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Original Contribution

Nrf2-heme oxygenase-1 axis in mucoepidermoid carcinoma of the lung: Antitumoral effects associated with down-regulation of matrix metalloproteinases

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ABSTRACT

Lung mucoepidermoid carcinoma (MEC) is a very poorly characterized rare subtype of non-small-cell lung cancer (NSCLC) associated with more favorable prognoses than other forms of intrathoracic malignancies. We have previously identified that heme oxygenase-1 (HO-1, encoded by *HMOX1*) inhibits MEC tumor growth and modulates the transcriptome of microRNAs. Here we investigate the role of a major upstream regulator of HO-1 and a master regulator of cellular antioxidant responses, transcription factor Nrf2, in MEC biology. Nrf2 overexpression in the NCI-H292 MEC cell line mimicked the phenotype of HO-1 overexpressing cells, leading to inhibition of cell proliferation and migration and down-regulation of oncogenic miR-378. *HMOX1* silencing identified HO-1 as a major mediator of Nrf2 action. Nrf2- and HO-1 overexpressing cells exhibited strongly diminished expression of multiple matrix metalloproteinases and inflammatory cytokine interleukin-1β, which was confirmed in an NCI-HO-1 xenograft model. Overexpression of HO-1 altered not only human MMP levels in tumor cells but also murine MMP levels within tumor microenvironment and metastatic niche. This could possibly contribute to decreased metastasis to the lungs and inhibitory effects of HO-1 on MEC tumor growth. Our profound transcriptome analysis and molecular characterization of the mucoepidermoid lung carcinoma helps to understand the specific clinical presentations of these tumors, emphasizing a unique antitumoral role of the Nrf2-HO-1 axis.

1. Introduction

Nrf2 (nuclear factor erythroid 2-related factor 2, encoded by *NFE2L2*) is a redox-sensitive transcription factor driving major molecular responses to protect cells against oxidative and electrophilic stress. Under physiological conditions, Nrf2 is sequestered within the cytoplasm while bound to its negative regulator Keap-1 (Kelch-like erythroid-derived cap-n-collar homology-associated protein-1), which serves as an adapter for cullin-3-dependent E3 ubiquitin ligase directing Nrf2 for proteasomal degradation. Upon stress, the Nrf2-Keap-1 complex is disrupted through modifications of key sensory cysteine residues of Keap-1 by reactive oxygen species or electrophiles [1]. In the nucleus, Nrf2

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dimerizes with small Maf protein and binds to the antioxidant response element (ARE) or electrophile response element (EpRE) to transactivate transcription of a broad array of cytoprotective genes, including phase 2 detoxifying enzymes and antioxidant genes such as NAD(P)H:quinone oxidoreductase-1 (NQO-1), glutathione S-transferase (GST), and heme oxygenase-1 (HO-1), an inducible isoform of the enzyme that degrades pro-oxidant heme into biliverdin, ferrous iron, and carbon monoxide.

Nrf2 plays a dual role in tumor growth and its action is largely context-dependent. On one hand, regarding tumor initiation, Nrf2 deficiency has been associated with increased susceptibility to carcinogenesis in multiple models and thus the transcription factor is a central target for chemoprevention [2,3]. On the other, aberrant Nrf2 activation enhances tumorigenic potential, not only through its cytoprotective effects, but also by actively promoting cancer cell proliferation, angiogenesis, and metastasis [4]. Similar oncogenic properties have been attributed to the principal Nrf2 target protein HO-1 in many tumor types [5]. In lung cancers, the neoplasms causing the largest number of cancer-related deaths worldwide, Nrf2 gain-of-function and/or Keap1 loss-of-function mutations often occur [6]. Accordingly, high intratumoral levels of Nrf2 and HO-1 have been associated with poor clinical outcomes in most common types of lung tumors [7,8]. Nevertheless, we have recently identified a particular subtype of non-small-cell lung carcinoma (NSCLC) where HO-1 acts rather as a tumor suppressor, inhibiting cancer cell proliferation, migration, tumor growth, and angiogenesis [9].

Mucoepidermoid carcinoma (MEC) of the lung is a very rare form of intrathoracic malignancy originating from the submucosal bronchial glands and affecting approximately 0.2% of lung cancer patients [10]. Interestingly, this subtype is associated with more favorable prognoses than other NSCLCs, as the MEC tumors usually have lower grades and are diagnosed at lower clinical stages, allowing more effective surgical procedures [11]. Yet very little is known about the microenvironment and molecular characteristics of this subtype. Besides our recent profiling of microRNAs in the NCI-H292 MEC cell line overexpressing *HMOX1* [9], there is one clinical study showing that low-grade pulmonary MECs are characterized by significantly attenuated expression of the matrix metalloproteinases (MMPs) MMP-2 and MMP-9 in comparison to typical lung cancer [12]. MMPs are proteolytic enzymes degrading extracellular matrix components, which play an important role in tumor growth and progression [13]. Accordingly, we found that the expression levels of MMP-2 were associated with tumorigenic potential in NCI-H292 xenografts [9], indicating that the regulation of MMPs may be of importance for the growth of MEC.

Here we investigate the molecular consequences of activation of the Nrf2-HO-1 axis in a model of human mucoepidermoid carcinoma of the lung. We show its atypical antitumoral actions in this subtype of NSCLC and identify the down-regulation of MMPs as one of the potential mechanisms contributing to the phenotype.

2. Materials and methods

2.1. Ethics statement

All experiments were carried out in accordance with good animal practice and were approved by the I Local Ethical Committee for Animal Research at the Jagiellonian University, Krakow.

2.2. Cell culture

The human NSCLC cell lines NCI-H292 (mucoepidermoid carcinoma, purchased from ATCC), A549 (adenocarcinoma, purchased from ATCC), and NCI-H460 (large cell carcinoma, purchased from ATCC) were cultured in RPMI 1640 (PAA), and SK-MES-1 (squamous cell carcinoma, purchased from ATCC) was cultured in MEM (Gibco), each supplemented with 10% fetal bovine serum (PAA) and penicillin (100 U/mL)/streptomycin (10 μg/mL) (Sigma) (pen/strep) under standard culture conditions: 37 °C, 5% CO_2, 95% humidity. NCI-H292 cell lines overexpressing *NFE2L2* or *HMOX1* were developed earlier in our laboratory as described in [14] and [9], respectively.

2.3. Measurement of Nrf2 transcriptional activity

Cells were transfected with ARE-Luc plasmid using Lipofectamine2000 (Invitrogen) according to the vendor's protocol, and Luc activity was measured as described earlier [15].

2.4. Measurement of cell proliferation and migration in vitro

Cell proliferation was determined using BrdU incorporation assay (cell proliferation ELISA, Roche) according to the manufacturer's protocol. A scratch assay for measurement of cell migration was performed as in [14].

2.5. Assay of ALDH activity

Cells were collected during trypsinization and stained using an ALDEFLUOR Kit (StemCell Technologies) according to the vendor's protocol. Flow cytometric analysis was performed on BD LSR II (the cytometry platform of the CBM UPR4301, CNRS, Orléans, France).

2.6. Quantitative RT-PCR

RNA was isolated using QIAzol (Qiagen) reagent according to the manufacturer's instructions and reverse-transcribed into cDNA using a RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas) and nCodeVilo (Invitrogen) for analysis of miRNA. Real-time PCR was performed using QuantiTect SYBR Green (Qiagen) or SYBR Green JumpStart Taq Ready Mix (Sigma) on a Light Cycler 480 II (Roche) or a StepOne Plus (Applied Biosystems) platform. Gene expression was calculated according to delta Ct or delta delta Ct methods with EF2 and U6 as reference genes for mRNA and miRNA analysis, respectively.

2.7. siRNA transfection

Cells were seeded in 24-well plates at low confluency (30–50%) and transfected with 50 nM chemically modified siRNA targeted at HO-1 (Stealth RNAi *HMOX1* siRNA, Invitrogen) or control siRNA (Stealth RNAi Negative Control siRNA, Invitrogen) using 1 μl/well of Lipofectamine2000 transfection reagent (Invitrogen) according to the protocol of the supplier.

2.8. Microarray gene expression profiling

NCI-H292 cells, control and overexpressing *HMOX1*, were cultured for 24 h in serum-deprived medium after reaching confluence. Then the cells were washed with PBS and lysed in QIAzol Lysis Reagent (Qiagen) and RNA was isolated by a modified Chomczynski method following the manufacturer's recommendations. The RNA was quantified using a NanoDrop-1000 spectrophotometer and quality was monitored with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was hybridized to Agilent Whole Human Genome Oligo Microarrays (G4112F) and changes of expression of at least 1.5-fold ($p < 0.05$) were considered significant. GoMiner software was used for segregation of the genes into ontology groups. Datasets are available