Research Article

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ARID2 modulates DNA damage response in human hepatocellular carcinoma cells

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Background & Aims: Recent genomic studies have identified frequent mutations of AT-rich interactive domain 2 (*ARID2*) in hepatocellular carcinoma (HCC), but it is not still understood how ARID2 exhibits tumor suppressor activities.

Methods: We established the *ARID2* knockout human HCC cell lines by using CRISPR/Cas9 system, and investigated the gene expression profiles and biological functions.

Results: Bioinformatic analysis indicated that UV-response genes were negatively regulated in the *ARID2* knockout cells, and they were sensitized to UV irradiation. ARID2 depletion attenuated nucleotide excision repair (NER) of DNA damage sites introduced by exposure to UV as well as chemical compounds known as carcinogens for HCC, benzo[a]pyrene and $FeCl_3$, since xeroderma pigmentosum complementation group G (XPG) could not accumulate without ARID2. By using large-scale public data sets, we validated that *ARID2* knockout could lead to similar molecular changes between *in vitro* and *in vivo* settings. A higher number of somatic mutations in the *ARID2*-mutated subtypes than that in the *ARID2* wild-type across various types of cancers including HCC was observed.

Conclusions: We provide evidence that *ARID2* knockout could contribute to disruption of NER process through inhibiting the recruitment of XPG, resulting in susceptibility to carcinogens and potential hypermutation. These findings have implications for therapeutic targets in cancers harboring *ARID2* mutations.

Lay summary: Recent genomic studies have identified frequent mutations of ARID2, a component of the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex, in hepatocellular carcinoma, but it is not still understood how ARID2 exhibits tumor suppressor activities. In current study, we provided evidence that *ARID2* knockout could contribute to disruption of DNA repair process, resulting in susceptibility to carcinogens and potential hypermutation. These findings have far-reaching implications for therapeutic targets in cancers harboring *ARID2* mutations.

Keywords: Hepatocellular carcinoma; ARID2; DNA damage response; Nucleotide excision repair; SWI/SNF; XPC; CRISPR/Cas9; XPG; Benzo[a]pyrene.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most leading cause of cancer-related death [1]. Chronic hepatitis and cirrhosis due to hepatitis B virus, hepatitis C virus infection, alcohol use, and metabolic diseases are the most prevalent risk factors for HCC, but the molecular mechanisms underlying hepatocarcinogenesis are complicated and remain unclear [1]. Recent genome-wide sequencing has identified recurrent mutations of chromatin-remodeling factors (25–60%) as well as *TP53* (25–40%) and *CTNNB1* (25–40%) mutations in HCC. In the chromatin-remodeling factors, the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex is the most frequently mutated family (20–40%) [2–5]. The first large-scale exome sequencing of HCC revealed that 18.2% of individuals with HCV-associated HCC in the United States and Europe harbor AT-rich interactive domain 2 (*ARID2*) inactivating mutations, suggesting that *ARID2* is a tumor suppressor gene commonly disrupted in this tumor subtype [2]. Another study demonstrated *ARID2* mutation frequency is 8.0% of HCC patients with high alcohol intake [3]. In Japan, whole genome sequencing has shown that *ARID2* mutations are observed in 5.8% of HCC patients, although this is not significantly correlated with HCV infection or any other risk factor [4]. *ARID2* is also reported as a key gene in three other cancers; exome sequencing shows *ARID2* loss-of-function mutations in 7.4% of liver cancers displaying biliary phenotype [6], in 7.0% of malignant melanoma [7], and in 1.6% of ovarian cancers [8].

The SWI/SNF complex remodels the chromatin structure by the ATP-dependent regulation of DNA-histone interactions at the nucleosomes to modulate gene expression and DNA repair [9]. It contains more than ten subunits including either of two

Cancer

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alternative ATPases, Brahma (BRM) and Brahma-related gene-1 (BRG1). Two types of the SWI/SNF complex are identified in mammals, the BRG1-associated factors (BAF) and the polybromo-BRG1-associated factors (PBAF). They share a number of proteins, while differing in other components. The BAF complex includes two isoforms, ARID1A and ARID1B, which are interchangeable and not present simultaneously in the complex, whereas the PBAF complex contains ARID2 [9]. ARID1A and BRG1 are subunits composing the SWI/SNF complex, and loss-of-function mutations of these two genes have been detected in various types of malignancies, and attract great interest as promising targets based on the concept of synthetic lethality [10–12].

Although several papers have documented *ARID2* mutations in HCC and other cancers, it is not still understood how ARID2 plays tumor suppressor roles in carcinogenesis. We here established *ARID2*-deficient HCC cell lines by using CRISPR/Cas9 system, and then compared the gene expression profiles of them with those of their parental cell lines, implying that *ARID2* could be involved in DNA damage response.

Materials and methods

Cell culture and animal experiments

Hepatocellular cellular carcinoma cell lines (JHH4 and JHH5) were purchased from Health Science Research Resources Bank (Osaka, Japan), authenticated by short tandem repeat DNA fingerprinting (BEX Co. Ltd., Tokyo, Japan), and periodically checked for *Mycoplasma* contamination by using e-Myco Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Sungnam, Korea). They were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). All cell lines were cultivated in a humidified incubator at 37 °C in 5% carbon dioxide and collected with 0.05% trypsin-0.02% EDTA solution (Invitrogen). The NOD/SCID (NOD.CB17-*Prkdc*^{scid}/J) mice were purchased from Charles River Laboratories (Wilmington, MA). All mouse procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Establishment of ARID2-knockout HCC cell lines using a CRISPR/Cas9 system

Three CRISPR targeting sequences were designed based on the Opimized CRISPR Design web tool (http://crispr.mit.edu/), and listed in Supplementary Table 1 [13]. Oligos were cloned into the gRNA Cloning Vector (Addgene, plasmid #41824) following hCRISPR gRNA Synthesis Protocol [14], and transfected with the hCas9 (Addgene, plasmid #41815) by using Neon Transfection System (Invitrogen). Cells were selected in media containing 2 mg/ml G418 (Invitrogen) for three days, and then isolated by limiting dilution. The sub-clones were validated by sequencing the genomic DNA with a pair of primers (5′-CCAGCTTTTACTGGACCCTGTTG-3′ and 5′-TAAACAAGACAATGTTGCATAACG-3′).

Cell proliferation assay

Cells were plated at a density of 2×10^4 cells per well in 12-well plates and incubated at 37 °C. The number of the cells was estimated 24, 48 and 72 h later using an MTT assay (Dojindo, Kumamoto, Japan). For cell counting with IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, United Kingdom), cells were plated at a density of 2×10^3 cells per well in 96-well plates, and counterstained for nuclei with Hoechst 33342 solution (Dojindo).

Cell migration assay

The double-chamber migration assay was performed using Transwell chambers (24-well plate, 8 μm pores; BD Biosciences), essentially as described [15]. In brief, the lower chambers were filled with culture media without antibiotics. JHH4 (2.5×10^4 cells/well) or JHH5 (1.5×10^5 cells/well) cancer cells were seeded onto the upper chambers and incubated at 37 °C for 24 to 72 h. Then, cells on the upper surface of the filters were removed using cotton wool swabs. The remaining cells were then fixed with 100% methanol and stained with crystal violet dye, and the number of cells migrating into the lower surface was counted in three randomly selected high-magnification fields (100×) for each sample.

Tumor seeding

Cells were suspended in 100 μl Matrigel (BD Biosceiences, San Jose, CA) and subcutaneously injected into NOD/SCID mice. The volume of the growing tumors was monitored every three days.

Western blotting

Total protein was extracted from each cell line as previously described [15]. Next, 30 μg of protein from each sample was subjected to electrophoresis through 4–15% sodium dodecyl sulfate-polyacrylamide gels and transferred onto immobilon polyvinyldifluoride membranes (Millipore Corp., Bedford, MA). The membrane blots were blocked with 2% skimmed milk for an hour at room temperature and then incubated overnight at 4 °C with primary antibodies. Antibodies used in this study were enumerated in Supplementary Table 2. The appropriate secondary antibodies were added for an hour, and signals were detected by using an enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL) and LAS-3000 (Fujifilm, Tokyo, Japan).

RNA extraction and microarray analysis

Total RNA was extracted from cells by using RNeasy Protect Mini Kit (QIAGEN, Tokyo, Japan). The integrity of the obtained RNA was confirmed by using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Contaminating DNA was removed by digestion with RNase-Free DNase Set (QIAGEN). Complementary RNA was prepared from 100 ng of total RNA from each sample with 3′ IVT Express Kit (Affymetrix, Santa Clara, CA). The hybridization and signal detection of the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) were performed in accordance with the manufacturer's instructions.

Bioinformatics

The four microarray datasets of two pairs of the parental and knockout HCC cell lines were normalized by using the robust multiarray average method in R statistical software (version 3.0.3) and the Affymetrix Bioconductor package. To investigate how biological function changed after *ARID2* inactivation, Gene Set Enrichment Analysis (GSEA; version 2.2.0) with the molecular signatures database (MSigDB) gene sets (h: hallmark gene sets; version 5.0) was performed, as described previously [16]. Gene sets with a false discovery rate (FDR) <0.25 were considered statistically significant and presented in Fig. 1E and Supplementary Fig. 2. A customized gene set containing 399 genes with expression levels >two-fold downregulated by *ARID2* knockout in the JHH4 cells was constructed (Supplementary Table 3). Public sequencing-based gene expression and mutation data sets for human primary cancers were downloaded from the International Cancer Genome Consortium (ICGC) website (https://icgc.org/). The fold-change and *p* value calculation in differential expression analysis of cancer samples with or without *ARID2* mutations was carried out using R statistical software and the edgeR Bioconductor package. A customized gene set was compiled, which included 367 genes with a >two-fold downregulation and $p < 0.01$ in the *ARID2* mutated HCC specimens of the LIHC-US study (Supplementary Table 4).

UV irradiation

Cells were rinsed in phosphate-buffered saline (PBS) and exposed to 254 nm UV light at 120 mJ/cm^2 using the auto-crosslink mode of Stratalinker 2400 (Stratagene, La Jolla, CA). For localized micropore UV treatment, 5.0 μm isopore polycarbonate membrane filters (Millipore) was placed on top of the cell monolayer. After UV irradiation, cells were cultivated in the incubator for several time periods before processing for other assays.

Immunofluorescence

Cells were seeded onto small coverslips in 6-well plates and incubated at 37 °C for 24 h to allow cell attachment. The cells were fixed with 4% paraformaldehyde at 4 °C for 15 min, permeabilized with 0.1% Triton X-100 for five min followed by

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