RESEARCH ARTICLE

Asiatic Acid Promotes p21WAF1/CIP1 Protein Stability through Attenuation of NDR1/2 Dependent Phosphorylation of p21WAF1/CIP1 in HepG2 Human Hepatoma Cells

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Abstract

Previous studies have suggested anti-tumor effects of asiatic acid in some human cancer cell lines. This agent is reported to increase the levels of p21WAF1/CIP1 in human breast cancer cell lines. However, the molecular mechanisms have not been established. Here we report that asiatic acid up-regulates p21WAF1/CIP1 protein expression but not the level of p21WAF1/CIP1 mRNA in HepG2 human hepatoma cells. Furthermore, we found that the asiatic acid induced increase of p21WAF1/CIP1 protein was associated with decreased phosphorylation (ser-146) of p21WAF1/CIP1. Knockdown of NDR1/2 kinase, which directly phosphorylates p21WAF1/CIP1 protein at ser-146 and enhances its proteasomal degradation, increased the levels of p21WAF1/CIP1 protein and eliminated the regulation of p21WAF1/CIP1 stability by asiatic acid. At the same time, the expression of NDR1/2 kinase decreased during treatment with asiatic acid in HepG2 cells. Moreover, asiatic acid inhibited the proliferation of HepG2 cells, this being attenuated by knockdown of p21WAF1/CIP1. In conclusion, we propose that asiatic acid inhibits the expression NDR1/2 kinase and promotes the stability of p21WAF1/CIP1 protein through attenuating NDR1/2 dependent phosphorylation of p21WAF1/CIP1 in HepG2 cells.

Keywords: Asiatic acid - p21WAF1/CIP1 - stability - NDR1/2 - phosphorylation

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Introduction

More than 60% of currently used anti-tumor drugs are natural origin or derived from natural compounds (Cragg et al., 2005). Plant-derived compounds are important source of these nature derived anti-tumor agents. Nearly 3000 plant species have been reported for the treatment of cancer (Altinbas et al., 2012; Boreddy et al., 2013). Some plant based anticancer agents have been allowed to enter the clinical trial, such as paclitaxel, vincristine, vinblastine, topotecan and irinotecan (Nobili et al., 2009). Asiatic acid, a pentacyclic triterpene found in the tropical medicinal plant Centella asiatica, has antioxidant, anti-inflammatory and neuroprotective properties (Huang et al., 2012; Guo et al., 2013). Some studies have reported the anti-tumor effect of asiatic acid. For example, asiatic acid was reported to enhance apoptosis and cell cycle arrest by promoting the activity of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells (Hsu et al., 2005). By generation of ROS, asiatic acid could induce apoptosis in human melanoma cells (Park et al., 2005; Xu et al., 2012). Asiatic acid could disturb the endoplasmic reticulum and alterations in calcium homeostasis to induce cell death (Gurfinkel et al., 2006). Furthermore, asiatic acid has also been shown to inhibit the angiogenic effects of VEGF (Kavitha et al., 2011).

The cyclin-dependent kinase inhibitor 1A, p21WAF1/CIP1, is known as an important inhibitor of cell proliferation by its interactions with complexes of cyclins and cyclin-dependent kinases (CDK) (Xu et al., 2012; Kan et al., 2013). p21WAF1/CIP1 is also involved in the regulation of apoptosis (Russo et al., 2013). It has been reported that apoptosis and cell cycle arrest induced by asiatic acid was associated with the increasing levels of p21WAF1/CIP1 (Hsu et al., 2005), but the underlying molecular mechanism was still unclear. In this study, we investigated the molecular mechanisms that regulate the levels of p21WAF1/CIP1 by asiatic acid.

Materials and Methods

Reagents

Asiatic acid, MG-132 and Anti-MYC Agarose were

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purchased from Sigma-Aldrich. Antibody of p21$^{WAF1/CIP1}$, β-actin and MYC-tag were from Cell Signaling Technology. Anti-phospho-p21$^{WAF1/CIP1}$ (ser-146) and anti-phosphoserine were from Abcam. Anti-NDR1/2, anti-NDR1 and anti-NDR2 were from Santa Cruz Technology. The real-time PCR Master Mix Kit was purchased from Takara. Small interference RNAs for NDR1 (sc-44366), NDR2 (sc-45828) and negative control (sc-37007) were obtained from Santa Cruz Technology.

Cell culture and treatment

Hepatocellular carcinoma G2 (HepG2) cells and 293T cells were obtained from the Institute of Cell and Biochemistry Research of Chinese Academy of Science. HepG2 cells and 293T cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37˚C, in a humidified atmosphere of 5% CO_2, supplemented with penicillin (100 UI/ml) and streptomycin (100 μg/ml).

RNA isolation and Reverse Transcription-PCR

Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer instructions. To examine p21$^{WAF1/CIP1}$ mRNA expression in HepG2 cells, RT-PCR assay was performed with Access RT-PCR kit (Promega). Real-time PCR was performed with 3 min incubation at 95°C and 40 amplification cycles (95°C, 10 s; 56°C, 15 s; 72°C, 35 s). β-actin was used as the normalization control. The amplification and data acquisition were run on a real-time PCR system (ABI Prism 7500, Applied Bio-systems, Foster City, CA, USA) for SYBR green PCR master mix. The primer sequences used were: β-actin: 5'-GTACCACTGGCATCGTGATGG ACT-3' (forward), 5'-CCGCTCATTGCCAATGGTGAT-3' (reverse); p21$^{WAF1/CIP1}$: 5'- GCAGACCAGCATGACAGAT TT-3' (forward), 5'- GGATTAGGGCTTCCTCTTGGA-3' (reverse).

Vector construction and transfection

MYC-DDK-tagged p21$^{WAF1/CIP1}$ expression vector was from OriGene. To get mutated p21$^{WAF1/CIP1}$ (ala-146), site-directed mutagenesis kit (Stratagene, La Jolla, CA) was performed to get the mutation from ser-146 to ala-146 (AGC to GCG). Mutagenic primers were used: 5'-CGGCGGCAGACCGCGATGACAGATTTC-3' (forward), 5'-GAAATCTGTCATCGCGGTCTGCCGC CG-3' (reverse). Transfection of siRNA and expression vector in HepG2 cells: After plating for 24 h, cells at 60% confluence were transfected with siRNA and expression vector using ipofectin2000 (Life Technologies). Six hours later, the medium was refreshed and cultured for 24h or 48h. Then the cells were treated with asiatic acid.

Viral Production and Infection

Lentiviral shRNA expression plasmids were purchased from Sigma. Two different shRNAs designed against p21$^{WAF1/CIP1}$ mRNA, and the sequences are listed as follows: 5'-CCGGAGAGGTTCCTAAGAGTGCTGGCTCGAGC AGCACTCTTAGGAACCTCTTTTTTG-3' (sh-p21-1), 5'-CCGGGAGCGATGGAACTTCGACTTTCTCGAGA AAG-TCGAAGTTCCATCGCTCTTTTTG-3' (sh-2). Stable cell lines were established by the infection of target cells with lentiviruses viruses. Lentiviral vectors coding for shRNA were transfected in 293T cells in combination with packing vectors to obtain viral particles used to infect HepG2 cells. Lentiviruses viruses were harvested at 48 h after the transfection and added to the HepG2 cells with 10 mg/ml polybrene for 24 h infection. Cells were then selected with 3 mg/ml puromycin for 48 h.

Immunoprecipitation

The transfected HepG2 cells were treated with asiatic acid for 12 h. Then the cells were lysed in RIPA buffer. We centrifuged cell lysates at 16000×g, 4˚C for 10 min and collected supernatants. Next, the collected supernatants were incubated with Anti-MYC Agarose for 3 h. The agaroses were washed with RIPA buffer then boiled in 2× loading buffer for 5 min. Finally, the samples were subjected to Western Blot and detected with different antibodies.

Western blot analysis

Cells were lysed for 20 min on ice in RIPA buffer with 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 mM NaF, 2m M Na3VO4, and 5 mg/ml leupeptin. Then cell lysates were centrifuged at 16000×g for 10 min at 4 °C. Protein concentrations were estimated using protein-dye (Bio-Rad,). Samples were separated by SDS-PAGE and then blotted onto a PVDF membrane (Millipore). After blotted with primary antibodies overnight, peroxidase conjugated secondary antibodies were incubated with the membrane. Bound antibodies were detected by Chemiluminescent HRP Substrate (Millipore) and photographed by LAS-3000 luminescent image system (Fujifilm).

In vitro cell proliferation assay

Cell proliferation was examined by using a Cell Counting Kit-8 (Dojindo). Cells were plated and treated with DMSO vehicle control or asiatic acid (10 μM) in 96-well plates at 2,000 per well and cultured in growth medium. Every 24 h, CCK-8 (10 μL) was added to each well containing 100 μL of DMEM medium. Then the plate was incubated for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (Tecan).

Colony formation

HepG2 cells were digested to reconstitute the single-cell suspension and seeded into 6-well plate (200 cells per well). Cells were incubated (37°C, 5% CO_2) in DMEM (10% FBS) with DMSO vehicle control or asiatic acid (10 μM). Three weeks later, the colonies were fixed with 10% formalin for 15 min and stained with crystal violet for 20 min then washed with phosphate buffered solution (PBS). Colonies were counted after taking photographs. Colony formation efficiency was calculated as the number of colonies generated divided by total number of input cells.

Statistical analysis

All values are shown as mean ± SD. *P* values were calculated using student's two-tailed t test; $P < 0.05$ was considered significant.

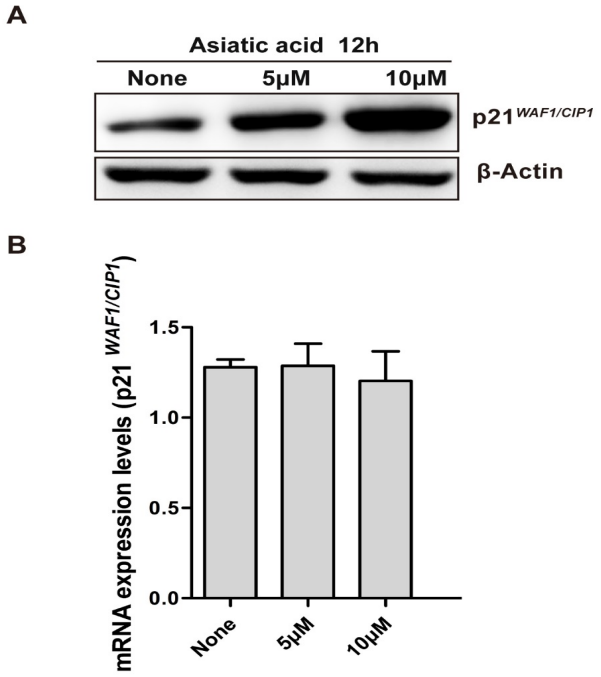

Figure 1. Effects of Asiatic Acid on the Stability of p21WAF1/CIP1 in HepG2 Cells. HepG2 cells were treated with asiatic acid for 12 hours with increased concentration. (A) The levels of p21WAF1/CIP1 and β-actin protein were examined by Western Blot. (B) The levels of p21WAF1/CIP1 mRNA were examined by real-time PCR

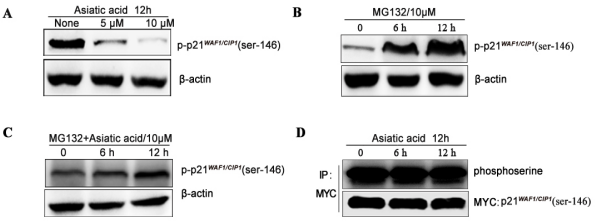

Figure 2. Asiatic Acid Inhibits the Phosphorylation of p21WAF1/CIP1 on Ser-146. (A) HepG2 cells were treated with asiatic acid for 12 hours with increased concentration. The levels of phosphorylated p21WAF1/CIP1 on ser-146 and β-actin protein were examined by Western Blot. (B) With the treatment of 10 μM MG-132, the levels of phosphorylated p21WAF1/CIP1 on ser-146 were examined by Western Blot. (C) HepG2 cells were treated with MG-132 (10 μM) in combination with asiatic acid (10 μM), the levels of p21WAF1/CIP1 were examined by Western Blot. (D) HepG2 cells were transfected with mutated p21WAF1/CIP1 (ala-146) for 24 hours and were treated with asiatic acid. IP was performed with MYC-beads followed by Western Blot. The intensity of phosphoserine of p21WAF1/CIP1 was quantified by densitometry and normalized with equal levels MYC- p21WAF1/CIP1 (ala-146)

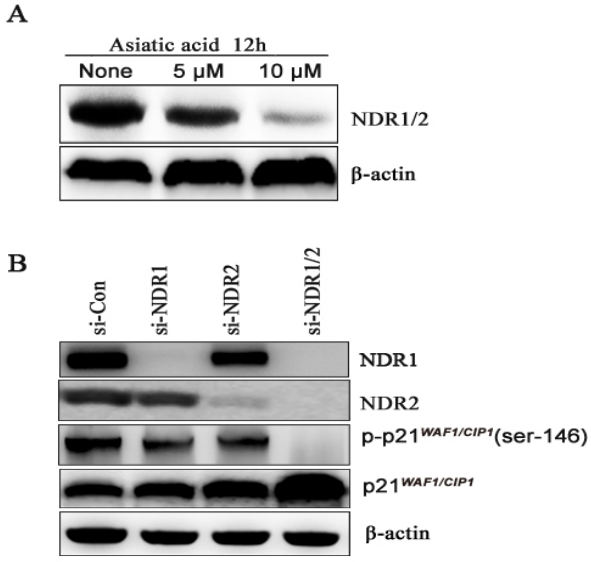

Figure 3. Asiatic Acid Down-regulates the Expression of NDR1/2 Kinase which Phosphorylates p21WAF1/CIP1 on Ser-146. (A) HepG2 cells were treated with asiatic acid for 12 hours with increased concentration. The levels of NDR1/2 kinase and β-actin protein were examined by Western Blot. (B) HepG2 cells were transfected with control siRNA, NDR1 siRNA alone, NDR2 siRNA alone or NDR1/2 (dual NDR1 and NDR2) siRNA for 48 hous. The expression of NDR1, NDR2, p-p21WAF1/CIP1 (phosphorylated p21WAF1/CIP1 on ser-146) and β-actin were analyzed by Western Blot

Results

Asiatic acid increases the levels of p21WAF1/CIP1 through enhancing the stability of p21WAF1/CIP1 protein

To confirm whether asiatic acid up-regulates the levels of p21WAF1/CIP1, HepG2 human hepatoma cells were treated with asiatic acid for 12 hours (0, 5 and 10 μM). As shown in Figure 1A, the levels of p21WAF1/CIP1 protein were up-regulated after the treatment with increasing concentrations of asiatic acid in HepG2 cells. We next examined the expression of p21WAF1/CIP1 mRNA by quantitative RT-PCR. The results show that there were no significant changes in p21WAF1/CIP1 mRNA under the treatment with different concentrations of asiatic acid (Figure 1B). P21WAF1/CIP1 was proved to be degraded through the proteasome pathway (Lee et al., 2007), thus we suppose that the regulation of p21WAF1/CIP1 protein levels by asiatic acid may be the results of stability regulation.

Asiatic acid inhibits the phosphorylation of p21WAF1/CIP1 at ser-146

Previous reports (Lee et al., 2007; Cornils et al, 2011) demonstrated that p21WAF1/CIP1 could be phosphorylated at ser-146 and ser-114. These could regulate the stability of p21WAF1/CIP1. As shown in Figure 2A, we found that the levels of phosphorylated p21WAF1/CIP1 (ser-146) decreased under the treatment with asiatic acid in HepG2 cells. Moreover, the phosphorylated p21WAF1/CIP1 (ser-146) clearly accumulated upon the treatment of MG-132 (Figure 2B). Differently, MG-132 weakly up-regulated the phosphorylated p21WAF1/CIP1 protein (ser-146) in the present of asiatic acid (Figure 2C). No commercial antibodies was used to recognize phosphorylated p21WAF1/CIP1 protein at ser-114, we mutated ser-146 of p21WAF1/CIP1 to alanine (ala-146) and transfected it into HepG2 cell lines. Then we examined the total levels of phosphoserine of MYC-tagged p21WAF1/CIP1 protein (ala-146) with a specific anti-phosphoserine antibody in asiatic acid treated HepG2 cells. The findings (Figure 2D) showed that serine-phosphorylation levels of p21WAF1/CIP1 protein (ala-146) were not changed under the treatment of asiatic acid. These results demonstrate that asiatic acid could inhibit the phosphoserine of p21WAF1/CIP1 at ser-146 but not ser-114.

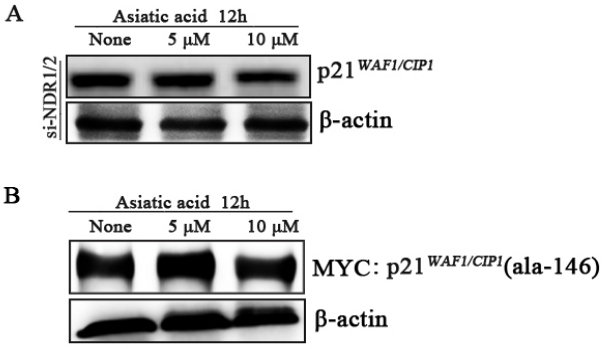

Figure 4. Knockdown of NDR1/2 Kinase Inhibits Asiatic Acid-induced Regulation of Stability of p21$^{WAF1/CIP1}$. (A) HepG2 cells were transfected with dual NDR1/2 siRNA for 48 hours and then were treated with asiatic acid for 12 hours. The expression of p21$^{WAF1/CIP1}$ and β-actin were analyzed by Western Blot. (B) HepG2 cells were transfected with dual NDR1/2 siRNA and mutated p21$^{WAF1/CIP1}$ (ala-146) for 24 hours, then treated with asiatic acid for 12 hours

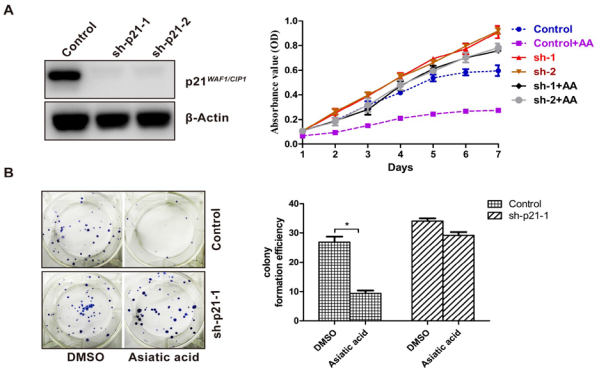

Figure 5. Asiatic Acid regulates Proliferative Activity in HepG2 Cells. (A) The expression of p21$^{WAF1/CIP1}$ was analyzed by Western Blot in HepG2 cells with stable expression of control-shRNA and two different shRNA targeting p21$^{WAF1/CIP1}$ (Control, sh-p21-1 and sh-p21-2). Growth curves for HepG2 cells that stable express control-shRNA, shRNA targeting p21$^{WAF1/CIP1}$ and with the treatment of DMSO or asiatic acid (AA). (B) Representative photographs of colony formation efficiency in HepG2 cells. Quantification of the colony formation efficiency in HepG2 cells. *$p < 0.0002$ compared with the DMSO control group

Asiatic acid down-regulates the expression of NDR1/2 kinase which phosphorylates p21$^{WAF1/CIP1}$ at ser-146

Both isoforms of NDR1/2 kinase were proved to phosphorylate p21$^{WAF1/CIP1}$ at ser-146 and promote its degradation (Cornils et al, 2011). We examined the expression of NDR1/2 kinase in HepG2 cells treated with asiatic acid. A significant decrease of NDR1/2 kinase expression was found in HepG2 cells (Figure 3A). Specific knockdown of NDR1 or NDR2 resulted in increased levels of p21$^{WAF1/CIP1}$ protein and decreased levels of phosphorylated p21$^{WAF1/CIP1}$ protein (ser-146). In addition, dual knockdown of NDR1/2 significantly up-regulated the levels of p21$^{WAF1/CIP1}$ and inhibited the phosphorylation of p21$^{WAF1/CIP1}$ (ser-146) (Figure 3B). These results imply that asiatic acid might inhibit the expression of NDR1/2 kinase and then reduce the phosphorylation of p21$^{WAF1/CIP1}$ (ser-146) to enhance its stability.

Knockdown of NDR1/2 kinase inhibits asiatic acid-induced regulation of p21$^{WAF1/CIP1}$ stability

In order to further investigate the function of asiatic acid in the regulation of p21$^{WAF1/CIP1}$ stability, dual NDR1/NDR2 knockdown was performed in HepG2 cells before asiatic acid treatment. We found that asiatic acid could not regulate the stability of p21$^{WAF1/CIP1}$ protein after the knockdown of NDR1/2 in HepG2 cells (Figure 4A). Furthermore, the levels of MYC-tagged p21$^{WAF1/CIP1}$ (ala-146) protein were also not changed by asiatic acid in MYC-tagged p21$^{WAF1/CIP1}$ (ala-146) transfected HepG2 cells (Figure 4B). These results further support a role for asiatic acid in the regulation of the stability of p21$^{WAF1/CIP1}$ through inhibiting NDR1/2 kinase dependent phosphorylation of p21$^{WAF1/CIP1}$.

Asiatic acid regulates proliferative activity of HepG2 cells

P21$^{WAF1/CIP1}$ acts as a downstream effector of multiple tumor suppressor pathways for promoting anti-proliferative activities (Abbas et al., 2009). We performed proliferation assays (CCK8) to evaluate the cell proliferative activity of HepG2 cells with the treatment of asiatic acid. As shown in Figure 5A, asiatic acid significantly reduced the proliferative activity of HepG2 cells ($p< 0.0001$). Knockdown of p21$^{WAF1/CIP1}$ using Lentiviral-mediated shRNA significantly increased the proliferative activity of HepG2 cells ($p< 0.003$). Differently, asiatic acid did not significantly inhibit the proliferation of HepG2 cells after stable knockdown of p21$^{WAF1/CIP1}$ ($p> 0.28$) (Figure 5A). Furthermore, colony formation assay was also performed to assess the survival rate of HepG2 cells in the present of asiatic acid. Colony formation of HepG2 cells significantly reduced ($p < 0.0002$) with the treatment of asiatic acid. In p21$^{WAF1/CIP1}$ stable knockdown HepG2 cells, asiatic acid weakly reduced the colony formation ($p >0.01$).

Discussion

For thousands of years, natural products have been applied to treat and prevent the human cancers (Mondal et al, 2012). Importantly, nearly 100 plant-derived compounds are currently undergoing clinical trials (Harvey, 2008). Meanwhile, the molecular mechanisms and targets of some compounds are still unclear. Asiatic acid, a plant-derived triterpenoid compound, has anti-tumor effect in some human cancer cell lines (Huang et al., 2012; Guo et al., 2013). Asiatic acid was reported to inhibit cell growth and promote cell apoptosis through mitochondrial death cascade in colon cancer cells (Tang et al., 2007). Asiatic acid could also induce apoptosis in human melanoma cells by generation of ROS (Park et al., 2005; Xu et al., 2012). Down-regulation of the expression and secretion of VEGF by asiatic acid could inhibit the angiogenesis of endothelial cells (Kavitha et al., 2011). Moreover, asiatic acid could inhibit liver fibrosis by blocking TGF-beta/Smad signaling in vivo and in vitro (Tang et al., 2012).

Cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ is a potent cyclin-dependent kinase inhibitor. P21$^{WAF1/CIP1}$ binds to and inhibits the activity of cyclin-dependent kinases

complexes and thus functions as a regulator of cell cycle progression at G1 (Phalke et al., 2012). Recently, a study has found that apoptosis and cell cycle arrest induced by asiatic acid was associated with the increasing levels of $p21^{WAF1/CIP1}$, but the underlying molecular mechanism was still unclear (Hsu et al., 2005). In the present study, we found that asiatic acid increased the levels of $p21^{WAF1/CIP1}$ protein but not the mRNA in HepG2 human hepatoma cells. This implies that asiatic acid may regulate the stability of $p21^{WAF1/CIP1}$.

Several researchers have proved that phosphorylation of $p21^{WAF1/CIP1}$ enhanced the degradation of $p21^{WAF1/CIP1}$ protein through proteasome pathway. For example, ser-114 phosphorylation of $p21^{WAF1/CIP1}$ by glycogen synthase kinase 3β (GSK-3β) promotes its proteasomal degradation after UV Irradiation. Moreover, Human NDR1/NDR2 kinases were reported to phosphorylate $p21^{WAF1/CIP1}$ on ser-146 and regulate the stability of $p21^{WAF1/CIP1}$ (Cornils et al, 2011). We found that levels of phosphorylated $p21^{WAF1/CIP1}$ protein (ser-146) decreased under the treatment of asiatic acid in HepG2 cells. Differently, phosphorylated $p21^{WAF1/CIP1}$ protein (ser-146) accumulated upon the treatment of MG-132 and this effect was not shown in the present of asiatic acid. These results imply that asiatic acid regulates the stability of $p21^{WAF1/CIP1}$ by inhibitory phosphorylation of $p21^{WAF1/CIP1}$ at ser-146. Our data also showed that phosphorylation of $p21^{WAF1/CIP1}$ (ser-114) was not responsible for asiatic acid-induced regulation of $p21^{WAF1/CIP1}$ stability by using a specific anti-phosphoserine antibody and immunoprecipitation assays with mutated $p21^{WAF1/CIP1}$ (ala-146).

We also found a significant decrease of NDR1/2 kinase expression in HepG2 cells with the treatment of asiatic acid. Dual NDR1/2 siRNA knockdown showed significantly reduced phosphorylation of $p21^{WAF1/CIP1}$ (ser-146) and increased levels of $p21^{WAF1/CIP1}$. Further study showed that the levels of mutated $p21^{WAF1/CIP1}$ (ala-146) protein were not changed during the treatment of asiatic acid. Moreover, asiatic acid could not regulate the stability of $p21^{WAF1/CIP1}$ protein after the knockdown of NDR1/2 in HepG2 cells. These results further support a role for asiatic acid in the regulation of $p21^{WAF1/CIP1}$ stability by regulating the phosphorylation of $p21^{WAF1/CIP1}$ (ser-146). Importantly, proliferation assay and colony formation assay have shown that asiatic acid significantly inhibited the proliferative activity of HepG2 and stable knockdown of $p21^{WAF1/CIP1}$ inhibited this effect. In conclusion, our results reveal that asiatic acid enhances the stability of $p21^{WAF1/CIP1}$ via down-regulating the levels of NDR1/2 kinase that phosphorylates $p21^{WAF1/CIP1}$ at ser-146 and inhibits the proliferation of HepG2 cells.

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