

Daintain/AIF-1 accelerates the activation of insulin-like growth factor-1 receptor signaling pathway in HepG2 cells

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Abstract. Daintain/allograft inflammatory factor-1 (AIF-1), as a novel inflammatory factor, has been reported to accelerate the proliferation and migration of breast cancer cells. However, the effect of daintain/AIF-1 on hepatocarcinogenesis remains unclear. In order to explore the effect of daintain/AIF-1 on the progression of hepatocellular carcinoma (HCC), enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) were performed to examine the secretion and gene expression of (IGF)-1, IGF-2 and IGFBP-3. The expression of IGF-1R and its downstream targets was evaluated by western blotting. In addition, the proliferation and cell-cycle progression of HepG2 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) and flow cytometric analysis. The results showed that HepG2 cells subjected to daintain/AIF-1 treatment revealed an obvious increase in the secretion of IGF-1 and IGF-2, and a reduction in the secretion of IGFBP-3. Moreover, daintain/AIF-1 accelerated the activation of IGF-1-induced IGF-1R and its downstream AKT signaling pathway, and subsequently promoted the activation of cyclin D1 pathway, thus accelerating the progression of the cell cycle and eventually promoting the proliferation of HepG2 cells. In conclusion, daintain/AIF-1 promoted the proliferation of HepG2 cells by accelerating the activation of IGF-1R and its downstream

signaling pathway, which confirms that daintain/AIF-1 plays a crucial role in the development of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common lethal cancers worldwide (1). Following the diagnosis of liver cancer, approximately 20% patients benefit from curative surgical therapies such as liver resection and transplantation. Accumulating evidence has shown that insulin-like growth factors (IGF-1 and IGF-2) and their receptors (IGF-1R) are involved in the progression of cancers (2-5). The interaction between IGF-1 or IGF-2 and IGF-1R plays a pivotal role in tumorigenesis and the proliferation of cancer cells due to promotion of the cell-cycle progression (6). The mRNA expression level of IGF-1 in human HCC tissues is much lower than that in normal liver tissues, whereas, IGF-2 and IGF-1R reveal the overexpression in animal models of hepatocarcinogenesis and in HCC tissues (7). Mounting evidence confirms that the IGFs/IGF-1R axis is crucial in the development of HCC (7,8).

Daintain was first isolated from porcine intestine (9) and allograft inflammatory factor-1 (AIF-1) was first identified in heterotopic cardiac allografts of rats (10). Daintain has a high homology with AIF-1, thus this peptide has been designated as daintain/AIF-1. Daintain/AIF-1 can be constitutively expressed in monocytes and macrophages, and is involved in macrophage activation (11,12). It plays a crucial role in many autoimmune diseases (9,13-15). In previous studies, daintain/AIF-1 was confirmed to promote the proliferation and migration of breast cancer cells (16,17). However, whether daintain/AIF-1 has any impact on the progression of HCC remain unknown.

The association of inflammation with cancer has been previously suggested (18). A clear example of inflammation-associated cancer is HCC. Although mounting evidence is gathered to explore its molecular mechanisms, an accurate molecular connection between inflammation and HCC remains elusive. It has been demonstrated that inflammatory factors such as TNF- α , IL-6 and IL-1 α contribute to the progression of HCC (19,20). Daintain/AIF-1, as a novel inflammatory factor, has been found to be expressed in activated Kupffer

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Table I. Primer sequences and reaction conditions of RT-PCR.

Genes	Primers	Sequences (5'-3')	Annealing temperature (°C)	Cycle no.
<i>IGF-1</i>	Forward	GCATTGTGGATGAGTGTTC	53	30
	Reverse	GGCTCCTCCTACATTCTGTA		
<i>IGF-2</i>	Forward	GAGCTCGAGGCGTTCAGG	58	30
	Reverse	GTCTTGGGTGGGTAGAGCAATC		
<i>IGFBP-3</i>	Forward	ATATGGTCCCTGCCGTAGA	55	30
	Reverse	AAATCGAGGCTGAGCCAG		
<i>GAPDH</i>	Forward	CAAGGTCATCCATGACAACCTTTG	56	25
	Reverse	GTCCACCACCCTGTTGCTGTAG		

IGF, insulin-like growth factor; RT-PCR, reverse transcription polymerase chain reaction.

cells lining the walls of liver sinusoids (21,22). Therefore, we hypothesized that daintain/AIF-1 may be involved in the development of HCC. Based on this hypothesis, the effects of daintain/AIF-1 on the activation of IGF-1R and its downstream signaling pathway in HepG2 cells were investigated. The results revealed that daintain/AIF-1 accelerated the activation of IGF-1-induced IGF-1R and its downstream signaling pathway.

Materials and methods

Reagents and antibodies. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), propidium iodide (PI), Triton X-100 and recombinant human IGF-1 were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human daintain/AIF-1 was prepared by our laboratory according to previous instructions (22). An Annexin V-FITC apoptosis detection kit was obtained from the eBiyuntian Institute of Biotechnology (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). Primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell line and culture. HepG2 cells were purchased from the China Center for Type Culture Collection (CCTCC) and cultured into Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/l streptomycin at 37°C in an incubator supplied with 5% CO₂.

Secretion of IGF-1, IGF-2 and IGFBP-3. HepG2 cells were plated in 6-well plates and incubated with daintain/AIF-1 at various concentrations in serum-free medium for 72 h. The cell supernatants were then collected to examine the secretion of IGF-1, IGF-2 and IGFBP-3 using commercial ELISA kits.

Reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed to cDNA using a RevertAid™ cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's

instructions. The primer sequences and reaction conditions of *IGF-1*, *IGF-2*, *IGFBP-3*, *IGF-1R* and *GAPDH* are shown in Table I. The PCR products were evaluated by 2% agarose gel electrophoresis.

Western blot analysis. Cell samples were lysed in 20 µl cell lysis buffer containing 1 mM PMSF. The extracted proteins were separated by 12% SDS-PAGE and transferred to PVDF membrane by 2-h electroblotting. The blots were blocked in 5% non-fat dry milk for 1 h at room temperature, and then incubated overnight with corresponding primary antibodies at 4°C. The membrane was washed with TBS containing 0.05% Tween-20 (TBS-T) three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The blots were then developed with the enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Rockford, IL, USA). The optical density of the protein bands was assessed using Image J software.

Cell proliferation assays. Cell proliferation was evaluated by an MTT assay. For the MTT assay, the cells were plated in 96-well plates at a density of 2.5x10³ cells/well and incubated overnight with standard culture medium. The cells were then starved for 24 h in serum-free medium. After 24-h cell culture, the original medium was replaced with fresh serum-free medium containing 40 µg/l IGF-1 in the presence or absence of daintain/AIF-1. After incubation for another 24 h, the medium was replaced with 100 µl MTT (0.5 mg/ml) and incubated at 37°C for 4 h. After 4 h, the MTT solution was removed and 100 µl DMSO was added into each well. After agitation for 10 min, the absorbance of each well was measured by a microplate reader (Tecan Sunrise, Salzburg, Austria) at a wavelength of 570 nm.

Cell cycle analysis. In order to investigate the distribution of the cell cycle, HepG2 cells were seeded in 6-well plates at a density of 1x10⁶ cells/well. After adhesion, the cells were starved for 24 h in serum-free DMEM. The original medium was then replaced with fresh serum-free medium containing 40 µg/l IGF-1 in the presence or absence of daintain/AIF-1. After incubation for 24 h, the cells were collected and analyzed by flow cytometer (FC500; Beckman Coulter, La Brea, CA, USA).

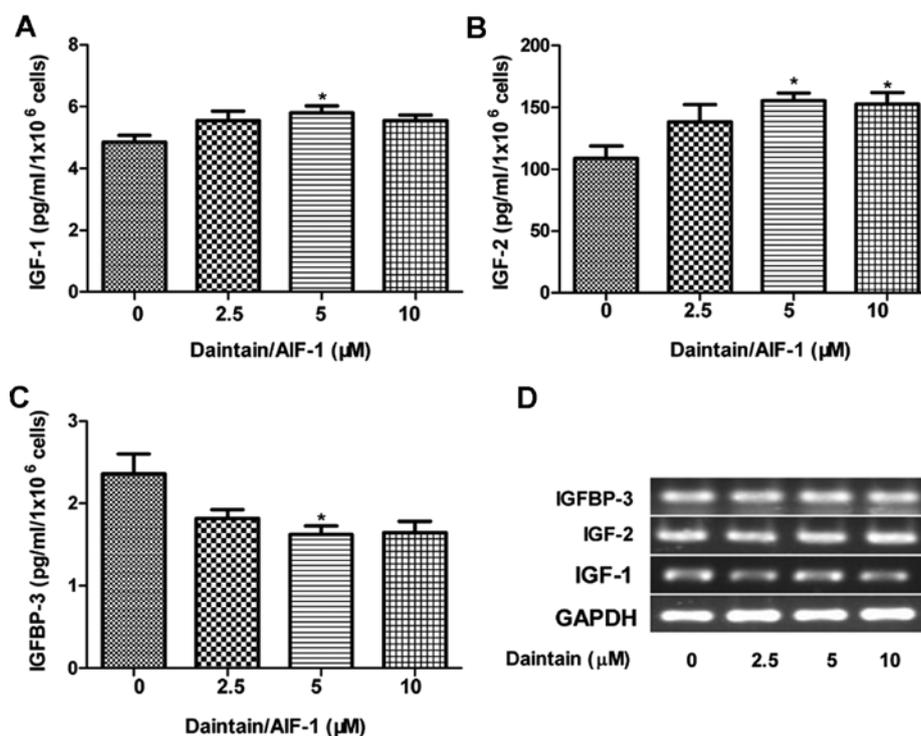


Figure 1. Daintain/AIF-1 accelerates the secretion of IGF-1, IGF-2 and IGFBP-3 in HepG2 cells, but has no impact on their gene expression. HepG2 cells were treated with daintain/AIF-1 at designated concentrations for 72 h in serum-free medium. Then, cell-free medium was collected to assess the secretion of (A) IGF-1, (B) IGF-2) and (C) IGFBP-3 by commercial ELISA kits. The mRNA expression of IGF-1, IGF-2 and IGFBP-3 (D) in daintain/AIF-treated cells was evaluated. Data were presented as mean \pm SD. A significant difference was considered at * $P < 0.05$ when compared with the control. AIF-1, allograft inflammatory factor-1; ELISA, enzyme-linked immunosorbent assay; IGF, insulin-like growth factor.

Statistical analysis. The experiments were repeated at least three times. The data were presented as mean \pm SD and calculated using the Student's t-test using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). A statistically significant difference was considered at $P < 0.05$.

Results

Daintain/AIF-1 promotes the secretion of IGF-1, IGF-2 and IGFBP-3 in HepG2 cells, but fails to modulate their gene expression. Accumulating data have demonstrated that alteration of the autocrine/paracrine loops involving IGFs and IGFBP-3 is associated with the proliferation of HCC cells (8,23-25). Therefore, we examined the effects of daintain/AIF-1 on the production of IGFs and IGFBP-3 in the present study. Enhanced secretion of IGF-1 and IGF-2 was observed due to the stimulation of daintain/AIF-1 (Fig. 1A and B). By contrast, daintain/AIF-1 at the concentration of 5 μ M obviously decreased the secretion of IGFBP-3 (Fig. 1C). However, semi-quantitative RT-PCR analysis clearly showed that daintain/AIF-1 had no obvious effect on the gene expression of IGF-1, IGF-2 and IGFBP-3 (Fig. 1D).

Daintain/AIF-1 accelerates the activation of IGF-1-induced IGF-1R in HepG2 cells. Western blot analysis was conducted to examine the effect of daintain/AIF-1 on the IGF-1-induced activation of IGF-1R. As shown in Fig. 2A and C, IGF-1 induced an obvious phosphorylation of IGF-1R and daintain/AIF-1 accelerated IGF-1-induced activation of IGF-1R

in a dose-dependent manner (Fig. 2B and D). Similarly, the increased level of phospho-IGF-1R was observed due to the co-incubation of IGF-1 and daintain/AIF-1. HepG2 cells treated with IGF-1 (40 μ g/l) for 3 h in the presence of 5 μ M daintain/AIF-1 resulted in the maximum increase in phospho-IGF-1R.

Daintain/AIF-1 enhances the activation of IGF-1-induced IGF-1R downstream signaling pathway in HepG2 cells. To determine the role of daintain/AIF-1 in the activation of IGF-1-induced IGF-1R, activation of the downstream signaling pathway of IGF-1R was also examined by western blot analysis. Consistent with the results shown in Fig. 3, IGF-1 enhanced the expression of phospho-AKT, which was further promoted by daintain/AIF-1 in a dose-dependent manner. Similarly, daintain/AIF-1 at the increasing concentration resulted in an elevated expression of phospho-pS6K1. Moreover, in the presence of IGF-1, daintain/AIF-1 stimulation enhanced the expression of cyclin D1, CDK4 and phosphorylated Rb, but had no effect on the expression of cyclin E although IGF-1 alone resulted in an increased expression of cyclin E.

Daintain/AIF-1 promotes IGF-1-induced proliferation of HepG2 cells. As shown in Fig. 4A, IGF-1 significantly accelerated the cell growth (115% of the control) and daintain/AIF-1 promoted IGF-1-induced cell proliferation. The flow cytometric analysis revealed that IGF-1 stimulation decreased the proportion of HepG2 cells in the G0/G1 phase (from 67.7 \pm 5.4 to 54.0 \pm 5.1%), but increased the proportion

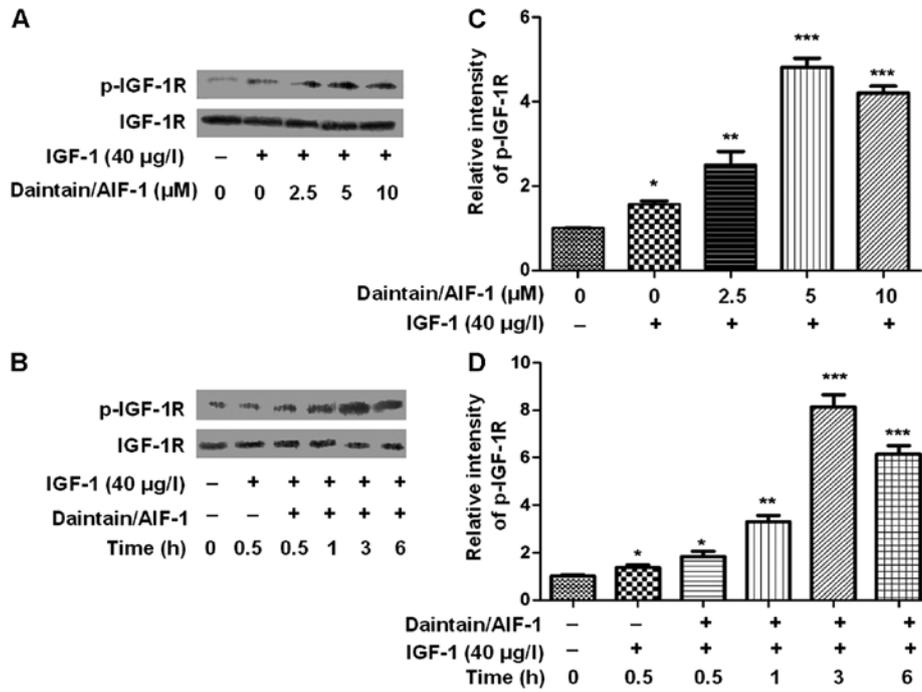


Figure 2. Daintain/AIF-1 accelerates the activation of IGF-1R and its downstream signaling pathways in the presence of IGF-1. HepG2 cells were treated with daintain/AIF-1 at the designated concentrations for 24 h in serum-free medium. The daintain/AIF-1-treated HepG2 cells were then stimulated with 40 µg/l IGF-1 for another 15 min and collected to examine the protein expression levels of phospho-IGF-1R and total IGF-1R by (A) western blot analysis. The relative expression or fold-changes of phospho-IGF-1R after 15 min IGF stimulation was statistically analyzed (C) by measuring the optical density of the protein bands using Image J software. The daintain/AIF-1-treated HepG2 cells were then incubated with 40 µg/l IGF-1 at various time-points and the expression levels of phospho-IGF-1R and total IGF-1R were evaluated by western blot analysis (B). The relative expression or fold-changes of phospho-IGF-1R during IGF-1 stimulation at various time-points was statistically analyzed (D) by measuring the optical density of protein bands using Image J software. AIF-1, allograft inflammatory factor-1; IGF, insulin-like growth factor. Data were expressed as mean ± SD, and asterisks indicated the statistically significant differences (*P<0.05, **P<0.01 and ***P<0.001) when compared with the control.

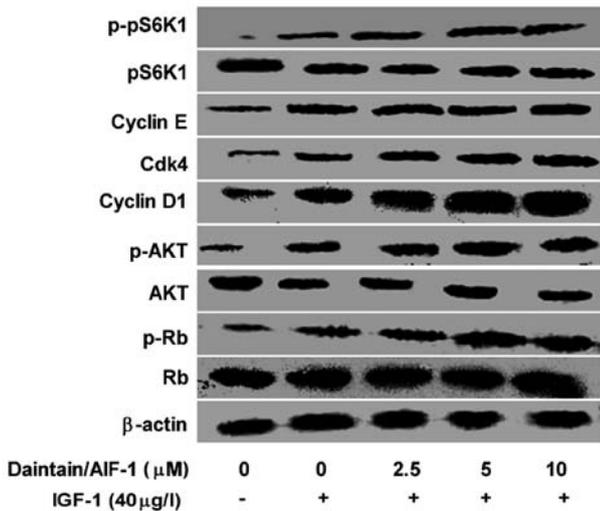


Figure 3. HepG2 cells were treated with daintain/AIF-1 at designated concentrations for 24 h in serum-free medium and then incubated with 40 µg/l IGF-1 for another 3 h. After treatment, the cell proteins were isolated and subjected to the examination of downstream targets of IGF-1R such as p-AKT, p-pS6K1, p-Rb, cyclin E and D1 and CDK4 by western blot analysis. AIF-1, allograft inflammatory factor-1; ELISA, enzyme-linked immunosorbent assay; IGF, insulin-like growth factor.

of HepG2 cells in S phase (from 21.9±1.4 to 31.5±2.8%) and G2/M phase (from 12.1±2.4 to 14.3±3.0%) (Fig. 4B). More significant changes in the cell cycle were observed due to

the combinatorial application of daintain/AIF-1 and IGF-1. In the presence of IGF-1 (40 µg/l), daintain/AIF-1 reduced the ratio of HepG2 cells in the G0/G1 phase and enhanced the proportion of HepG2 cells in the S and G2/M phase in a dose-dependent manner (Fig. 4B).

IGF-1R is involved in the promotion of daintain/AIF-1 on IGF-1-induced HepG2 cell proliferation. To confirm whether IGF-1R plays a role in the promotion of daintain/AIF-1 on the IGF-1-induced proliferation of HepG2 cells, the anti-IGF-1R antibody was used to suppress the expression of IGF-1R. The results showed that the anti-IGF-1R neutralizing antibody decreased the proliferation of HepG2 cells under the induction of IGF-1. Furthermore, the promotion effect of daintain/AIF-1 on IGF-1-induced cell proliferation was eliminated due to the blockage of IGF-1R. Of note, after the blockage of IGF-1R, treatment of HepG2 cells with daintain/AIF-1 only caused a slight increase in the cell number (Fig. 5).

Discussion

Although previous findings have demonstrated that daintain/AIF-1 promotes breast cancer cell proliferation via the activation of the NF-κB/cyclin D1 pathway, (16) the precise mechanisms of daintain/AIF-1 for promoting cancer cell proliferation are not completely understood. In the present study, we employed HepG2 cell lines to investigate the

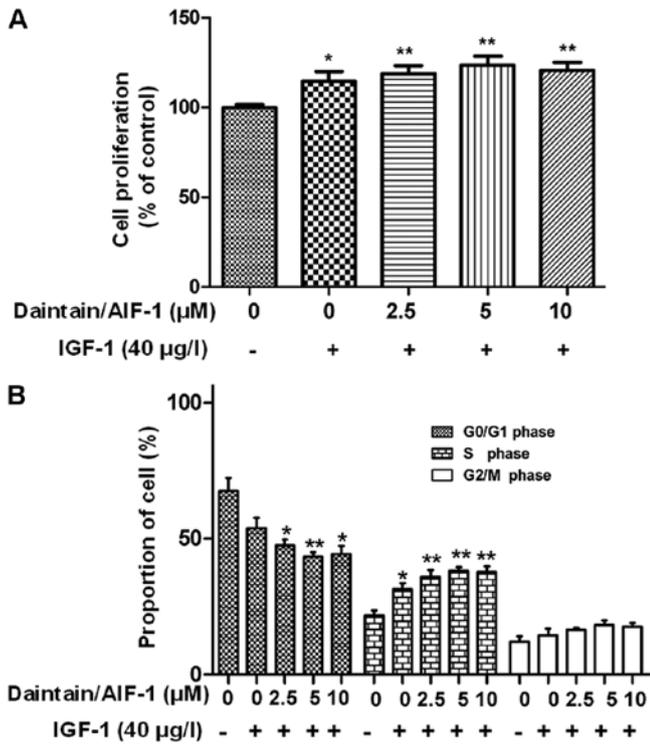


Figure 4. Daintain/AIF-1 promotes IGF-1-induced HepG2 cell proliferation and cell-cycle progression. (A) Cell proliferation was analyzed by MTT. (B) The distribution of the cell cycle was analyzed by flow cytometry. Data are presented as mean ± SD, and asterisks indicated the statistically significant differences (*P<0.05, and **P<0.01) when compared with the control. AIF-1, allograft inflammatory factor-1; IGF, insulin-like growth factor.

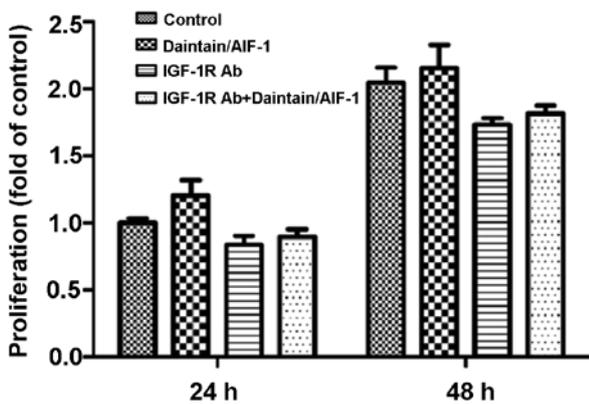


Figure 5. The blockage of IGF-1R inhibits IGF-1-induced HepG2 cell proliferation and eliminates the promotion effect of daintain/AIF-1 on IGF-1-induced HepG2 cell proliferation. HepG2 cells were treated with daintain/AIF-1 (5 μM) or anti-IGF-1R antibody (10 ng/ml) alone for 24 and 48 h, or a combinatorial application of daintain/AIF-1 and anti-IGF-1R antibody for 24 and 48 h. Subsequently, 40 μg/l IGF-1 was incubated with the pretreated cells for another 3 h. Following treatment, the cells were collected to analyze the cell proliferation by MTT assay. AIF-1, allograft inflammatory factor-1; IGF, insulin-like growth factor.

molecular mechanism of daintain/AIF-1 on promoting cell proliferation. Our results showed that daintain/AIF-1 is closely associated with the activation of IGF-1-induced IGF-1R and its downstream signaling pathway.

The IGF/IGF-1R axis has been previously found to play a critical role in the development of HCC (3,7,8). IGF-1R is predominantly activated by its ligands, such as IGF-1 and IGF-2. The bioactivity of IGFs can be modulated by IGFBPs due to the high affinity to IGF-1 and IGF-2 (26). IGFBPs can sequester IGF from IGF-1R, thereby attenuating the bioactivity of these growth factors. IGFBP-3 accounts for the majority of circulating IGF (3,27,28). Treatment of HCC cells with recombinant IGFBP-3 can lead to a significant reduction in cell proliferation by inhibiting the activation of IGF-1-induced IGF-1R, ERK and AKT signaling pathways (23). In our study, daintain/AIF-1 enhanced the secretion of IGF-1 and IGF-2, reduced the secretion of IGFBP-3, but had no impact on their gene expression. On the basis of these results, daintain/AIF-1 may be involved in the activation of IGF-1R signaling pathway in HepG2 cells through the elevated IGF-1 and IGF-2.

The activation of IGF-1R is a vital process in the promotion of cell proliferation. Therefore, we monitored IGF-1-induced activation of IGF-1R in the presence of daintain/AIF-1 by western blot analysis. Treatment of HepG2 cells with daintain/AIF-1 for 24 h significantly enhanced IGF-1-induced tyrosine phosphorylation of IGF-1R in a dose- and time-dependent manner.

When IGF-1R is stimulated, the tyrosine kinase activates its downstream signaling pathways, including the Ras/MAPK and PI3K/AKT pathways. The phosphorylation of MAPK can induce a subsequent increase in cell proliferation, and the activation of PI3K/AKT can lead to the modulation of mammalian target of rapamycin (mTOR), thus resulting in translational adaptation (29-31). mTOR can regulate cyclin D1 expression and Rb phosphorylation, and the inhibition of mTOR can arrest cells in the G0/G1 phase of the cell cycle (32,33). Based on our study, daintain/AIF-1 revealed an obvious enhancement on the IGF-1-induced phosphorylation of PI3K/AKT and its downstream target pS6K1. pS6K1 is a key downstream element of the AKT signaling pathway, and the de-phosphorylation of pS6K1 can block the G1 cell cycle progression (34). We have also demonstrated that the incubation of HepG2 cells with daintain/AIF-1 upregulated the expression of cyclin D1, CDK4 and phosphorylated Rb in the presence of IGF-1. Cyclin D1 is important in the transition from G1 to S phase (35,36). Cyclin D1 can couple with CDK4 or CDK6 to form cyclin D1/CDK complexes, thus correspondingly resulting in the activation of CDKs and the phosphorylation of Rb (37,38). Hypophosphorylated Rb can abrogate the inhibitory effect of Rb on the cell-cycle progression and accelerate cell entry into the S phase (39).

The physiological function of daintain/AIF-1 on promoting IGF-1-induced activation of the IGF-1R signaling pathway was confirmed by the MTT assay. The results showed that daintain/AIF-1 markedly promoted IGF-1-induced HepG2 cell proliferation. Data from the flow cytometric analysis also revealed that daintain/AIF-1 accelerated HepG2 cell entry into the S and G2/M phase, which is consistent with our previous observation that daintain/AIF-1 promoted cyclin D1 expression and Rb phosphorylation.

In our study, the blockage of IGF-1R using the anti-IGF-1R antibody inhibited IGF-1-induced HepG2 cell proliferation and eliminated the promotion effect of daintain/AIF-1 on IGF-1-induced HepG2 cell proliferation. These

results suggest that the IGF/IGF-1R signaling pathway plays a crucial role in the promotion function of daintain/AIF-1 on IGF-1-induced HepG2 cell proliferation. However, daintain/AIF-1 may have other pathways to promote HepG2 cell proliferation besides the activation of IGF-1R signaling pathway. Moreover, we observed the accelerated migration of HepG2 cells in the presence of IGF-1. However, daintain/AIF-1 failed to modulate IGF-1-induced cell migration (data not shown).

Taken together, according to our current studies and knowledge, daintain/AIF-1 induces activation of the IGF/IGF-1R axis and its downstream signaling pathways by regulating IGF-1, IGF-2 and IGFBP-3 secretion and enhancing IGF-1R sensitivity in the presence of IGF-1 stimulation, thereby resulting in the proliferation of HepG2 cells. However, more studies are required to elucidate the exact mechanisms of daintain/AIF-1 involved in the activation of the IGF-1R signaling pathway and its downstream signaling pathways.

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