RASSF1A-mediated regulation of amphiregulin via the Hippo pathway in hepatocellular carcinoma

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ABSTRACT

Ras association domain family 1 isoform A (RASSF1A) is a tumor suppressor and the methylation thereof is known to be involved in many human cancers including hepatocellular carcinoma (HCC). RASSF1A has been shown to suppress tumors via activation of components of the Hippo tumor suppressor pathway, including mammalian STE20-like kinase (MST). Amphiregulin (AREG), a target gene for Yes-associated protein (YAP), is a known oncogenic component of the Hippo pathway; however, the tumor suppressive effect of RASSF1A on AREG in regards to regulation of the Hippo pathway remains unclear in HCC.

In this study, overexpression of RASSF1A in HCC cells (Hep3B, SK-Hep1, and PLC/PRF/5), which lack functional RASSF1A, significantly inhibited cell proliferation and induced apoptosis by activating the Hippo pathway. Consequently, overexpression of RASSF1A inhibited the oncogenic functions of YAP, leading to a significant reduction in AREG secretion via regulation of the Hippo pathway. In human study, greater expression of RASSF1A was observed in chronic hepatitis (CH)/cirrhosis than in HCC, whereas expression of YAP and AREG was higher in 81% and 86% of HCC than corresponding CH/cirrhosis, respectively. Furthermore, a gradual decrease in RASSF1A protein expression was detected as multistep hepatocarcinogenesis progressed from CH/cirrhosis, dysplastic nodules (DNs), toward HCCs, while the protein expression of YAP and AREG gradually increased. These findings give evidence to the regulation of YAP and AREG (YAP signature) by RASSF1A in human multistep hepatocarcinogenesis. In conclusion, our results suggest that RASSF1A might play a crucial role in HCC suppression by regulating AREG expression via activation of the Hippo tumor suppressor pathway, leading to inactivation of oncogenic YAP.
**INTRODUCTION**

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of cancer-related death (1). Recently, several genetic and epigenetic alterations were shown to be related with hepatocarcinogenesis, including frequent promoter hypermethylation of several tumor suppressor genes, such as Ras association domain family 1 isoform A (RASSF1A) (2). In HCC, epigenetic alterations, such as global DNA hypomethylation or CpG island hypermethylation, are critical mechanisms that affect the loss and gain of DNA methylation, respectively (3). Accordingly, inactivation of RASSF1A by DNA methylation has been shown to be involved in the development of many human cancers, including lung cancer, cervical squamous cell carcinoma, breast cancer, and HCC (4-6).

As a tumor suppressor, RASSF1A is known to play critical roles in cell cycle regulation, microtubule stability, and apoptosis (5, 6). Recently, RASSF1A was also shown to play an important role in the Hippo tumor suppressor pathway. RASSF1A induces phosphorylation and activation of the pro-apoptotic mammalian STE20-like kinases (MST), a major component of the Hippo pathway; moreover, RASSF1A sustains phosphorylation of MST leading to apoptosis through activation of the Hippo pathway (7). Thereby, yes-associated protein (YAP), an oncogenic component of the Hippo pathway, is phosphorylated and inactivated by RASSF1A (8). In contrast, when YAP, an oncogenic transcription coactivator, is dephosphorylated, it translocates into the nucleus and subsequently upregulates the transcription levels of oncogenes, such as CTGF, Gli2, and amphiregulin (AREG) genes, inducing cell proliferation, cell survival, and cell migration (9, 10). In addition, YAP has been identified as an independent prognostic marker for overall survival and disease-free survival in HCC (11).

AREG, an EGF family member, is reported to be a downstream effector of the Hippo pathway and a direct target of YAP (12). AREG (YAP signature) is readily induced in the regenerating liver and acts as a potent mitogenic and anti-apoptotic factor for normal
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hepatocytes. AREG is secreted in many cancer cells including human HCC cells, such as Hep3B and PLC/PRF/5 cells (13), and was shown to mediate protection against liver injury in a mouse model (14). AREG promotes a variety of effects depending on the cellular context, including evasion of apoptosis, tissue invasion, and self-sufficiency in growth signals, all of which are involved in tumor development and progression (15). In a previous study, Castillo J et al. demonstrated that recombinant AREG treatment on HCC cells stimulates cell growth and confers apoptosis resistance. They also showed that knockdown of AREG by specific small interfering RNAs in HCC cells inhibited cell growth and reduced their ability to resist apoptosis (13). Additionally, in colorectal cancer, AREG positive status was shown to be significantly correlated with tumor invasion depth, distant metastases, and nerve invasion, indicating an association between AREG levels and invasive growth (16). Moreover, previous clinical research supporting the role of AREG in cancer development and progression demonstrated that AREG might serve as a prognostic and/or a predictive biomarker (17, 18). Although a few studies have reported on the effects of RASSF1A on the Hippo pathway components involving YAP (7, 19), the relationship between RASSF1A and AREG, a target gene of YAP, is not fully understood in HCC.

To investigate the effect of RASSF1A on AREG via regulation of the Hippo pathway, we overexpressed RASSF1A protein in HCC cells and evaluated the expressions thereof in human tissues by western blot and immunohistochemical analysis. As a result, we were able to demonstrate that RASSF1A overexpression reduces AREG production leading to apoptosis via activation of the Hippo pathway in HCC.

MATERIALS AND METHODS

Cell culture and 5-aza-2'-deoxycytidine and protein transport inhibitor treatment
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HepG2 (HB8065), Hep3B (HB8064), Huh7 (PTA 8561), SK-Hep1 (HTB-52), PLC/PRF/5 (CRL-8024), and HeLa (CCL-2) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in DMEM (Gibco-BRL, MD, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. For all experiments, cells were maintained at 37 °C in a humidified 5% CO2 incubator. 5-aza-2’-deoxycytidine (5-AZA) was purchased from Sigma (MO, USA) and protein transport inhibitor was purchased from Becton Dickinson (CA, USA).

Genomic DNA extraction, modification, and methylation-specific PCR

Genomic DNA was isolated from HCC cells using the Get pure DNA Kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer’s protocol. Briefly, HCC cells were harvested and lysed with lysis buffer containing 10 μL of proteinase K solution. Cell lysates were incubated at 65 °C for 10 min, added to 50 μL of precipitation solution I and resuspended in precipitation solution II. Samples were then centrifuged at 13,000 rpm for 5 min, after which the supernatants were transferred to fresh tubes and precipitated with ethanol and DNA elution was performed. Eluted genomic DNA was dissolved in TE buffer and was quantified by NanoDrop ND-100 (Thermo Fisher Scientific, MA, USA). Genomic DNA modification was performed using the EZ DNA modification kit (Zymo-Research, CA, USA) according to the manufacturer’s instructions. Next, 1 μg of extracted DNA was mixed in 50 μL of sodium bisulfate containing 5 μL of dilution buffer and then incubated for 15 min at 37 °C. The incubated samples were mixed with 100 μL of CT conversion reagent and incubated for 15 h at 50 °C under a dark condition. The DNA samples were then put on ice for 10 min, transferred into Zymo-Spin IC columns treated with 400 μL of binding buffer, and centrifuged at 13,000 rpm for 30 s. After discarding the flow-through, the columns were washed with wash buffer. After washing, 200 μL of desulphonation buffer was added to the samples, which were
then incubated at room temperature for 15 min and centrifuged at 13,000 rpm for 30 s. Each column was then washed twice with 200 uL of wash buffer. Extracted DNA was then used to determine the methylation status of RASSF1A by methylation specific PCR (MSP) as previously described (3, 20).

**Total RNA extraction and cDNA synthesis**

Total cellular RNA from HCC cells was extracted with TRizol reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer’s protocol. RNA concentration and purity were determined by Nanodrop, measuring fluorescence at 260 nm and 280 nm. Absence of genomic DNA contamination was confirmed by PCR of total RNA. Transcription into cDNA was performed using the High Capacity RNA to cDNA kit (Applied Biosystems Inc., Foster City, USA) according to the manufacturer’s instructions.

**Reverse transcriptase PCR (RT-PCR)**

Standard RT-PCR was performed using primer sequences for RASSF1A (forward: 5’-GATGAAGCCTGTGTAAGAACCGTCCT-3’ and reverse: 5’-CAGATTGCAAGTTCACCTGCCACTA-3’), AREG (forward: 5’-TGCTGGATTGGACCTCAATG-3’ and reverse: 5’-TCCCGAGGACCGTTCACCTAC-3’) and β-actin (forward: 5’-TGTGGCATCCACGAAACTAC-3’ and reverse: 5’-GGAGCAATGATCTTGATCTTCA-3’). PCR was performed using AccuPower PCR Premix (Bioneer, CA, USA) according to the manufacturer’s instructions. The amplified fragment corresponding to RASSF1A was 280 bps and that corresponding to AREG was 163 bps. Approximately 1-2 uL of cDNA was used for PCR amplification. The PCR conditions for RASSF1A, AREG and β-actin (internal control) were as follows: an initial 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C; 30 s at 64 °C for RASSF1A, 59 °C for AREG and 64 °C for β-actin; 30 s at 72 °C; and a final elongation for 10 min at 72 °C. The PCR products were
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separated by electrophoresis on a 2% agarose gel.

Transfection

For transient transfection of wild type RASSF1A, we used the pEGEF-FLAG-RASSF1A plasmid kindly donated from Professor Dea Sik Lim. For transfections, Hep3B, SK-Hep1, and PLC/PRF/5 cells were cultured for 24h in 6-well plates, to which 1 ug of DNA was added along with 2 uL of Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer’s recommendations. The cells were harvested at the indicated times.

MTT assay

HCC cells transfected with the RASSF1A plasmid (RASSF1A) or control plasmid (mock) were cultured in 6-well plates and allowed to settle for 24 h before transfection with Lipofectamine 2000. The cells were then treated with 2 ng/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma) for 4 h at 37 °C. The monolayer was suspended in 0.1 mL of DMSO and its absorbance at 570 nm was read by an ELISA reader. The control value corresponding to untreated cells was taken as 100%, and the viability of treated samples was expressed as a percentage of the control.

Annexin V-FITC and PI staining and FACS analysis

Apoptosis was evaluated using the Annexin V-FITC apoptosis detection kit (BD bioscience Pharmingen). Briefly, cells were washed twice in PBS, collected and resuspended in 100 uL of 1 x Annexin V binding buffer. Next, 5 uL of Annexin V-FITC conjugate and 10 uL of PI buffer were added to each sample. The cells were then incubated at room temperature for 15 min under a dark condition. After adding 200 uL of the 1 x Annexin V binding buffer, cells were analyzed using a FACSscan flow cytometer (BD bioscience Pharmingen).
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ELISA

Hep3B and PLC/PRF/5 cells were seeded in a 6-well plate, transfected with mock or RASSF1A plasmid and then incubated for 72 h. Conditioned medium (CM) was obtained after culture of cells for the indicated time periods. The concentration of AREG in the CM was measured by the AREG DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions. Capture antibody was diluted in phosphate-buffered saline (PBS) and used to coat the wells of immunoplates (Costar) overnight at room temperature. The coated plates were then washed and blocked with 1% bovine serum albumin (BSA) in PBS prior to addition of the samples thereto. Thereafter, biotinylated detection antibody and HRP conjugated streptavidin were added, and HRP colorimetric substrate (sigma) development was assessed by absorbance at 450 nm.

Human tissue samples and pathological examination

Human liver tissue samples from 22 HCCs and 15 dysplastic nodules (DNs) were studied, and each sample consisted of tissue from the HCC or DN in addition to corresponding non-tumoral liver tissue (Supplementary Table 1). The tissues were obtained from 20 males and 7 females, the mean age of whom was 54±9.7 years (mean±SD, range 27-68 years). The etiologies of liver disease were hepatitis B-virus (n=24, 89%), hepatitis C-virus (n=1, 3.7%), alcohol (n=1, 3.7%), and unknown (n=1, 3.7%). The specimens were fixed in formalin, and representative sections were submitted for histologic examination and immunohistochemistry. In addition, fresh liver tissues were sampled from the same cases, snap frozen in liquid nitrogen and stored at -70°C. Differentiation of HCC was evaluated according to Edmondson-Steiner grade, and included one (5%) grade I HCC, fourteen (64%) grade II HCCs, and seven (31%) grade III HCCs. The average tumor size was 4.8±2.0 cm (mean±SD, range 2.0-9.0 cm). DN
cases were consisted of 5 cases of low grade DNs (LGDNs) and 10 cases of high grade DNs (HGDNs). Non-tumoral liver tissues exhibited cirrhosis in 15 cases and chronic hepatitis in 12 cases. Liver specimens were provided by the Liver Cancer Specimen Bank, National Research Resource Bank Program, Korea Science, and Engineering Foundation of the Ministry of Science and Technology.

Representative sections of formalin-fixed paraffin-embedded tissues were used for immunohistochemistry. Paraffin sections were first deparaffinized in xylene for 60 min, and then rehydrated with graded alcohol. Endogenous peroxidase activity was quenched in a 3% hydrogen peroxide/methanol solution for 20 min. For antigen retrieval, sections were boiled in 100 mM of sodium citrate, at a pH of 6.0, for 12 min in a microwave oven. Monoclonal primary antibodies to RASSF1A (eBioscience) and YAP (Santa Cruz), as well as polyclonal primary antibody to AREG (R&D systems), diluted to 1:50, 1:100, and 1:20 dilutions, respectively, were applied to the sample sections, which were then allowed to incubate overnight at 4°C. After washing with TBS, the sections were incubated with secondary antibody using the DAKO EnVision Rabbit/Mouse kit or secondary goat antibody conjugated HRP for 20 min at room temperature, and then developed with diaminobenzidine (DAKO). Finally, the sections were counterstained with hematoxylin, dehydrated using graded alcohol and cleared in xylene.

Histoscores for RASSF1A, YAP, and AREG were evaluated as like followings. Staining intensity and distribution among cells were scored as 0, 1, 2, 3, and 4 for negative, weak, moderate, positive, and strong staining, respectively. Intensity and distribution scores were multiplied together for interpretation.

**Western blot analysis**

Human HCC cells and tissues were lysed with RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged at 13,200 rpm for 15 min at
4 °C. Supernatants were then collected and used to determine protein concentrations using the Bradford method. Protein samples were resolved on a 10% SDS–polyacrylamide gel and then transferred to nitrocellulose membranes (Bio-RAD, CA, USA). The membranes were incubated with appropriate antibodies. We used the following antibodies: mouse monoclonal anti-human RASSF1A (1:1000; eBioscience, CA, USA), goat polyclonal anti-human AREG (1:500; R&D systems, MN, USA), rabbit polyclonal anti-human p-YAP (1:1000; Cell signaling, MA, USA), mouse monoclonal anti-human YAP (1:1000; Santa Cruz, CA, USA), rabbit polyclonal anti-human p-MST (1:1000; Cell signaling), rabbit polyclonal anti-human MST (1:1000; Cell signaling), rabbit polyclonal anti-human caspase 3 (1:1000; Cell signaling), and rabbit polyclonal anti-human β-actin antibody (1:1000; Cell signaling). Membranes were incubated with their respective antibodies overnight, followed by incubation with secondary horseradish peroxidase (HRP) conjugated antibody (Cell signaling) for 1 h at room temperature. Membrane-bound antibodies were detected using the luminol reagent kit ECL system (Santa Cruz). Densitometric scanning of band intensities were quantified by Quantity One (Bio-Rad, USA).

Statistical methods

Statistical analysis was carried out using PASW statistics software (version 18.0, SPSS Inc., Chicago, IL, USA) and assessed using the Mann-Whitney test and t-test as deemed appropriate. Correlations for RASSF1A, YAP, and AREG expression were estimated using Spearman’s correlation coefficient. All p-values corresponded to two-sided tests and a p-value of less than 0.05 was considered statistically significant.

RESULTS
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The methylation status of RASSF1A in HCC cells

To discover the methylation status of RASSF1A in HCC cells, MSP was performed in five human HCC cells, including HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5 cells. HeLa cells, which are known to exhibit unmethylated RASSF1A expression, were used as a positive control. The results revealed methylation of RASSF1A in all five HCC cells (Figure 1A). For further confirmation, Hep3B and SK-Hep1 cells were treated with 5 uM or 10 uM 5-AZA, a demethylating agent, for 48 h and harvested, after which the demethylation of RASSF1A was determined by RT-PCR. Increased mRNA levels in RASSF1A were detected in 5-AZA treated cells in comparison to untreated cells (Figure 1B). These results indicated that RASSF1A was inactivated by methylation in HCC cells.

The effect of RASSF1A overexpression on HCC cell proliferation and apoptosis

To investigate the effect of RASSF1A overexpression on cell proliferation and apoptosis in Hep3B, SK-Hep1, and PLC/PRF/5 cells, which lack functional RASSF1A; MTT assay, Annexin V-FITC and PI staining were performed, respectively. MTT assay revealed that cell proliferation in HCC cells transfected with RASSF1A plasmid (RASSF1A) was markedly inhibited, compared to cells transfected with control plasmid (mock) or untreated cells, after 72 h of transfection. Hep3B cells transfected with RASSF1A plasmid showed a significant inhibition of cell proliferation in comparison to mock transfectant or untreated cells at 24 h ($p=0.005$), 48 h ($p=0.031$), and 72 h ($p=0.023$). SK-Hep1 cells transfected with RASSF1A plasmid also showed a significant inhibition of cell proliferation at 24 h ($p=0.001$), 48 h ($p=0.028$), and 72 h ($p=0.0004$), compared to mock transfectant or untreated cells, while PLC/PRF/5 cells displayed significant inhibition of cell proliferation only at 48 h ($p=0.001$) and 72 h ($p=0.047$) in cells transfected with RASSF1A plasmid. Hep3B, SK-Hep1, and PLC/PRF/5 cells transfected with mock plasmid showed a similar level of cell proliferation with untreated cells. After 72 h, cell
proliferation rates of mock and RASSF1A groups were, respectively, 298 ±18.45% and 206 ±17.65% in Hep3B cells; 307 ±4.58% and 101 ±3.16% in SK-Hep1 cells; and 222 ±4.75% and 185 ±10.89% in PLC/PRF/5 cells (Figure 2A).

Annexin V-FITC and PI staining with SK-Hep1 and PLC/PRF/5 demonstrated that overexpression of RASSF1A induced apoptosis in both cells, and a significant induction of apoptosis was shown in SK-Hep1 cells, by 14.95 ±1.47 fold at 48 h (p=0.001) and 37.12 ±1.95 fold at 72 h (p<0.0001). Although statistically insignificant in PLC/PRF/5 cells, apoptosis was increased by 1.827 ±1.14 fold at 48 h and 1.91 ±3.27 fold at 72 h in PLC/PRF/5 cells transfected with RASSF1A plasmid (Figure 2B). To confirm apoptosis by overexpression of RASSF1A, we analyzed activation of caspase 3. Cleaved caspase 3, an active form of caspase 3, was detected and increased in all cells transfected with RASSF1A plasmid, compared to HCC cells transfected with mock plasmid, indicating an induction and increase in apoptosis due to RASSF1A overexpression (Figure 2C). Taken together, these results suggested that RASSF1A overexpression induced apoptotic cell death, leading to inhibition of cell proliferation in HCC cells that lack functional RASSF1A.

Activation of the Hippo pathway by RASSF1A overexpression in HCC cells

In order to identify activation of the Hippo pathway in HCC cells transfected with RASSF1A plasmid, the phosphorylation status of MST and YAP, major components of the Hippo pathway, was analyzed by western blot. Phosphorylation of MST and YAP was significantly increased in HCC cells transfected with RASSF1A plasmid, leading to the activation of MST and inactivation of YAP after 48 h (Figure 3A). The protein levels of RASSF1A, p-MST, and p-YAP in Hep3B cells transfected with RASSF1A plasmid, respectively, were 5.70 ±1.73 fold (p=0.042), 2.47 ±0.29 fold (p=0.013), and 2.17 ±0.23 fold (p=0.012) higher than those of cells transfected with mock plasmid. The protein levels of RASSF1A,
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p-MST, and p-YAP in SK-Hep1 cells transfected with RASSF1A plasmid, respectively, were 4.47 ±1.27 fold ($p=0.047$), 4.17 ±1.26 fold ($p=0.048$), and 2.63 ±0.40 fold ($p=0.019$) higher than those of cells transfected with mock plasmid. The protein levels of RASSF1A, p-MST, and p-YAP in PLC/PRF/5 cells transfected with RASSF1A plasmid, respectively, were 7.09 ±0.76 fold ($p=0.005$), 2.73 ±0.25 fold ($p=0.006$), and 2.61 ±0.20 fold ($p=0.005$) higher than those of cells transfected with mock plasmid. Overexpressed RASSF1A protein initiated the phosphorylation and subsequent activation of MST at 12 h, which gradually increased up to 72 h. YAP, a downstream target of MST, began to be phosphorylated by overexpression of RASSF1A at 48 h, and its phosphorylation was sustained up to 72 h in a time dependent manner (Figure 3B). These results suggested that RASSF1A overexpression activates the Hippo tumor suppressor pathway by phosphorylating MST and YAP, leading to the activation of MST and inactivation of YAP in HCC cells. Therefrom, we discerned that phosphorylation and inactivation of YAP by RASSF1A overexpression might affect its oncogenic function as a transcriptional coactivator, leading to expression of its downstream targets.

RASSF1A overexpression inactivates YAP and decreases AREG secretion via regulation of the Hippo pathway

To investigate the regulation of AREG, an oncogenic downstream target of YAP, by RASSF1A in HCC cells, AREG expression in HCC cells transfected with RASSF1A or mock plasmid were evaluated by RT-PCR and western blot. The mRNA levels of AREG were significantly decreased in Hep3B, SK-Hep1, and PLC/PRF/5 cells after RASSF1A overexpression (Figure 4A). The mRNA level of AREG gradually decreased over 72 h in a time dependent manner in Hep3B cells transfected with RASSF1A plasmid (Figure 4B). In order to determine the effect of RASSF1A overexpression on the protein expression levels of AREG, Hep3B and PLC/PRF/5 cells were treated with protein transport inhibitor, which inhibits AREG
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secretion from cells and allows for the accumulation of intracellular AREG for detection by western blot. SK-Hep1 cells were excluded from the western blot analysis for the detection of AREG due to their low level of endogenous AREG protein expression (21). Similar to the results for AREG mRNA levels, the protein levels of AREG were significantly decreased in Hep3B and PLC/PRF/5 cells transfected with RASSF1A plasmid. In addition, increased phosphorylation of YAP was also detected in HCC cells transfected with RASSF1A plasmid (Figure 4C). To confirm the inhibitory effect of RASSF1A overexpression on AREG expression, we measured AREG secretion in the conditioned medium (CM) of HCC cells by ELISA in a time dependent manner. The levels of secreted AREG were decreased in the CM of both HCC cells transfected with RASSF1A plasmid (Figure 4D). To exclude the possibility that the decreased number of cells led to reduced secretion of AREG, the CM of HCC cells were harvested prior to when the reduction of cell proliferation began in cells transfected with RASSF1A plasmid in comparison to those transfected with mock plasmid. These data demonstrated that RASSF1A overexpression downregulates AREG expression and secretion by phosphorylation and inactivation of YAP, a transcription coactivator of AREG, via activation of the Hippo pathway.

The protein expressions of RASSF1A, YAP, and AREG in human multistep hepatocarcinogenesis

To verify the in vitro results, the protein expressions of RASSF1A, YAP, and AREG were evaluated in the human liver tissues of 22 HCC patients, using western blot (Figure 5A and 5B). All 22 human HCC cases (100%) showed significant downregulation of RASSF1A expression, compared to their corresponding non-tumoral liver tissues of chronic hepatitis (CH)/cirrhosis. YAP protein expression exhibited a dramatic increase in 19 cases (82%) (nos. 1-5, 7-13, 15-16, and 18-22) of HCC tissues and average ratio of the YAP intensity of
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HCC/non-tumor detected by western blot was over 2 folds (2.02±0.97 fold, mean±SD), while 3 cases (no. 6, 14, and 17) showed similar expression levels of YAP protein between HCCs and their corresponding non-tumoral liver tissues (YAP intensity of HCC/non-tumor was 0.72-0.91). Significant upregulation of AREG in the HCC tissues was detected in 18 cases (86%) (nos. 1-13, 15, 17-18, and 20-21) and the average ratio of AREG intensity of HCC/non-tumor was 1.36±0.25 fold (mean±SD). While 4 cases (nos. 14, 16, 19, and 22) showed no significant difference in AREG expression in HCC tissues, compared to corresponding non-tumoral liver tissue of CH/cirrhosis (AREG intensity of HCC/non-tumor was 0.96-1.00) (Supplementary Table 2).

For further investigation of human multistep hepatocarcinogenesis, immunohistochemistry for RASSF1A, YAP, and AREG was performed in CH/cirrhosis, LGDNs, HGDNs, and HCC tissues (Figure 5C and 5D). The immunohistochemical analysis revealed a tendency of gradual decrease in RASSF1A expression as multistep hepatocarcinogenesis progressed toward HCC, and CH/cirrhosis tissue showed the highest histoscore of RASSF1A, which was significantly higher compared to HGDN and HCC (p=0.009 and p=0.03, respectively) (Figure 5C). Whereas the protein expression of YAP and AREG showed a tendency of gradual increase in multistep hepatocarcinogenesis with the highest expression level in HCC. YAP histoscores were significantly higher in HCCs than in CH/cirrhosis, LGDNs, and HGDNs (p<0.05 in all) and YAP histocores of HGDNs were significantly higher than in CH/cirrhosis (p=0.0005). Additionally the nuclear and cytoplasmic expression of YAP protein was evaluated in each lesion of multistep hepatocarcinogenesis, nuclear expression of YAP protein was significantly dominant compared to cytoplasmic expression in HCCs (p=0.0002), whereas nuclear expression of YAP protein was similar to that of cytoplasm in CH/cirrhosis, LGDNs, and HGDNs (Supplementary Figure 1). AREG histoscores also showed the highest
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level in HCCs, which was significantly higher compared to CH/cirrhosis, LGDNs, and HGDNs ($p<0.05$ in all) (Figure 5C). Furthermore, the relationship between YAP and AREG histoscores showed a significant positive correlation ($p=0.001$, $r=0.408$), while that between RASSF1A and YAP histoscores exhibited a significant negative correlation ($p=0.043$, $r=-0.236$) (Supplementary Figure 2). These results indicated that RASSF1A, YAP, and AREG (YAP signature) are involved in human multistep hepatocarcinogenesis. Accordingly, the data obtained from human HCC tissues were consistent with our in vitro results, which showed that RASSF1A overexpression regulates AREG.

DISCUSSION

RASSF1A with loss of heterozygosity is reported to be silenced through CpG promoter methylation in several tumors including lung, bladder, breast, and liver cancers (3-6, 22). It has been also reported that hypermethylation of RASSF1A, BLU, and FHIT on chromosome 3 is a very common process in early event of HCC by studying HCC samples and HCC cells treated with 5-AZA (23). The study of DNA methyltransferase has been revealed to play an important role in hypermethylation of RASSF1A in HCC cells treated with deacetylase inhibitor, panobinostat (24). Recently, micro RNA-602 has been reported to inhibit the tumor suppressive function of RASSF1A via decrease of RASSF1A expression in HeG2 cells overexpressed HBX protein (25). Previously, RASSF1A was also shown to play an important role in activating the Hippo tumor suppressor pathway via interactions with MST and LATS, leading to inactivation of the transcription coactivator YAP (26). Moreover, YAP is known to be an independent prognostic marker for overall survival and disease-free survival in HCC (11); meanwhile, AREG, its oncogenic target, has also been reported to serve as a prognostic and/or a predictive biomarker of cancer development and progression (17, 18).
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In this study, methylation of RASSF1A was observed in all five HCC cells, including HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5 cells, compared to HeLa cells which are known to exhibit unmethylated RASSF1A. Although partially unmethylated RASSF1A was slightly detected in Hep3B cells, protein expression of RASSF1A was not detected in all five HCC cells. Moreover, overexpression of RASSF1A inhibited cell proliferation and induced apoptosis in Hep3B cells, suggesting a tumor suppressive effect of RASSF1A in HCC cells that lack functional RASSF1A.

RASSF1A has been shown to suppress tumors via activation of components of the Hippo tumor suppressor pathway, including MST. RASSF1A directly binds to MST through SARAH motif, where it phosphorylates and activates MST (19). Activated MST subsequently phosphorylates LATS1, which then induces YAP phosphorylation, leading to inactivation of its oncogenic properties (27). When phosphorylated YAP binds to 14-3-3 or CK1δ/ε, its role as a transcription coactivator is inhibited by cytosolic retention or ubiquitination and degradation, resulting in the loss of its oncogenic potential to transactivate its downstream targets (9, 28). To determine whether overexpression of RASSF1A activates the Hippo pathway in HCC cells in which RASSF1A is methylated, the phosphorylation status of MST was evaluated in Hep3B cells transfected with RASSF1A plasmid. Our results revealed that RASSF1A induced phosphorylation of MST, which reportedly activates the Hippo pathway (29, 30). Accordingly, methylation and inactivation of the RASSF1A gene in HCC may contribute to hepatocarcinogenesis by inactivation of the Hippo tumor suppressor pathway, resulting in dephosphorylation and activation of YAP. YAP has been reported to be an oncogenic transcription coactivator that induces oncogenes, such as AREG, CTGF, and Gli2, to increase cell proliferation and survival (9, 10, 12). In this study, overexpression of RASSF1A reduced the expression of AREG at both the mRNA and the protein levels in HCC cells with methylated RASSF1A, inducing decreased secretion of AREG to conditioned medium (CM) in vitro.
**RASSF1A downregulates AREG by activation of Hippo pathway**

In hepatocarcinogenesis, AREG is reported to play an important role in cell growth and apoptosis resistance in HCC (12, 31). Previously, AREG was shown to induce epidermal growth factor receptor dependent intracellular signaling, which stimulates proliferation and resistance to TGF-β-induced apoptosis, in AREG treated HCC cells. This suggests that AREG expression may play a role in the protection of HCC cells against apoptosis. Furthermore, silencing of AREG in HCC cells reportedly induces apoptosis by upregulating Bim expression (13). At the moment, the transcription factor that mediates AREG expression remains to be further investigated, as the AREG promoter does not contain a TEAD-binding element, which is different from the canonical pathway (12). A novel demethylating agent, zebularine, was shown to induce apoptosis in HCC cells by reversing hypermethylation of RASSF1A (32, 33). Thus, epigenetic regulation of RASSF1A may provide potential targets for liver cancer therapies. It is suggested that AREG, which is a YAP signature known to regulate HCC proliferation and resistance to apoptosis, could be another potential target for liver cancer therapies (13).

Human hepatocarcinogenesis is known to be a multistep process (3). DNs are considered precancerous lesions of HCC, and can be divided into LGDN and HGDN according to their cellular and structural atypia (34). For investigation of human multistep hepatocarcinogenesis, protein expression levels for RASSF1A, YAP, and AREG were investigated in CH/cirrhosis, LGDNs, HGDNs, and HCC tissues. As multistep human hepatocarcinogenesis progressed from CH/cirrhosis, LGDNs, HGDNs, and finally HCCs, there was a tendency of gradual decrease in RASSF1A expression. Whereas the protein expression of YAP and AREG gradually increased as multistep hepatocarcinogenesis progressed with the highest expression level in HCCs. The nuclear expression of YAP, indicating that YAP is dephosphorylated and oncogenically activated was significantly increased compared to cytoplasmic expression in HCC. The protein expression levels of RASSF1A, YAP, and AREG in HCCs and corresponding CH/cirrhosis were detected by western blot as well as immunohistochemistry, and both methods showed consistent
RASSF1A downregulates AREG by activation of Hippo pathway

results. In majority of HCC cases, RASSF1A protein expression was lower and the protein expression of YAP and AREG was higher compared to corresponding CH/cirrhosis tissues, although some cases demonstrated similar expression levels of these proteins between HCC and corresponding CH/cirrhosis (Supplementary Table 2). Furthermore, the relationship between YAP and AREG histoscores showed a significant positive correlation, while that between RASSF1A and YAP signature histoscores exhibited a significant negative correlation. These results indicated that RASSF1A, YAP, and AREG (YAP signature) are involved in human multistep hepatocarcinogenesis. Actually, the RASSF1A protein expression of this study was consistent with our previous report, in which a gradual increase in RASSF1A methylation was seen along with progression of multistep hepatocarcinogenesis, wherein the methylation frequency for RASSF1A was higher in early HCC than in CH/cirrhosis and RASSF1A protein expression was remarkably decreased in progressed HCC compared to cirrhotic CH/cirrhosis (3).

Through our study, RASSF1A was shown to suppress AREG via regulation of the Hippo pathway in vitro, and in human multistep hepatocarcinogenesis. Therefore, RASSF1A is suggested to play an important role in HCC suppression by regulating AREG expression via activation of the Hippo tumor suppressor pathway, leading to inactivation of oncogenic YAP.

FIGURE LEGENDS

Figure 1. RASSF1A is methylated in HCC cells. (A) RASSF1A methylation status of HCC cells was measured by MSP. (B) HCC cells were treated with 5 uM or 10 uM 5-AZA for 48 h. RASSF1A mRNA levels were measured by RT-PCR. UT; untreated cells, 5-AZA; 5-AZA treated cells.
RASSF1A downregulates AREG by activation of Hippo pathway

**Figure 2.** RASSF1A overexpression downregulates cell proliferation and induces apoptosis in HCC cells. (A) Cell proliferation of HCC cells transfected with RASSF1A or mock plasmid were detected by MTT assay after 4 h of incubation. *p<0.05 for mock and RASSF1A. (B) Quantitative analysis of Annexin V-FITC and PI staining was performed using SK-Hep1 and PLC/PRF/5 cells transfected with RASSF1A or mock plasmid for the indicated time period. *p<0.05 for mock and RASSF1A. (C) Protein levels of caspase 3 active forms were assessed in HCC cells transfected with RASSF1A or mock plasmid by western blot analysis. M; Mock, R; RASSF1A, active c-3; active form of caspase 3. β-actin was used as a loading control.

**Figure 3.** The Hippo pathway was activated by RASSF1A overexpression in HCC cells. (A) Protein levels of YAP signature in HCC cells transfected with RASSF1A or mock plasmid were analyzed by western blot analysis. Quantitation results of three repeated western blot analyses of HCC cells were assessed by Quantity One. *p<0.05 between mock and RASSF1A. (B) Protein levels of YAP signature in Hep3B cells transfected with RASSF1A plasmid were detected by western blot analysis in a time dependent manner up to 72 h. M; Mock, R; RASSF1A. β-actin was used as a loading control.

**Figure 4.** Downregulation of AREG by RASSF1A overexpression in HCC cells (A) The mRNA levels of AREG in HCC cells transfected with RASSF1A or mock plasmid were assessed with RT-PCR. HCC cells were incubated for 48 h. (B) RT-PCR was performed with Hep3B cells transfected with RASSF1A or mock plasmid in a time dependent manner up to 72 h to analyze mRNA levels of AREG. (C) Protein levels of AREG, p-YAP, and YAP in HCC cells transfected with RASSF1A or mock plasmid were detected by western blot analysis. (D) AREG release in HCC cells transfected with RASSF1A or mock plasmid were measured by ELISA for the indicated times. Confirmation of RASSF1A overexpression was examined by western blot
**Figure 5.** Downregulation of RASSF1A and upregulation of YAP and AREG proteins in human multistep hepatocarcinogenesis. (A) Protein levels of RASSF1A, YAP, and AREG in tissues from 22 cases of human HCC and corresponding chronic hepatitis (CH)/cirrhosis were assessed by western blot analysis. (B) The protein expression level of RASSF1A, YAP, and AREG in human tissues was quantified by Quantity One. β-actin was used as a loading control. (C) The protein expression levels of RASSF1A, YAP and AREG detected by immunohistochemistry were compared among different lesions of human multistep hepatocarcinogenesis. *Indicates a statistically significant difference (p<0.05). (D) Representative immunohistochemical findings for RASSF1A, YAP, and AREG were shown in human multistep hepatocarcinogenesis. (Original magnification X 200). C; chronic hepatitis (CH)/cirrhosis, T; hepatocellular carcinoma, LGDN; low grade dysplastic nodule, HGDN; high grade dysplastic nodule, HCC; hepatocellular carcinoma, IHC; immunohistochemistry.

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**REFERENCES**

RASSF1A downregulates AREG by activation of Hippo pathway


RASSF1A downregulates AREG by activation of Hippo pathway


Figure 2

A

Hep3B

SK-Hep1

PLC/PRF/5

% of cell proliferation

% of cell proliferation

% of cell proliferation

0h 24h 48h 72h

0h 24h 48h 72h

0h 24h 48h 72h

Control Mock RASSF1A

Control Mock RASSF1A

Control Mock RASSF1A

B

SK-Hep1

PLC/PRF/5

% of apoptosis

% of apoptosis

0 20 40 60 80 100

0 20 40 60 80 100

48h 72h

48h 72h

Mock RASSF1A

Mock RASSF1A

Mock RASSF1A

p = 0.001*
p < 0.001*

C

Hep3B PLC/PRF/5 SK-Hep1

Mock R

Mock R

Mock R

kDa

55

36

55

36

55

36

RASSF1A active c-3 β-actin
Figure 3

A. Western blot analysis of RASSF1A, p-MST, MST, p-YAP, YAP, and β-actin in Hep3B, SK-Hep1, and PLC/PRF/5 cells treated with mock or RASSF1A.

B. Time-course analysis of RASSF1A, p-MST, MST, p-YAP, YAP, and β-actin expression in Hep3B cells treated with RASSF1A.
Figure 4

A

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280bp
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B

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RASSF1A
AREG
β-actin

C

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kDa
55
36
72
55
55
55
55
36
36

RASSF1A
β-YAP
YAP
AREG
β-actin

D

Hep3B

**Relative AREG secretion level**

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**% of cell proliferation**

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PLC/PRF5

**Relative AREG secretion level**

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**% of cell proliferation**

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RASSF1A-mediated regulation of amphiregulin via the Hippo pathway in hepatocellular carcinoma

Ei Yong Ahn, Ji Su Kim, Gi Jeong Kim, et al.

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