Redox Biology 17 (2018) 246–258

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

High CD44 expression mediates p62-associated NFE2L2/NRF2 activation in breast cancer stem cell-like cells: Implications for cancer stem cell resistance

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ARTICLE INFO

Keywords:
CD44
Cancer stem cell (CSC)
NFE2L2/NRF2
Reactive oxygen species (ROS)
Stress resistance
p62

ABSTRACT

Cluster of differentiation 44 (CD44) is the most common cancer stem cell (CSC) marker and high CD44 expression has been associated with anticancer drug resistance, tumor recurrence, and metastasis. In this study, we aimed to investigate the molecular mechanism by which CD44 and nuclear factor erythroid 2-like 2 (NFE2L2; NRF2), a key regulator of antioxidant genes, are linked to CSC resistance using $CD44^{high}$ breast CSC-like cells. NRF2 expression was higher in $CD44^{high}$ cell populations isolated from doxorubicin-resistant MCF7 (ADR), as well as MCF7, MDA-MB231, and A549 cells, than in corresponding $CD44^{low}$ cells. High NRF2 expression in the $CD44^{high}CD24^{low}$ CSC population (ADR44P) established from ADR cells depended on standard isoform of CD44. Silencing of *CD44* or overexpression of CD44 resulted in the reduction or elevation of NRF2, respectively, and treatment with hyaluronic acid, a CD44 ligand, augmented NRF2 activation. As functional implications, *NRF2* silencing rendered ADR44P cells to retain higher levels of reactive oxygen species and to be sensitive to anticancer drug toxicity. Moreover, *NRF2*-silenced ADR44P cells displayed tumor growth retardation and reduced colony/sphere formation and invasion capacity. In line with these, CD44 significantly colocalized with NRF2 in breast tumor clinical samples. The molecular mechanism of CD44-mediated NRF2 activation was found to involve high p62 expression. CD44 elevation led to an increase in p62, and inhibition of p62 resulted in NRF2 suppression in ADR44P. Collectively, our results showed that high CD44 led to p62-associated NRF2 activation in $CD44^{high}$ breast CSC-like cells. NRF2 activation contributed to the aggressive phenotype, tumor growth, and anticancer drug resistance of $CD44^{high}$ CSCs. Therefore, the CD44-NRF2 axis might be a promising therapeutic target for the control of stress resistance and survival of $CD44^{high}$ CSC population within breast tumors.

1. Introduction

Cancer stem cells (CSCs), a small population of cancer cells, possess self-renewal and differentiation capacity similar to normal stem cells. In 1997, Bonnet and Dick observed that only the $CD34^{+}/CD38^{-}$ cells from acute myeloid leukemia (AML) patients were capable of tumor initiation in immune-deficient mice [1]. Since this discovery, CSCs have been identified in other types of cancer, including breast, brain, and lung cancer, and they are believed to be responsible for tumor relapse after therapy [2–4]. One common property between adult stem cells and CSCs is a low level of reactive oxygen species (ROS). Hematopoietic stem cells retain a lower level of ROS than their mature lineage, and this low ROS level is associated with stem-cell resistance to an oxidative environment [5,6]. Like hematopoietic stem cells, CSC subpopulations from several human as well as murine breast tumors contained lower ROS levels than corresponding non-tumor cells [7]. Because of the low ROS level, CSCs were relatively more resistant to radiation-induced DNA damage and cell death than non-tumor cells. Diehn *et al.* [7] also found that breast CSCs express high levels of antioxidant proteins, such as glutathione peroxidase-1 (GPX1), catalase, and superoxide dismutase-2 (SOD2) when compared to non-tumor cells, indicating a differential expression of ROS-scavenging genes in CSCs. In addition,

Abbreviations: AKR, aldo-keto reductase; ARE, antioxidant response element; CD44, cluster of differentiation 44; CSC, cancer stem cell; GCLC, glutamate-cysteine ligase catalytic subunit; GPX, glutathione peroxidase; GSH, glutathione; HO-1, heme oxygenase-1; HA, hyaluronic acid; KEAP1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinase; MDR1, multidrug resistance protein 1; NFE2L2/NRF2, nuclear factor erythroid 2-like 2; NQO-1, NAD(P)H quinone oxidoreductase-1; OCT4, octamer-binding transcription factor 4; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; SOX2, sex determining region Y-box 2

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https://doi.org/10.1016/j.redox.2018.04.015
Received 21 February 2018; Received in revised form 6 April 2018; Accepted 13 April 2018
Available online 26 April 2018

enhanced expression of genes encoding ROS metabolism and drug efflux transporters elicited CSC resistance against anticancer treatment [8–10].

The multifunctional glycoprotein cluster of differentiation 44 (CD44) is a receptor for extracellular matrix (ECM) components, primarily hyaluronic acid (HA), and is the most common CSC marker in multiple types of cancers. This cell-surface glycoprotein, encoded by a single and highly conserved gene, exists in a variety of isoforms resulting from alternative splicing and post-translational modification [11,12]. Among these, the standard isoform (CD44s), which is the smallest CD44 isoform, is encoded exclusively by the constant exons. Inclusion of the variant exons that are located between the constant exons produces CD44 variant isoforms (CD44v). CD44s is widely expressed in most animal cells, whereas CD44v is present only on some epithelial cells, including malignant epithelial cells [11,12]. Although CD44 is involved in normal physiological events, such as cell-cell interaction and cell-matrix interaction, its high expression has been associated with tumor initiation and progression. Through the binding to cytoskeletal proteins, CD44 induces actin-cytoskeletal remodeling, which is involved in cancer cell motility, migration, and invasion [13]. Particularly, lines of evidence indicate that high CD44 expression is related to the CSC phenotype in many types of tumors. As few as 100 $CD44^+$ cells from breast tumors were found to be capable of initiating a heterogeneous tumor in vivo, and a single $CD44^+$ cell isolated from xenograft tumors demonstrated self-renewal properties when transplanted into mice [14,15]. $CD44^{high}$ prostate cancer cells showed increased mRNA levels of the stemness marker octamer-binding protein 3/4 (OCT3/4) and β-catenin, and had stronger proliferative, tumorigenic, and metastatic capacity than the corresponding $CD44^{low}$cells [16]. $CD44^{high}$ gastric cancer cells were reported to be resistant to cisplatin and 5-fluorouracil chemotherapy and to upregulate hedgehog signaling, which promotes the CSC phenotype [17]. As mentioned earlier, numerous studies have shown that CD44 is the most reliable CSC marker in multiple types of tumors; however, the molecular function of CD44 for the CSC phenotype is not fully understood.

Nuclear factor erythroid 2-like 2 (NFE2L2/NRF2) protects cells against oxidative damage by upregulating the expression of various genes encoding multiple antioxidant proteins (e.g., glutathione [GSH] synthesis and regeneration enzymes), phase 2 detoxifying enzymes (e.g., aldo-keto reductase [AKR]), and drug efflux transporters [18,19]. When cells are exposed to oxidative stress, NRF2 dissociates from its inhibitor Kelch-like ECH-associated protein 1 (KEAP1), and this allows NRF2 to translocate into the nucleus where it binds to the antioxidant-response elements (AREs) in its target genes [20,21]. In addition to KEAP1-mediated regulation, p62 is involved in NRF2 activation as a non-canonical regulation pathway. Originally, p62 was identified as a linker protein that associates ubiquitinated proteins with the autophagy system, and is now getting attention for its role in ROS defense. Specifically, p62 protein has been found to bind to specific KEAP1 residues by competing with NRF2 and thereby, p62 elevation can result in NRF2 elevation [22,23]. Additionally, p62 has been shown to induce autophagic degradation of KEAP1 protein, which also leads to p62-mediated NRF2 activation [24].

Over the last few decades, numerous studies have focused on the protective effects of NRF2 in normal cells [25,26]. However, accumulating evidence suggests that NRF2 hyperactivation provides a favorable environment for cancer cells to facilitate tumor growth and survival. High NRF2 expression in cancer cells could result in growth enhancement and chemoresistance by upregulating its target genes, including GSH-generating enzymes and drug efflux transporters [27,28]. Our previous studies also showed that *NRF2* silencing in different types of cancer cells could decrease tumor growth and enhanced sensitivity to anticancer treatments [29–31]. In particular, considering the direct involvement of NRF2 in cellular ROS regulation and anticancer drug resistance, the possible contribution of NRF2 to CSC biology remains to be addressed. We previously showed that constitutive activation of NRF2 was closely correlated with anticancer drug resistance in CSC-enriched spheroid breast and colon cancer cells [32,33]. In this study, in an attempt to investigate the direct association of NRF2 with CSC phenotype, we established a $CD44^{high}$ breast CSC-like system, and investigated the role of NRF2 activation in CSC-like properties in breast CSCs.

2. Materials and methods

2.1. Reagents

Antibodies recognizing sex determining region Y-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4), p62, microtubule-associated proteins 1A/1B light chain 3B (LC3B), multidrug resistance protein-1 (MDR1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and CD44 were from Cell Signaling Technology (Danvers, MA, USA). NRF2, KEAP1, lamin B and β-tubulin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated CD44 and phycoerythrin (PE)-conjugated CD24 antibodies were from Biolegend (San Diego, CA, USA). The CD44s plasmid was obtained from Addgene (Cambridge, MA, USA). The lentiviral expression plasmids for human *NRF2* short hairpin RNA (shRNA), Mission™ Lentiviral Packaging Mix, hexadimethrine bromide, puromycin, doxorubicin, daunorubicin, hyaluronic acid, 4-methylumbelliferone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich (Saint Louis, MO, USA). Propidium iodide (PI) was purchased from Biolegend. 6-Carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) were purchased from Life Technologies (Carlsbad, CA, USA). The SYBR premix ExTaq system was obtained from Takara (Otsu, Japan). Cyto-ID autophagy detection kit 2.0 was obtained from Enzo Life Science (Farmingdale, NY, USA).

2.2. Cell culture

The human breast carcinoma cell line MCF7 and MDA-MB231 were purchased from the American Type Culture Collection (Rockville, MD, USA). Doxorubicin-resistant cell line MCF7/ADR was gifted by Dr. Keon Wook Kang (Seoul National University, Republic of Korea). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS; HyClone) and penicillin/streptomycin (WelGene Inc., Daegu, Republic of Korea). The human lung carcinoma cell line A549 was obtained from ATCC. These cells were maintained in RPMI 1640 with 10% fatal bovine serum and penicillin/streptomycin. The cells were grown at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Sphere culture of cancer cells

Cells were plated at a density of 20,000 cells/mL in 100 mm ultralow attachment plates (Corning Costar Corp., Cambridge, MA, USA) and were grown in a serum-free DMEM and Nutrient Mixture F-12 medium supplemented with B27 (1:50, Life Technologies), 20 ng/mL epithelial growth factor (EGF), 20 ng/mL basic fibroblast growth factor (R&D System, Minneapolis, MN, USA), 5 μg/mL bovine insulin (Cell Application Inc., San Diego, CA, USA), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), and penicillin/streptomycin (HyClone) as described previously [34]. Cells were grown for 3 days for sphere formation.

2.4. Production of shRNA lentiviral particles

Lentiviral particles were produced in HEK 293T cells following the transfection of the cells with the relevant shRNA expression plasmid and Mission™ Lentiviral Packaging Mix as described previously [35]. Briefly, HEK 293T cells in Opti-MEM (Life Technologies) were transfected with 1.5 μg pLKO.1-*NRF2* shRNA, (5′-CCGGGCTCCTACTGTGAT