RASSF1A-mediated amphiregulin regulation in hepatocellular carcinoma via Hippo pathway

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감사의 글

많이 부족한 저에게 실험을 하고 공부를 할 수 있게 해주시고 제 졸업논문이 완성 되기까지 아낌 없는 지도와 배려를 베풀어 주신 박영년 교수님께 깊은 감사를 드립니다.

제 졸업논문을 지도해 주신 김호근 교수님, 최진섭 교수님께 감사의 말씀을 드립니다. 이 논문이 나오기까지 모든 노력과 수고를 아끼지 않으시고 도와주신 김지수 선생님께 깊은 감사를 드립니다. 제 실험에 아낌없이 큰 도움을 주신 임대식 교수님께 감사를 드립니다. 옆에서 여러 가지 조언을 아끼지 않으시고 도움을 주신 김세훈 교수님 그리고 김현기, 최준정, 방근배, 차종훈 선생님께 감사를 드립니다. 언제나 옆에서 도움을 주고 응원을 해준 병리학교실의 유정은, 나득채, 김광일 선생님께 감사를 드립니다.

마지막으로, 제가 무사히 모든 과정을 마칠 수 있게 도와준 저희 가족들께 감사를 드리고 하나님께 감사를 드립니다.

그 동안 도와주셔서 감사합니다

TABLE OF CONTENTS

ABSTRACT 1
I. INTRODUCTION 4
II. MATERIALS AND METHODS 9
  1. Genomic DNA extraction, modification, and methylation-specific PCR (MSP) 9
  2. Western blot analysis 10
  3. Total RNA extraction and cDNA synthesis 11
  4. Reverse transcriptase PCR (RT-PCR) 11
  5. Cell culture and 5-aza-2'-deoxycytidine (5-AZA) and protein transport inhibitor treatment 12
  6. Transfection 13
  7. Tissue preparation 13
  8. MTT assay 14
  9. Annexin V-FITC and PI staining and FACS analysis 14
  10. Statistical methods 14
III. RESULT 16
  The methylation status of RASSF1A in HCC cells 16
  Decreased cell proliferation by RASSF1A overexpression in HCC cells 17
  Overexpression of RASSF1A activated Hippo pathway in HCC cells 20
  Downregulation of AREG expression by RASSF1A expression in HCC cells 23
  RASSF1A, YAP, and AREG expression in human HCC tissues 25
IV. DISCUSSION 28
V. CONCLUSION 32
REFERENCES 34
ABSTRACT (in KOREAN) 40

LIST OF FIGURES

Figure 1. RASSF1A is methylated in HCC cells. 17
Figure 2. Effect of RASSF1A expression on cell proliferation and apoptosis in HCC cells..20
Figure 3. The activation of Hippo pathway by RASSF1A overexpression in HCC cells..22
Figure 4. AREG downregulation in HCC cells overexpressed with RASSF1A. 24
Figure 5. RASSF1A, YAP and AREG expression in human HCC tissues....27

ABSTRACT

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Ras association domain family 1 isoform A (RASSF1A) is a tumor suppressor and its methylation is involved in many human cancers including hepatocellular carcinoma (HCC). RASSF1A induces tumor suppressive effect by phosphorylation and activation of Hippo pathway kinase cascade. The activated kinase cascade leads to inhibition of yes-associated protein (YAP), a potent transcription coactivator playing a critical role as an oncogenic component inducing tumorigenic effect in the Hippo pathway. Amphiregulin (AREG) is one of the target genes of YAP that induces cell proliferation and anti-apoptotic effect on cancer cells. Here, we demonstrate the regulation of AREG by RASSF1A overexpression through the Hippo pathway.

Methylation of RASSF1A was examined in HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5 HCC cells. Overexpression of RASSF1A reduced cell proliferation significantly in Hep3B and SK-Hep1 cells at 24h, 48h, and 72h and PLC/PRF/5 cells at 48h and 72h ($p< 0.05$). RASSF1A overexpression also

induced apoptosis in SK-Hep1 ($p < 0.05$) and PLC/PRF/5 cells. Phosphorylation of MST1/2 and YAP, which is a component of the Hippo pathway, was detected in Hep3B, SK-Hep1, and PLC/PRF/5 cells with overexpressed RASSF1A. As a result, we have also examined reduced AREG expression in Hep3B, SK-Hep1, and PLC/PRF/5 cells with overexpressed RASSF1A. Furthermore, in human liver tissues, RASSF1A expression was higher in non-tumor liver tissues than in HCC tissues, but AREG and YAP expressions were higher in HCC tissues than in non-tumor liver tissues.

Our results suggest that RASSF1A expression in HCC cells inhibit cell proliferation and induce apoptosis. Increased phosphorylation of MST and YAP in HCC cells with overexpressed RASSF1A, revealed the activation of the Hippo pathway. We have also found the decrease of AREG levels in HCC cells with overexpressed RASSF1A, explaining the inhibition of YAP, which is a transcription coactivator of AREG. From the *in vivo* study results, we have showed that the detection of RASSF1A, YAP, and AREG in HCC tissues support the regulation of AREG by RASSF1A. Thus, our results support that RASSF1A-mediated downregulation of AREG activates the tumor suppressive Hippo pathway by phosphorylation of YAP.

Key words: RASSF1A, AREG, YAP, Hippo pathway, HCC

ABBREVIATION

RASSF1A: Ras association domain family 1 isoform A
HCC: hepatocellular carcinoma
YAP: yes associated protein
HCV: hepatitis C virus
HBV: hepatitis B virus
5-AZA: 5-aza-2'-deoxycytidine
MAPs: microtubule associated proteins
MST1/2: mammalian Ste20–like kinase 1/2
LATS1: large tumor suppressor homolog 1
SARAH: Salvador/Rassf/Hippo
CK1δ/ε: casein kinase 1 δ/ε
MSP: methylation-specific polymerase chain reaction
RT-PCR: reverse transcriptase polymerase chain reaction

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I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of cancer-related death[1]. Hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, and cirrhosis caused by alcoholic liver disease are the main risk factors for HCC[2]. Recently, several genetic and epigenetic alterations are known to be related with hepatocarcinogenesis, including frequent promoter hypermethylation of several tumor suppressor genes such as p16[3], E-cadherin[4] and RASSF1A[5] or mutations of p53[6], Rb1[7], and β-catenin[8] proteins. This epigenetic alteration is a critical mechanism involving both losses and gains of DNA methylation by global DNA hypomethylation or CpG island hypermethylation in HCC, respectively[9]. The transcriptional silencing of tumor suppressor genes and hypermethylation in specific CpG island sequences mediated by DNA methylation have been frequently detected in many types of human cancers [10, 11].

RASSF1A, a tumor suppressor gene, is located at chromosome 3p21.3.

Inactivation of RASSF1A by DNA methylation is involved in the development of many human cancers including lung cancer, cervical squamous cell carcinoma, breast cancer, and HCC[5, 12, 13]. As a tumor suppressor, RASSF1A has been verified to play critical roles in cell cycle regulation, microtubule stability, and apoptosis[13, 14]. A recent study has indicated that RASSF1A regulates cell cycle at the G1-S checkpoint and modulates the expression levels of cyclin D1[15]. Subsequent study confirmed the mechanism of cell cycle regulation through JNK pathway inhibition[16]. RASSF1A is also known to localize at microtubules and promote their stabilization by binding to microtubule associated proteins (MAPs) that directly bind with tublin[17-20]. It is also reported that RASSF1A is involved in fas ligand mediated apoptosis by binding to the YAP-p73 complex[21] and regulating BAX activity by interacting with the apoptosis-1 (MOAP-1) modulator[22]. RASSF1A is also involved in the Hippo pathway, which is a tumor suppressive pathway inhibiting oncogenic transcription coactivator YAP[23].

In the Hippo pathway, RASSF1A binds to and phosphorylates MST1/2, the orthologs of the *Drosophila* Hippo kinase, through the Salvador/Rassf/Hippo (SARAH) domain activating MST1/2[24]. A recent work showed that MST1/2 knockdown significantly decreased RASSF1A mediated apoptosis[23], which indicates the critical role of MST1/2 in RASSF1A-induced apoptosis. Activated MST1/2 induces phosphorylation of the downstream target, large tumor suppressor homolog 1 (LATS1) kinase. The hydrophobic motif of LATS1 is phosphorylated by MST1/2, and the activation loop is autophosphorylated[25].

Activated LATS1 phosphorylates YAP, which is one of the downstream target of LATS1, inhibiting the transcriptional activity of YAP.

Originally, YAP functions as an oncogene and YAP gene locus is known to be amplified in human cancers including intracranial ependymomas, oral squamous cell carcinomas, and medulloblastomas inducing oncogenic property[12, 26-28]. In a recent study, YAP has been identified to be an independent prognostic marker for overall survival and disease-free survival by analyzing 177 pairs of HCC and normal samples with clinical records[27].

In the Hippo pathway, YAP is directly phosphorylated in five HXRXXS consensus motifs by LATS1/2[29]. This phosphorylation of YAP results in its association with 14–3–3 proteins inducing cytoplasmic retention.[30]. This regulatory mechanism is utilized in YAP regulation by cell density and is likely conserved in *Drosophila*[31]. Recently, subsequent phosphorylation by another kinase, possibly casein kinase 1 (CK1δ/ε), phosphorylates YAP on S381 primes activating a phosphodegron and causing the recruitment of β-TRCP and E3 ubiquitin ligase leading to polyubiquitination and degradation of YAP[29]. Eventually, the spatial separation or the ubiquitination of YAP from transcription factors and target gene promoters lead to tumor suppressive effect[31].

On the other hand, when YAP is dephosphorylated, it translocates to the nucleus and then upregulates the transcription levels oncogenes such as of CTGF, GLi2, and AREG genes, inducing cell proliferation, cell survival, and

cell migration [30, 31]. For example, dephosphorylated YAP binds to the TEAD making YAP-TEAD complex, which directly targets CTGF and plays a role in YAP-induced proliferation and anchorage independent growth[32].

In a previous report, AREG, an EGF family member, is reported to be a downstream effector of the Hippo pathway and a direct target of YAP[33]. Its overexpression is implicated in normal organ proliferation and malignancy. AREG is secreted in many cancer cells including Hep3B and PLC/PRF/5[34, 35] and mediates protection against liver injury in a mouse model[36]. In breast, AREG is a key mediator of estrogen-induced pubertal epithelial morphogenesis[37]. It can promote diverse effects by depending on the cellular context, such as evasion of apoptosis, tissue invasion, and self-sufficiency in growth signals, all involved in tumor development and progression[38]. *In vitro* studies performed in tumor cell lines treated with AREG or with specific small interfering RNAs (siRNAs) to silence AREG gene expression reported that AREG played a crucial role in the proliferation and survival of transformed cells[39]. Also, a 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[40, 41].

Although many studies have been done conducted on RASSF1A and YAP, the relationship between AREG with RASSF1A is still not fully understood. The aim of this study is to identify the relationship between RASSF1A and AREG through YAP signature, suggesting the regulatory mechanism of AREG by

overexpressing RASSF1A. To achieve this aim, we first confirmed the methylation levels of RASSF1A in HCC cells and subsequently, we overexpressed RASSF1A in HCC cells to evaluate the effect of RASSF1A. As a result, we examined the tumor suppressive effect induced by overexpression of RASSF1A in HCC cells, such as decreased cell proliferation and increased apoptosis. We evaluated the function of RASSF1A by investigating the phosphorylation and activation of the Hippo pathway leading to the regulation of AREG. Based on our results, we suggest that RASSF1A may play a crucial role in tumor suppression regulating AREG expression through the Hippo pathway.

II. MATERIALS AND METHODS

1. Genomic DNA extraction, modification, and methylation-specific PCR (MSP)

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 proteinase K solution. Cell lysates were incubated at 65 ℃ for 10 min, added to 50 μL of precipitation solution I and resuspended in precipitation solution II. Samples were centrifuged at 13,000 rpm for 5 min and 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 ug of extracted DNA was mixed in 50 uL of sodium bisulfate containing 5 uL of dilution buffer and then incubated for 15 min at 37 ℃. The incubated samples were mixed with 100 uL of CT conversion reagent and incubated for 15 h at 50 ℃ in the dark condition. The DNA samples were put on ice for 10 min, transferred into Zymo-Spin IC columns treated with 400 uL 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 uL of desulphonation buffer were added to the sample and incubated at room temperature for 15 min, and then centrifuged at 13,000 rpm for 30 s. Each column was washed twice with 200 uL of wash buffer. Extracted DNA was used to determine the methylation status of RASSF1A by MSP as previously described[9].

2. Western blot analysis

Cells were harvested, centrifuged, and lysed with cell lysis RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were centrifuged at 13,200 rpm for 15 min at 4 ℃. Supernatants were collected and used to determine protein concentration 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) antibody, rabbit polyclonal anti-human p-YAP (1:1000; Cell signaling, MA, USA), mouse polyclonal 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). The membranes were incubated with their antibodies overnight followed by incubation with secondary horseradish peroxidase conjugated antibody (Cell signaling) for 1h at room temperature. Membrane-bound antibodies were detected by using the luminol reagent kit ECL system (Santa Cruz).

3. Total RNA extraction and cDNA synthesis

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

4. Reverse transcriptase PCR (RT-PCR)

Standard RT-PCR was performed using the primer sequence for RASSF1A (forward: 5′-GATGAAGCCTGTGTAAGAACCGTCCT-3′ and reverse: 5´-CA GATTGCAAGTTCACCTGCCACTA-3′), AREG (forward: 5′-TGCTGGATTG GACCTCAATG-3′ and reverse: 5′-TCCCGAGGACGGTTCACTAC-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. An 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. PCR conditions for RASSF1A, AREG, and β-actin (internal control) were as follows: initial 5 min at 95 ℃ and then 30 cycles of 30 s at 95 ℃; 30 s at 64 ℃ for RASSF1A, 59 ℃ for AREG, and 64 ℃ for β-actin; 30 s at 72 ℃; and a final elongation for 10 min at 72 ℃. The PCR products were separated by electrophoresis on a 2% agarose gel.

5. Cell culture and 5-aza-2'-deoxycytidine (5-AZA) 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) 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. In all experiments, these cells were maintained at 37 ℃ in a humidified 5% CO_2 incubator. 5-AZA was purchased from Sigma (MO, USA) and protein transport inhibitor was purchased from Becton Dickinson (CA, USA).

6. Transfection

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

7. Tissue preparation

The tissue samples of 14 HCCs were provided by the Liver Cancer Specimen Bank, a part of the National Research Resource Bank Program, which is run by the Korea Science and Engineering Foundation under the Ministry of Science and Technology. The samples were consisted of HCC tumor and non-tumor sections. The patient population consisted of 10 males and 4 females and their ages were 53 ±12.9 years (mean±SD). The average tumor size was 4.4±2.8 cm. The differentiation of HCC was evaluated according to the Edmondson-Steiner grading system, which revealed 3 patients in grade I – II and 11 patients in grade III - IV.

8. MTT assay

HCC cells with overexpressd RASSF1A or mock vector were cultured in 6-well plates and were allowed to settle for 24h before transfection with lipofectamin 2000. The cells were treated with 2ng/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) for 4h at 37 ℃. The monolayer was suspended in 0.1 mL of DMSO and the 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.

9. 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, resuspended in 100 uL of 1 x Annexin V-FITC binding buffer, and added to 5 uL of Annexin V-FITC conjugate and 10 uL of PI buffer. The cells were then incubated at room temperature for 15 min in the dark condition. After adding 200 uL of 1 x Annexin V-FITC binding buffer, the cells were analyzed using a FACScan flow cytometer (BD bioscience Pharmingen).

10. Statistical methods

Statistical analysis was carried out using PASW statistics (version 18.0, SPSS

Inc., Chicago, IL, USA) and assessed using the Mann-Whitney test, t-test, chi-square test, as deemed appropriate. All *P*-values corresponded to two-sided tests and a *P*-value less than 0.05 was considered statistically significant.

III. RESULT

The methylation status of RASSF1A in HCC cells

To find out the methylation status of RASSF1A in HCC cells, methylation specific PCR (MSP) was performed in 5 human HCC cells such as HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5. HeLa cells, which are known to have unmethylated RASSF1A expression, were used as a positive control. The result revealed that RASSF1A is methylated in all five HCC cells (Figure 1A). As a further confirmation, Hep3B and SK-Hep1 cells were treated with 5 uM or 10 uM 5-AZA, a demethylating agent, for 48h and were harvested to determine the demethylation of RASSF1A by RT-PCR. Increased mRNA level of RASSF1A indicated demethylation of RASSF1A (Figure 1B). This result confirmed that RASSF1A was inactivated by methylation in HCC cells.

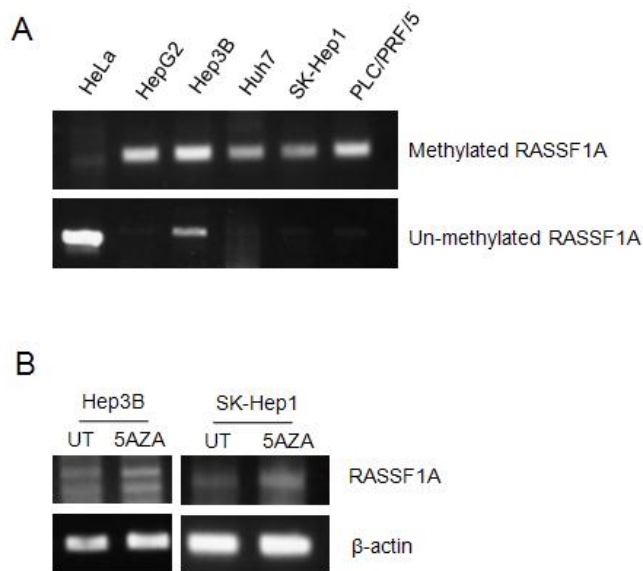


Figure 1. RASSF1A methylation in HCC cells. (A) RASSF1A methylation status was measured by MSP. (B) HCC cells were treated with 5 uM or 10 uM 5-AZA for 48h. RASSF1A mRNA levels were measured by RT-PCR. UT: untreated cells, 5-AZA: 5-AZA treated cells

Decreased cell proliferation by RASSF1A overexpression in HCC cells

Since cell viability is the net result of cell proliferation and apoptosis, we analyzed the cell proliferation and apoptosis of HCC cells to investigate the effect of RASSF1A overexpression on cell viability in HCC cells. The result of MTT assay revealed that cell proliferation in HCC cells overexpressed with RASSF1A vector was markedly reduced compared with cells overexpressed with mock vector or untreated cells for 72h of transfection. Hep3B cells showed

a significantly lower level of cell proliferation while overexpressed with RASSF1A vector at 24h (p=0.005), 48h (p=0.031), and 72h (p=0.023). SK-Hep1 cells also showed a significantly lower level of cell proliferation at 24h (p=0.001), 48h (p=0.028), and 72h (p=0.0004) in cells with overexpressed RASSF1A. On the other hand, PLC/PRF/5 displayed significant lower level of cell proliferation only at 48h (p=0.001) and 72h (p=0.047) in cells with overexpressed RASSF1A. Hep3B, SK-Hep1, and PLC/PRF/5 cells, which were overexpressed with mock vector, showed a similar level of cell proliferation with untreated cells. After 72hours, Cell proliferation rates at mock and RASSF1A group were 298±18.45% and 206±17.65% in Hep3B, respectively; 307±4.58% and 101±3.16% in SK-Hep1, respectively; 222±4.75% and 185±10.89% in PLC/PRF/5, respectively (Figure 2A). To investigate the effect of RASSF1A overexpression on apoptosis, Annexin V and PI staining was performed with SK-Hep1 and PLC/PRF/5 cells and we confirmed a significant increase of apoptosis in SK-Hep1 cells overexpressed with RASSF1A by 14.95±1.47 fold at 48h (p=0.001) and 37.12±1.95 fold at 72h (p<0.0001). Although PLC/PRF/5 cells showed no statistical significance, apoptosis was increased by 1.827±1.14 fold at 48h and 1.91±3.27 fold at 72h in PLC/PRF/5 cells overexpressed with RASSF1A (Figure 2B). Furthermore, cleaved caspase3, an active form of caspase 3, was detected using western blot to investigate the induction of apoptosis by RASSF1A expression in HCC cells. Hep3B, SK-Hep1, and PLC/PRF/5 cells overexpressed with RASSF1A showed significant

increase of cleaved caspase-3 expression compared to HCC cells overexpressed with mock vector, indicating an increase of apoptosis in HCC cells overexpressed with RASSF1A (Figure 2C). Taken together, these results suggested that RASSF1A expression inhibited cell viability in HCC cells overexpressed with RASSF1A.

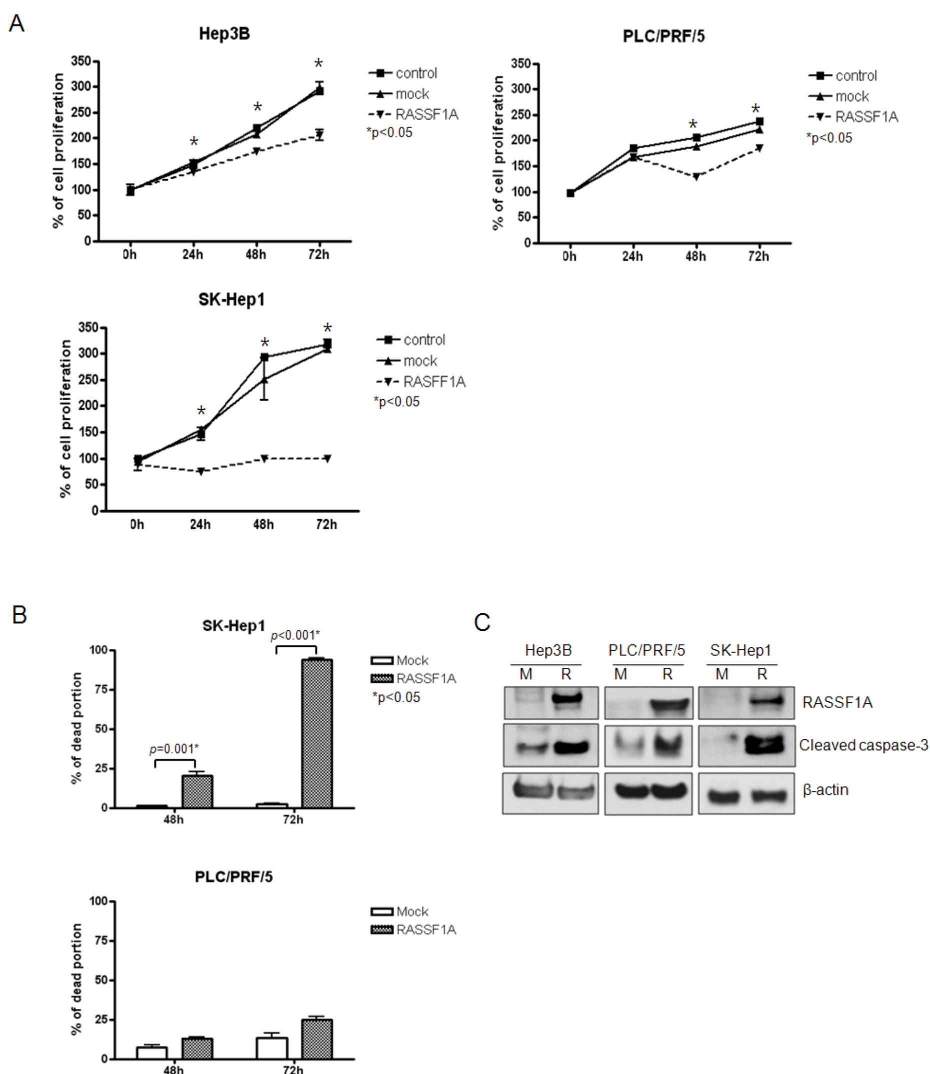
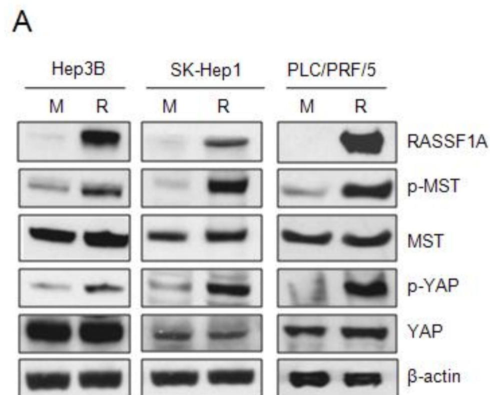


Figure 2. Effect of RASSF1A expression on cell proliferation and apoptosis in HCC cells. (A) Cell proliferation of HCC cells overexpressed with RASSF1A vector or mock vector. MTT was detected after 4h of incubation on HCC cells. *$p < 0.05$ for mock vector and RASSF1A. (B) Quantitative analysis of Annexin V-FITC and PI staining on SK-Hep1 and PLC/PRF/5 cells overexpressed with RASSF1A vector or mock vector for the indicated time period. *$p < 0.05$ for mock vector and RASSF1A. (C) Protein levels of active form of caspase-3 in HCC cells overexpressed with RASSF1A vector or mock vector. M: mock, R: RASSF1A. β-actin was used as a loading control.

Overexpression of RASSF1A activated Hippo pathway in HCC cells

In order to identify the activation of the Hippo pathway in HCC cells overexpressed with RASSF1A, the phoshphorylation status of the Hippo pathway components was analyzed with western blot. Phosphorylation of MST and YAP significantly increased after 48h of transfection in HCC cells overexpressed with RASSF1A (Figure 3A). The RASSF1A, p-MST, and p-YAP protein levels of Hep3B cells overexpressed with RASSF1A 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 cells overexpressed with mock vector. The RASSF1A, p-MST and p-YAP protein levels of SK-Hep1 cells overexpressed with RASSF1A is 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 cells overexpressed with mock vector. The RASSF1A, p-MST and p-YAP

protein levels of PLC/PRF/5 cells overexpressed with RASSF1A 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 cells overexpressed with mock vector. Overexpression of RASSF1A revealed gradual increase of the phosphorylation levels of MST1/2 and YAP for 72h in HCC cells overexpressed with RASSF1A in a time dependant manner (Figure 3C). These results showed that RASSF1A overexpression in HCC cells activated the Hippo pathway by phosphorylating MST1/2 and YAP. Eventually, our result demonstrated that activated MST1/2 phosphorylated YAP inhibiting its activity.



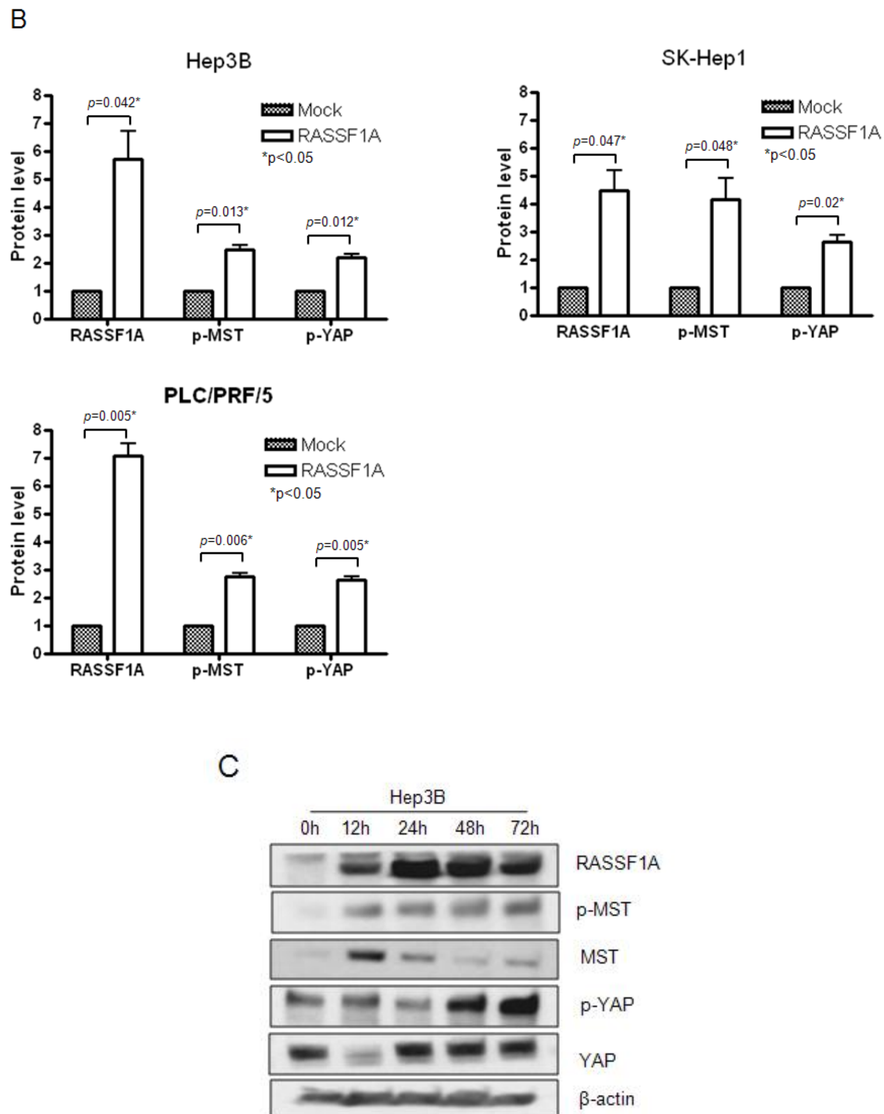


Figure 3. Activation of the Hippo pathway by RASSF1A overexpression in HCC cells. (A) Protein levels of YAP signature in HCC cells with overexpressed RASSF1A or mock vector. (B) Quantitation result of three repeated western blot analysis. *$P < 0.05$ between mock and RASSF1A. (C)

Protein levels of YAP signature in Hep3B cells with overexpressed RASSF1A in a time dependant manner for up to 72h. M: Mock, R: RASSF1A. β-actin was used as a loading control.

Downregulation of AREG expression by RASSF1A expression in HCC cells

To examine the regulation of AREG by RASSF1A in HCC cells, RT-PCR and western blot were performed to detect AREG expression in HCC cells overexpressed with RASSF1A or mock vector. The mRNA levels of AREG were significantly decreased in Hep3B, SK-Hep1, and PLC/PRF/5 cells after overexpression with RASSF1A (Figure 4A). In Hep3B cells, the mRNA level of AREG was gradually decreased by 72h in a time dependant manner when overexpressed with RASSF1A (Figure 4B). In order to detect the protein levels of AREG, Hep3B and PLC/PRF/5 cells were treated with protein transport inhibitor, which inhibits AREG secretion from the cells and accumulate AREG for detection. SK-Hep1 cells were excluded due to the lack of endogenous AREG protein expression. The protein levels of AREG were significantly decreased in Hep3B and PLC/PRF/5 cells overexpressed with RASSF1A. In addition, increased phosphorylation of YAP was also detected in HCC cells overexpressed with RASSF1A (Figure 4C). These results displayed that RASSF1A overexpression downregulated AREG expression by phosphorylation and inactivation of YAP, which is the transcription coactivator of AREG.

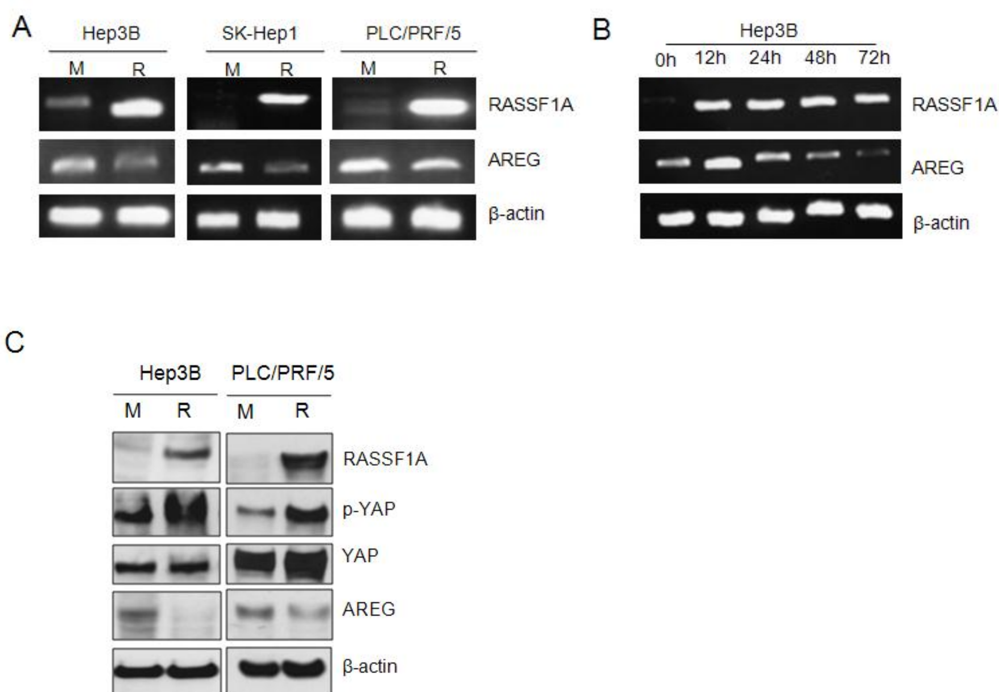
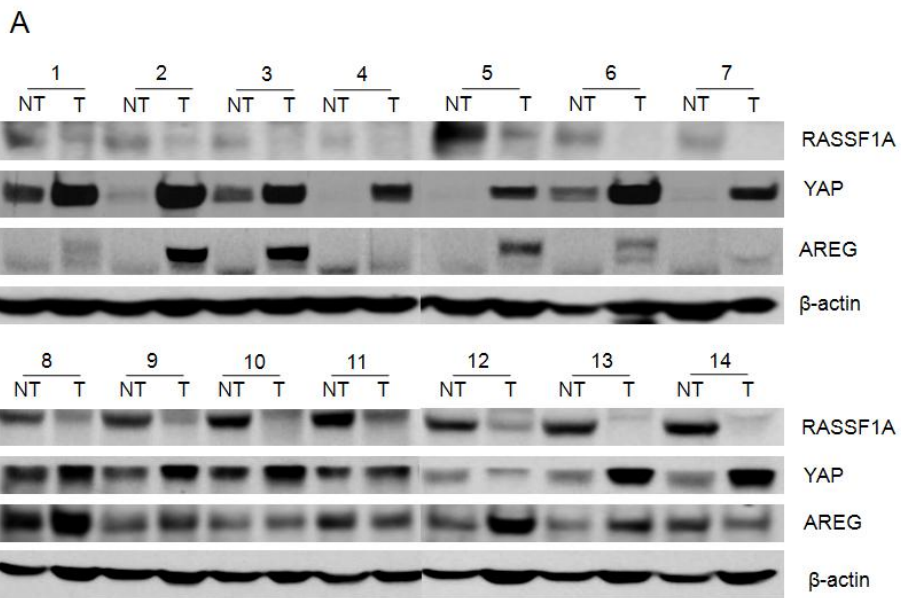


Figure 4. AREG downregulation in HCC cells with overexpressed RASSF1A. (A) The mRNA levels of AREG in HCC cells overexpressed with RASSF1A vector or mock vector for 48h. (B) The mRNA levels of AREG in Hep3B cells overexpressed with RASSF1A vector or mock vector in a time dependent manner for 72h. The mRNA levels of RASSF1A, AREG, and β-actin were measured by RT-PCR. (C) Protein levels of AREG and phosphorylation levels of YAP in HCC cells were overexpressed with RASSF1A vector or mock vector. M: Mock, R: RASSF1A. β-actin was used as a loading control.

RASSF1A, YAP, and AREG expression in human HCC tissues

To confirm the previous *in vitro* results, we evaluated RASSF1A, YAP and AREG expressions in human HCC tissues using western blot. A total of 14 cases of human tissues were analyzed with western blot (Figure 5A) and 13 of 14 cases of the HCC tissues showed downregulation of the RASSF1A expression compared to non-tumor tissues. The upregulation of AREG in the HCC tissues were detected in 11 of 14 cases (nos. 1-9, 12 and 13). The YAP expression was also evaluated in the HCC tissues and 13 cases (nos. 1-11 and 13-14) displayed an increased expression of YAP and 1 case (no. 12) showed no difference in the YAP expression between the HCC tissues and non-tumor tissues. In this study, 9 of 14 cases showed consistency with our previous *in vitro* data, which was downregulation of RASSF1A and increased AREG and YAP expressions in HCC. It was also predicted that the RASSF1A expression in non-tumor tissues might indicate activation of the Hippo pathway (Figure 5B). These results implicated RASSF1A methylation in HCC tissues induce the expression of AREG and YAP. It also demonstrates that the RASSF1A expression downregulated the AREG expression in non-tumor liver tissues by inhibiting YAP.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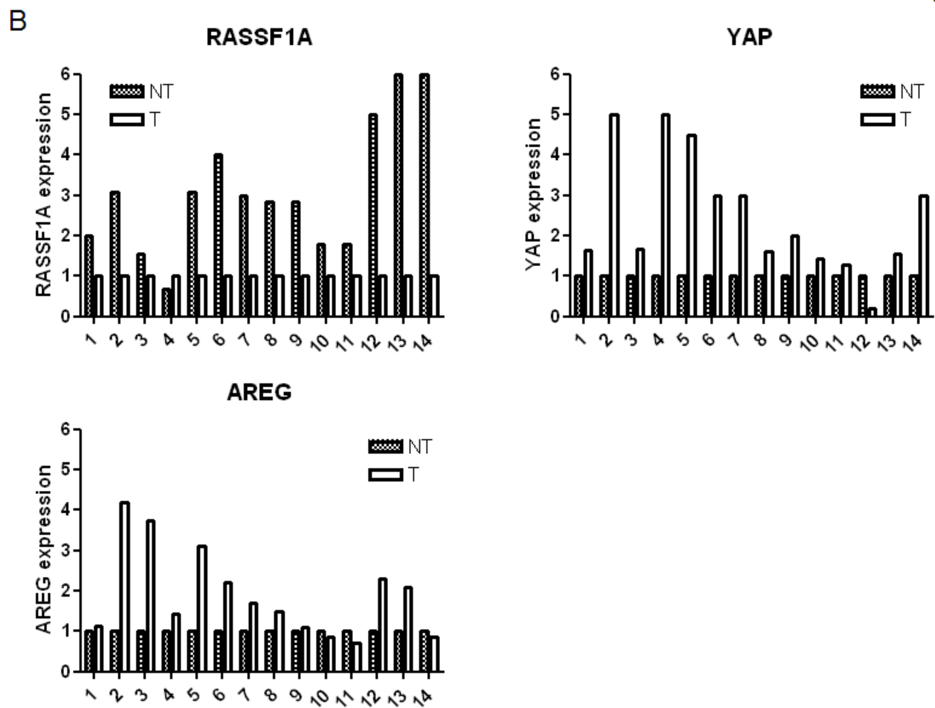
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Figure 5. RASSF1A, YAP, and AREG expressions in human HCC tissues. (A) Protein levels of RASSF1A, YAP and AREG in 14 cases of human HCC tissues paired with non-tumor cases. (B) Protein level quantification of RASSF1A, AREG, and YAP. NT: non-tumor, T: HCC. β-actin was used as a loading control.

IV. DISCUSSION

RASSF1A was identified as a tumor suppressor with biological functions such as inhibiting cell migration, arresting cell cycle progression, and inducing apoptosis[13]. Epigenetic inactivation of RASSF1A has been reported in many human cancers including lung, bladder, breast, and liver cancers[42]. In this study, we have identified the inactivation of RASSF1A by detecting the methylation status of RASSF1A by the MSP method in 5 HCC cells including HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5. All the HCC cells that we screened showed methylation of RASSF1A (Figure 1A). Although partially un-methylated RASSF1A was detected in Hep3B cells, we have confirmed that no protein expression was detected in western blot analysis (Figure 2B, 3A). To further confirm that RASSF1A was inactivated by methylation in Hep3B and SK-Hep1 cells, the HCC cells were treated with a demethylation agent, 5-AZA. As a result, demethylated RASSF1A was detected in Hep3B and SK-Hep1 cells when treated with 5-AZA (Figure 1B). These results supports that RASSF1A is methylated in HCC cells, which is a consistent result with previous report[12]. Although we have identified methylation of RASSF1A in HCC cells, a study will be needed to understand the biological mechanism that induces methylation of RASSF1A.

The Hippo pathway, which is a tumor suppressive pathway, is expected to be inactivated in HCC cells with methylated RASSF1A[43]. To investigate whether

the expression of RASSF1A is able to reactivate the Hippo pathway resulting tumor suppressive effect, we have overexpressed RASSF1A in HCC cells. As a result, decreased cell proliferation and increased apoptosis was examined in HCC cells overexpressed with RASSF1A, which demonstrates the tumor suppressive effect of RASSF1A (Figure 2).

RASSF1A directly binds to MST1/2 through SARAH motif, where it phosphorylates and activates MST1/2[24]. Activated MST1/2 phosphorylates LATS1, which induces YAP phosphorylation[44]. YAP is an oncogenic transcription coactivator activating oncogenes that induce cell proliferation or survival, such as CTGF, GLi2, and AREG. [30, 31, 33]. Inactivation by cytosolic retention or degradation by ubiquitination occurs when phosphorylated YAP binds to 14-3-3 or CK1δ/ε, respectively[31, 45]. We have evaluated the increase of phosphorylation status of MST1/2 and YAP by western blot analysis in HCC cells overexpressed with RASSF1A. This result reveals that MST1/2 is activated by RASSF1A[46] and phosphorylation of YAP indicates inhibition of oncogenic activity of YAP by cytosolic retention or ubiquitination (Figure 3). Therefore, we suggest that methylation of RASSF1A gene in HCC may contribute to the tumorigenic effect by YAP dephosphorylation. In addition, together with the cell proliferation assay result, it supports the previous report that RASSF1A induces apoptosis by promoting MST1/2 phosphorylation[23, 25].

The mRNA level of AREG was significantly decreased in RASSF1A overexpressed HCC cells. This result indicates that AREG is regulated by

RASSF1A in a transcriptional level through inhibition of YAP transcription coactivator (Figure 4A, B). As a result, significant reduction of AREG protein level was detected in HCC cells with RASSF1A overexpression (Figure 4C). These results support that RASSF1A has an important role in regulating of AREG.

Finally, the detection of RASSF1A, YAP and AREG expression in HCC tissues confirms the *in vitro* data. Previous studies of HCC related methylation revealed an increase of RASSF1A methylation as a progress in hepatocarcinogenesis[9]. In agreement with this report, we have also observed a low level of RASSF1A protein expression in HCC tissues. On the other hand, a high expression of AREG and YAP proteins was detected in HCC tissues indicating a high level of cell proliferation and migration. Although dephosphorylation of YAP was not detected in HCC tissues (data not shown), a significantly high expression level of total YAP in HCC tissues compared to that of non-tumor tissues indicated that total YAP protein was highly upregulated in HCC tissues, which is a consistent result with a previous report[27]. Therefore, the present data regarding the induction of AREG by highly expressed YAP also explains that AREG is the downstream target of the transcription coactivator YAP. However, the AREG promoter does not contain a TEAD-binding element and the transcription factor mediating AREG expression remains to be further investigated[33]. Although the mechanism of RASSF1A regulating AREG is still unclear we have shown the regulation of AREG by RASSF1A.

From this study, we have found that RASSF1A overexpression induces the tumor suppressive effect by activating the Hippo pathway, which is a consistent result with previous studies related to decreased cell proliferation and increased apoptosis by RASSF1A overexpression. We suggest that AREG downregulation by RASSF1A overexpression provides a better understanding of the biological significance of RASSF1A in HCC development and suggests potential targets for the development of cancer therapies.

V. CONCLUSION

In this present study, to elucidate the mechanism of AREG regulation by RASSF1A, we investigated the Hippo pathway activation in HCC cells. In this study the phosphorylation level of MST1/2 and YAP was examined. Cell proliferation and apoptosis level were included to examine the tumor suppressive effect of Hippo pathway induced by RASSF1A. Finally, to confirm the *in vitro* results we examined the expression of RASSF1A, YAP AREG in human HCC tissues.

1. RASSF1A was methylated in HepG2, Hep3B, Huh7, SK-Hep1, and PLC/PRF/5 cell lines. Increased mRNA level of RASSF1A was detected in HCC cells treated with demethylating agent, which indicates the methylation of RASSF1A in HCC cells.

2. Cell proliferation was significantly reduced in Hep3B, SK-Hep1, and PLC/PRF/5 cells with overexpressed RASSF1A. Apoptosis was increased in SK-Hep1 and PLC/PRF/5 cells with overexpressed RASSF1A. Activation of caspase-3 was increased in Hep3B, SK-Hep1, and PLC/PRF/5 cells with overexpressed RASSF1A.

3. Phosphorylation of MST1/2 and YAP was increased in HCC cells with overexpressed RASSF1A.

4. The mRNA and protein levels of AREG were reduced and the phosphorylation of YAP was increased in Hep3B, SK-Hep1, and PLC/PRF/5 cells with overexpressed RASSF1A.

5. In the *in vivo* study, 9 out of 14 cases of HCC tissues with RASSF1A inactivation showed high level of AREG and YAP expression. On the other hand RASSF1A was highly expressed and the expression level of YAP and AREG was lower in non-tumor liver tissues compared to HCC tissues.

In conclusion, we have identified RASSF1A as a potential tumor suppressor in HCC and the inhibition of AREG resulted by phosphorylation and inactivation of YAP by RASSF1A. Therefore we can suggest that, we have identified RASSF1A as a regulator of AREG through Hippo pathway.

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ABSTRACT (in KOREAN)

간세포암종에서 Hippo pathway를 통한 RASSF1A의
AREG 발현 조절

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종양 억제인자인 Ras associated domain 1 A (RASSF1A) 의 메틸화에 의한 불활성화는 간세포암종 및 여러 인체 암과 연관되어 나타나고 있는 현상이다. RASSF1A는 Hippo pathway의 키나아제 연쇄반응의 인산화와 활성화를 유도하여 종양인자인 yes associated protein (YAP) 부전사인자를 억제 시킴으로써 종양 억제 효과를 유발한다. AREG는 YAP의 표적 유전자로 암세포의 세포증식을 일으키고 암세포의 세포고사를 억제하는 효과를 나타내는 유전자이다. 본 연구는 RASSF1A의 과발현이 Hippo pathway를 통해 AREG를 조절 한다는 것을 증명하고자 한다.

Methylation-specific PCR (MSP) 방법으로 간암세포주에서 RASSF1A가 메틸화에 의해 불활성화가 되어 있는 것을 확인하였다. RASSF1A에 의한 AREG 조절을 확인하기 위해서 간암세포주에 RASSF1A를 과발현을 시켰다. RASSF1A가 과발현된 간암세포주에서

세포증식은 감소하였으며 세포고사는 증가하였고, Hippo pathway의 구성원인 MST1/2와 YAP의 인산화는 증가하였다. 그 결과 RASSF1A 과발현에 의하여 AREG의 발현이 감소함을 확인하였다. 더욱이 인체 조직에서 RASSF1A의 발현은 간암 조직에서보다 정상조직에서 높게 나타났으나 AREG와 YAP은 정상조직에 비해서 간암조직에서 더 높게 나타났다.

따라서 본 연구에서 RASSF1A에 의한 AREG 발현 감소가 YAP의 인산화에 의한 부전사인자 역할을 억제함으로써 Hippo pathway의 종양 억제효과를 유도한다는 것을 증명하였다.

핵심되는 말 : RASSF1A, Hippo pathway, YAP, AREG, 간세포암종