Epigenetic Changes Induced by Reactive Oxygen Species in Hepatocellular Carcinoma: Methylation of the E-cadherin Promoter

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Background & Aims: **In addition to genetic alterations, epigenetic changes underlie tumor progression and metastasis. Promoter methylation can silence tumor suppressor genes, and reactive oxygen species (ROS) promote DNA damage, although the relationship between ROS and epigenetic changes in cancer cells is not clear. We sought to determine whether ROS promote hypermethylation of the promoter region of E-cadherin, a regulator of the epithelial-to-mesenchymal transition, in hepatocellular carcinoma (HCC) cells.** ***Methods:*** **HCC cells were exposed to H_2O_2 or stably transfected to express Snail, a transcription factor that down-regulates E-cadherin expression. E-cadherin and Snail expression levels were examined by real-time reverse-transcriptase polymerase chain reaction and immunoblot analyses. The methylation status of E-cadherin was examined by methyl-specific polymerase chain reaction, bisulfite sequencing, and chromatin immunoprecipitation. The interactions between Snail, histone deacetylase 1, and DNA methyltransferase 1 were assessed by immunoprecipitation/immunoblot and immunofluorescence analyses. ROS-induced stress, E-cadherin expression, Snail expression, and E-cadherin promoter methylation were confirmed in HCC tissues by immunoblot, immunohistochemistry, and methyl-specific polymerase chain reaction analyses.** ***Results:*** **We demonstrated that ROS induce hypermethylation of the E-cadherin promoter by increasing Snail expression. Snail induced DNA methylation of the E-cadherin promoter by recruiting histone deacetylase 1 and DNA methyltransferase 1. In human HCC tissues, we observed a correlation among ROS induction, E-cadherin down-regulation, Snail up-regulation, and E-cadherin promoter methylation.** ***Conclusions:*** **These findings provide novel mechanistic insights into epigenetic modulations induced by ROS in the process of carcinogenesis. They are potentially relevant to understanding the activity of ROS in silencing various tumor suppressor genes and in subsequent tumor progression and metastasis.**

DNA methylation is the most frequent epigenetic alteration seen in mammalian genomes, and it frequently mediates transcriptional repression.[1,2] Recently, evidence has emerged that both genetic and epigenetic changes underlie carcinogenesis.[3] Especially, hypermethylation of CpG islands in promoter regions of tumor suppressor genes is frequently seen in tumor cells.[4,5] Reactive oxygen species (ROS) also have been suggested to participate in tumor progression by promoting DNA damage and/or altering cellular signaling pathways.[6] It was recently proposed that ROS are involved in tumor metastasis, a complex process including epithelial-to-mesenchymal transitions, migration, invasion, and angiogenesis in the tumor region.[7] ROS also control expression of matrix metalloproteinases (MMPs), mitogen-activated protein kinase (MAPK), and Ras pathway activation and can also down-regulate E-cadherin expression.[8–10]

A correlation between hypermethylation of the promoter of E-cadherin, which encodes a cell adhesion molecule and is considered a tumor suppressor, and E-cadherin down-regulation has been reported in several cancers.[11,12] Loss of E-cadherin expression occurs in the primary step of metastasis, namely, the loss of cellular adhesion.[13,14] E-cadherin is down-regulated by the transcription factor Snail and silenced by promoter hypermethylation.[15] Decreased E-cadherin expression is correlated with epithelial-to-mesenchymal transitions, metastasis, and poor prognosis in hepatocellular carcinoma (HCC).[16,17] In addition, as in other cancers, DNA methylation within the promoter of tumor suppressor genes including E-cadherin has been reported in HCC.[18–22] Because muta-

Abbreviations used in this paper: 5-aza-dC, 5-aza-2′-deoxycytidine; BS, bisulfite sequencing; ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; GPx1, glutathione peroxidase 1; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MSP, methyl-specific polymerase chain reaction; PMS, phormazane methylsulfate; SOD, superoxide dismutase; Prx, peroxiredoxin; ROS, reactive oxygen species.

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tions in E-cadherin are sporadic in human tumors and reexpression of E-cadherin in metastasis is often observed, epigenetic regulation of E-cadherin expression could be significant in tumor progression.[15] However, until now, despite the fact that E-cadherin is a good candidate for epigenetic regulation, there has been no demonstration that ROS induce CpG methylation of the E-cadherin promoter in cancer cells. To elucidate the link between ROS and DNA methylation in HCC, we used the E-cadherin promoter as a model of ROS-induced epigenetic change.

In this report, we analyzed promoter regulation via Snail and methylation and identified a link between these 2 factors. In HCC cells, ROS induced the up-regulation of Snail expression and the methylation of the E-cadherin promoter. Specifically, Snail induced methylation of CpG sites in the E-cadherin promoter. We also investigated alterations by ROS in Snail expression and E-cadherin promoter methylation in human HCC tissue specimens. Overall, we showed that ROS induced methylation of the E-cadherin promoter via Snail.

Materials and Methods

Tissue Specimens and Histopathologic Examination

Seventy-six HCCs and corresponding non-HCC tissues were collected from Severance Hospital, Yonsei University College of Medicine, and from Samsung Medical Center, Sungkyunkwan University School of Medicine (Seoul, Korea). Tissue samples from resected liver specimens were snap frozen in liquid nitrogen and stored at −70°C. Informed consent was obtained from each patient, and tissue collection was approved by each institutional review board. The patient population consisted of 57 males and 19 females, ranging in age from 25 to 76 years (51 ± 11 years, mean ± standard deviation). All non-HCC liver tissues showed hepatitis B virus-associated chronic hepatitis or cirrhosis. Representative sections were subjected to routine histologic examination and evaluated in terms of differentiation, tumor size, tumor capsule formation, vascular invasion, and intrahepatic metastasis. Differentiation was graded according to Edmondson and Steiner's criteria.[23] Normal liver tissue from 5 patients with metastatic colonic carcinoma served as controls. Tissue specimens were from the Tissue Bank of the Samsumg Medical Center and the Liver Cancer Specimen Bank from the National Research Resource Bank Program of the Korea Science and Engineering Foundation in the Ministry of Science and Technology.

Cell Culture

Hep3B (human hepatoma cells), Huh7 (human hepatoma cells), A431 (human epidermoid carcinoma cells), and HT29 (human colorectal adenocarcinoma cells) were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). For ROS treatments, the media were daily changed to fresh media with 10% FBS, and cells were incubated 4 days with 300 μmol/L H_2O_2, 10 μmol/L phormazane methylsulfate (PMS), or 20 μmol/L menadione. In some experiments, cells were pretreated with 5 mmol/L N-acetylcysteine, 1 μmol/L 5-aza-2′-deoxycytidine, and 10 μmol/L LY294002 for 30 minutes before addition of H_2O_2, PMS, or menadione. To establish stable Huh7 or Hep3B lines expressing human Snail, we inserted the Snail complementary DNA (cDNA) sequence into the pCMV/HA (Clontech Laboratories Inc, Mountain View, CA) vector and transfected Huh7 or Hep3B cells either pCMV/HA as a control or pCMV/HA-Snail using Fugene6 (Roche Applied Science, Penzberg, Germany). Puromycin (InvivoGen, San Diego, CA) was used to select colonies. To establish inducible Hep3B cell lines expressing human Snail, we inserted the HA-Snail sequence into the pTRE-tight (Clontech Laboratories Inc) vector and transfected Hep3B cells inserting regulator plasmid pTet-On, either pTRE-tight as a control or pTRE-tight/HA-Snail. Colonies were screened as described in manufacturer's protocols (Clontech Laboratories Inc). For Snail shRNA experiments, Huh7 and Hep3B cells were transfected with the pLKO.1 vector expressing Snail short hairpin RNA (shRNA), and then stably transfected cell lines (Huh7-SNAi and Hep3B-SNAi) were selected with puromycin. All other reagents were purchased from Sigma Chemical Co (St. Louis, MO).

Methylation-Specific Polymerase Chain Reaction

One microgram genomic DNA was treated with sodium bisulphate using the One Day MSP Kit (IN2GEN, Seoul, Korea). Modified genomic DNA was analyzed with methylation-specific polymerase chain reaction (MSP) E-cadherin primers as described.[24]

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed as described[11] with modifications. Prior to formaldehyde cross-linking, cells were treated with 10 mmol/L dimethyl adipimidate and 0.25% dimethyl sulfoxide in phosphate-buffered saline (PBS) for 45 minutes. Cells were cross-linked with formaldehyde for 15 minutes. In all cases, chromatin was sheared to an average length of 0.4 to 1 kilobase (kb) using Bioruptor (Cosmo Bio Co Ltd, Tokyo, Japan). Immunoprecipitations of cross-linked chromatin were carried out with commercial antibodies. PCR amplification products were quantified by real-time PCR (ABI 7300, Applied Biosystems) with specific primers for E-cadherin promoters. PCR amplification was normalized to DNA collected after sonication (input fraction).