione (GSH) levels. The effect of CD44v on cisplatin sensitivity was evaluated in vitro with the MTS assay.

Results: EGFR signaling due to *EGFR* mutation increased ROS levels in transfected HEK293T cells. The expression of CD44v isoforms was found to be inversely correlated with basal ROS levels in *EGFR* mutation–positive NSCLC cell lines. Knockdown of CD44v induced depletion of intracellular GSH and increased ROS levels in *EGFR*-mutated NSCLC cells that express CD44v at a high level ($CD44v^{high}$). In addition, depletion of GSH by treatment with buthionine-[*S, R*]-sulfoximine induced marked accumulation of ROS and enhanced the cytotoxicity of cisplatin in $CD44v^{high}$ *EGFR*-mutated NSCLC cells but not in corresponding $CD44v^{low}$ cells. This enhancement of cisplatin cytotoxicity by GSH depletion was prevented by treatment with the antioxidant *N*-acetyl-L-cysteine. Knockdown of CD44v also enhanced cisplatin cytotoxicity in $CD44v^{high}$ *EGFR* mutation–positive NSCLC cells but not in $CD44v^{low}$ cells.

Conclusion: Our results thus implicate CD44v in redox adaptation and as a potential target for treatment in $CD44v^{high}$ *EGFR*-mutated NSCLC cells.

1. Introduction

Many types of cancer cells show increased levels of reactive oxygen species (ROS) compared with normal cells [1–3]. A moderate increase in ROS levels promotes cancer cell survival and cancer progression through activation of various oncogenic signaling pathways [4,5], whereas pronounced accumulation of ROS can result in irreversible oxidative damage to cells [6]. Maintenance of redox balance is therefore crucial for cancer cells.

Although the exact mechanisms responsible for intrinsic oxidative stress in cancer cells remain unclear, ROS production has been found to be induced by expression of oncogenes such as those for RAS, BCR-ABL, and MYC proteins [7]. The epidermal growth factor receptor (EGFR) is frequently overexpressed in various tumor types [8,9], and activation of this receptor by its ligand EGF elicits an increase in ROS levels [10,11]. In non–small cell lung cancer (NSCLC), mutations of the *EGFR* gene result in ligand-independent activation of the receptor and have been identified as oncogenic drivers [12–15]. However, it has remained unknown whether activation of EGFR signaling by such mutations triggers ROS generation in NSCLC cells.

CD44 is a major adhesion glycoprotein for the extracellular matrix and participates in various physiological and pathological processes, including cancer development [16–22]. CD44 variant (CD44v) isoforms contribute to defense against oxidative stress by up-regulating the

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synthesis of reduced glutathione (GSH), the primary intracellular antioxidant. CD44v-positive cancer cells are therefore resistant to oxidative stress and have also been shown to be resistant to platinum-based chemotherapy [23]. We have now examined the relation between oncogenic EGFR signaling and CD44v expression in the maintenance of redox balance in *EGFR* mutation–positive NSCLC cells. We also investigated whether changes to redox status mediated through CD44v affect the sensitivity to chemotherapy in NSCLC cells harboring *EGFR* mutations.

2. Materials and methods

2.1. Cell culture and reagents

Human NSCLC cell lines (PC9, H1650, HCC827, A549, H23, H2122, H1437, H1573, H1944) and HEK293T cells were obtained from American Type Culture Collection, and 11–18 cells were kindly provided by Y. Maehara (Kyushu University). PC9, H1650, and HCC827 cells harbor an activating in-frame deletion in exon 19 (Ex19del) of *EGFR*, whereas 11–18 cells harbor an activating L858R mutation in exon 21 of *EGFR*. All other NSCLC cell lines are wild type for *EGFR*. The various cell lines were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) or Dulbecco's modified Eagle's medium (Gibco), each supplemented with 10% fetal bovine serum. All cells were maintained under a humidified atmosphere of 5% CO_2 at 37 °C. For EGF treatment, cells were cultured to 80% to 90% confluence, washed with phosphate-buffered saline, and cultured overnight in serum-free medium before exposure to recombinant human EGF (Peprotech, Rocky Hill, NJ, USA). Erlotinib (Selleck Chemicals, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) (Wako, Osaka, Japan) and stored at −20 °C for a maximum of 2 weeks. Buthionine-[*S, R*]-sulfoximine (BSO), *N*-acetyl-L-cysteine (NAC), and cisplatin were obtained from Cayman Chemical (Ann Arbor, MI, USA), Sigma-Aldrich (St. Louis, MO, USA), and Nichi-Iko (Toyama, Japan), respectively.

2.2. Plasmid transfection

Plasmids encoding an Ex19del (E746–A750) form of human EGFR (pBabe-19del) or wild-type human EGFR (pBabe-EGFR-WT) were obtained from Addgene and subjected to amplification by the polymerase chain reaction with PrimeSTAR GXL DNA Polymerase (Takara Bio, Otsu, Japan). The amplification products were verified by sequencing and were ligated into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) between the Xho I and Kpn I sites with the use of an In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA). HEK293T cells were transfected with the resulting expression vectors for 48 h with the use of the Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA).

2.3. RNA interference

Cells were cultured for 24 h before transient transfection for 48 h with small interfering RNAs (siRNAs) mixed with the RNAiMAX reagent (Life Technologies). The sequences of siRNA (chimeric RNA-DNA) duplexes (Japan Bioservice, Saitama, Japan) were as follows: CD44 #1, 5′-AAAUGGUCGCUACAGCAUCTT-3′ and 5′-GAUGCUGUAGCGAC CAUUUTT-3′; CD44 #2, 5′-GUAUGACACAUAUUGCUUCTT-3′ and 5′-GAAGCAAUAUGUGUCAUACTT-3′; and control, 5′-AGCAAAUCGCGUG CAUCAUTT-3′ and 5′-AUGAUGCACGCGAUUUGCUTT-3′. All data presented were obtained with the CD44 #1 siRNA, but similar results were obtained with the CD44 #2 siRNA.

2.4. Flow cytometric analysis

Cells were stained with rat monoclonal antibodies to human CD44v9 (Cosmo Bio, Tokyo, Japan) for 30 min at 4 °C before flow cytometric analysis with a FACS Verse instrument (BD Biosciences, Tokyo, Japan).

2.5. Immunoblot analysis

Cells were washed with phosphate-buffered saline, lysed with SDS sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl [pH 6.8], and protease and phosphatase inhibitor cocktails), mixed with bromophenol blue and dithiothreitol (final concentration,100 mM), and incubated at 95 °C for 5 min. Protein was quantitated with the BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of lysate protein were subjected to SDS-polyacrylamide gel electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride membrane, and the membrane was then exposed to rabbit polyclonal antibodies to Y1068-phosphorylated human EGFR, to EGFR, to xCT, to β-actin (Cell Signaling Technology, Danvers, MA, USA), or to the cytoplasmic region of CD44 (CD44cyto) (Trans Genic, Fukuoka, Japan). Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare UK, Amersham, England), Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific), and a ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

2.6. Measurement of ROS levels

The intracellular ROS concentration was determined on the basis of dichlorofluorescein (DCF) staining. Cells were incubated with 5 μM H_2DCFDA (Life Technologies) for 15 min at 37 °C, after which fluorescence was measured with the use of a PerkinElmer ARVO X3 (PerkinElmer Japan, Kanagawa, Japan) or FACS Verse (BD Biosciences) instrument.

2.7. Measurement of GSH

The intracellular concentration of GSH was measured with the use of a GSH-Glo Glutathione Assay (Promega, Madison, WI, USA), which is based on the conversion of a luciferin derivative to luciferin by glutathione S-transferase in the presence of GSH. The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of GSH in the sample and was measured with a Flexstation3 luminometer system (Molecular Devices, Sunnyvale, CA, USA) at 24 h after cells (3×10^3 per well) were seeded in a 96-well plate. The assay results were normalized with the use of a GSH standard solution provided with the kit.

2.8. Cytotoxicity assay

Cells seeded in a 96-well plate were exposed to drugs for the indicated times, after which viability was determined with the MTS assay as performed with the use of a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

2.9. Sequencing analysis

DNA was extracted from NSCLC cell lines (PC9, H1650, and 11–18) with the use of an All Prep Kit (Qiagen,Valencia, CA, USA) and was amplified by the polymerase chain reaction with primers that target exon 2 of the NF-E2–related factor 2 gene (*NRF2*), the region in which mutations that confer constitutive activation of NRF2 have been