Effects of the augmenter of liver regeneration on the biological behavior of hepatocellular carcinoma

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ABSTRACT

الأهداف: الاستفادة من (siRNA) المستهدف (hALR) ومضاد (McAb) (hALR) لتثبيط وظيفة (hALR)، وذلك من أجل تحديد ما إذا كان نمو السرطان الكبدي يتأثر بواسطة (siRNA) المستهدف (hALR) ومضاد (hALR McAb) عبر تثبيط ظهور (hALR).

الطريقة: أجريت هذه الدراسة بمختبر الأحياء الدقيقة للأمراض المعدية – وزارة التعليم – معهد التهاب الكبد – جامعة تشونقكينق الطبية – الصين، خلال الفترة ما بين يناير 2005م إلى مايو 2007م. قمنا بنقل (siRNA) بلاميزد (pSIALR-A) المستهدف (DNA) التابع ل(hALR) وبلازميد التحكم غير المعالج (pSIALR-B) إلى خلايا (HepG2) على التوالي. بعد ذلك تم اكتشاف تكاثر خلايا (HepG2) ونمو الورم بعد معالجتها بواسطة (pSIALR-A) ومضاد (hALR McAb) في الفئران.

النتائج: تم ظهور بناء بلازميد (pSIALR-A) و(pSIALR-B). يحفز (pSIALR-A) ظهور (hALR) في خلايا (HepG2) بوضوح. يستهدف (siRNA) مضاد (hALR) ومضاد (hALR McAb) لتثبيط وظيفة (hALR) بوضوح و نمو خلايا (HepG2). يستهدف (siRNA) مضاد (hALR) ومضاد (hALR McAb) و يثبط بوضوح نمو الطعم الأجنبي للورم في خمسة من الفئران. يثبط مضاد (hALR McAb) بشكل واضح النمو الاستقلالي لخلايا (HepG2).

خاتمة: توضح نتائجنا أن مضاد (hALR McAb) يثبط النمو الاستقلالي لخلايا الورم الكبدي. إضافة إلى ذلك، يحافظ (hALR) النمو الاستقلالي لخلايا لورم الكبدي عبر آلية إنتاج ذاتية.

Objectives: To take advantage of the small interfering ribonucleic acid (siRNA) targeting the human augmenter of liver regeneration (hALR) and anti-hALR monoclonal antibody (McAb) to inhibit the function of hALR, and to demonstrate whether the growth of hepatoma is influenced by siRNA targeting hALR and anti-hALR McAb through inhibiting expression of hALR.

Methods: This study was conducted in the Key Laboratory of Molecular Biology for Infectious Diseases, Ministry of Education, Institute for Viral Hepatitis, Chongqing Medical University, China, between January 2005 and May 2007. We transfected siRNA plasmid pSIALR-A, which targeted the complementary deoxyribonucleic acid (cDNA) of hALR and the unrelated control plasmid pSIALR-B into human hepatocellular liver carcinoma cell line (HepG2) cells. Then, the proliferation of HepG2 cells, after being treated with pSIALR-A and anti-hALR McAb was detected. The growth of the xenograft tumor was observed after being treated with pSIALR-A and anti-hALR McAb in nude mice.

Results: We successfully constructed expressing plasmid pSIALR-A and pSIALR-B. The pSIALR-A inhibited the expression of hALR in HepG2 cells significantly. The siRNA targeting hALR and anti-hALR McAb inhibited obviously the growth of HepG2 cells *in vitro*. siRNA targeting hALR and anti-hALR McAb significantly inhibited the growth of xenograft tumor in 5 nude mice. Anti-hALR McAb inhibited apparently the autonomous growth of HepG2 cells.

Conclusions: Our results demonstrated that anti-hALR McAb inhibited the autonomous growth of hepatoma cells obviously, moreover, hALR maintained the autonomous growth of hepatoma cells *in vitro* through an autocrine mechanism.

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The augmenter of liver regeneration (ALR), a heat-stable hepatotrophic growth factor, was first discovered and cloned from rat livers.[1,2] The ALR plays an important role in stimulating liver regeneration, liver damage repair, and survival of hepatocytes.[3-5] Its protein administration induces an increase in the mitochondrial gene expression, and enhances cytochrome content and the oxidative phosphorylation capacity of liver-derived mitochondria.[6] High dose recombinant human augmenter of liver regeneration (rhALR) is effective in inhibiting gene expression of metalloproteinase-1 (TIMP1), and reversing fibrosis in experimental liver fibrosis.[7,8] It also plays an important role in liver regeneration by regional regulation of natural killer (NK) cells.[9] The ALR may protect against failure of regeneration by inhibition of hepatic NK cell activity in acute liver injury.[10] As a growth factor and as immunoregulator, it controls the mitochondrial transcription factor A (MTF) expression and the lytic activity of liver-resident NK cells.[11] Diabetes was reversed in a significantly greater proportion of treatment of fetal pancreas and ALR-treated recipients, than those animals treated with transport protein alone. The local delivery of ALR may be used as an adjunct to treatment of fetal pancreas transplantation to improve the rate of success.[12] The expression of ALR in renal tissue is active, and may take part in mediating the regeneration of tubular epithelial cells in acute renal failure.[13] Research indicated that ALR could stimulate the proliferation of hepatoma cells in a dose-dependent manner *in vitro*.[14,15] Moreover, ALR could increase the proliferation of cells and protect cells against damage after the ALR expression plasmid was transfected into hepatoma cells.[14,15] It also reported increased ALR expression in cirrhotic liver compared with normal liver tissue.[16] In hepatocellular carcinoma (HCC) and cholangiocellular carcinoma, ALR messenger ribonucleic acid (mRNA) expression was also significantly enhanced compared with normal liver tissue, but expression levels did not differ from the matching non-neoplastic tissue in the same patient.[16] In our laboratory, we demonstrated that ALR was related to carcinogenesis of HCC. It could stimulate the proliferations of cell line QGY and human hepatocellular liver carcinoma cell line (HepG2) cells in a dose-dependent manner *in vitro*, but had no effect on primary rat hepatocytes, which demonstrated that there existed different receptors on the normal hepatocytes and hepatocarcinoma cells.[17] It was shown by immunohistochemistry that the staining of ALR was strongly positive in the tissue of patients with HCC, however, it was weakly positive in few cells of normal rats.[18] Other data showed the existence of ALR receptor in hepatocytes and hepatoma cells, and the proliferation promotion effect of ALR was mediated by a specific receptor.[19] These results indicated that ALR was produced in an autocrined way in the carcinogenesis of HCC. However, the association of ALR and carcinogenesis of HCC was not fully understood. Our objective in this study is to investigate whether the biological behavior of HCC was influenced by short interference RNA (siRNA) targeting hALR and anti-ALR McAb on protein and nuclei acid level *in vitro* and *in vivo*. This study provides the experimental evidence for hALR directly taking part in the development of HCC, which furthers the understanding of the mechanism of carcinogenesis of HCC.

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Methods. The current study was conducted in the Key Laboratory of Molecular Infectious Diseases, Chongqing Medical University, China. The DNA maker was obtained from Takara Biotech (Dalian, China). Lipofectamine 2000 transfection reagent was purchased from LIFE Company (Invitrogen, California, USA). Reagents for immunocytochemical staining were provided by Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd (Beijing, China). The RNaesy Mini Kit and EndoFree Plasmid Maxi Kit was provided by Qiagen (Dusseldorf, Germany). Access reverse transcription-polymerase chain reaction (RT-PCR) system was provided by Promega (Madison, USA), fetal bovine serum was from Hyclone Laboratories (Logan, USA). The ^{3}H-thymidine (^{3}H-TdR) (1 mci/ml) came from Chinese Atomic Energy Institute (Beijing, China) anti-hALR McAb, ascites induced by SP2/0 cells were provided by the Institute for Viral Hepatitis, Chongqing University of Medical Sciences.[20] The HepG2 cells were provided by the Institute for Viral Hepatitis, Chongqing University of Medical Sciences. The Pgenesil-1 (with GFP) was purchased from Wuhan Genesil Biotechnology Co. Ltd (Wuhan, China). All PCR primers were synthesized by Shanghai Ding'an Biological Company (Shanghai, China).

Female pathogen-free BALC/C nude mice (20-22 g, 4 weeks old) purchased from the National Rodent Laboratory Animal Resources, Shanghai Branch, Shanghai, China, were used in this study. The mice were maintained under specific pathogen-free conditions in a facility with standard rodent chow, water, and a 12-hour light/dark cycle in Chongqing University of Medical Science. The experiments were performed according

to the national regulations, and approved by the local animal experiments ethical committee.

Expression of hALR in HepG2 cells by immunocytochemistry. The primary antibody was 1:500 anti-ALR McAb, A 1:500 ascites induced by SP2/0 cells served as negative control. Biotinylated goat anti-mouse antibody was used as secondary antibody. The immunohistochemical staining procedure was carried out according to the cell Signaling Technology's Protocol.

Construction and identification of expressing siRNA plasmid of hALR. Selection of hALR siRNA and siRNA design. The siRNA selection was designed as recommended and derived from the coding sequence of the hALR gene (AY550027) with the position of the 187-207 nucleotide. The target sequences (5'-3') and the siRNA duplex were designated as Sequence A.

5'-GATCCGAGCTTCATAAGGCGCATGC TTCAAGACGGCATGCGCCTTATGA AGCTCTTTTTTGTCGACA-3'

In addition, the non-specific siRNA duplex used as control were designated as Sequence B.

5'-GATCCGAGCTTCATAAGGCGCATGC TTCAAGACGGCATGCGCCTTATGA AGCTCTTTTTTGTCGACA-3'

Sequence A and B identified were BLASTed against the Genbank database.

Sequence A and B containing no homology to the non-hALR sequence were synthesized by Genesil Company. A 5'-TTCAAGACG-3' was used as 9-nt loop, and a stretch of 6 deoxyadenosines as transcription terminator. All short hairpin RNAs (shRNA) were designed according to the structure of siRNA sense strand-loop-siRNA antisense strand, and inserted into Pgenesil-1 between BamH I site and Hind III site.

Construction and identification of pSIALR-A and pSIALR-B. Construction and identification of siRNA plasmid of hALR (pSIALR-A and pSIALR-B) were performed by Wuhan Genesil Biotechnology Co. Ltd, Wuhan, China. The pSIALR-A and pSIALR-B were extracted from bacteria according to the Qiagen instruction from the manufacturer.

Transfection and identification of function of recombinant plasmid. The recombinant plasmid pSIALR-A and pSIALR-B were transfected into HepG2 cells using LipofectamineTM 2000 transfection reagent according to the manufacturer's protoco1 (pSIALR-A/HepG2 and pSIALR-B/HepG2). At 24-hours after transfection, the cells were examined for the presence of GFP by an inverted fluorescent microscope.

Expression of hALR in HepG2 cells after transfection by immunocytochemistry. At 48-hours after transfection, expression of hALR was evaluated by immunocytochemistry using reagents for immunocytochemical staining according to the instruction of the manufacturer. The primary antibody was 1:500 anti-hALR McAb, 1:500 ascites induced by SP2/0 cells as negative control.

Expression of hALR mRNA in HepG2 cells before and after transfection by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). For RT-PCR analysis, the total RNA was extracted from HepG2 cells at 48-hours after transfection pSIALR-A and pSIALR-B, and HepG2 cells without transfection using a RNeasy Mini kit according to the manufacturer instruction. The following specific oligonucleotide primers were used:

hALR: 5'-ATGCGGACCCAGCAGAAGC-3' (upstream)
5'-CTTAGTCACAGGAGCCGTCCTT-3' (downstream)
Amplimer 379bp

ß-actin: 5'-CAAAGACCTGTACGCCAACA-3' (upstream)
5'-GAAGCATTTGCGGTGGAC-3' (downstream)
Amplimer 255bp.

Housekeeping gene ß-actin was used as an internal control. According to the supplier's manual, RT-PCR reactions were carried out in automated thermocyclers by incubation at 48°C for 45 minutes, followed by 2 minutes heating at 95°C. Amplifications were carried out by 40 cycles of incubation at 94°C for 0.5 minutes, 60°C for one minute, and 68°C for 2 minutes, with a final extension step of incubation at 68°C for 7 minutes. The amplified 2µl DNA fragments were analyzed by 2% (weight/volume) agarose gel electrophoresis. After electrophoresis and scanning, all PCR product bands were analyzed, and relative mRNA expression was estimated by normalization with ß-actin.

^{3}H-TdR incorporation approach. Proliferation of HepG2 cells after transfection. The HepG2 cells were plated into 96-well plates 24-hours prior to transfection (5×10^{3}/well). The HepG2 cells were divided into 2 groups (12 replicates in each group), and transfected with the recombinant plasmid pSIALR-A in one group, and pSIALR-B in the other group, according to the manufacturer's protocol of Lipofectamine TM 2000 transfection reagent. At 48-hours after transfection, ^{3}H-TdR (1µCi/well) was added to the culture medium. At 12-hours after adding the ^{3}H-TdR, the incubation was terminated. The cells were trypsinized and lysed with trysin/EDTA, and collected from the acetic fiber filter. The filter was dried and transferred into a liquid scintillation counter, and the radioactivity (counts per minute value) was measured by the liquid scintillation counter (Beckmen Co, Hamburg, Germany). Cell proliferation was assessed after quantification of ^{3}H-TdR incorporation.

Data were expressed in cpm as the mean ± SD of 12 replicates, and were representative of at least 3 separate experiments.

Proliferation of HepG2 cells treated with anti-hALR McAb. The HepG2 cells were divided into 3 groups. Each group was treated with anti-ALR McAb, ascites induced by SP2/0 cells as negative control, and phosphate buffered saline (PBS) as blank control. The final dilution for these 2 antibodies was 1:125, 1:250, 1:500 and 1:1000 (6 replicates in each final dilution). At 24-hours and 48-hours after the HepG2 cells was being plated, the culture medium was replaced with the same final dilution for these antibodies. At 60-hours after being plated, ^{3}H-TdR (1μCi/well) was added to the culture medium. At 12-hours after adding ^{3}H-TdR, the incubation was terminated. The cells were collected from the acetic fiber filter. The filter was transferred into a liquid scintillation counter and radioactivity (cpm value) was measured by the liquid scintillation counter (Beckmen Co, Hamburg, Germany).

Mouse tumor model. The effect of hALR siRNA at 48-hours after transfection on mouse tumor growth (pSIALR-A/HepG2 and pSIALR-B/HepG2) were harvested. Ten nude mice were divided into pSIALR-A/HepG2 group and pSIALR-B/HepG2 group randomly. Each group had 5 mice. In each group, subcutaneous tumors were induced by inoculation of 1.2×10^{6} cells transfected with different plasmids in the flank of each mouse. Tumor growth was measured every 3 days using a digital caliper by an observer blinded to treatment allocation. Tumor volume was calculated as, $1/6\pi \times$ longest diameter × shortest diameter^{2}.[21] At 30 days after inoculation, the animals were sacrificed and tumor tissue was excised.

Effect of anti-hALR McAb on mouse tumor growth. A $1.0\text{x}10^{7}$ HepG2 cells were resuspended in 1:500 anti-hALR McAb and 1:500 ascites was induced by SP2/0 cells. Ten nude mice were divided into 1:500 anti-hALR McAb group and 1:500 ascites induced by SP2/0 cells group randomly. Each group had 5 mice. Subcutaneous tumors were induced by inoculation of 1×10^{6} cells in the flank of the mice. Every other day, each group of mice were treated with 1:500 anti-hALR McAb or 1:500 ascites induced by SP2/0 cells by 0.2 ml intraperitoneal (ip) bolus injection. Tumor volume was calculated as above. At 30 days after inoculation, the mice were sacrificed, and the tumor tissue was excised.

Statistical analysis. Experimental results were expressed as mean ± SD. Statistical analysis was performed by student's t-test. A *p*-value less than 0.05 was considered statistically significant. All statistical calculations were performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL., USA) software package.

Results. Expression of hALR in HepG2 cells by immunocytochemistry and siRNA of hALR inhibited hALR expression on the level of protein and nucleotide after transfection. As mentioned earlier, although ALR were expressed in the tissue of patients with hepatocellular carcinoma, we should determine the expression of ALR in HepG2 cells first before observing whether siRNA of ALR inhibits proliferation of HepG2 cells. To detect the expression of hALR in HepG2 cells, we performed immunocytochemistry on HepG2 cells. Staining cells revealed that hALR were expressed in HepG2 cells (**Figure 1a**), while the staining of cells incubated in ascites induced by SP2/0 cells as primary antibody was negative (**Figure 1b**). After the expression of hALR in HepG2 cells was determined, we took the steps to design and construct recombinant plasmid pSIALR-A and pSIALR-B (**Figure 2**). Following that step, recombinant plasmids were transfected into HepG2 cells. Owing to the gene of green fluorescent protein being cloned in the recombinant plasmid, green fluorescent protein in HepG2 cells transfected with recombinant plasmid was initially tested by inverted fluorescent microscope to demonstrate effect of transfection. The green fluorescence signal spread in the

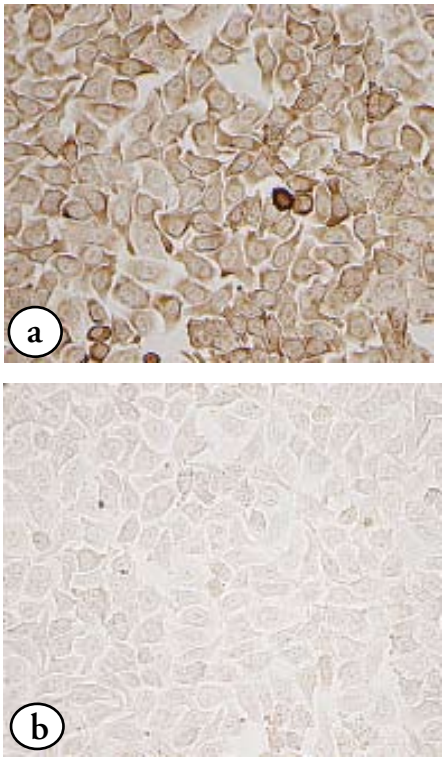

Figure 1 - Expression of human augmenter of liver regeneration (hALR) in HepG2 cells (original magnification ×200). **a**) Immunocytochemical staining showed there were positive cells in the presence of hALR. **b**) The staining of HepG2 cells incubated in ascites induced by SP2/0 cells as primary antibody was negative.

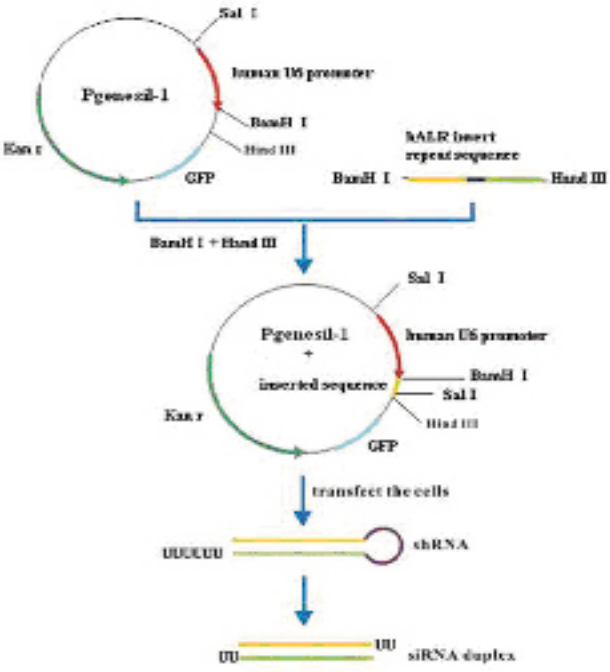

Figure 2 - Construction of recombinant plasmid based on the Pgenesil-1 plasmid backbone.

cytoplasm of HepG2 cells at 24 hours after transfection (**Figures 3a & 3b**). Then decreased expression of hALR in HepG2 cells transfected with pSIALR-A compared with negative control was detected by immunocytochemistry (**Figures 4a & 4b**). When expressed as a ratio with ß-actin, the level of hALR protein expression was reduced to approximately 17% compared with negative control by RT-PCR analysis (**Figure 5**). These data demonstrated that siRNA of hALR could effectively and specifically inhibit human hALR expression in cultured HepG2 cells on the level of protein and nucleotide, which suggested that we had successfully created the target plasmid. The proliferation of HepG2 cells was inhibited on protein and nuclei acid levels *in vitro*. After testing the ability of recombinant plasmid pSIALR-A to inhibit the expression of hALR, we next examined the ability of pSIALR-A to inhibit proliferation of HepG2 cells. First of all, HepG2 cells were divided in 2 groups, transfected with the recombinant plasmid pSIALR-A and pSIALR-B. After transfection, proliferation of HepG2 cells was detected by the ^{3}H-TdR incorporation approach. The ^{3}H-TdR incorporation of pSIALR-A/HepG2 cells was decreased compared with the incorporation of pSIALR-

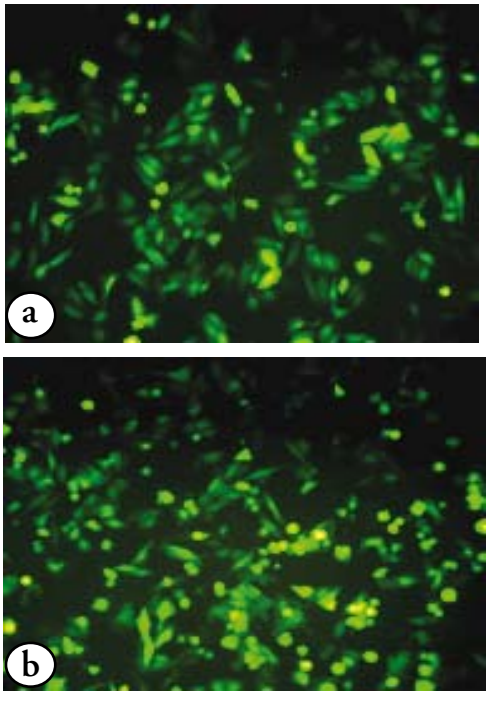

Figure 3 - Green fluorescent protein in HepG2 cells transfected with recombinant plasmid (inverted fluorescent microscope, original magnification×200). The green fluorescence signal spread in the cytoplasm of HepG2 cells at 24 hours after transfection: **a**) transfection with pSIALR-A, **b**) transfection with pSIALR-B.

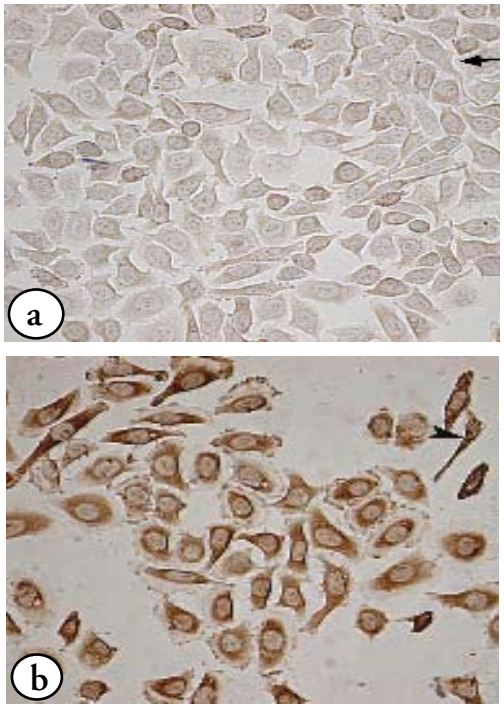

Figure 4 - Immunocytochemistry showed hALR expression was inhibited by hALR siRNA. (original magnification x200). **a**) Reduced expression of hALR in HepG2 cells transfected with pSIALR-A. **b**) No reduction of hALR expression in HepG2 cells transfected with pSIALR-B.

B/HepG2 cells (67687 ± 6548 versus 104807 ± 5713), which demonstrated that hALR siRNA could inhibit proliferation of HepG2 cells. Data were expressed in cpm as the mean ± SD of 12 replicates, and were representative of 3 separate experiments (p<0.01 [**Figure 6**]). Since the proliferation of HepG2 cells could be inhibited by siRNA of hALR on nuclei acid level, we next tested wether the proliferation of HepG2 cells could be inhibited by anti-hALR McAb on protein level. The HepG2 cells were divided into 3 groups. Each group was treated with a different dilution of anti-ALR McAb, ascites induced by SP2/0 cells as negative control, and PBS as blank control. After HepG2 cells was treated, proliferation of HepG2 cells was detected by the ^{3}H-TdR incorporation approach. Proliferation of HepG2 cells was inhibited by 1:125, 1:250, and 1:500 anti-hALR McAb for 72 hours, compared with the same final dilution of negative control (**Table 1**). Taken together, these results showed that the proliferation of HepG2 cells was inhibited on protein and nuclei acid levels *in vitro*. The growth of hepatoma partially was inhibited in mouse tumor model after hALR siRNA, and anti-hALR McAb suppressing function of hALR. As mentioned above, pSIALR-A could inhibit proliferation of HepG2 cells, our next step was to determine the effect of hALR siRNA on mouse tumor growth.

In this model, nude mice were divided into the pSIALR-A/HepG2 group, and pSIALR-B/HepG2 group randomly. After transfection with recombinant plasmid, pSIALR-A/HepG2 and pSIALR-B/HepG2 were harvested. Then, subcutaneous tumors were induced by inoculation of pSIALR-A/HepG2 and pSIALR-B/HepG2 in each group. The tumors became palpable at 5-10 days after inoculation of the tumor cells. Tumor sizes were significantly smaller in the HepG2 pSIALR-A group than in the HepG2 pSIALR-B group from 12-30 days after inoculation (**Table 2**) (**Figure 7a**). Tumors were measured at the indicated time points. Volume (mm^3) is expressed as the mean ± SD of 5 animals per group. Tumor volume of HepG2/pSIALR-A group has a significant difference from the HepG2/pSIALR-B group at p<0.05. At 30 days after inoculation, the animals were sacrificed and tumor

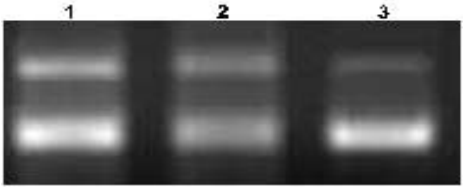

Figure 5 - Levels of hALR mRNA expression in HepG2 cells were detected by reverse transcription polymerase chain reaction. Lane 1: level of hALR mRNA expression in HepG2 cells transfected with pSIALR-B. Lane 2: level of hALR mRNA expression in HepG2 cells. Lane 3: level of hALR mRNA expression was decreased dramatically by 83% in HepG2 cells transfected with pSIALR-A compared with that in HepG2 cells transfected with pSIALR-B.

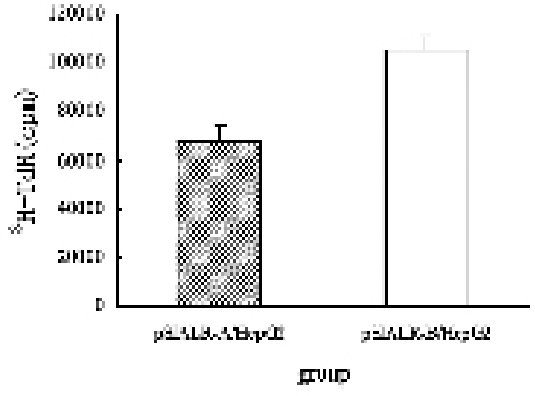

Figure 6 - Proliferation of HepG2 cells was inhibited on protein and nuclei acid levels in vitro. The ^{3}H-TdR incorporation of pSIALR-A/HepG2 cells was decreased compared with incorporation of pSIALR-B/HepG2 cells, p<0.01.

Table 1 - The effect of anti-hALR McAb on proliferation of HepG2 cells detected by ^{3}H-TdR incorporation approach.

Final dilution	Anti-hALR McAb group (cpm)	Ascites induced by SP2/0 cells group (cpm)
1:125	176600 ± 8127*	208577 ± 11501
1:250	91900 ± 18821#	197290 ± 22062
1:500	150403 ± 21757*	326846 ± 81409
1:1000	384838 ± 6948	395991 ± 45671
Blank control	402256 ± 17170	

cpm - counts per minute, hALR - human augmenter of liver regeneration, McAb - monoclonal antibody, ^{3}H-TdR - ^{3}H-thymidine *p<0.01. ^{3}H-TdR incorporation was decreased compared with the same final dilution of negative control, while final dilution of anti-hALR McAb being 1:25, 1:250, and 1:500.

Table 2 - Comparison of tumor growth in mice inoculated with HepG2/pSIALR-A, and HepG2/pSIALR-B.

Days after inoculation	HepG2/pSIALR-A	HepG2/pSIALR-B
9	0.03 ± 0.01	0.03 ± 0.01
12	0.16 ± 0.11*	0.53 ± 0.23
15	0.58 ± 0.14*	1.32 ± 0.30
18	1.24 ± 0.36*	2.43 ± 0.75
21	1.43 ± 0.47*	2.74 ± 0.96
24	1.49 ± 0.36*	3.00 ± 0.98
27	1.98 ± 0.50*	3.56 ± 1.35
30	2.14 ± 0.57†	5.66 ± 0.99

Significant difference from the HepG2/pSIALR-B group at *p<0.05, and †p<0.01

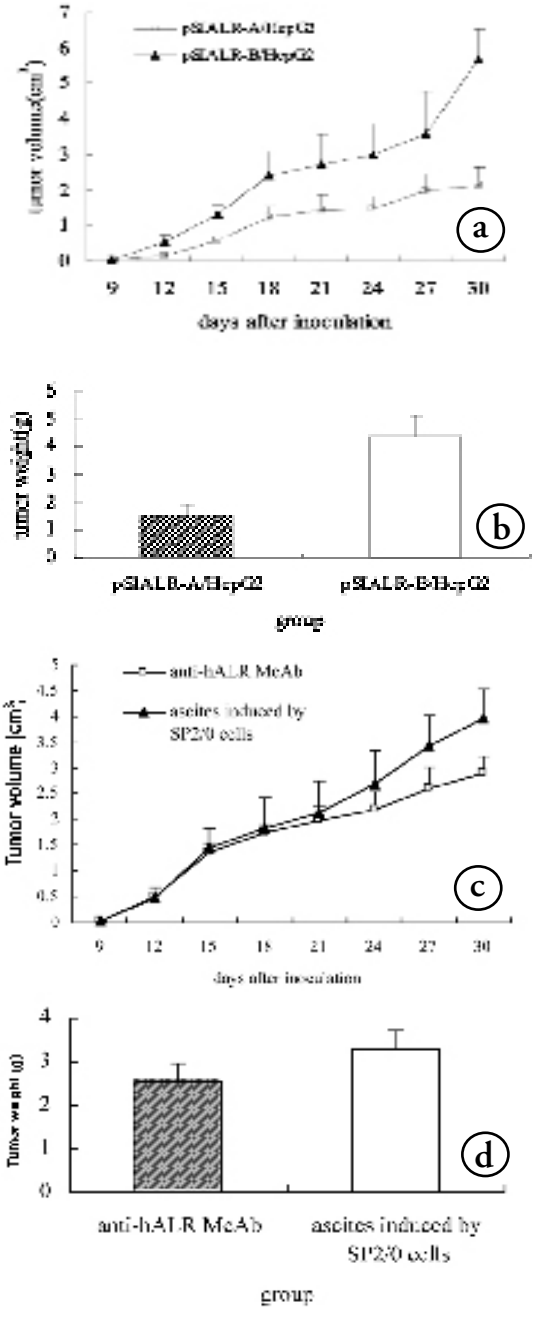

Figure 7 - The growth of hepatoma was partially inhibited in mouse tumor model after hALR (human augmenter of liver regeneration) siRNA suppressed the expression of hALR and anti-hALR McAb (monoclonal antibody) neutralized the secretion of hALR. **a**) Tumor sizes were significantly smaller in HepG2/pSIALR-A group than in HepG2/pSIALR-B group from 12 to 30 days after inoculation. **b**) Tumor weight was significantly lighter in HepG2/pSIALR-A group than in HepG2/pSIALR-B group at 30 days after inoculation, $p<0.01$. **c**) Tumor sizes were significantly smaller in anti-hALR McAb group than in ascites induced by SP2/0 cells group from 27 to 30 days after inoculation. **d**)Tumor weight was significantly lighter in anti-hALR McAb group than in ascites induced by SP2/0 cells group at 30 days after inoculation, $p<0.01$.

tissue was weighed. Tumors were significantly lighter in HepG2/pSIALR-A group than in HepG2/pSIALR-B group at 30 days after inoculation, 1.48 ± 0.44 g versus 4.39 ± 0.78 g (**Figure 7b**). Weight (g) is expressed as the mean ± SD of 5 animals per group. Tumor weight of HepG2/pSIALR-B group at $p<0.01$.

After observing that anti-hALR McAb could inhibit proliferation of HepG2 cells, we took steps to determine the effect of anti-hALR McAb on mouse tumor growth. In this model, nude mice were divided into 1:500 anti-hALR McAb group, and 1:500 ascites induced by SP2/0 cells group. The HepG2 cells were harvested and resuspended in 1:500 anti-hALR McAb, and 1:500 ascites induced by SP2/0 cells. Subcutaneous tumors were induced by inoculation of HepG2 cells in each group of mice. Every other day, each group of mice were treated with 1:500 anti-hALR McAb and 1:500 ascites induced by SP2/0 cells. The tumors became palpable at 3-10 days after inoculation of the tumor cells. Tumor sizes were significantly smaller in the anti-hALR McAb group than in the SP2/0 group from 27-30 days after inoculation (**Table 3**) (**Figure 7c**). Tumors were measured at the indicated time points. Volume (mm^3) is expressed as the mean ± SD of 5 animals per group, $p<0.05$. At 30 days after inoculation, the animals were sacrificed and tumor tissue was weighed. Tumors were significantly lighter in the anti-hALR McAb group than in the ascites induced by SP2/0 cells group at 30 days after inoculation, 2.53 ± 0.44 versus 3.28 ± 0.43, (**Figure 7d**) Weight (g) is expressed as the mean ± SD of 5 animals per group, $p<0.01$. To sum up, these results suggested the growth of hepatoma partially was inhibited in nude mouse tumor model after hALR siRNA and anti-hALR McAb suppressing function of hALR.

Discussion. Hepatocellular carcinoma, a critical disease threatening human health, ranks eighth in

Table 3 - Comparison of tumor growth in mice injected with anti-hALR McAb and ascites induced by SP2/0 cells.

Days after inoculation	Anti-hALR McAb group	Ascites induced by SP2/0 cells group
9	0.024 ±0 .01	0.029 ± 0.02
12	0.52 ± 0.16	0.48 ± 0.21
15	1.37 ± 0.17	1.45 ± 0.38
18	1.74 ± 0.16	1.84 ± 0.58
21	1.97 ± 0.23	2.13 ± 0.61
24	2.18 ± 0.35	2.69 ± 0.66
27	2.59 ± 0.43*	3.42 ± 0.59
30	2.91 ± 0.33#	3.97 ± 0.55

hALR - human augmenter of liver regeneration, McAb - monoclonal antibody

frequency among human cancers worldwide, mainly in Asia, Africa, and southern Europe. In some parts of Asia and Africa the prevalence is more than 100/100000 population.[22] Although many approaches, such as surgical resection, transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), radiotherapy, and liver transplantation were developed to treat it, and the effective and survival rates were increased, a large number of patients died from recurrence and metastasis.[23-27] The reason for carcinogenesis of HCC is not clear. Until now, a lot of cytokines have been discovered to be associated with carcinogenesis of HCC. Furthermore, some of them were used to treat HCC as a means of biotherapy. However, present biotherapy cannot obtain satisfactory results. More and more researchers had begun to realize that the occurrence of tumors is a complex course induced by multiple genes, and multiple steps. It is necessary to explore unknown cytokines' function in carcinogenesis of HCC to understand carcinogenesis of HCC better, and explore new methods for the therapy of HCC.

The RNA interference (RNAi), a new technique developed in the late 20th century, is an evolutionarily conserved phenomenon, and involves generation of active siRNA. The siRNA mediates degradation of the complementary homologous RNA (post-transcriptional gene silencing -PTGS). The RNAi has been promptly developed into a powerful tool for studying protein function in tumors.[28-32] In the present study, we utilized the RNAi technique to inhibit proliferation of hepatoma cells through hALR siRNA degrading hALR expression.

In our laboratory, we demonstrated that ALR was associated with carcinogenesis of HCC. It was not clear that the specific function of ALR in carcinogenesis of HCC. Therefore in this study, we further demonstrated that siRNA targeting hALR and anti-hALR McAb inhibited the growth of hepatoma partially on protein and nuclei acid level *in vitro* and *in vivo*.

In this study, expressing siRNA plasmid pSIALR-A, which targeted the cDNA of hALR, and the unrelated control plasmid pSIALR-B were constructed after expression of hALR in HepG2 cells was observed. The RNAi plasmid pSIALR-A and the unrelated control plasmid pSIALR-B were transfected into HepG2 cells. After transfection into cells, hairpin was changed into siRNA, which would combine with hALR mRNA. After combination, the siRNA degraded hALR mRNA, inducing gene silence. Our study showed the siRNA targeting hALR suppressed the expression of hALR in a sequence-specific manner. The level of hALR mRNA expression was decreased dramatically by 83% in HepG2 cells transfected with pSIALR-A compared with that in HepG2 cells transfected with pSIALR-B. Immunocytochemistry showed expression of hALR was reduced in HepG2 cells transfected with pSIALR-A. The growth of hepatoma partially was inhibited on protein and nuclei acid level *in vitro* and *in vivo* after hALR siRNA and anti-hALR McAb suppressing function of hALR. Inhibition of the growth of exnograft tumor in nude mice by hALR siRNA was observed from the twelfth day after inoculation, on the contrary, inhibition of the growth of exnograft tumor by anti-hALR McAb was observed from the twenty-seventh day after inoculation. These results showed that hALR siRNA suppressed hALR on nuclei acid level at 48 or 72 hours after transfection, and inhibited the growth of exnograft tumor earlier than anti-ALR McAb. Anti-hALR McAb inhibited the growth of exnograft tumor through neutralization of hALR locally. Inhibition of the growth of exnograft tumor was not observed early as the anti-hALR McAb could not penetrate to every cell to neutralize all hALR in exnograft tumor through i.p. bolus injection at few times; the hALR produced by hepatoma cells stimulated cells proliferation in an extracellular way, and an intracellular way as well.[33] Anti-hALR could not inhibit intracellular endocrined hALR. According to the findings *in vitro*, the final concentration of anti-hALR was determined *in vivo*, however, the final concentration used *in vivo* was probably not the proper concentration. Therefore, inhibition of the exnograft tumor was observed after repeating i.p. bolus injection.

Our results showed that the growth of hepatoma was inhibited by suppressing hALR on nucleic acid and protein level, which indicated hALR was one of the factors promoting the development of HCC. The hALR participate in the autonomous growth of hepatoma cells *in vitro* through an autocrine mechanism. Moreover, 2 means of biotherapy (hALR siRNA and anti-ALR McAb) were utilized to inhibit the growth of the tumor. Our results also provided the experimental evidence for hALR as therapeutic target of liver cancer.

Although our study has proven that hALR play an important role in promoting malignant proliferation of HepG2 by autocrine way, and that the proliferation of HepG2 was apparently inhibited by specific siRNA and McAb, the application of specific siRNA and McAb will be limited in clinical therapy in the future, because of the target of siRNA and the heterology of McAb. In order to overcome these problems, it will be necessary to search the receptor of hALR and its blocking agents in future studies.

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