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Silencing of *Hint1*, a novel tumor suppressor gene, by promoter hypermethylation in hepatocellular carcinoma

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ABSTRACT

The Hint1 protein, a member of the histidine triad (HIT) family, is highly conserved in diverse species and ubiquitously expressed in mammalian tissues. Previous studies in mice provided evidence that *Hint1* may be haploinsufficient with respect to its function as a tumor suppressor. In the present study, we investigated the aberrant methylation of *Hint1* and explored possible relationships between aberrant methylation and clinicopathological features in hepatocellular carcinoma (HCC). Hypermethylation of *Hint1* was evaluated by the methylation specific PCR (MSP) method in 40 patients with HCC (tumor and paired adjacent non-tumor tissues) from Taiwan, 22 cases of normal liver tissue (14 from Taiwan and 8 from the US). HINT1 expression in tissues was detected by immunohistochemistry. The frequencies of hypermethylation of *Hint1* in tumor, paired adjacent non-tumor and normal liver tissue were 55.0%, 37.5% and 9.1%, respectively. A statistically significant inverse association was found between *Hint1* methylation status and expression of the HINT1 protein in tumor tissues ($p = 0.003$). The relationship between *Hint1* methylation status and clinical features and other, previously measured biomarkers was also analyzed. *p16* hypermethylation was statistically significantly associated with *Hint1* methylation status ($p = 0.035$). There were no correlations between *Hint1* methylation and hepatitis B (HBV) or hepatitis C (HCV) infection status or aflatoxin B_1 (AFB_1-) and polycyclic aromatic hydrocarbons (PAHs)-DNA adduct levels. These results suggest that promoter hypermethylation of *Hint1* may play a role in hepatocarcinogenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, and a leading cause of death in many countries. The epidemiology of HCC has marked demographic and geographic variations, occurring mainly in Africa and Asia. However, the incidence is also increasing in the United States and Europe [1]. The major risks for the development of HCC have been identified as chronic hepatitis B virus (HBV) and hepatitis virus C (HCV) infections and several dietary or environmental factors, including aflatoxin B_1 (AFB_1) and polycyclic aromatic hydrocarbons (PAHs). HBV and HCV infections and AFB_1 exposure are responsible for approximately 80% of all HCCs [2,3]. As with other cancers, the development of HCC is a complex, multistep process [4]. The molecular pathogenesis of HCC appears to involve multiple genetic aberrations in the molecular control of hepatocyte proliferation, differentiation and death and the maintenance of genomic integrity. This process is influenced by the cumulative acti-

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Abbreviations: Hint1, hit nucleotide binding protein-1; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; AFB_1, aflatoxin B_1; PAHs, polycyclic aromatic hydrocarbons.

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vation and inactivation of oncogenes, tumor suppressor genes, cell cycle control genes and other genes.

The HINT protein, a member of the histidine triad (HIT) family, is highly conserved in diverse species and ubiquitously expressed in mammalian tissues. The HIT protein superfamily consists of at least three subfamilies: Hint, Fhit and Ga1T [5]. In previous studies, *Hint1* deleted mice had a marked increase in susceptibility to chemical carcinogen-induced gastric tumors [6], mammary tumors and ovarian tumors [7]. In addition, with aging, *Hint1* deleted mice displayed an increase in the occurrence of a variety of spontaneous tumors including HCC [7]. These studies in mice also provided evidence that *Hint1* may be haploinsufficient with respect to its function as a tumor suppressor gene. Mechanistic studies indicate that *Hint1* can play a role in apoptosis and *p53* expression [8] and that it can bind to and inhibit several transcription factors including MITF, USF2 and β-catenin and also inhibit AP-1 activity by binding to POSH [9]. It had been reported that *Hint1* is transcriptionally silenced in some human non-small cell lung cancer (NSCLC) cell lines and that increased expression of *Hint1* inhibits growth of the NSCLC cell lines H522 and H538 [10]. Similar effects have been seen in colon cancer cells [9].

During the past decade, extensive studies in the field of epigenetics have brought an awareness that not only genetic, but also epigenetic changes, play a very important role in carcinogenesis [11,12]. DNA methylation is one of the best understood epigenetic mechanisms; hypermethylation of normally unmethylated CpG islands, which are CpG dinucleotide-rich areas located mainly in the promoter regions of many genes, correlate with loss of transcription and loss of gene function [13]. In HCC, a growing number of genes have been identified as undergoing aberrant promoter hypermethylation, suggesting that promoter hypermethylation is an important molecular mechanism for hepatocarcinogenesis. These include the genes *p16, p15* and *RASSF1A* [14,15]. These epigenetic changes have also been implicated as early events in the development of HCC [16–18]. In recent studies, we found promoter hypermethylation of *Hint1* in a subset of both colon cancer and HCC cell lines [9,19].

With the above findings as a background, in the present study, we investigated the *Hint1* promoter methylation profile in HCC and paired adjacent non-tumor DNA samples from patients and also explored the correlation between *Hint1* methylation status and other biomarkers and clinical parameters.

2. Materials and methods

2.1. Patient population and data on clinical parameters

The study samples consisted of 40 frozen dissected tumor and paired adjacent non-tumor tissues, collected in the Department of Surgery, National Taiwan University Hospital. Informed consent was obtained from patients, and the study was approved by the appropriate institutional review committees. Demographic data and clinicopathological characteristics were obtained from hospital charts, and HBV and HCV status was determined by immunoassay (see Table 1). The criteria for HCC grade are based on the World Health Organization classification [20]. Fourteen normal control liver tissues were obtained from subjects affected with intrahepatic stones, liver cysts, and other non-cancerous diseases identified at the National Taiwan University Hospital. Eight US normal control liver tissues were from subjects affected with heart disease identified at Columbia Presbyterian Hospital in New York City.

2.2. Immunohistochemical detection of Hint1 protein in paraffin-embedded sections

Detection of the HINT1 protein in 5 μm paraffin-embedded sections used a commercial polyclonal antibody (ProteinTech Group Inc. Campbell Park Dr., Chicago, IL). The specificity of the anti-Hint1 polyclonal antibody was confirmed by Western blot analysis of a human breast cancer cell line MCF-7 whole cell lysate and a *Hint1* gene knocked out (Hint1−/−) mouse embryonic fibroblast (MEF) cell line whole cell lysate. The Hint1 antibody is highly specific since there is only one major band (13 kD for HInt1) detected from the MCF-7 whole cell lysate (data not shown) After deparaffinization and rehydration in graded ethanol, the slides were immersed in 10 mM citric acid (pH 6.0) and microwaved for 10 min at 400 W. Staining was carried out according to the manufacture's instruction: the primary antibody (1:400 dilution) was added and sections were incubated overnight at 4 °C. This was followed by adding the secondary antibody and ABC reagent and DAB (both ABC and DAB kits were from Vector Laboratories, Burlingame, CA). Slides were then counterstained with Harris hematoxylin (Sigma, St. Louis, MO). The following categories were used for scoring: intensity of staining, none (0), mild (1), moderate (2), strong (3); and percentage of positive staining, <5% (0), 5–25% (1), 25–50% (2), >50% (3) of cells [21]. Combining intensity and percentage staining resulted in the following score 0–1; negative (−); 2–6 positive (+). Liver sections from wild type (*Hint1*+/+) and *Hint1* knocked out (*Hint1*−/−) mice [7] were used as positive and negative controls.

2.3. Real time RT-PCR

To test Hint1 expression in normal liver tissues, Western blot and real time RT-PCR was carried out on 3 US normal liver tissues. Primers used in the RT-PCR: hHint1-E1-F1: 5′-TTCTTCCGAGCCTCTCCTCT-3′; hHint1-E1-R1: 5′-GA CGATACCCACCTCAGCAG-3′. GAPDH-F, 5′-AACTT TGGCAT TGTGGAAGG-3′; GAPDH-R, 5′-ACACATTGGG GGTAGGAA CA-3′. A 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for testing.

2.4. DNA extraction

DNA was isolated from frozen tissue samples, as previous described [22]. Briefly, tissue was placed in liquid nitrogen and pulverized with a blender. The tissue powder was lysed with a DNA lysing buffer (10 mM Tris, 10 mM NaCl, 0.1% sodium dodecyl sulfate at pH 7.9, and 200 μg/ml proteinase K). DNA was isolated by RNase treatment, phenol/chloroform extraction and ethanol precipitation.