NEOPLASIA
www.neoplasia.com

Volume 11 Number 8 August 2009 pp. 763–770 763

Distinct HIC1-SIRT1-p53 Loop Deregulation in Lung Squamous Carcinoma and Adenocarcinoma Patients[1,2]

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Abstract

A HIC1-SIRT1-p53 circular loop in which hypermethylation in cancer 1 (HIC1) represses the transcription of SIRT1 that deacetylates and inactivates p53 thus leading to HIC1 inactivation has been identified in cell and animal models. However, the alteration and prognostic effects of HIC1-SIRT1-p53 circular loop have never been demonstrated in human cancer patients. We examine the HIC1-SIRT1-p53 alterations in 118 lung cancer patients to define their etiological roles in tumorigenesis. We found that patients with lung squamous cell carcinoma with low p53 acetylation and SIRT1 expression mostly showed low HIC1 expression, confirming deregulation of HIC1-SIRT1-p53 circular loop in the clinical model. Interestingly, the expression of deleted in breast cancer 1 (DBC1), which blocks the interaction between SIRT1 deacetylase and p53, led to acetylated p53 in patients with lung adenocarcinoma. However, epigenetic alteration of *HIC1* promoter by posttranslational modifications of histones and promoter hypermethylation favoring the compacted chromatin production attenuated the transcriptional induction by acetylated p53. Importantly, lung cancer patients with altered HIC1-SIRT1-p53 circular regulation showed poor prognosis. Our data show the first valid clinical evidence of the deregulation of HIC1-SIRT1-p53 loop in lung tumorigenesis and prognosis. Distinct status of p53 acetylation/deacetylation and HIC1 alteration mechanism result from different SIRT1-DBC1 control and epigenetic alteration in lung squamous cell carcinoma and lung adenocarcinoma.

Neoplasia (2009) 11, 763–770

Introduction

Non–small cell lung cancer (NSCLC) represents a heterogeneous group of cancers consisting mainly of squamous cell carcinoma (SCC) and adenocarcinoma (AD) [1]. The 5-year survival rate has been 10% to 15% for the past two decades and differs in various tumor subtypes [2]. Therefore, an understanding of distinct differences of the molecular mechanisms in NSCLC subtypes may follow subtly different pathways to tumorigenesis and is urgently needed for the development of effective personalized therapeutic modalities and diagnostic approaches.

Our genome-wide loss of heterozygosity studies showed a high deletion frequency at the chromosomal regions 17p13.1-13.3 in NSCLC [3–5]. As chromosome 17p13 harbors multiple tumor suppressor genes, such as *p53* and hypermethylation in cancer 1 (*HIC1*), the founding of loss of chromosomal region 17p13.1-13.3 drew our attention to the p53-HIC1-SIRT1 circular control previously proposed in cell and animal models [6]. The HIC1 protein is a sequence-specific transcriptional repressor [7] and a *bona fide* tumor suppressor [8]. One of its repression targets is the SIRT1 NAD^+-dependent deacetylase,

Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; AD, adenocarcinoma; DBC1, deleted in breast cancer 1; H3K9me, histone H3 methylated at Lys9; H3K9ac, histone H3 acetylated at Lys9; HIC1, hypermethylation in cancer 1; MeCP2, methyl CpG binding protein 2; NSCLC, non–small cell lung cancer; SCC, squamous cell carcinomas; SAHA, suberoylanilide hydroxamic acid

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[1]This work was supported in part by grant NSC96-2628-B-006-048-MY3 from the National Science Council and grant DOH97-TD-G-111-035 from the Department of Health (The Executive Yuan, Republic of China).

[2]This article refers to supplementary materials, which are designated by Tables W1 to W3 and Figure W1 and are available online at www.neoplasia.com.

Received 12 March 2009; Revised 4 May 2009; Accepted 6 May 2009

DOI 10.1593/neo.09470

which is important for chromatin silencing, gene regulation, metabolism, and longevity [9]. SIRT1 modulates p53-mediated transcriptional activation and apoptosis in cells responsive to various stresses, and its deacetylase activity is required for these SIRT1-mediated effects on p53 [10,11]. In addition, *HIC1* is a direct transactivating target of active acetylated p53, which binds to the p53-responsive elements in *HIC1* promoter [12,13]. A circular regulatory loop among HIC1, SIRT1, and p53, in which HIC1 directly represses the transcription of SIRT1 that deacetylates and thereby inactivates p53 and leads to HIC1 inactivation, has been identified in cell and animal models [6]. In addition, the deleted in breast cancer 1 (DBC1) protein has recently been demonstrated to block the interaction between SIRT1 deacetylase and p53 resulting in the increase of p53 acetylation [14,15].

The above-mentioned control loops are all proposed in cell models. However, the detailed functional effects of HIC1-SIRT1-p53 circular loop have never been demonstrated in human cancer patients. Because HIC1 is highly expressed in normal lung tissue [16] and *HIC1* and *SIRT1* knockout mice show lung epithelial carcinoma and lung defects, respectively [17,18], we now performed a comprehensive analysis of HIC1, SIRT1, p53, and DBC1 alterations and their clinical correlation study in 118 patients with NSCLC to explore whether there is a clinical link between HIC1-SIRT1-p53 loop and to determine how HIC1 inactivation is achieved in human NSCLC.

Materials and Methods

Subjects

Paired tumor and normal lung tissues were obtained from 118 patients with NSCLC who were recruited at the Taipei Veterans General Hospital between 2002 and 2004 after obtaining appropriate institutional review board permission and informed consent from the patients. Overall survival was calculated from the day of surgery to the date of death or the last follow-up. The mean follow-up period was 37.4 months (range, 1-66 months). For the methylation assay, genomic DNA from primary lung tumor tissues was prepared using proteinase K digestion and phenol-chloroform extraction. For the RNA expression assay, total RNA was prepared from paired tumor lung and normal lung tissues using Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Immunohistochemical Analysis

Paraffin blocks of tumors were sectioned into 5-μm slices and then processed using standard deparaffinization and rehydration techniques. Antibodies used and their experimental conditions are summarized in Table W1. Staining was scored 3, 2, 1, or 0 if more than 70%, between 36% and 70%, between 5% and 35%, or less than 5%, respectively, of tumor cell nuclei or cytoplasm were positively stained for SIRT1 and HIC1. The score of 1 or 0 indicated the presence of little or no SIRT1 and HIC1. Acetylated p53 stains were recorded as expression when more than 25% of tumor cells were positive.

Tissue Western Blot Analysis

High-quality protein for tissue Western blot analysis was extracted from 97 tumors. Immunoblot analysis was performed using the conditions described in Table W1. For HIC1 and SIRT1 proteins, tumor cells that exhibited target protein expression less than 50% that of internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were deemed to have an abnormal pattern. For p53, the acetylation ratio was calculated as acetylated p53/total p53, and a ratio less than 50% was evaluated as low p53 acetylation.

Messenger RNA Expression Analysis

Reactions were carried out in a volume of 25 μl with 1 μl of cDNA and 0.25 pmol of primers in a DNA thermal cycler. Tumor cells that exhibited HIC1 messenger RNA (mRNA) expression, which were normalized with GAPDH as the internal control, less than 50% that of normal cells were deemed to have an abnormal pattern. In addition, real-time reverse transcription–polymerase chain reactions (RT-PCRs) were performed to measure DBC1 expression using standard conditions in the ABI Prism 7900HR (Applied Biosystems, Foster City, CA). The ΔCT values, normalized relative to *β-actin* mRNA levels, were calculated as relative expression levels using the SDS software (Applied Biosystems).

Methylation-Specific PCR Assay

The primers for the methylation-specific PCR are listed in Table W2. Positive control samples with unmethylated DNA from IMR90 normal lung cell and *Sss*I methyltransferase-treated methylated DNA were included for each set of PCR. The hypermethylation genes were defined as the amplification of more M products than U products from the tumor sample.

Lung Cancer Cell Lines

Lung cancer cell lines A549 and CL1-0 were maintained in Dulbecco's modified Eagle's medium and H226 cells were maintained in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Eugene, OR). The cells were maintained at 37°C in a humidified incubator containing 5% CO_2 in air.

5-Aza-2′-Deoxycytidine and Suberoylanilide Hydroxamic Acid Treatment of Lung Cancer Cells

The A549 human lung AD cells were plated at 10^5 per 100-mm culture dish on the day before treatment. The cultures were treated for three doubling times with 2 μM 5-aza-2′-deoxycytidine (5-aza-dC) and for 48 hours with 5 μM suberoylanilide hydroxamic acid (SAHA). The cells were then harvested for methylation-specific PCR, RT-PCR, and Western blot assays.

Chromatin Immunoprecipitation–PCR Assay

A549 cells were cross-linked with 1% formaldehyde for 15 minutes at 37°C and stopped by the addition of glycine to a final concentration of 0.125 M. Sonicate lysate on ice to shear DNA to lengths between 200 and 800 bp. Subsequent steps were performed with the chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. Chromatin was immunoprecipitated for 16 hours at 4°C using antibodies described in Table W1. *HIC1* and *GAPDH* primers used for PCR are listed in Table W2.

Statistical Analysis

Pearson χ^2 test was used to compare the frequency of HIC1, SIRT1, DBC1, and p53 alterations in patients with NSCLC having different clinicopathologic parameters. Type III censoring was performed on subjects who were still alive at the end of the study. Survival curves were calculated according to the Kaplan-Meier method,