Inhibition of autophagy suppresses liver tumorigenesis

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Directed by Professor Kwang-Hyub Han

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ABSTRACT

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Introduction: Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults and the second leading cause of cancer-related deaths worldwide. Many studies have shown that autophagy is associated with liver cancer. Atg7 is a critical component for the formation of autophagosome and required for autophagy processes. To investigate the role of autophagy in liver cancer, we utilized Atg7 knock-down transgenic mouse models.

Methods: Transposon vectors encoding Atg7 shRNA were constructed. Transposons encoding Atg7 shRNA were mixed with those encoding $Hras^{G12V}$ and shp53 and then injected into the lateral tail veins of 5-week-old C57BL/6 mice. The development of liver cancer was observed grossly and histologically at 5 weeks post hydrodynamic injection. Expression of genes related to autophagy process was assessed using western blotting and immunohistochemistry.

Results: Atg7 expression significantly decreased in NIH3T3 cells transfected

with short hairpin RNA downregulating Atg7 (shAtg7). We observed that GFP-LC3 puncta was reduced by shAtg7 in Hep3B cell with starvation. We found that the size and numbers of tumor nodules significantly decreased in HCC mouse model with Atg7 knockdown.

Conclusion: Our data indicate that knockdown of Atg7 led to a significant decrease in tumroigenesis in a murine HCC model. Inhibiting the autophagosome formation is expected to be a therapeutic option for liver cancer.

Key words: autophagy, autophagosome, Atg7, HCC(Hepatocellular carcinoma), hydrodynamic transfection

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I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults and the second leading cause of cancer-related deaths worldwide.[1,2] Chronic infection of hepatitis B and C, cirrhosis and alcohol abuse are leading causes, as well as metastases of HCC development from tumors elsewhere in the body.[3] Most HCCs are resistant to the clinically approved drug, sorafenib which extends lifespan of HCC patients by just 2.8 months on average with various side effects.[4]

Autophagy is a self-eating process, which recycles long-lived or misfolded proteins, protein aggregates, and damaged organelles to necessary component for cellular homeostasis in eukaryotic cells.[5,6] There are three major forms of autophagy: macroautophagy, microautophagy and chaperon-mediated autophagy.[7,8] In the case of macroautophagy, an isolated membrane, phagopore expands and wraps around portions of the cytoplasm to form a double-membraned vesicle called an autophagosome and then fuses with a lysosome to form an autolysosome which degrades inner particles by lysosomal proteases.[9]

Although the features of autophagy have been actively investigated, the role of autophagy in tumorigenesis has not been clearly revealed. The role of autophagy is different in each tissue and tumorigenesis.[10] Especially, the dual roles as tumor promoter and tumor suppressor have been reported in HCC. The suppressor of cancer by autophagy has been studied through p62, Atg5 and Atg7 knockout mice models. The studies indicated that deletion of these genes related with the autophagy process induces tumor development and progression.[11] On the contrary, autophagy as tumor promotor is activated by stress including hypoxic environment and growth factor deprivation, promoting the survival of cancer cells.

Atg7 has an important role in the Atg5-Atg12 complex and LC3/Atg8-conjugation system during the autophagy process. Recent studies have shown that autophagy may have a major role in HCC development.[7] The liver specific Atg5 or Atg7 knockout mice models showed liver damage and adenomas. Furthermore, BECN1 deletion promoted hepatitis B virus related HCC, and the deletion has been observed in human cancer.[12]

Ras signaling is involved in promoting cell proliferation and cell survival.[13] And the continuous overexpression of oncogenic Ras increased autophagy.[14] Ras-dependent autophagy can increase glycolysis, improve mitochondrial function, and renders tumor cells resistant to anoikis.[15] In a lung cancer mice model developed by expression of oncogenic Kras, deletion of Atg7 led to reduced tumor growth. [16,17] Ras is activated in human HCC patient samples in different stages of hepatocarcinogenesis.[13]

Previous studies showing tumor suppressive role of autophagy in liver were conducted by deleting Atg7 or Atg5 in normal hepatocytes. We wanted to find out the role of autophagy in established HCC. Thus, in this study, we utilize the Hras ($Hras^{G12V}$) and short hairpin p53 (shp53) HCC mouse model that we have previously developed and investigated the role autophagy in HCC by suppressing Atg5 or Atg7.[18,19]

II. MATERIALS AND METHODS

1. Plasmids

The cDNA encoding an activated human Hras (HrasG12V) was PCR amplified using pBABE puro H-Ras V12 (Addgene, plasmid #9051) as a template with the following primer pairs: Forward 5'-TTGAATTCGCCACCATGACGGAATATAAGCTGGTGGTGG-3' and reverse 5'-TTGAATTCTTAGGAGAGCACACACTTGC-3'. The amplified products were then digested with EcoRI and were cloned into pT2/EGFP following digestion of the plasmid with the same restiriction enzyme to remove the EGFP cDNA. The resulting plasmid is referred to as pT2/HrasG12V.

pT2/shp53/GFP4, a transposon vector encoding a short hairpin RNA against tumor suppressor P53 was a gift from Dr. John Ohfelt and is referred to as pT2/shp53 throughout the paper.

Plasmid, pt2/C-Luc//PGK-SB13 encoding SB transposase under the control of the phosphoglycerate kinase (PGK) promotor and also harboring a transposon expressing the firefly luciferase was a kind gift from Dr. John Ohfelt.

2. Knockdown of Atg7 in vitro and in vivo

For knockdowns of Atg7, the miR-E based shRNA expression cassette was used. The whole miR-E knockdown cassette expressing Renilla Luciferase shRNA (shREN) was PCR amplified from SGEN (a kind gift from Dr Johannes Zuber), and subsequently substituted for the miR-30 based knockdown cassette in pT3-EFDsM (a kind gift from Dr Scott Lowe) using BlpI and MluI restriction enzyme sites. The ORF encoding EGFP was then substituted for the dsRED coding region in the vector using BsrGI and BlpI

restriction enzyme sites. The resulting plasmid is thus designed to simultaneously express EGFP and miR-E-based shREN (referred to as “pT3-EF1a EGFP-shREN). shRNAs were selected by the sensor rule previously described. The sequences of 97mer oligos coding for the respective shRNAs are provided in Table1.

PCR was performed using a 97-mer oligo as a template with the following primers, forward: 5’- TGAACTCGAGAAGGTATAT TGCTGTTGACAGTGAGCG-3’, and reverse: 5’-TCTCGAATTC TAGCCCCTTGAAG TCCGAGGCAGTAGGC-3’. The resulting PCR products were subsequently substituted for the shREN coding region in pT3-EF1a EGFP-shREN using EcoRI and XhoI restriction enzyme sites. Transposon plasmids simultaneously expressing EGFP and miR-E-based shRNAs were used for hydrodynamic injection.

Table 1. The sequences of 97mer oligos coding for the respective shRNAs

shRNA	97mer Oligo sequence
Atg7.728	5’-TGCTGTTGACAGTGAGCGAAAGGTCAAAGGACAAA GATAATAGTGAAGCCACAGATGTATTATCTTTGTCCTTTG ACCTTGTGCCTACTGCCTCGGA-3’
Atg7.3511	5’-TGCTGTTGACAGTGAGCGAACGCAGTGTTGTCATGGA GTATAGTGAAGCCACAGATGTATACTCCATGACAACACT GCGTCTGCCTACTGCCTCGGA-3’
Atg7.1536	5’-TGCTGTTGACAGTGAGCGACAAGCGAAAGCTGGTCA ATAGTGAAGCCACAGATGTATTGATGACCAGCTTTCGCT TGCTGCCTACTGCCTCGGA-3’
Atg7.159	5’-TGCTGTTGACAGTGAGCGACCAGAAGAAGTTGAACG AGTATAGTGAAGCCACAGATGTATACTCGTTCAACTTCTT CTGGGTGCCTACTGCCTCGGA-3’

3. Transfection, Protein harvest from tumor tissue and cells, Western blotting.

NIH3T3 cells were used for in vitro experiments. Cells were plated 3 x 10^5 cells per well on 6 well plate one day prior to transfection so that the cells were approximately 80% confluent on the day of transfection. Cells were transiently transfected with 2 μg of DNA using 6μl FuGENE® HD Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cells were transfected with pT3 vectors as constructed previously and then were harvested 2 days post-transfection using the 10X RIPA buffer (Cell Signaling, Denvers, MA, USA) according to the manufacturer's instructions. Cells were briefly washed with PBS to remove residual media. 200μl of 1X RIPA buffed and diluted in DW were added to each well and then were incubated on ice for 5 minutes. Afterwards, cells were scrapped with a scrapper and transferred to a new tube. For 30-60 minutes, cells were placed on ice, and extracts were centrifuged for 10 minutes at 14,000 RPM in a cold microcentrifuge. Finally, supernatants were removed for use. Cells were harvested as described above.

To detect various related proteins from tumor tissue, a proper size of tumor sample was homogenized and digested in 1X RIPA buffer, which includes PMSF(Fluka, Switzerland) (final concentration is 1 Mm), Aprotinin (Sigma, Steinheim, Germany) (final concentration is 2 μg/ml), phosphatase inhibitor cocktail solution (GeneDEPOT, Barker, TX, USA) and DTT(Invitrogen, Calsbad, CA, USA) (final concentration is 2Mm), on ice for 1 hour. After the digestion, samples were centrifuged for 25 minutes at 14,000 RPM in a cold microcentrifuge. Then, supernatants were removed for use. Western blot experiments were performed using standard methods. All the used anti-bodies for western blot are described in Table 2.

Table 2. Antibodies used in western blotting

Antibody	Origin	Ratio	회사
GAPDH	Rabbit	1:3000	CST #2118
Atg7	Rabbit	1:1000	Wako #013-22831

4. Experimental animals

Male 5- to 6-week-old wild-type C57BL/6 mice were purchased from Orientbio (Seongnam, Korea). The injected mice were housed in a specific pathogen-free (SPF) barrier area. The temperature (22°C) and humidity (55%) were controlled constantly. Water (RO water) and food (PMI) were supplied. All experiments using live mice were performed in strict accordance with the Guidelines and Regulations for the Care and Use of Laboratory Animals in AAALAC-accredited facilities, and were approved by the Animals Policy and Welfare Committee of the Yonsei University College of Medicine.

A hydrodynamic injection was performed. The plasmids HrasG12V and shp53.1224, shREN, shAtg7.728, shAtg7.1536, shAtg7.159, pPGKSB13 were prepared with endotoxin-free Maxi Kits (Qiagen, Hilden, Germany). Mice of the same body weight each received the same molar amount of transposons, regardless of the types of transposons. For double transgenic groups, half-molar amounts of transposons for each transgene were mixed together. DNA mixtures of transposons and transposase-encoding vector (i.e., pPGKSB13) were suspended in lactated Ringer's solution and subsequently injected into the lateral tail vein of male 5- to 6-week-old mice (0.1 ml/g body weight) in less than 7 seconds. Mice were randomly assigned to hydrodynamic injection. Livers were harvested at 5 weeks following hydrodynamic transfection unless specified otherwise.

The mice were sacrificed humanely by zoletil/xylazine ansthesia. Midline laparotomy of the mice was carried out, with blood sampling from inferior vena cava and the extraction of liver. Apart of the extracted liver specimen was immersed in 10% neutral buffered formalin and fixed in paraffin. The other part of the specimen was cut into sections and flash frozen in liquid nitrogen.

5. Histological analysis

After euthanizing the mice, their livers were removed and rinsed in PBS. Samples collected from the livers were fixed in 10% Neutral-Buffered formalin. Fixed tissue samples were embedded in paraffin. Four-μm liver sections were placed and heat-fixed onto slide glass. The specimens were deparaffinized in xylene and dehydrated in a graded alcohol series. The liver sections were stained with either hematoxylin/eosin.

6. Immunohistochemistry (IHC)

Formalin fixed paraffin embedded liver tissue were sectioned at 4um. The liver tissue sections were deparaffinized in xylene briefly and rehydrated through a gradual decrease in ethanol concentration. The antigen epitopes on the tissue sections were then unmasked a using Sodium Citrate buffer (pH6.0). The tissue section slides were then treated with 3% hydrogen peroxide to remove any endogenous peroxidases. Blocking was performed at room temperature using a VECSTASTAIN ABC kit blocking solution(Vector Laboratories, Bulingame, CA, USA) for 30 minutes. The sections were incubated overnight at 4℃ in a humidified chamber using various primary antibodies at incubated dilutions. All the used anti-bodies for western blot are described in Table 3.

After the primary incubation, sections were washed thoroughly in PBS before incubating with biotinylated secondary antibody solution raised against the primary antibody initially used for 30 minutes at room temperature. After thorough washes with PBS, the sections were treated with freshly VECTASTAIN ABC reagent (Vector Laboratories, CA, USA) for 30 minutes at room temperature. After thorough washes with PBS, sections were then treated with freshly prepare DAB substrate (Vector Laboratories, CA, USA) and followed awaiting an adequate signal to develop before stopping the reaction in water. Finally, sections were then lightly counter-stained with hematoxylin, dehydrated through a gradual increase in ethanol concentration, cleared in xylene and mounted in Entellan (Merck, Darrnstadt, Germany)

Table 3. Antibodies used in IHC

Antibody	Origin	Ratio	Antibody information
GFP	Rabbit	1:400	CST #8558
ATG7	Rabbit	1:100	CST #2555

III. RESULTS

1. Knockdown of Atg7 using short hairpin RNA in NIH3T3 and Hep3B cells

Ras upregulates Atg5/7 and induce autophagic activity.[20] We assumed autophagy might play a role in the development of HCC induced by $Hras^{G12V}$ and shp53. To test this, we chose to down-regulate ATG7 gene using short hairpin RNA (shRNA) in the murine HCC model induced by $Hras^{G12V}$ and shp53. We checked for knockdown effect by various Atg7 shRNAs in NIH3T3 cells. Transfection efficiencies of plasmids expressing individual shRNA were compared using a co-expressed reporter protein, GFP [Fig. 1A]. We found that the knockdown of Atg7 was efficiently achieved by expression of Atg7 shRNA among which shAtg7.1536 has the best efficacy for knockdown of Atg7 [Fig. 1B].

To investigated whether knockdown of Atg7 suppresses autophagy activity, GFP-LC3 was expressed in Hep3B cells with starvation after transfection with shAtg7. GFP-LC3 puncta increased in Hep3B cells transfected with shREN (control shRNA) due to starvation, however expression of shAtg7.1536 significantly reduced GFP-LC3 puncta formation in starved Hep3B [Fig. 1C].

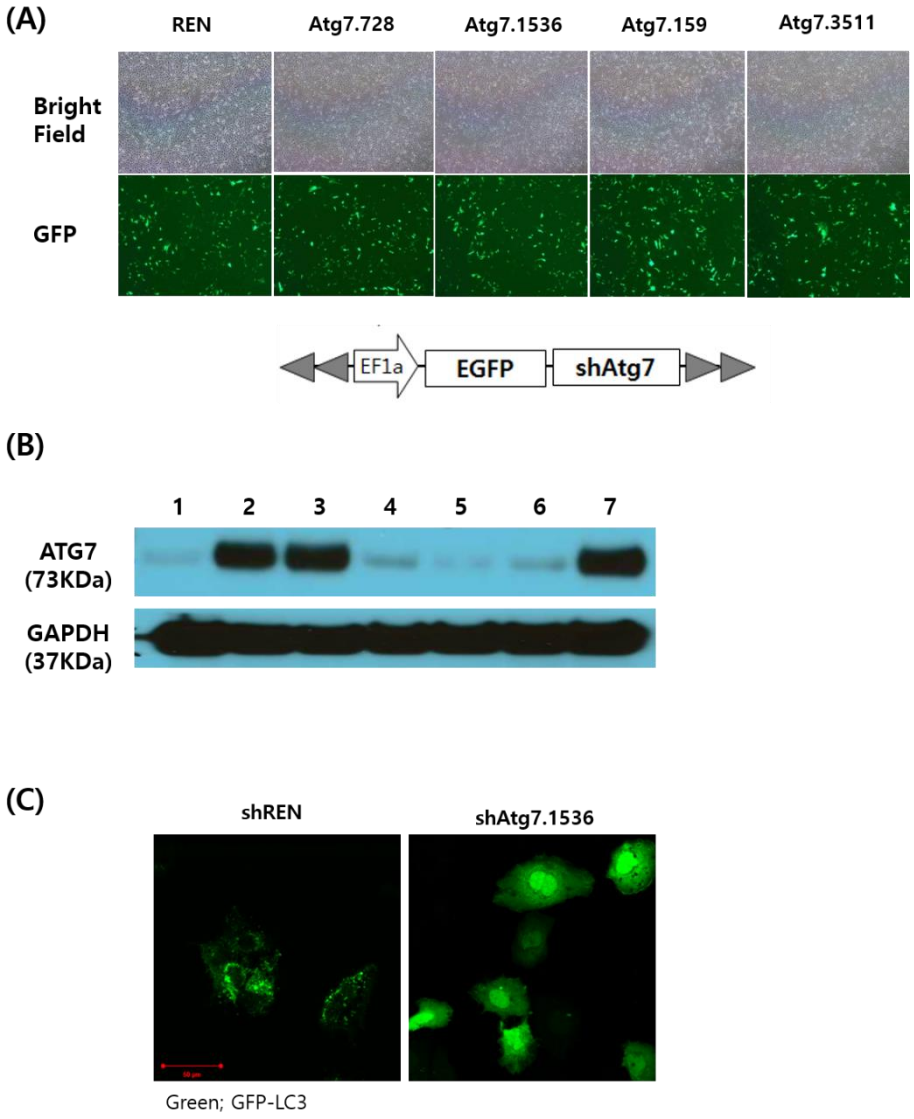


Figure 1. The effect of transposon vector encoding Atg7 knockdown. **(A)** Images of transfected NIH3T3 cells with various shAtg7 were obtained using a microscope (Olympus, Tokyo, Japan) with a 10X objective. Scale bar, 200μm). **(B)** The expression levels of the Atg7 were analyzed by western blotting with anti-Atg7 and anti–GAPDH (control) antibodies. (1; Non-transfection, 2; Atg7 overexpression, 3 to 7; co-expression of Atg7 with

shRNA, 3; shREN, 4; shAtg7.728, 5; shAtg7.1536, 6; shAtg7.159, 7; shAtg7.3511) **(C)** Representative images of transfected Hep3B using a confocal microscope. Scale bars, 100 μm. Hep3B cells in culture stably expressing GFP-LC3 and shREN(control) or shAtg7.1536 with 4 hours of starvation.

2. Knockdown of Atg7 suppresses hepatocarcinogenesis in mice.

Atg7 deletion in normal liver induces adenoma accompanied with hepatic inflammation.[21] To investigate the role of autophagy in our HCC model, we delivered transposons encoding various Atg7 shRNA in the liver together with transposons encoding $Hras^{G12V}$ and shp53 [Fig. 2A]. Expression of Atg7 shRNA inhibited tumorigenensis in mice induced by $Hras^{G12V}$ and shp53 when livers were analyzed at 5 weeks PHI, except for the shAtg7.728 [Fig. 2B]. Average number and size of tumor nodules in each group is shown in Table 4. Liver weight per body weight (LW/BW), often used to evaluate tumor burden in liver, was significantly reduced in shAtg7.1536 group compared with control group [Fig. 2C].

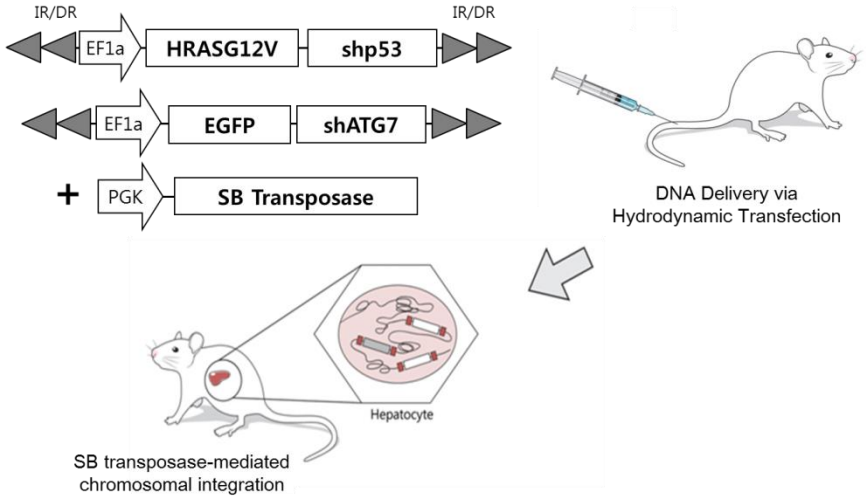
Table 4. Tumor nodules in mouse models

	Average of number of nodules	
Group	Below 3mm	Over 3mm
shREN	*>10	**TMTC
shAtg7.728	*>10	*>10
shAtg7.1536	2 ± 0.5	0
shAtg7.159	3 ± 0.5	0

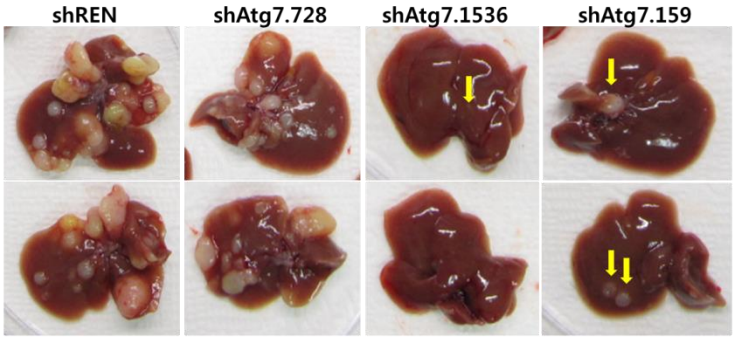
*: more than 10

**: Too many to count

(A)



(B)



(C)

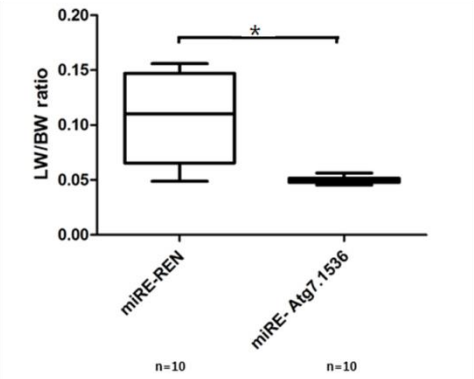


Figure 2. To investigate the role of Atg7 on malignant liver cancer. (A) Schematic illustration of the experimental procedure. The transposons were mixed with plasmids encoding Sleeping Beauty (SB) transposase and were hydrodynamically delivered to the liver (see the Materials and Methods section). Once in cells, the SB transposase is expressed and binds to the IR/DRs of the transposons. The enzyme subsequently cleaves the transposons at the sites of IR/DRs and integrates them at the new location within the host genome, allowing stable expression of the transgenes. (B) Liver tumors induced by $Hras^{G12V}$ and shp53 (control), $Hras^{G12V}$, shp53 and each shAtg7 plasmids. (C) Liver weight/body weight (LW/BW) ratio of mice expressing shREN and shAtg7.1536. The graph represents the mean ± SEM (n=10 livers per group) (*, $P < 0.0005$)

3. Degree of Atg7 expression in tumor nodules in mice liver.

To verify Atg7 gene knockdown in tumors, we investigated GFP and Atg7 expression levels in tumor nodules using IHC. In liver tumors expressing control shRNA (shREN), GFP and Atg7 expression were invariably detected. Tumors in Atg7 knockdown groups also generally exhibited GFP expression while Atg7 expression was not detected. Occasionally, tumors in these groups showed Atg7 expression. Because these tumors were found GFP-negative, we presume that transposon encoding shAtg7 was not successfully integrated into the genome of tumor-initiating cells of the tumors and thus they failed to express Atg7 shRNA. [Fig. 3].

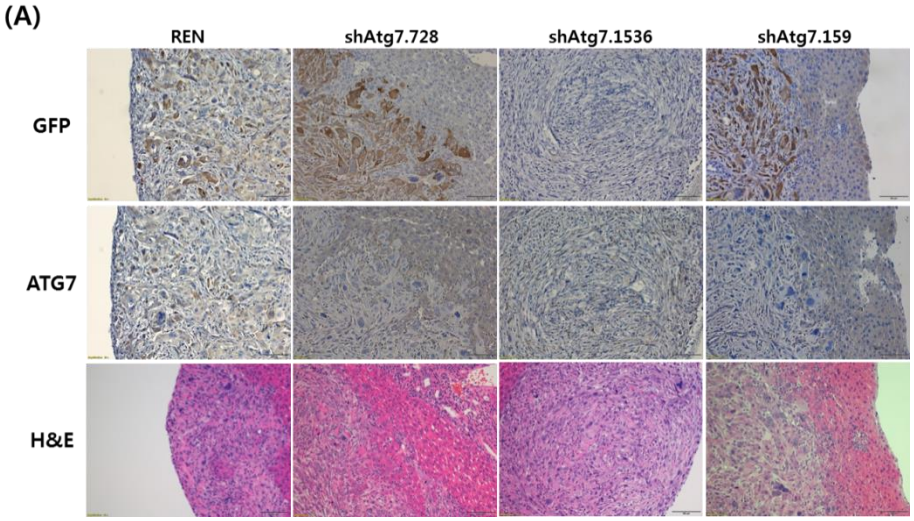


Figure 3. Expression of the Atg7 gene in established mouse model liver. Representative liver sections at the time of 5 weeks PHI. To test activity of constructed shAtg7 and degree of autophagosome accumulation. (A) Immunohistochemical staining for GFP and Atg7 in liver tissue sections (original magnification, x200). shAtg7.1536 is GFP negatively, but there was increased in Atg7 expression.

IV. DISCUSSION

Autophagy maintains homeostasis in normal liver. It is induced by stressful environments such as starvation.[22] Autophagy converts damaged organelles or proteins into amino acids, glucose, and etc to supply cells with energy resources.[23]

Many researchers have investigated the effects of autophagy in liver cancer. But the results often conflict with each other. Autophagy can both inhibit and promote tumor development in liver. The role of autophagy seems to be different depending on the stages of carcinogenesis in liver cancer. Autophagy inhibits the neoplastic transformation of hepatocytes during the very early stage of liver carcinogenesis through the removal of damaged and cytotoxic macromolecules. However, it contributes to tumor growth during later stages of carcinogenesis by supplying neoplastic cells with nutrients such as amino acids, free fatty acids (FFA) and glucose to meet the high metabolic demands of cancer cell.[23,24]

Immunohistochemistry data showed a lower expression level of LC3 in tumor than that in human normal liver tissue. Expression level of LC3 after the chemotherapy was upregulated. Therefore, they thought that targeting of autophagy can more effective clinical therapy in HCC patients.[25] Moreover, the results of protein expression level of atg5 and LCB and the data of transmission electron microscopy (TEM) showed that autophagy was activated in human fibrosis liver than normal liver.[26]

Ras signaling is involved in cell growth and cell proliferation. The signaling pathway is also related to tumor invasion, metastasis, and angiogenesis. Mutation in Ras can help to predict diagnosis and treat HCC patients.[27] $Hras^{G12V}$ and knockdown of p53 induce HCC in mouse liver, and we employed this mouse model for this study.

Our data showed that suppression of tumorigenesis in the liver was caused by knockdown of Atg7, which significantly decreased the numbers and sizes of liver cancer nodules. IHC analysis confirmed that Atg7 expression was absent in tumors expressing shAtg7. shAtg7.1536 was found to be the best suppressor of liver cancer in our study. Consistent with the finding from the gross morphology, microscopic examination revealed only a few small neoplastic lesions in livers expressing shAtg7.1536.

Recently, numerous articles showed autophagy process may help survival of the cancer cell. They investigated how cancer cells could survive by autophagy in many stressful situations such as a low oxygen condition. Autophagy is also up-regulated when growth factor is depleted and metabolic stress increases. Furthermore, hypoxic environment increases autophagy which leads to enhanced survival of cancer cell.[28]

In summary, inhibition of autophagy activity through knockdown of Atg7 suppressed tumorigenesis in HCC mouse model induced by Hras and shp53. It will be worthwhile to investigate whether the autophagy target treatment can help tumor suppression in liver cancer.

V. CONCLUSION

In this study, expression of various shAtg7 genes has displayed suppression of tumorigenesis in HCC mouse models induced by expression of $Hras^{G12V}$ and p53 shRNA. Based on these experimental results, inhibition of the autophagic activity through knockdown of Atg7 may be helpful to suppress hepatocellular carcinoma.

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ABSTRACT(IN KOREAN)

자가포식 저해작용을 통한 간암발생 억제 연구

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간세포암은 성인에서 가장 흔한 원발성 간암이며, 전 세계적으로 암 관련 사망의 두 번째 주요 원인이다. 자가포식은 많은 연구자들의 연구를 통해 기작은 밝혀진바 있으나, 그 작용의 양상이 조직이나 질병에 따라 다르게 나타난다고 보고되고 있다. 최근 간 특이적으로 자가포식 관련 유전자들을 결손 시킨 마우스를 이용한 연구들을 통해서, 자가포식이 간암과 밀접한 관계가 있다고 밝히고 있다. 특히나 간암연구에서는 자가포식에 있어 자가소화포를 형성하는데 관여하는 단백질인 Atg5나 Atg7을 결손 시킨 형질변형 마우스 모델을 이용해 통해 자가포식의 활성에 따라 간암생성에 영향을 미친다는 것을 밝히고 있다.

자가포식이 간암형성에 있어 어떠한 역할을 하는지 확인하기 위해, 자가소화포 형성에 있어서 중요한 역할을 하는 Atg7이 억제될 경우 간암 형성에 어떠한 영향을 미치는지 확인하고자 하였다. 이전의 실험을 통해 간암 마우스 모델인 Hras 유전자, shp53 유전

자와 함께 Atg7 유전자를 억제시키는 다양한 유전자를 혈관에 주입하는 방법과 *Sleeping Beauty* 트랜스포존 시스템을 사용하였다. 이 방법을 이용하여 Atg7 유전자가 마우스의 간세포의 염색체로 삽입되어 지속적으로 발현하게 한다. 유전자를 주입하고 5주가 지난 시점에 마우스의 간 조직을 획득하여 간암 형성 정도를 비교하였다.

다양한 Atg7 발현 억제 유전자를 사용하였으나, Atg7.156을 억제한 경우가 가장 간암 형성 억제에 있어 좋은 효과를 보였다. 조직, 형태학적 관찰 결과, Atg7.1536의 종양부위에서는 Atg7이 억제되지 않았다는 것을 확인할 수 있었다.

본 연구에서는 Hras 유전자와 p53 유전자가 억제된 마우스에 간 특이적으로 Atg7 유전자의 억제 효과에 따라 나타나는 간암형성의 억제 확인을 하였다. 이를 통해 자가포식 활성을 저해하는 것이 간암 치료로서 긍정적인 역할을 할 수 있다고 생각된다.

핵심되는 말: 자가포식, 자가소화포, Atg7, HCC(Hepatocellular carcinoma), DNA 혈관 주입