Oncogenes and Tumor Suppressors

RASSF1A-Mediated Regulation of AREG via the Hippo Pathway in Hepatocellular Carcinoma

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Abstract

RASSF1A-mediated regulation of AREG via the Hippo pathway in 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 to hepatocarcinogenesis, including frequent promoter hypermethylation of several tumor suppressor genes, such as Ras association domain family 1 isoform A (RASSF1A; ref. 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 proapoptotic mammalian STE20-like kinases (MST; ref. 5), 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 downstream effector of the Hippo pathway and a direct target of YAP (12). AREG (YAP signature) is readily induced by RASSF1A in human multistep hepatocarcinogenesis. Mol Cancer Res; 11(7); 748–58. ©2013 AACR.
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 and colleagues showed that recombinant AREG treatment on HCC cells stimulates cell growth and confers apoptosis resistance. They also showed that knockdown of AREG by specific siRNAs in HCC cells inhibited cell growth and reduced their ability to resist apoptosis (13).

In addition, 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 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).

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 show 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**

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. The cells were grown in Dulbecco’s modified Eagle’s medium (Gibco-BRL) 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 and protein transport inhibitor was purchased from Becton Dickinson.

**Genomic DNA extraction, modification, and methylation-specific PCR**

Genomic DNA was isolated from HCC cells using the Get pure DNA Kit (Dojindo Molecular Technologies) according to the manufacturer’s protocol. Briefly, HCC cells were harvested and lysed with lysis buffer containing 10 μL of protease K solution. Cell lysates were incubated at 65°C for 10 minutes, added to 50 μL of precipitation solution I, and resuspended in precipitation solution II. Samples were then centrifuged at 13,000 rpm for 5 minutes, after which the supernatants were transferred to fresh tubes and precipitated with ethanol and DNA elution was conducted. Eluted genomic DNA was dissolved in Tris–EDTA (TE) buffer and was quantified by NanoDrop ND-100 (Thermo Fisher Scientific). Genomic DNA modification was conducted using the EZ DNA Modification Kit (Zymo-Research) 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 minutes at 37°C. The incubated samples were mixed with 100 μL of CT conversion reagent and incubated for 15 hours at 50°C under a dark condition. The DNA samples were then put on ice for 10 minutes, transferred into Zymo-Spin IC columns treated with 400 μL of binding buffer, and centrifuged at 13,000 rpm for 30 seconds. After discarding the flow-through, the columns were washed with wash buffer. After washing, 200 μL of desulfonation buffer was added to the samples, which were then incubated at room temperature for 15 minutes and centrifuged at 13,000 rpm for 30 seconds. Each column was then washed twice with 200 μL 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) according to the manufacturer’s protocol. RNA concentration and purity were determined by NanoDrop, measuring fluorescence at 260 and 280 nm. Absence of genomic DNA contamination was confirmed by PCR of total RNA. Transcription into cDNA was conducted using the High Capacity RNA to cDNA Kit (Applied Biosystems Inc.) according to the manufacturer’s instructions.

**Reverse transcriptase PCR**

Standard reverse transcriptase PCR (RT-PCR) was conducted using primer sequences for RASSF1A (forward: 5′-GATGAAAGCCTTGTAAGAACCGTCTC-3′ and reverse: 5′-CAGATTGCAAGTTTACCTGCACCTA-3′) and AREG (forward: 5′-TGCTGGATTGGACCTCAATG-3′ and reverse: 5′-TCCGGAGGACGTTCTACACTAC-3′), and β-actin (forward: 5′-TGTTGGATCCAGAAACTAC-3′ and reverse: 5′-GGAGCAATGTCTTGATCTTC-3′). PCR was conducted using AccuPower PCR Premix (Bioneer) 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 to 2 μL of cDNA was used for PCR amplification. The PCR conditions for RASSF1A, AREG, and β-actin were: initial 5 minutes at 95°C followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 64°C for RASSF1A, 59°C for AREG and 64°C for β-actin; 30 seconds at 72°C; and a final elongation for 10 minutes at 72°C. The PCR products were 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
Prof. Dea Sik Lim (Korea Advanced Institute for Science and Technology, Daejeon, South Korea). For transfections, Hep3B, SK-HeP1, and PLC/PRF/5 cells were cultured for 24 hours in 6-well plates, to which 1 μg of DNA was added along with 2 μL 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 hours before transfection with Lipofectamine 2000. The cells were then treated with 2 ng/mL of MTT (Sigma) for 4 hours at 37°C. The monolayer was suspended in 0.1 mL of dimethyl sulfoxide (DMSO) and its absorbance at 570 nm was read by an ELISA reader. The control value corresponding to untreated samples 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 Biosciences Pharmingen). Briefly, cells were washed twice in PBS, collected and resuspended in 100 μL of 1 × Annexin V–binding buffer. Next, 5 μL of Annexin V–fluorescein isothiocyanate (FITC) conjugate and 10 μL of propidium iodide (PI) buffer were added to each sample. The cells were then incubated at room temperature for 15 minutes under a dark condition. After adding 200 μL of the 1 × Annexin V–binding buffer, cells were analyzed using a FACScan flow cytometer (BD Biosciences Pharmingen).

**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 hours. Conditioned medium was obtained after culture of cells for the indicated time periods. The concentration of AREG in the conditioned medium was measured by the AREG DuoSet ELISA Kit (R&D Systems) according to the manufacturer’s instructions. Capture antibody was diluted in 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 in PBS before addition of the samples thereto. Thereafter, biotinylated detection antibody and horseradish peroxidase (HRP)-conjugated streptavidin were added, and HRP colorimetric substrate (Sigma) development was assessed by absorbance at 450 nm.

**Human tissue samples and pathologic examination**

Human liver tissue samples from 22 HCCs and 15 dysplastic nodules were studied, and each sample consisted of tissue from the HCC or dysplastic nodule in addition to corresponding nontumoral liver tissue (Supplementary Table S1). 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 1 (5%) grade 1 HCC, 14 (64%) grade 2 HCCs, and 7 (31%) grade 3 HCCs. The average tumor size was 4.8 ± 2.0 cm (mean ± SD; range, 2.0–9.0 cm). Dysplastic nodule cases were consisted of 5 cases of low-grade dysplastic nodules (LGDN) and 10 cases of high-grade dysplastic nodules (HGDN). Nontumoral 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 minutes and then rehydrated with graded alcohol. Endogenous peroxidase activity was quenched in a 3% hydrogen peroxide/methanol solution for 20 minutes. For antigen retrieval, sections were boiled in 100 mmol/L of sodium citrate, at a pH of 6.0, for 12 minutes in a microwave oven. Monoclonal primary antibodies to RASSF1A (eBioscience) and YAP (Santa Cruz Biotechnology), 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 minutes at room temperature and then developed with diaminobenzidine (Dako). Finally, the sections were counterstained with hematoxylin, dehydrated using graded alcohol, and cleared in xylene. Histo scores 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 radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Roche). Lysates were centrifuged at 13,200 rpm for 15 minutes 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–PAGE and then transferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated with appropriate antibodies. We used the following antibodies: mouse monoclonal anti-human RASSF1A (1:1,000;
eBioscience), goat polyclonal anti-human AREG (1:500; R&D Systems), rabbit polyclonal anti-human p-YAP (1:1,000; Cell Signaling), mouse monoclonal anti-human YAP (1:1,000; Santa Cruz Biotechnology), rabbit polyclonal anti-human p-MST (1:1,000; Cell Signaling), rabbit polyclonal anti-human caspase-3 (1:1,000; Cell Signaling), and rabbit polyclonal anti-human β-actin antibody (1:1,000; Cell Signaling). Membranes were incubated with their respective antibodies overnight, followed by incubation with secondary HRP-conjugated antibody (Cell signaling) for 1 hour at room temperature. Membrane-bound antibodies were detected using the luminol reagent kit ECL system (Santa Cruz Biotechnology). Densitometric scanning of band intensities were quantified by Quantity One (Bio-Rad).

Statistical analysis
Statistical analysis was carried out using PASW statistics software (version 18.0, SPSS Inc.) and assessed using the Mann–Whitney test and t test as deemed appropriate. Correlations for RASSF1A, YAP, and AREG expression were estimated using Spearman correlation coefficient. All P values corresponded to two-sided tests and a P value of less than 0.05 was considered statistically significant.

Results
The methylation status of RASSF1A in HCC cells
To discover the methylation status of RASSF1A in HCC cells, MSP was conducted in 5 human HCC cells, including HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5 cells.

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 or 10 μmol/L 5-AZA for 48 hours. RASSF1A mRNA levels were measured by RT-PCR. UT, untreated cells; 5-AZA, 5-AZA–treated cells.

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 hours of incubation. B, quantitative analysis of Annexin V–FITC and PI staining was conducted 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.
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 conducted, respectively. MTT assay revealed that cell proliferation in HCC cells transfected with RASSF1A plasmid (RASSF1A) was markedly inhibited, compared with cells transfected with control plasmid (mock) or untreated cells, after 72 hours of transfection. Hep3B cells transfected with RASSF1A plasmid showed a significant inhibition of cell proliferation, compared with cells transfected with control plasmid (mock) or untreated cells, after 72 hours of transfection. Hep3B cells transfected with RASSF1A plasmid showed a significant inhibition of cell proliferation only at 48 hours ($P = 0.001$) and 72 hours ($P = 0.047$) 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 hours, 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 (Fig. 2A).

Annexin V–FITC and PI staining with SK-Hep1 and PLC/PRF/5 showed 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 hours ($P = 0.001$) and 37.12– ± 1.95–fold at 72 hours ($P < 0.0001$). Although statistically insignificant in PLC/PRF/5 cells, apoptosis was increased by 1.827– ± 1.14–fold at 48 hours and 1.91– ± 3.27–fold at 72 hours in PLC/PRF/5 cells transfected with RASSF1A plasmid (Fig. 2B).
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 hours. B, RT-PCR was conducted with Hep3B cells transfected with RASSF1A or mock plasmid in a time-dependent manner up to 72 hours 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 analysis (middle). Cell proliferation of HCC cells were confirmed by MTT assay (bottom). M, mock; R, RASSF1A. β-Actin was used as a loading control.
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 with HCC cells transfected with mock plasmid, indicating an induction and increase in apoptosis due to RASSF1A overexpression (Fig. 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

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 analysis. 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 hours (Fig. 3A). The protein levels of RASSF1A, p-MST, and p-YAP in Hep3B cells transfected with RASSF1A plasmid, respectively, were 5.70-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, p-MST, and p-YAP in PLC/PRF/5 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 SK-Hep1 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 hours, which gradually increased up to 72 hours. YAP, a downstream target of MST, began to be phosphorylated by overexpression of RASSF1A at 48 hours, and its phosphorylation was sustained up to 72 hours in a time-dependent manner (Fig. 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. Therefore, 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 analysis. The mRNA levels of AREG were significantly decreased in Hep3B, SK-Hep1, and PLC/PRF/5 cells after RASSF1A overexpression (Fig. 4A). The mRNA level of AREG gradually decreased over 72 hours in a time-dependent manner in Hep3B cells transfected with RASSF1A plasmid (Fig. 4B). 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 secretion from cells and allows for the accumulation of intracellular AREG for detection by Western blot analysis. 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 (Fig. 4C). To confirm the inhibitory effect of RASSF1A overexpression on AREG expression, we measured AREG secretion in the conditioned medium of HCC cells by ELISA in a time-dependent manner. The levels of secreted AREG were decreased in the conditioned medium of both HCC cells transfected with RASSF1A plasmid (Fig. 4D). To exclude the possibility that the decreased number of cells led to reduced secretion of AREG, the conditioned medium of HCC cells were harvested before the reduction of cell proliferation began in cells transfected with RASSF1A plasmid in comparison with those transfected with mock plasmid. These data showed that RASSF1A overexpression downregulates AREG expression and secretion by phosphorylation and inactivation of YAP, a transcriptional 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 patients with HCC, using Western blot analysis (Fig. 5A and B). All 22 human HCC cases (100%) showed significant downregulation of RASSF1A expression, compared with their corresponding nontumoral liver tissues of chronic hepatitis/cirrhosis. YAP protein expression...
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 HCC/nontumor detected by Western blot analysis was over 2-fold (2.02–0.97-fold; mean ± SD), whereas 3 cases (nos. 6, 14, and 17) showed similar expression levels of YAP protein between HCCs and their corresponding nontumoral liver tissues (YAP intensity of HCC/nontumor 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/nontumor was 1.36–0.25-fold (mean ± SD). Although 4 cases (nos. 14, 16, 19, and 22) showed no significant difference in AREG expression in HCC tissues, compared with corresponding nontumoral liver tissue of chronic hepatitis/cirrhosis (AREG intensity of HCC/nontumor was 0.96–1.00; Supplementary Table S2).

For further investigation of human multistep hepatocarcinogenesis, immunohistochemistry for RASSF1A, YAP, and AREG was conducted in chronic hepatitis/cirrhosis, LGDNs, HGDNs, and HCC tissues (Fig. 5C and D). The immunohistochemical analysis revealed a tendency of gradual decrease in RASSF1A expression as multistep hepatocarcinogenesis progressed toward HCC, and chronic hepatitis/cirrhosis tissue showed the highest histoscore of RASSF1A, which was significantly higher compared with HGDN and HCC (P = 0.009 and P = 0.03, respectively; Fig. 5C). Although 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 chronic hepatitis/cirrhosis, LGDNs, and HGDNs (P < 0.05 in all) and YAP histoscores of HGDNs were significantly higher than in chronic hepatitis/cirrhosis (P = 0.0005). In addition, 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 with cytoplasmic expression in HCCs (P = 0.0002), whereas nuclear expression of YAP protein was similar to that of cytoplasm in chronic hepatitis/cirrhosis, LGDNs, and HGDNs (Supplementary Fig. S1). AREG histoscores also showed the highest level in HCCs, which was significantly higher compared with chronic hepatitis/cirrhosis, LGDNs, and HGDNs (P < 0.05 in all; Fig. 5C).

Furthermore, the relationship between YAP and AREG histoscores showed a significant positive correlation (P = 0.001; r = 0.408), whereas between RASSF1A and YAP histoscores exhibited a significant negative correlation (P = 0.043; r = –0.236; Supplementary Fig. S2). 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 LOH 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, miRNA-602 has been reported to inhibit the tumor suppressive function of RASSF1A via decrease of RASSF1A expression in HepG2 cells with 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).

In this study, methylation of RASSF1A was observed in all 5 HCC cells, including HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5 cells, compared with 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 5 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 in vitro.

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 EGF 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). Dysplastic nodules 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 chronic hepatitis/cirrhosis, LGDNs, HGDNs, and HCC tissues. As multistep human hepatocarcinogenesis progressed from chronic hepatitis/cirrhosis, LGDNs, HGDNs, and finally HCCs, there was a tendency of gradual decrease in RASSF1A expression. Although 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 with cytoplasmic expression in HCC. The protein expression levels of RASSF1A, YAP, and AREG in HCCs and corresponding chronic hepatitis/cirrhosis were detected by Western blot analysis as well as immunohistochemistry, and both methods showed consistent results. In majority of HCC cases, RASSF1A protein expression was lower and the protein expression of YAP and AREG was higher compared with corresponding chronic hepatitis/cirrhosis tissues, although some cases showed similar expression levels of these proteins between HCC and corresponding chronic hepatitis/cirrhosis (Supplementary Table S2). Furthermore, the relationship between YAP and AREG expression via regulation of the Hippo pathway (12) was shown to suppress AREG–induced apoptosis, in AREG-treated HCC cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: E.Y. Ahn, J.S. Kim, Y.N. Park
Development of methodology: E.Y. Ahn, J.S. Kim, Y.N. Park
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