SIAH1 causes growth arrest and apoptosis in hepatoma cells through ß-catenin degradation-dependent and -independent mechanisms

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Abstract. We have previously shown that expression of *SIAH1* is frequently down-regulated in HCCs and associated with their advanced stages. It has been shown that *SIAH1* functions in the phosphorylation-independent degradation of ß-catenin and induces apoptosis and growth arrest. To examine if the effects of *SIAH1* overexpression depend on the altered ß-catenin signaling pathway, we transferred the *SIAH1* gene into three hepatoma cell lines with different genetic backgrounds: HepG2 (mutant ß-catenin), SNU475 (mutant AXIN1), and Huh7 cells (wild type ß-catenin and AXIN1). SIAH1 significantly decreased aberrant ß-catenin signal in HepG2 and SNU475 cells and induced growth arrest and apoptosis. However, SIAH1 also induced apoptosis in Huh7 cells, which retained a normal membranous distribution pattern of ß-catenin. Immunoblotting study demonstrated that SIAH1 also reduces the amount of PEG10 protein, which is known to be frequently overexpressed in HCC and to promote cell proliferation. These data suggest that PEG10 is another target protein of SIAH1 to induce apoptosis in hepatoma cells. Our results should lead to a better understanding of the relationship between deregulation of ß-catenin signals and hepatocarcinogenesis. Further investigations into the mechanisms by which *SIAH1* promotes apoptosis and suppresses cell growth should also allow for the discovery of new therapeutic strategies.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. Epidemiological studies have revealed that major causes of HCC are exposure to carcinogens such as aflatoxin B1, cirrhosis of any etiology, or chronic infection to hepatitis B or C. Molecular approaches have disclosed involvement of several genetic alterations in hepatocellular carcinogenesis, including mutation of *TP53*, *ß-catenin* and *AXIN1* (1-3). Among these, mutation of *ß-catenin* or *AXIN1* has been shown to result in nuclear accumulation of ß-catenin protein. Accumulated ß-catenin is known to associate with the Tcf/Lef family of transcriptional factors thereby activating target genes, such as c-myc and cyclin D1. Immunohistochemical analysis revealed that ß-catenin is aberrantly accumulated in nearly 50% of HCCs (3,4), suggesting that alteration of the ß-catenin signaling pathway is one of the most common and major mechanisms of the development of HCC.

We previously performed a fine deletion mapping of chromosome 16 to search for a novel tumor suppressor gene for HCC and identified *SIAH1*, which is located at a commonly deleted region 16q12.1 (5). Although we did not find any somatic mutation of *SIAH1*, its expression was markedly down-regulated, especially in cases harboring loss of heterozygosity (LOH) at the *SIAH1* locus. Moreover, decreased expression of *SIAH1* was associated with the advanced stage of HCCs, suggesting that inactivation of SIAH1 plays an important role in HCC progression (5). *SIAH1* is a human homolog of the *Drosophila* seven in absentia (sina), required for formation of the R7 photoreceptor cells during eye development (6). It has been suggested that *SIAH1* is one of the downstream effectors of p53 and induces apoptosis. The *SIAH1* product has a RING finger domain and interacts with ubiquitin conjugating enzyme (E2), promoting the degradation of various target proteins via the ubiquitin-proteasome pathway (7). Among these targets are several oncogenic or anti-apoptotic proteins, including ß-catenin, PEG10, and Bag-1 (8,9).

ß-catenin is a multifunctional protein that plays an important role in the transduction of Wnt signals and in the intercellular adhesion by linking the cytoplasmic domain of cadherin (10). In general, the cytoplasmic level of ß-catenin is kept low by the phosphorylation-dependent degradation. The current model suggests that the Axin and adenomatous polyposis coli (APC) gene products serve as scaffolds to facilitate the phosphorylation of ß-catenin by glycogen synthase kinase 3ß (GSK3ß) (11,12). Phosphorylated ß-catenin is then targeted

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Abbreviations: HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; RT-PCR, reverse transcription-PCR

Key words: SIAH1, hepatocellular carcinoma, ß-catenin, PEG10

for ubiquitin-mediated degradation. In colorectal or liver cancers, genetic alterations in APC, *ß-catenin* or *AXIN1* are considered to play important roles in carcinogenesis by increasing cytoplasmic and nuclear ß-catenin.

However, ß-catenin level is also regulated by a phosphorylation-independent degradation, which is initiated by an increase in *SIAH1* expression. SIAH1 interacts sequentially with APC, Siah interacting protein (SIP), Skp1, and Ebi (13). Ebi binds directly to ß-catenin and initiates a subsequent proteosome-mediated degradation (13). Therefore, inactivation of *SIAH1* could cause the aberrant accumulation of ß-catenin. Inactivating mutations of *SIAH1*, which cause the accumulation of ß-catenin, has in fact been reported in gastric cancer (14). Since the aberrant accumulation of ß-catenin is also observed in HCC cases that do not harbor mutations of ß-catenin or *AXIN*, SIAH1 inactivation may be another participating factor in the accumulation of ß-catenin, and thus the development of HCC.

In this study, we induced the *SIAH1* gene into hepatoma cells with or without accumulation of ß-catenin to examine if the apoptosis and cell cycle arrest induced by SIAH1 depend on the altered ß-catenin signals. We also analyzed the influence of SIAH1 on protein levels of ß-catenin and possible other SIAH1 targets. Our data may raise novel insights into mechanisms of hepatocellular carcinogenesis and suggest that gene transfer of *SIAH1* might be a promising therapeutic modality for HCC.

Materials and methods

Tissue samples and cells. We obtained tumors and corresponding non-cancerous liver tissues with informed consent from 100 patients who underwent hepatectomy for HCC at Kyoto University Hospital. All tumors were diagnosed histopathologically as HCC. Human hepatoma cells HepG2, Alexander and Huh7 were obtained from the American Type Culture Collection (Manassas, VA). SNU449 and SNU475 were obtained from the Korea cell line bank. All cell lines were grown in monolayers in appropriate media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO) and maintained at 37˚C in air containing 5% CO_2.

Preparation of DNA and RNA. Cancerous and non-cancerous liver tissues were stored at -80˚C until analysis. Each specimen was serially sectioned in 10-μm slices and stained with H&E to define the analyzed regions. To avoid cross-contamination of cancer and non-cancerous cells, we collected cancerous tissues by micro-dissection. Genomic DNA was extracted by use of a QIAamp tissue kit (Qiagen K.K., Tokyo, Japan) after proteinase K digestion. Total RNA was extracted with Trizol reagent (Invitrogen, Tokyo, Japan) from frozen specimens and cells.

RT-PCR. RT-PCR experiments were carried out in 20 μl volumes of PCR buffer (Takara, Tokyo, Japan), with 4 min at 94˚C for denaturing followed by 20 (for GAPDH) or 30 (for SIAH1) cycles at 94˚C for 30 sec, 56˚C for 30 sec and 72˚C for 30 sec in the GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, CA). Primer sequences were as follows: for GAPDH, forward 5'-GACAACAGCCTCAAGATCATCA-3' and reverse 5'-GGTCCACCACTGACACGTTG-3' and for SIAH1, forward 5'-CGTCAGACTGCTACAGCATT-3' and reverse 5'-TCCATAGCCAAGTTGCGAATG-3'.

Quantitative real-time PCR. Quantitative real-time PCR on the Sequence Detection System 7700 (Applied Biosystems) was performed as described elsewhere (15) with some modifications. Primers and probes for SIAH1 were designed through use of primer express software (SIAH1 TaqMan-F, 5'-GTTGGTGCCAGGACCGG-3'; SIAH1 TaqMan-R, 5'-TGGGATGGTGGACACTTCG-3'; SIAH1 Probe, 5'-TTTCCGCCTTCAGAAATGAGCCGTC-3', Applied Biosystems). We selected GAPDH as an internal control, and the primers and probes were provided by Applied Biosystems. Quantitative real-time PCR was performed with 5 μl of each reverse transcription sample, 12.5 μl of Universal PCR Master mix, 0.9 μmol/l of each primer, and 0.43 μmol/l of the TaqMan probe (Applied Biosystems) in a final volume of 25 μl. The thermocycler conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. The results of quantitative real-time PCR were described according to the comparative $\Delta\Delta$Ct (threshold cycle) method. The assessment of $\Delta\Delta$Ct was as described previously (5).

Construction and infection of adenovirus. Generation and preparation of adenovirus-expressing SIAH1 has been explained elsewhere (8). Cells were infected with the viral solutions and incubated at 37˚C for 1 h, with brief agitation every 15 min. Fresh medium was added to the culture and the plates were returned to the 37˚C incubator.

MTT assay. Cell viability was evaluated with the Cell counting kit-8 according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). Each hepatoma cell line was seeded at $1x10^4$ cells/well in six-well culture plates, 24 h before viral infection. Cells were infected with Ad-SIAH1 or Ad-LacZ at an MOI of 0, 20, 50 and 100. Absorbance of triplicate dishes of each treatment was measured at 96 h after viral infection, and presented as mean ± SD from at least three independent experiments.

Flow cytometry. Cells were plated at a density of $1x10^5$ cells in six-well culture plates 24 h before infection at 100 MOI of Ad-SIAH1 or Ad-LacZ. The cells were trypsinized 48 h after infection, collected in PBS and fixed in 70% cold ethanol. After RNase treatment, we stained cells with propidium iodide (50 μg/ml) in PBS. Flow cytometry was performed on a Becton-Dickinson FACScan and analyzed by ModFit software (Verity Software House). The percentages of nuclei in subG1 population were determined from at least 20,000 ungated cells.

TUNEL assay. Detection of apoptotic cells by fluorescence microscopy was performed using the ApopTag-kit (Oncor) according to the manufacturer's protocol.

TCF4 reporter assay. The reporter plasmids were transfected with FuGENE6 reagent (Boehringer) according to the supplier's

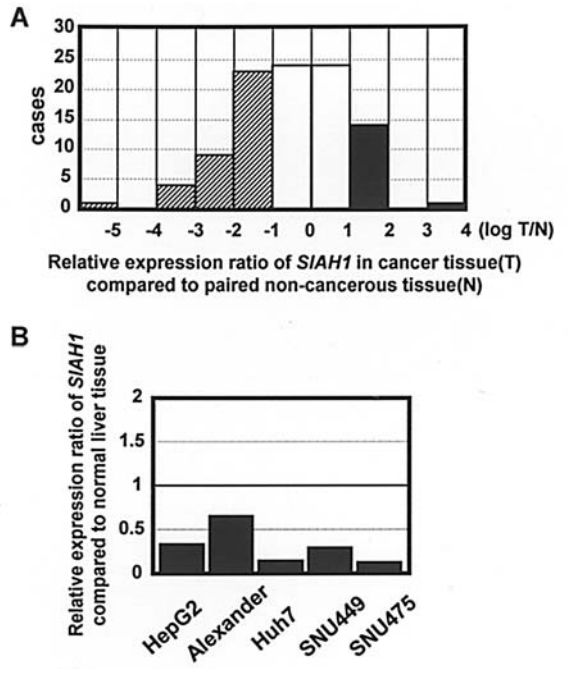

Figure 1. mRNA expression and LOH of *SIAH1* in HCCs and hepatoma cells. (A) mRNA expression of *SIAH1* in 100 HCCs. Expression was determined by quantitative RT-PCR and shown as a histogram. X-axis is logarithmic expression ratio of *SIAH1* in cancerous tissue as compared to their corresponding non-cancerous tissue. Diagonally striped bars indicate cases with decreased expression below half level of control non-cancerous tissue. Solid bars indicate cases with increased expression greater than 2-fold. (B) Expression of *SIAH1* in five hepatoma cell lines. mRNA extracted from normal liver tissue mixture was used as universal control.

recommendations. Reporter assays were carried out using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol. Reporter plasmids contain four copies of TCF4-specific DNA-binding sequence (TBE2) or its mutant sequence (TBE2m) upstream of the luciferase gene in pGL3-Basic vector (Promega) (16). A plasmid vector, pRL-TK (Promega), was co-transfected with each reporter construct to normalize for transfection efficiency. Luciferase activity was measured and presented as mean ± SD from at least three independent experiments.

Immunoblotting. Cell extracts were prepared using lysis buffer [500 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 7.4), and 1 mM DTT], with Complete Protease Inhibitor Cocktail (Boehringer-Mannheim, Mannheim, Germany). Proteins were separated by 10% SDS-PAGE, followed by immunoblotting with a mouse anti-ß-catenin (Transduction Laboratories, Lexington, KY, USA), a mouse anti-c-Myc (4A6) (cell signaling solution), a goat anti-ß-actin (Sigma) and a rabbit anti-PEG10. Horseradish peroxidase-conjugated goat anti-mouse IgG, horseradish peroxidase-conjugated rabbit anti-goat IgG and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as the secondary antibodies for the ECL Detection System (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunocytochemistry. Cultured cells replaced on four chamber slides were fixed with PBS containing 4% Paraformaldehyde for 15 min, then rendered permeable with PBS containing 0.1% Triton X-100 for 3 min at 4˚C. We covered cells with 2% BSA in PBS for 30 min at room temperature to block non-specific antibody-binding sites. Then the cells were incubated with a mouse anti-ß-catenin antibody diluted at 1:250 in the blocking solution. Antibodies were stained with a goat anti-mouse secondary antibody conjugated to rhodamine (Leinco), and viewed with a Zeiss AxioVert 135 (Zeiss, Hallbergmoos, Germany).

Table I. Expression ratio (T/N ratio) and LOH of *SIAH1*.

LOH at *SIAH1*	T/N ratio of *SIAH1*	
	<0.5	>2.0
(-)	8	12
(+)	29	3

Results

Expression of SIAH1 is frequently down-regulated in HCC. We previously showed that expression of *SIAH1* is down-regulated in 9 of 10 HCC cases, which harbor LOH at the *SIAH1* locus. To examine if expression of *SIAH1* is commonly decreased in HCCs, we evaluated the expression of *SIAH1* mRNA by quantitative real-time PCR in 100 HCCs. The mean T/N expression ratio among 100 HCCs was 0.69. Thirty-seven tumors showed down-regulation (T/N ratio <0.5) of *SIAH1*, whereas only 15 tumors showed up-regulation (T/N ratio >2.0, Fig. 1A). Intriguingly, we found LOH at *SIAH1* locus in 29 out of 37 HCCs with decreased expression of *SIAH1*, while only 3 HCCs showed LOH among 15 HCCs with elevated expression of *SIAH1* (Table I). There was a statistically significant association between LOH at the *SIAH1* locus and suppressed expression of *SIAH1* (chi-square test, P<0.0001). These data confirmed that expression of *SIAH1* is often down-regulated in HCCs, especially in tumors with LOH at the *SIAH1* locus.

We next evaluated the expression of *SIAH1* in five hepatoma cell lines by quantitative RT-PCR. The expression level of *SIAH1* mRNA was reduced in all five cell lines examined. In HepG2, Huh7, SNU449 and SNU475, the expression of *SIAH1* was less than half the mean expression level of *SIAH1* observed in non-cancerous liver tissue (Fig. 1B).

Introduction of SIAH1 inhibits growth of hepatoma cells irrespective of aberrant accumulation of ß-catenin. Among the cells with decreased expression of *SIAH1*, we previously showed genetic alterations of ß-catenin degradation pathway in HepG2, SNU449 and SNU475 cells: deletion of exon 3 and 4 of *ß-catenin* in HepG2; mutation of *ß-catenin* in SNU449 cells; and homozygous deletion of the *AXIN1* gene in SNU475 cells. Consistently, immunocytochemical staining demonstrated aberrant accumulation of ß-catenin in these cells. On the other hand, Huh7 cells, retaining wild-type APC, ß-catenin, and AXIN1, showed staining of ß-catenin specifically at plasma membranes (Fig. 5B). Since SIAH1 has been reported to

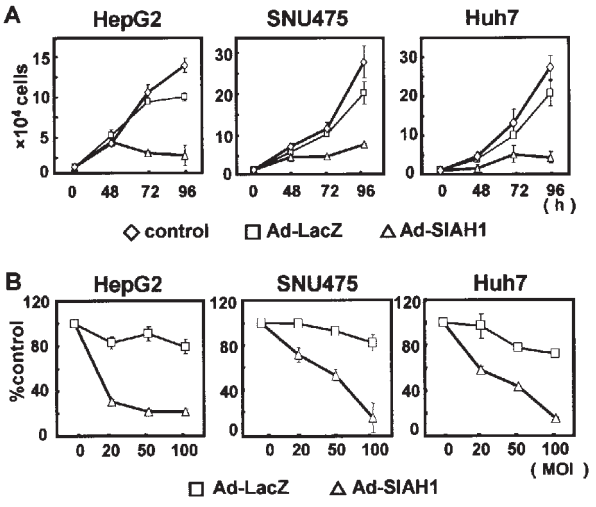

Figure 2. Effects of *SIAH1* transfer on cell growth and viability in HepG2, SNU475 and Huh7 cells. (A) Growth curves after infection with Ad-SIAH1 or Ad-LacZ at 100 MOI (mean ± SD). Growth curves of cells without infection are shown as controls. (B) Assessment of cell viability by MTT assay at 96 h after infection with Ad-SIAH1 or Ad-LacZ at MOIs of 20, 50, 100 (mean ± SD).

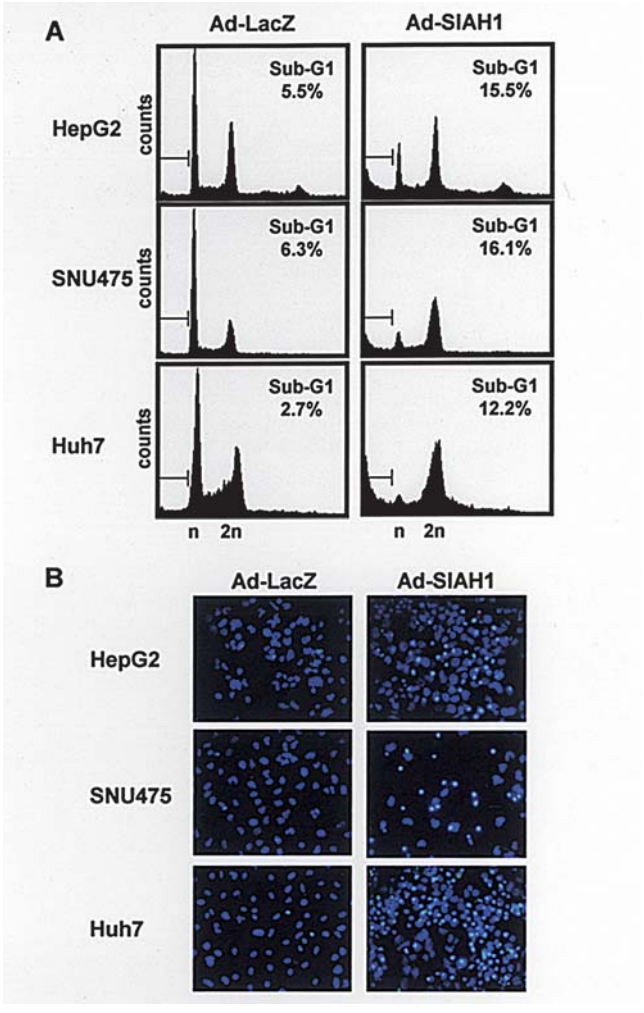

Figure 3. Transfection with Ad-SIAH1 increases apoptosis and G2/M populations in HepG2, SNU475, and Huh7 cells. (A) Flow-cytometric analysis of cell cycle and sub-G1 populations at 48 h after infection with Ad-SIAH1 or Ad-LacZ (each at 100 MOI). The bar shows the region of sub-G1 population. (B) TUNEL assays at 48 h after infection with Ad-SIAH1 or Ad-LacZ (each at 100 MOI) followed by immunofluorescence. The photographs are merged images of DAPI (blue) and fluorescein-conjugated nucleotide (green) incorporated into fragment DNA. Nuclei stained with green were considered to be apoptotic fragments.

degrade ß-catenin irrespective of its phosphorylation status in mammalian cells, we hypothesized that re-introduction of SIAH1 into hepatoma cells with accumulated ß-catenin might cause growth suppression by its degradation. To test this hypothesis, we evaluated the effect of *SIAH1* gene transfer by the recombinant adenoviruses encoding SIAH1 (Ad-SIAH1) to HepG2, SNU475 and Huh7 cells. We excluded SNU449 cells from the analysis, because of low infection efficiency by the adenovirus.

Firstly, we counted cell numbers of each cell line at 0, 48, 72 and 96 h after infection of Ad-LacZ (adenoviruses encoding ß-galactosidase) or Ad-SIAH1, or without infection. Cell growth of HepG2 and SNU475, but also Huh7, was signifi-cantly inhibited when infected with Ad-SIAH1, whereas cells infected with Ad-LacZ or without infection exponentially increased their numbers to reach subconfluency by 96 h (Fig. 2A).

We also assessed the cell viability by MTT assay after *SIAH1* gene transfer in these hepatoma cell lines. We introduced Ad-SIAH1 and Ad-LacZ to these cell lines at a multiplicity of infection (MOI) of 20, 50, and 100 and cell viability was determined at 96 h after infection. In all cell lines examined, the cell viability demonstrated a dose-dependent decrease when infected with Ad-SIAH1, while infection with Ad-LacZ had little influence (Fig. 2B). These data indicate that introduction of *SIAH1* inhibits cell growth and decreases viability in hepatoma cells independently of aberrant accumulation of ß-catenin.

SIAH1 causes G2/M growth arrest and promotes apoptosis in hepatoma cells. To further elucidate the mechanism of the growth suppression by Ad-SIAH1, we analyzed cell cycle profiles of HepG2, SNU475 and Huh7 cells after adenovirus mediated transfer of the *SIAH1* or *LacZ* genes. In all cells, cell cycle analysis by flow-cytometry showed an increased peak corresponding to G2/M population after introduction of the *SIAH1* gene, suggesting that a substantial portion of cells had arrested at G2/M phase. Moreover, the number of cells in sub-G1 populations significantly increased in all cell lines when infected with Ad-SIAH1, compared to the numbers after introduction of the *LacZ* gene (Fig. 3A). These data suggest that SIAH1 also promotes apoptosis in these cells.

To confirm that the increased sub-G1 populations reflect apoptosis, HepG2, SNU475 and Huh7 cells were subjected to a TUNEL assay following infection with Ad-SIAH1 or Ad-LacZ. The assay corroborated that the number of apoptotic cells after the transfer of the *SIAH1* gene was significantly greater than those observed with introduction of the *LacZ* gene (Fig. 3B). Again, we observed the increased apoptosis mediated by SIAH1 in all hepatoma cells regardless of their different genetic backgrounds.

SIAH1 gene transfer reduces elevated TCF4 transcriptional activity in hepatoma cells. To explore the effect of *SIAH1*

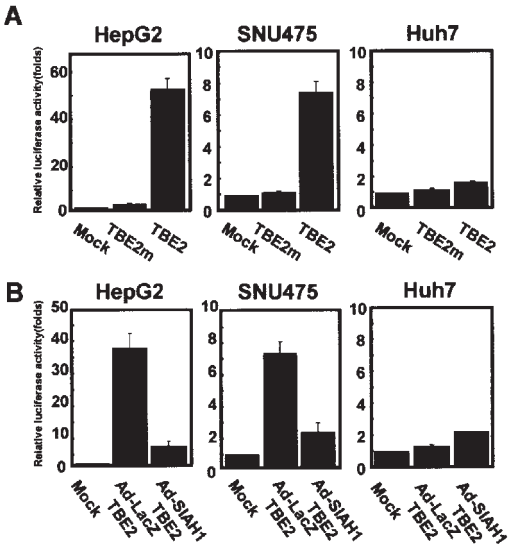

Figure 4. TCF4-specific transcriptional activity is suppressed by *SIAH1* gene transfer. (A) TCF4 reporter gene assays in HepG2, SNU475 and Huh7 cells. Relative luciferase activity (mean ± SD) was calculated after reporter plasmids, which contained four copies of TCF4-binding element (TBE2), its mutant sequence (TBE2m) or a control plasmid (mock) was transfected into each cell line. (B) Effects of Ad-SIAH1 on TCF4-specific transcriptional activity. Cells infected with Ad-LacZ or Ad-SIAH1 at MOI 100 were subsequently transfected with a reporter plasmid containing the TCF4-binding element (TBE2).

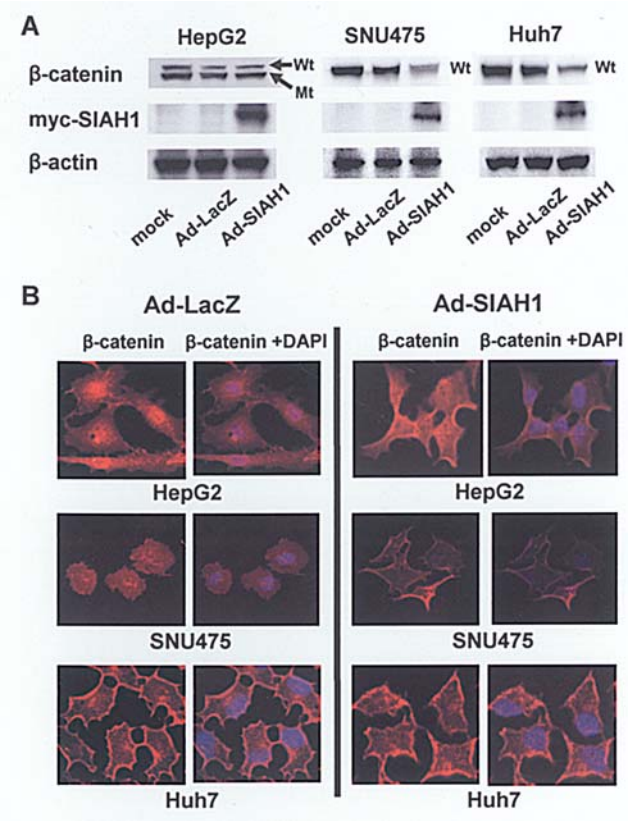

Figure 5. Effects of *SIAH1* gene transfer on the amount of ß-catenin protein and its subcellular localization. HepG2, SNU475 and Huh7 cells were infected with Ad-SIAH1 or Ad-LacZ at MOI 100 for 48 h. (A) Whole cell lysates from each cell line were analyzed by immunoblotting using anti-ß-catenin, anti-myc tagged SIAH1, and anti-ß-actin antibodies. Immunoblotting of cells without infection are shown as controls (mock). Wt, wild-type ß-catenin; Mt, deletion mutant type ß-catenin. (B) Immunocytochemical staining of ß-catenin. Nuclei are counter-stained with DAPI.

introduction on ß-catenin signal, we first evaluated the TCF4 transcriptional activity in HepG2, SNU475 and Huh7 cells by a reporter gene assay using the reporter plasmid containing four copies of the TCF4-binding motif (TBE2). Consistent with the genetic alterations of *ß-catenin* (HepG2) or *AXIN1* (SNU475), the assay demonstrated >50-fold increase of the TCF4 transcriptional activity in HepG2 cells and approximately 7-fold increase in SNU475 cells. On the other hand, Huh7 cells did not show any increase of the TCF4 transcriptional activity (Fig. 4A). We observed the elevated luciferase activity only with the reporter plasmid containing the wild-type TCF4-binding motif, but not with the mutant motif (TBE2m). These data confirm that the increase in transcriptional activity was TCF4-specific.

Next, we infected these cells with Ad-SIAH1 to examine the effects on the elevated ß-catenin signal. After 48 h of infection, cells were analyzed by the TCF4 reporter assay. In both HepG2 and SNU475 cells, infection with Ad-SIAH1 significantly reduced TCF4 transcriptional activity compared to the infection with Ad-LacZ (Fig. 4B). There was no significant difference between the infection with Ad-SIAH1 or Ad-LacZ in Huh7 cells. These results suggest that introduction of *SIAH1* reduced the aberrant nuclear accumulation of ß-catenin/TCF4 complex, and thus lowered TCF4 transcriptional activity in HepG2 and SNU475 cells.

SIAH1 decreases aberrantly accumulated nuclear ß-catenin. To elucidate the mechanism as to how overexpression of *SIAH1* reduces the TCF4 transcriptional activity, we examined the amount of ß-catenin protein after *SIAH1* gene transfer in HepG2, SNU475 and Huh7 cells. At 48 h after infection with Ad-SIAH1 or Ad-LacZ, whole cell lysates were subjected to immunoblot analysis. In all cells, infection with Ad-SIAH1 reduced the amount of wild-type ß-catenin compared to the amount after infection with Ad-LacZ or without infection. However, there was no reduction observed for mutant ß-catenin lacking 116 amino acids (25 to 140) in HepG2 cells, consistent with the previous report that SIAH1-mediated degradation of ß-catenin requires these amino acids (Fig. 5A).

We also evaluated the subcellular localization of ß-catenin protein by immunocytochemistry after the *SIAH1* or *LacZ* gene transfer. HepG2 cells, which harbor a deletion mutant of *ß-catenin*, showed a strong nuclear staining pattern, while SNU475 cells, which lack the *AXIN1* gene, showed a spotty cytoplasmic staining pattern with moderate nuclear staining. Intriguingly, after infection with Ad-SIAH1, nuclear ß-catenin staining in HepG2 and SNU475 cells almost disappeared. In SNU475 cells, ß-catenin was re-localized almost exclusively to the plasma membrane, although in HepG2 cells, a substantial amount of ß-catenin was still stained in the cytoplasm. The specific staining pattern of ß-catenin at the plasma membrane in Huh7 cells was not affected by the introduction of *SIAH1* (Fig. 5B).

These results suggest that the introduction of *SIAH1* reduces the amount of aberrantly accumulated ß-catenin in the nucleus by its degradation and/or other unknown mechanisms, and thus reduces the TCF4 transcriptional activity.

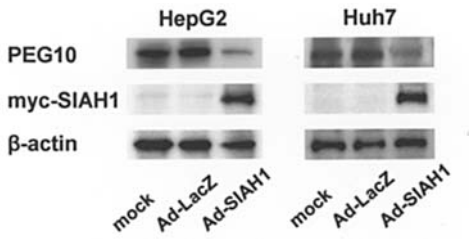

Figure 6. Induction of *SIAH1* decreases PEG10 protein. Whole cell lysates were collected from HepG2 and Huh7 cells at 48 h after infection with Ad-LacZ or Ad-SIAH1 and subjected to immunoblot analysis. Blots were probed with anti-PEG10, anti-myc tagged SIAH1, and anti-ß-actin antibodies.

SIAH1 also reduces another target protein, PEG10. Although degradation of ß-catenin might play important roles in the mechanisms by which SIAH1 causes apoptosis and growth arrest of hepatoma cells, there should be other mechanisms, as SIAH1 also induces apoptosis in Huh7 cells, which do not demonstrate abnormal accumulation of ß-catenin. SIAH1 has been reported to interact with several other oncogenic proteins, including c-Myb, PEG10 and Bag-1. Among these proteins, we previously showed that PEG10 is frequently overexpressed in HCCs, and that it promotes proliferation and prevents SIAH1-induced apoptosis of hepatoma cells. Since Huh7 cells are known to express high levels of PEG10, we hypothesized that the introduction of SIAH1 into Huh7 cells targets PEG10 protein for degradation. To explore this hypothesis, we examined intracellular levels of PEG10 protein by immunoblot analysis at 48 h after infection of Ad-SIAH1 in Huh7 cells. Infection with Ad-SIAH1 significantly reduced the amount of PEG10 protein compared with Ad-LacZ or no infection control (Fig. 6). These data suggest that growth suppression and apoptosis in Huh7 cells was mediated by degradation of PEG10 protein, instead of decreasing ß-catenin signals. We also examined PEG10 protein levels after Ad-SIAH1 infection in HepG2 cells. Introduction of SIAH1 significantly decreased PEG10 in HepG2 cells as well, suggesting that reduction of both ß-catenin signal and PEG10 protein levels synergically contributes to the growth suppression and apoptosis in these cells.

Discussion

Recent progress in molecular genetics and biology has disclosed the mechanisms of hepatocellular carcinogenesis. Accumulation of ß-catenin is observed in almost 50% of HCCs and the constitutively activated ß-catenin signal appears to play an important role in the development of HCC (4,17). Genetic alterations of *ß-catenin* and *AXIN1*, which impede the phosphorylation-dependent degradation of ß-catenin by TCF complex containing ß-TRCP, are considered to be the major cause of the aberrant accumulation (11,12). However, mutations of these molecules are identified only in approxi-mately one-fourth of HCCs, which are much less than cases with ß-catenin accumulation (2,3,18,19). Thus, we surmised that other mechanisms may be involved in the accumulation of ß-catenin. Recently, it has been shown that ß-catenin is also regulated by a phosphorylation-independent degradation, which involves APC, Skp1, SIP, Ebi and SIAH1 (13). Our previous study demonstrated frequent LOH and decreased expression of *SIAH1* in HCCs, especially in large or invasive tumors. These findings raise the possibility that SIAH1 inactivation also contributes to the abnormal accumulation of ß-catenin, and thus progression of HCC.

In this study, we first performed expression analysis of *SIAH1* in 100 HCC cases and confirmed that decreased expression of *SIAH1* is one of the most common findings in HCC cases, especially in cases with LOH. Although mutations of *SIAH1* have been reported in gastric cancers, inactivation by mutation does not seem to be a major mechanism in HCC, as we screened 50 HCC cases which showed LOH and/or reduced expression of *SIAH1* but did not identify any mutations (data not shown). Regarding the mechanism for suppression of the *SIAH1* gene, the hemi-allelic loss appears to play important roles, as we observed LOH at the *SIAH1* locus in the majority of cases with decreased *SIAH1* levels (78.3%, 29/37) and did not detect any aberrant methylation in promoter regions of the *SIAH1* gene (data not shown). A recent study has shown that LMP1, a viral oncogene of EBV, inhibits *SIAH1* expression at the transcriptional level, and so unknown viral products of HCV or HBV may down-regulate SIAH1 in HCCs (20).

SIAH1 contains a nuclear localization signal, forms the E3 ubiquitin ligase complex, and degrades a variety of nuclear target proteins, including ß-catenin. SIAH1 also has been reported to induce apoptosis in various types of cells, although major downstream effector molecules have not been identified. Several papers have reported that the elevated ß-catenin signal functions as an anti-apoptotic factor and stimulates cell proliferation of carcinoma cells. Thus, we hypothesized that SIAH1 also regulates ß-catenin in hepatoma cells and frequent inactivation of SIAH1 contributes to up-regulation of ß-catenin and thereby progression of HCC. As expected, overexpression of the *SIAH1* gene significantly induced apoptosis and inhibited cell proliferation in HepG2 or SNU475 cells, which contain accumulated ß-catenin in the nucleus. However, introduction of the *SIAH1* gene into Huh7 cells, which retain the normal distribution pattern of ß-catenin, also induced apoptosis and growth inhibition. Since SIAH1 also caused G2/M arrest in all cells examined, which is consistent with the previous report that SIAH1 causes disorganization of the mitotic program, the growth suppressive effect by Ad-SIAH1 is not a non-specific effect by the adenoviral toxicity.

We also examined whether SIAH1 could degrade ß-catenin protein and reduce its downstream signals. The reporter assays indicated the significant reduction in TCF4 transcriptional activity in HepG2 and SNU475 cells after infection with Ad-SIAH1. The TCF4 transcriptional activity in Huh7 cells was below significant levels and did not significantly change after the infection with Ad-SIAH1. In HepG2 and SNU475 cells, immunocytochemical studies demonstrated that accumulated ß-catenin in the nucleus almost disappeared and re-distributed to the plasma membranes after introduction of *SIAH1*. The change in the localization of ß-catenin may be due simply to the degradation of ß-catenin protein in the nucleus, because SIAH1 is known to have a nuclear localization signal and associate and destruct the target proteins primarily in the nucleus. The

other possibilities are that SIAH1 is directly involved in the export of ß-catenin from the nucleus, or SIAH1 inhibits the translocation of ß-catenin into the nucleus, although we currently do not have any supportive data for either hypothesis. We observed re-distribution of ß-catenin only in hepatoma cells with aberrant nuclear ß-catenin accumulation. However, immunoblotting studies demonstrated the significantly reduced ß-catenin protein levels also in Huh7 cells, which have an intact APC/AXIN/GSK3ß complex and show a normal membranous distribution pattern of ß-catenin.

Taken together, re-introduction of *SIAH1* into hepatoma cells decreases the amount of nuclear ß-catenin protein and reproduces the normal membranous distribution pattern of ß-catenin, resulting in the reduction of ß-catenin signals. These effects of SIAH1 on ß-catenin are more eminent in HepG2 and SNU475 cells, which show elevated ß-catenin signals, and are probably linked to the growth suppressive effects of SIAH1 on these cells. Because SIAH1 also inhibited the growth of Huh7 cells, which show only basal levels of ß-catenin signals, there would be mechanisms other than modulating ß-catenin signaling to induce cell cycle arrest and apoptosis. Since we still observed the decrease of total amount of ß-catenin protein in Huh7 cells, although SIAH1 did not affect the TCF4 transcriptional activity, the reduction of ß-catenin levels in the cytoplasm and plasma membranes might play some roles in the growth arrest and apoptosis through modifying cell adhesion signaling. However, the role of membranous and cytoplasmic ß-catenin as a part of adhesion signaling is not established and requires further investigation.

Another possible mechanism would be that SIAH1 targets anti-apoptotic proteins other than ß-catenin for degradation in Huh7 cells. Among several known target proteins of SIAH1, ß-catenin, PEG10 and Bag-1 have been reported to function as anti-apoptotic molecules. Because several groups including ours have reported that PEG10 is commonly overexpressed in HCCs and our previous data showed that the induction of the *PEG10* gene in hepatoma cells promoted cell growth, and knock-down of *PEG10* by antisense oligonucleotides suppressed cell proliferation (8,21,22), we regarded PEG10 protein as a candidate target protein. Immunoblotting showed that SIAH1 significantly reduced the amount of PEG10 protein in Huh7 cells, supporting the idea that SIAH1 inhibits the growth of these cells by promoting degradation of PEG10 protein. Since the infection with Ad-SIAH1 reduces PEG10 protein in HepG2 cells as well, ß-catenin and PEG10 are likely to be dual target proteins for degradation by SIAH1 in hepatoma cells.

In conclusion, our findings demonstrate that gene transfer of *SIAH1* into hepatoma cells efficiently induced apoptosis and inhibited their growth. Although aberrant ß-catenin signal was clearly down-regulated by SIAH1, the growth suppressive effect did not fully depend on ß-catenin, but also on other target proteins including PEG10. These data should lead to a better understanding of the relationship between deregulation of ß-catenin signals and hepatocarcinogenesis. It is also noteworthy that SIAH1 may be an effective and widely applicable therapeutic molecule for HCCs. Further investigation of the mechanisms by which *SIAH1* promotes apoptosis and suppresses cell growth should also allow for the discovery of new therapeutic strategies.

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