# SIAH1-induced p34$^{SEI-1}$ polyubiquitination/degradation mediates p53 preferential vitamin C cytotoxicity

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**Abstract.** Vitamin C is considered as an important anticancer therapeutic agent although this view is debatable. In this study, we introduce a physiological mechanism demonstrating how vitamin C exerts anticancer activity that induces cell cycle arrest and apoptosis. Our previous and current data reveal that p53 tumor suppressor is the prerequisite factor for stronger anticancer effects of vitamin C. In addition, vitamin C-mediated cancer cell cytotoxicity appears to be achieved at least partly through the downregulation of the p34$^{SEI-1}$ oncoprotein. Our previous study showed that p34$^{SEI-1}$ increases the survival of various types of cancer cells by inhibiting their apoptosis. Present data suggest that vitamin C treatment decreases the p34$^{SEI-1}$ expression at the protein level and therefore alleviates its anti-apoptotic activity. Of note, SIAH1, E3 ubiquitin ligase, appears to be responsible for the p34$^{SEI-1}$ polyubiquitination and its subsequent degradation, which is dependent on p53. In summary, vitamin C increases cancer cell death by inducing SIAH1-mediated polyubiquitination/degradation of the p34$^{SEI-1}$ oncoprotein in a p53-dependent manner.

## Introduction

Vitamin C (also known as an ascorbate or ascorbic acid) is an essential micronutrient and considered to function as an anticancer drug (1,2). Many studies have reported the importance of vitamin C cytotoxicity. However, the use of vitamin C as an anticancer therapeutic agent and its effectiveness remain debatable (3-6). In previous reports, 0.3-20 mM of vitamin C has been used for a pharmacological concentration in various cancer cell lines (4,7). This range of concentration is considered to effectively induce cancer cell death *in vitro* and to inhibit tumor growth *in vivo*. This can be achieved through intravenous (i.v.) or intraperitoneal (i.p.) injection, but not through oral dosing (4,8). Vitamin C is thought to modify the expression levels of the proteins that are involved in many different signaling pathways, which ultimately trigger cancer cells to be more susceptible to vitamin C (9,10). Most importantly, vitamin C has a significant advantage because it can selectively kill cancer cells but not normal cells in pharmacological concentrations. It is a crucial characteristic to be an ideal anticancer drug (11,12). Considering these facts, vitamin C can be regarded as an ideal therapeutic agent. However, its action mechanism has not yet been clearly defined. We therefore have attempted to elucidate the physiological mechanism how vitamin C can act as an anticancer agent. We previously showed that vitamin C stabilizes p53 by inducing MDM2 polyubiquitination/degradation. Stabilized p53 in turn aggravates intracellular oxidative stress and consequently causes cancer cells to be more sensitive to vitamin C (13). In this process, p53-dependent enhancement of vitamin C cytotoxicity is caused by increased ROS (reactive oxygen species) generation via a differentially regulated p53 transcriptional network (13). In addition, we found that the p34$^{SEI-1}$ protein level was decreased in response to vitamin C treatment. p34$^{SEI-1}$ has been shown to have multiple biological functions in cells (14-17). In particular, p34$^{SEI-1}$ plays vital roles in cell cycle as a transcriptional co-factor and in apoptosis as an anti-apoptotic oncoprotein (14,18,19). We previously showed that it inhibits cancer cell death by stabilizing XIAP (X-linked inhibitor of apoptosis protein), a potent inhibitor of apoptosis and inhibiting ROS-induced cell death through suppression of ASK1 (18,19). Therefore, p34$^{SEI-1}$ downregulation could be one of efficient means of suppressing tumorigenesis. On the basis of these observations, we hypothesized that decreased p34$^{SEI-1}$ might be responsible for the p53-mediated vitamin C cytotoxicity in cancer cells.

Considering the facts that vitamin C induces p53 activation and p34$^{SEI-1}$ downregulation at the protein level, we initially focused on the p53 function that controls the stability of the target proteins by regulating ubiquitination and subsequent

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*Abbreviations:* p34$^{SEI-1}$, 34 kDa protein encoding *SEI-1* (*Selected with Ink4a-1 as bait*) gene; SIAH1, seven in absentia homolog 1

*Key words:* vitamin C, p53, p34$^{SEI-1}$, SIAH1

proteasomal degradation among the p53-mediated many other regulating systems. This process can be mediated by three different types of enzymes (E1, E2 and E3) that are required for the ubiquitination-proteasomal pathway (20-23). Among them, the expression of SIAH1, E3 ubiquitin-protein ligase, is strongly dependent on p53 (24,25). It is a member of the SIAH family that belong to the ring domain ubiquitin ligases (26). SIAH1 is involved in numerous cellular processes including apoptosis, tumor suppression, cell cycle, axon guidance, transcription regulation and tumor necrosis factor signaling (27-30). In particular, SIAH1 negatively affects cancer cell survival and proliferation. Thus, deregulation of the SIAH1 expression is strongly related to cancer progression (31-33). Accordingly, SIAH1 activation could be an important factor to block tumorigenesis (33).

In this report, we propose that vitamin C treatment of cancer cells enhances p53 activity, which in turn induces the SIAH1-mediated polyubiquitination/degradation of the p34$^{SEI-1}$ oncoprotein and ultimately increases cancer cells cytotoxicity.

## Materials and methods

*Cell lines, cell culture and vitamin C preparation*. HCT116$^{+/+}$ (p53 wild-type) and HCT116$^{-/-}$ (p53 null-type) colon cancer cell lines were provided by Dr Bert Vogelstein (Johns Hopkins University, USA) and MCF7 was obtained from the ATCC (American Type Culture Collection, USA). Each cell line was cultured in DMEM (Dulbecco's modified Eagle's medium) medium (WelGENE, Korea) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (both from Gibco BRL, USA). Cells were cultured at 37˚C in a humidified atmosphere composed of 95% air and 5% $CO_2$. Vitamin C was adjusted to pH 7.0 using sodium hydroxide and it was prepared immediately prior to use.

*Western blotting*. Western blot analysis was performed as previously described (13). The antibodies used in this study were purchased as follows: p53 (Santa Cruz Biotechnology, sc-126, USA), phospho-p53 (Ser46) (Santa Cruz Biotechnology, sc-101764), phospho-p53 (Ser15) (Cell Signaling Technology, cat. no. 9284, USA), phosphor-p38MAPK (Thr180/Tyr182) (Cell Signaling Technology, cat. no. 9211), p21 (Santa Cruz Biotechnology, sc-397), Cdc25C (Santa Cruz Biotechnology, sc-6950), XIAP (Santa Cruz Biotechnology, sc-8789), Caspase 9 (Cell Signaling Technology, cat. no. 9508), Cleaved caspase 9 (Cell Signaling Technology, cat. no. 9505), p34$^{SEI-1}$ (Enzo Life Sciences, cat. no. ALX-804-645, Australia), SIAH1 (Santa Cruz Biotechnology, sc-5505), and γ-tubulin (Santa Cruz Biotechnology, sc-7396).

*Transfection*. Transfection was performed after plating cells in 60 mm$^3$ dish with 90% confluence of cells using Lipofectamine 2000 (Invitrogen, USA). To induce p53 overexpression, cells were transiently transfected with either a control vector (pcDNA3.1) or a p53 overexpressing vector (pcDNA3.1/p53) for 48-72 h. To suppress endogenous p53 level, cells were transfected with a control vector (pLKO.1) or a p53 shRNA silencing vector (pLKO.1/p53-shRNA) for 48 h (13). To induce overexpression of wild-type SIAH1 or mutant type SIAH1$^{C44S}$, cells were transfected with pCMV-SPORT6/SIAH1 or pCMV-SPORT6/SIAH1$^{C44S}$, respectively. pCMV-SPORT6 plasmid was used as a control vector. pCMV-SPORT6/SIAH1 plasmid (clone number hMU004814) was purchased from Korea Human Gene Bank (Medical Genomics Research center, KRIBB, Korea). pCMV-SPORT6/SIAH1$^{C44S}$ plasmid was constructed by introducing SIAH1$^{C44S}$ mutation into wild-type SIAH1 gene in pCMV-SPORT6/SIAH1 (34). Mutagenesis was achieved by using site directed mutagenesis method (Quick change site-directed mutagenesis kit, Stratagene, cat. no. 200519, USA) and the following primer pairs: SIAH1: forward, (5'-GTCTTTTTGAGTGTCC AGTCAGCTTTGACTATGTGTTAC-3'); and reverse (5'-GTAA CACATAGTCAAAGCTGACTGGACACTCAAAAAGAC-3'). To suppress the endogenous SIAH1 expression, cells were transfected with shRNA control vector (pLKO.1) or a SIAH1 silencing plasmid (pLKO.1/SIAH1-shRNA) for 48 h. For modulating of p34$^{SEI-1}$ expression level, cells were transfected with a control vector (pcDNA3.1), a p34$^{SEI-1}$ overexpressing vector (pcDNA3.1/p34$^{SEI-1}$), scrambled siRNA (scRNA) or p34$^{SEI-1}$ small interfering RNA (si-p34$^{SEI-1}$, 5'-CAGUGUGG CUGACAACUUACUGG-3') for 48-72 h. The expression level of each protein was examined by using western blot analysis.

*Flow cytometric analysis*. To detect the cell cycle arrest, vitamin C treated cells were harvested and washed three times with PBS (phosphate-buffered saline). Resulting cells were added with 70% ethanol immediately and maintained at 4˚C for 1-3 h to allow cell fixation. Cells were washed with PBS and then suspended in 0.1 mg/ml propidium iodide solution at 4˚C, followed by addition of 20 µl of 10 mg/ml RNase and incubation at 37˚C for 30 min. Stained samples were analyzed by using flow cytometry (BD Biosciences, USA), in which 1x10$^4$ cells were recorded for each sample. Apoptosis was assessed by Annexin V-binding and propidium iodide staining. Cells were harvested and washed three times with PBS and then incubated with 1 µl of Annexin V-fluorescein isothiocynate (FITC) and 2.5 µl of propidium iodide (Enzo, cat. no. ADI-ADK-700) for 15 min on ice in the dark and analyzed on a FACSCanto machine (BD Biosciences).

*Measurement of vitamin C cytotoxicity*. Cell viability was determined by employing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, USA) assay as described previously (13). HCT116 cells in 96-well plates were treated with 0.3 mM of vitamin C for 8 h. The resulting cells were then incubated with MTT for 4 h at 37˚C and the cell cytotoxicity was measured by using Perkin-Elmer Wallac 1420 Victor2™ microplate reader (Perkin-Elmer, USA). Cell death was measured by using trypan blue exclusion assay.

*Colony forming assay*. Cells were seeded at a density of 2x10$^2$ per 60 mm$^3$ dish and treated with 2 mM of vitamin C for 24 h after pretreatment with or without 2 mM of NAC (N-acetyl-L-cysteine) and then cultured for 17 days. Colonies were fixed with 3.7% formaldehyde and counted after staining with 0.05% crystal violet solution.

*Co-immunoprecipitation (co-IP) and ubiquitination assay*. Cell lysates were prepared by lysing cells in RIPA buffer with

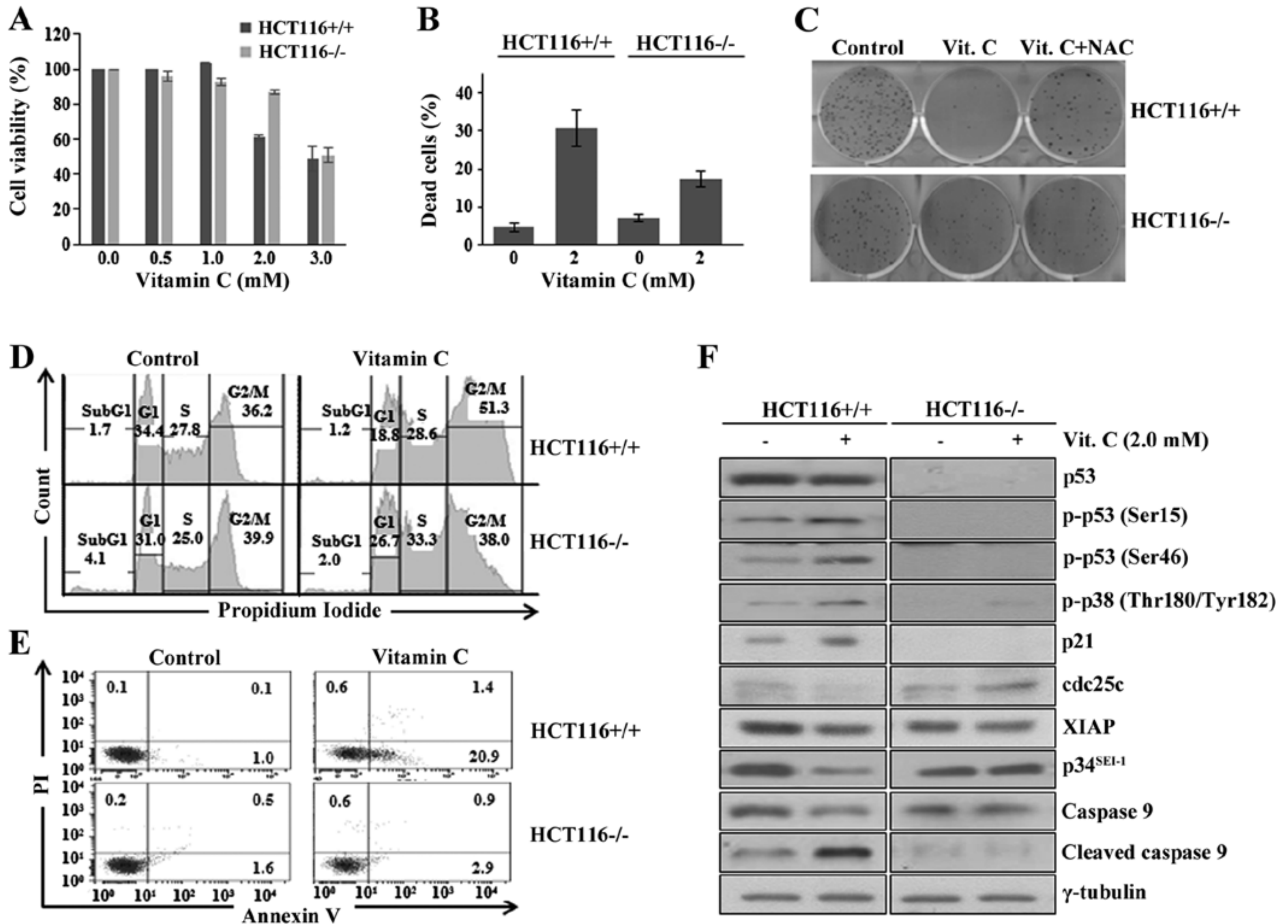


Figure 1. p53-dependent enhancement of vitamin C cytotoxicity. (A) $HCT116^{+/+}$ and $HCT116^{-/-}$ cells were treated with vitamin C at the indicated doses for 8 h and viability was assessed by using the MTT assay. (B) $HCT116^{+/+}$ and $HCT116^{-/-}$ cells were treated with 2 mM of vitamin C for 8 h and then cell death was analyzed by using Trypan blue exclusion. Bars represent the means ± SD from three independent experiments. $p<0.05$. (C) $HCT116^{+/+}$ and $HCT116^{-/-}$ cells were treated with 2 mM of vitamin C after pretreatment with or without 2 mM of NAC and then assessed for the colony formation with crystal violet staining 20 days later. (D) $HCT116^{+/+}$ and $HCT116^{-/-}$ cells were treated with 2 mM of vitamin C for 8 h and then harvested and incubated with propidium iodide followed by flow cytometry analysis. Numbers indicate the percentage of cells. Representative result is shown from three independent experiments. (E) HCT116 cells were treated with 2 mM of vitamin C for 8 h. The cells were evaluated for quantification of apoptosis by using Annexin V/propidium iodide followed by flow cytometry analysis. Representative images are shown from three independent experiments. (F) The expression levels of the proteins responding to the vitamin C treatment were analyzed using western blotting. γ-tubulin was used as a loading control.

protease inhibitors, followed by pre-clearing with protein A/G Sepharose (Santa Cruz Biotechnology, sc-2020). The pre-cleared lysates were incubated with either anti-$p34^{SEI-1}$ (Enzo Life Sciences, cat. no. ALX-804-645) or anti-SIAH1 (Santa Cruz Biotechnology, sc-5505) antibodies for 16 h at 4˚C with continuous agitation, in which the protein A/G Sepharose was added. The resulting complex with antibody and agarose A/G bead was centrifuged at 10,000 x g for 5 min and then washed with RIPA buffer three times. The proteins were eluted from the beads by boiling in an SDS sample buffer. They were then analyzed using a western blotting with the corresponding antibodies. For the $p34^{SEI-1}$ ubiquitination experiments, $HCT116^{+/+}$ or $HCT116^{-/-}$ cells were transfected with indicating plasmids. The cells were then treated with 20 $\mu$M of MG132 proteasome inhibitor (A.G. Scientific, cat. no. M-1157, USA) for 16 h and lysed with RIPA buffer containing protease inhibitors. The lysates were centrifuged to obtain cytosolic proteins. Ubiquitinated $p34^{SEI-1}$ was immunoprecipitated with anti-$p34^{SEI-1}$ antibody, followed by immunoblotting with anti-Ub antibody (Santa Cruz Biotechnology, sc-8017).

## Results

*Vitamin C cytotoxicity can be enhanced by p53.* To confirm the dependence of vitamin C induced cytotoxicity on p53 (13), the effect of p53 on vitamin C induced cytotoxicity was checked after p53 wild-type $HCT116^{+/+}$ and p53 null mutant-type $HCT116^{-/-}$ colon cancer cells were treated with indicated doses of vitamin C (Fig. 1A). Cell viability was significantly decreased in $HCT116^{+/+}$, suggesting that p53 enhances vitamin C induced cytotoxicity. Cell death of vitamin C-treated $HCT116^{+/+}$ was approximately two times higher than that of the $HCT116^{-/-}$ cells (Fig. 1B). Colony forming assay was employed to further examine the relationship between vitamin C cytotoxicity and p53. Colony formation of $HCT116^{+/+}$ cells was greatly decreased by vitamin C treatment compared to those

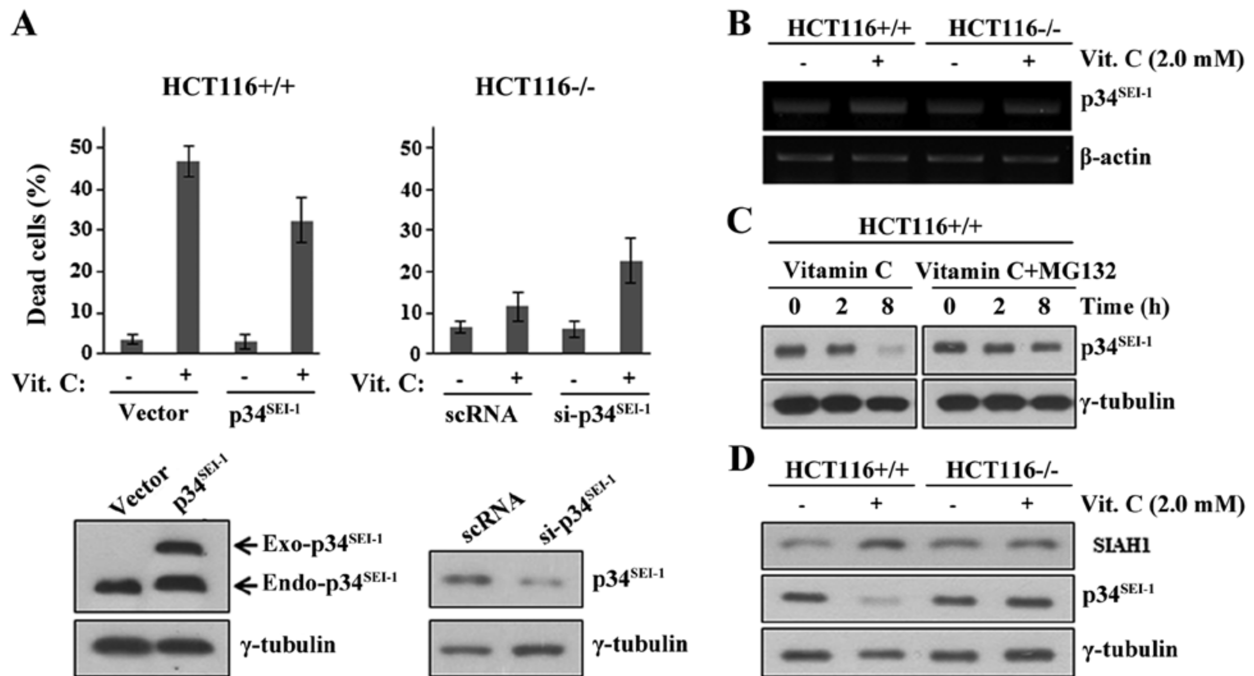


Figure 2. Involvement of $p34^{SEI-1}$ in vitamin C induced cancer cell death. (A) $HCT116^{+/+}$ cells were transfected with pcDNA3.1 control vector or $p34^{SEI-1}$ overexpressing pcDNA3.1/$p34^{SEI-1}$ plasmid for 48 h. $HCT116^{-/-}$ were also transfected with scRNA or $p34^{SEI-1}$ siRNA for 48 h. Transfected cells were treated with 2 mM of vitamin C and then analyzed by Trypan blue exclusion. The percentage of dead cells is shown as the mean values ± SD (n=3, $p<0.05$). The modulated $p34^{SEI-1}$ protein level was analyzed by western blotting. (B) $HCT116^{+/+}$ and $HCT116^{-/-}$ cells were cultured in media treated with or without 2 mM of vitamin C for 8 h and RT-PCR was employed to determine $p34^{SEI-1}$ mRNA level. β-actin was used a loading control. (C) $p34^{SEI-1}$ protein level was determined by western blotting after $HCT116^{+/+}$ cells were grown in the absence or presence of MG132 and treated with vitamin C for indicated times. (D) $HCT116^{+/+}$ and $HCT116^{-/-}$ cells were treated with 2 mM of vitamin C and protein levels of SIAH1 and $p34^{SEI-1}$ were analyzed by using western blotting. γ-tubulin was used as a loading control.

of control and $HCT116^{-/-}$ cells (Fig. 1C). The treatment of NAC antioxidant alleviated vitamin C-mediated decrease in colony forming of $HCT116^{+/+}$, which is consistent with our previous results (13). The data indicate that vitamin C kills cancer cells by enhancing ROS production in the presence of p53. Next question was how p53 can positively affect vitamin C induced cytotoxicity in cancer cells. Previous reports have shown that vitamin C affects various signaling pathways related with cell cycle and apoptosis (12,35-37). In our present study, vitamin C induced G2/M arrest at a higher level in $HCT116^{+/+}$ cells compared to that in $HCT116^{-/-}$ cells (Fig. 1D). Furthermore, much higher level of apoptosis was also induced in $HCT116^{+/+}$ than that of $HCT116^{-/-}$ cells in response to vitamin C treatment (Fig. 1E). These findings indicate that both cell cycle arrest and apoptosis was much more sensitive to vitamin C treatment in wild-type p53 expressing HCT116 cells. Based on these results, western blot analysis was performed to examine the changes in expression levels of genes that play vital roles in cell cycle arrest and apoptosis in vitamin C-treated HCT116 cell lines. Vitamin C treatment to $HCT116^{+/+}$ resulted in phosphorylation of p53 on serine 15 (p-p53 Ser15) and serine 46 (p-p53 Ser46) residues probably due to p38MAPK activation (Fig. 1F). This conclusion was supported by treatment of p38 inhibitor, SB203580, in which phosphorylation levels of both p38 and p53 (Ser46) were decreased (data not shown). It is well known that the phosphorylation of p53 on the serine 15 residue leads to p53 stabilization and activation and the phosphorylation of p53 on the serine 46 residue induces apoptosis (38,39) by activating p38MAPK via phosphorylation on two residues, threonine 180 and threonine 182 (Thr180/Tyr182) (13,40,41). The effect of vitamin C on cell cycle was checked by examining the expression levels of p21 and cdc25c, major cell cycle regulators. Vitamin C treatment increased and decreased p21 and cdc25c protein levels only in $HCT116^{+/+}$ cells, respectively. It suggests that vitamin C treatment induced G2/M cell cycle arrest in a p53-dependent manner. Next, we checked the altered expression levels of apoptosis relating proteins, XIAP, $p34^{SEI-1}$, and caspase 9. XIAP plays as a potent inhibitor of apoptosis by inhibiting caspase 3, 7 and 9 activation and its overexpression confers resistance to tumor chemotherapy (42,43). Interestingly, XIAP expression is known to be negatively regulated by p53 (44). Therefore, XIAP protein level was checked to examine involvement of XIAP in vitamin C-mediated apoptosis. XIAP expression was significantly decreased in $HCT116^{+/+}$ cells but not in $HCT116^{-/-}$ cells in response to vitamin C. According to our previous result, XIAP stability can be greatly increased by $p34^{SEI-1}$ (19). Very interestingly, $p34^{SEI-1}$ protein level was decreased only in $HCT116^{+/+}$ cells but not in $HCT116^{-/-}$ cells. It suggests that p53 is responsible for the downregulation of the XIAP and $p34^{SEI-1}$ proteins in response to the vitamin C treatment. This conclusion was confirmed by checking caspase 9 activation, one of direct targets of XIAP (45,46). Cleaved active form of caspase 9 was found to be increased in vitamin C-treated $HCT116^{+/+}$ but not in $HCT116^{-/-}$ (Fig. 1F). The data imply that vitamin C may initiate the apoptotic process through downregulating XIAP and $p34^{SEI-1}$ in p53 wild-type cells. Considering that $p34^{SEI-1}$ plays important roles in tumorigenesis as a vital regulator of

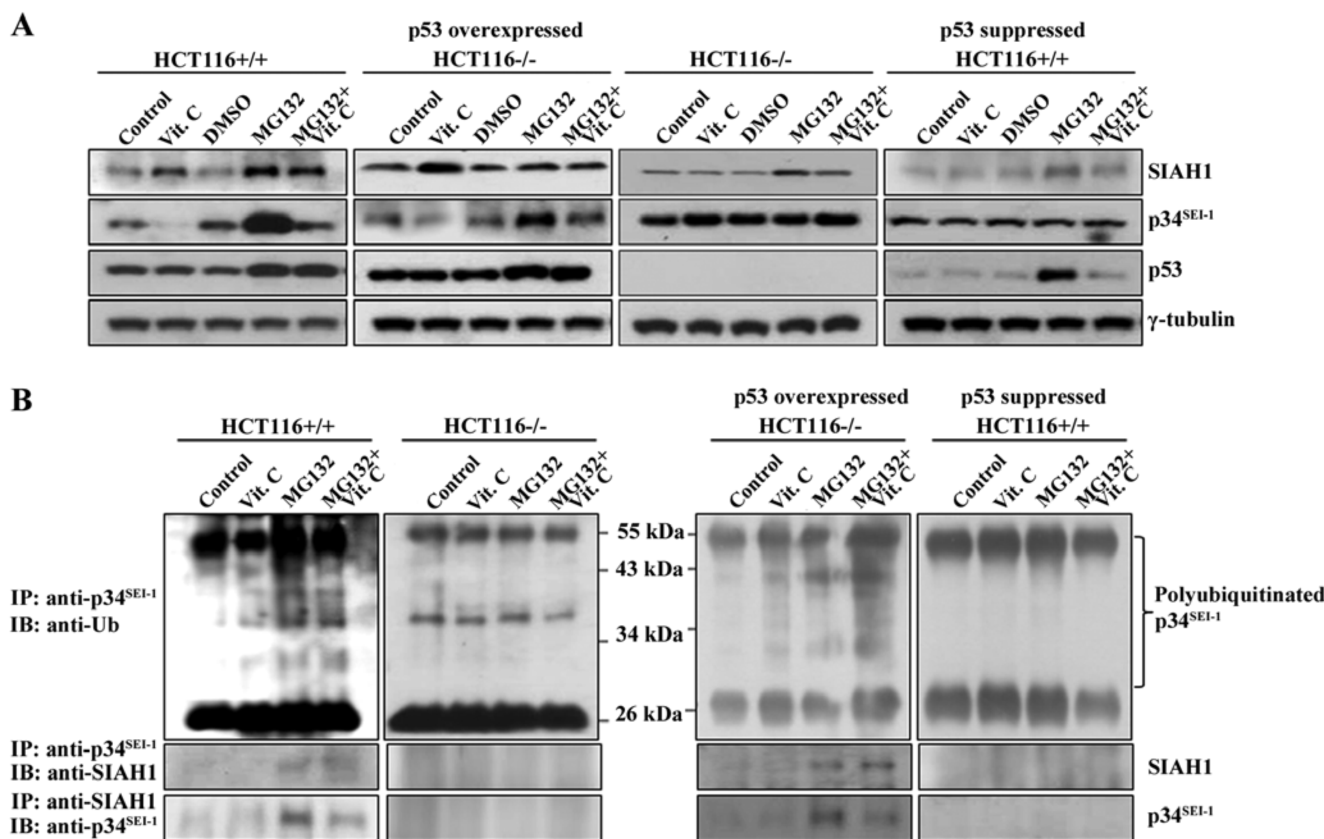


Figure 3. Polyubiquitination and degradation of p34$^{SEI-1}$ by vitamin C induced p53 activation. (A) HCT116$^{+/+}$ and HCT116$^{-/-}$ cells were transfected with p53 overexpressing pcDNA3.1/p53 or p53 suppressing pLKO/p53-shRNA vectors, respectively. Effect of p53 on the expression levels of the SIAH1 and p34$^{SEI-1}$ was examined using western blot analysis with the corresponding antibodies. The cells were pre-treated with DMSO or 20 $\mu$M of MG132, a 26S proteasome inhibitor for 1 h and then 2 mM of vitamin C were added for 8 h. γ-tubulin was used as a loading control. (B) HCT116$^{+/+}$ and HCT116$^{-/-}$ cells were transfected with p53 overexpressing pcDNA/p53 or p53 suppressing pLKO/p53-shRNA. Cells were pre-treated or with or without 20 $\mu$M of MG132 for 1 h and then treated with 2 mM of vitamin C for 8 h. The cell lysates were immunoprecipitated (IP) with anti-p34$^{SEI-1}$ antibody and immunoblotted (IB) with anti-ubiquitin.

cell cycle and apoptosis, we hypothesized that p34$^{SEI-1}$ might play critical roles in p53-dependent vitamin C-mediated cell cycle arrest and apoptosis.

*p34$^{SEI-1}$ is involved in vitamin C-induced cell death*. To determine whether p34$^{SEI-1}$ is involved in the p53-dependent vitamin C cytotoxicity, we examined whether the altered expression of p34$^{SEI-1}$ modulates the vitamin C-mediated cell death in a p53-dependent manner. In HCT116$^{+/+}$ cells, p34$^{SEI-1}$ overexpression alleviated cell death after vitamin C treatment compared with vector only transfected control cells (Fig. 2A). In contrast, p34$^{SEI-1}$ suppression in HCT116$^{-/-}$ cells increased cell death compared with the control cells (Fig. 2A). These observations indicate that the p34$^{SEI-1}$ negatively affects vitamin C-induced cell death. The next question was how vitamin C downregulates p34$^{SEI-1}$ expression in a p53-dependent manner. Our result showed that p34$^{SEI-1}$ mRNA level was not changed upon vitamin C treatment in either HCT116$^{+/+}$ or HCT116$^{-/-}$ cells (Fig. 2B). However, p34$^{SEI-1}$ protein level was greatly decreased at 8 h after vitamin C treatment but it was not changed by addition of MG132 (Fig. 2C). The data suggest that vitamin C downregulated p34$^{SEI-1}$ at the protein level proteasome-dependently. To identify the E3 ligase responsible for the p34$^{SEI-1}$ ubiquitination/degradation in the vitamin C treated cells, we initially focused on SIAH1 E3 ubiquitin ligase because SIAH1 expression is strongly dependent on p53 (24,32,34). Our data showed that SIAH1 protein level was significantly increased in HCT116$^{+/+}$ cells, but not in HCT116$^{-/-}$ cells upon treatment of vitamin C, in which inverse relationship was found between SIAH1 and p34$^{SEI-1}$ expression levels (Fig. 2D). This result implies that the SIAH1 might be responsible for the p34$^{SEI-1}$ degradation in p53 expressing cells in response to vitamin C treatment. Our collective data support the view that p34$^{SEI-1}$ is involved in vitamin C-induced cell death in p53 wild-type cells.

*Vitamin C induces polyubiquitination and degradation of p34$^{SEI-1}$ in a p53-dependent manner*. In order to reconfirm the essential function of p53 in the p34$^{SEI-1}$ downregulation via the SIAH1 E3 ligase, HCT116$^{-/-}$ and HCT116$^{+/+}$ cells were transfected with a p53 overexpressing vector (pcDNA3.1/p53) or a p53 silencing vector (pLKO.1/p53-shRNA), respectively, and then the SIAH1 and p34$^{SEI-1}$ expression levels were checked after the vitamin C treatment. Upon the reintroduction of the wild-type p53 into the p53-deficient HCT116$^{-/-}$, vitamin C was able to downregulate intracellular p34$^{SEI-1}$ such as the case of HCT116$^{+/+}$cells (Fig. 3A). On the contrary, the p53 silenced HCT116$^{+/+}$ cells exhibited the similar result to that of HCT116$^{-/-}$ cells (Fig. 3A). This result suggests that p53 is required for SIAH1 upregulation and p34$^{SEI-1}$ downregulation

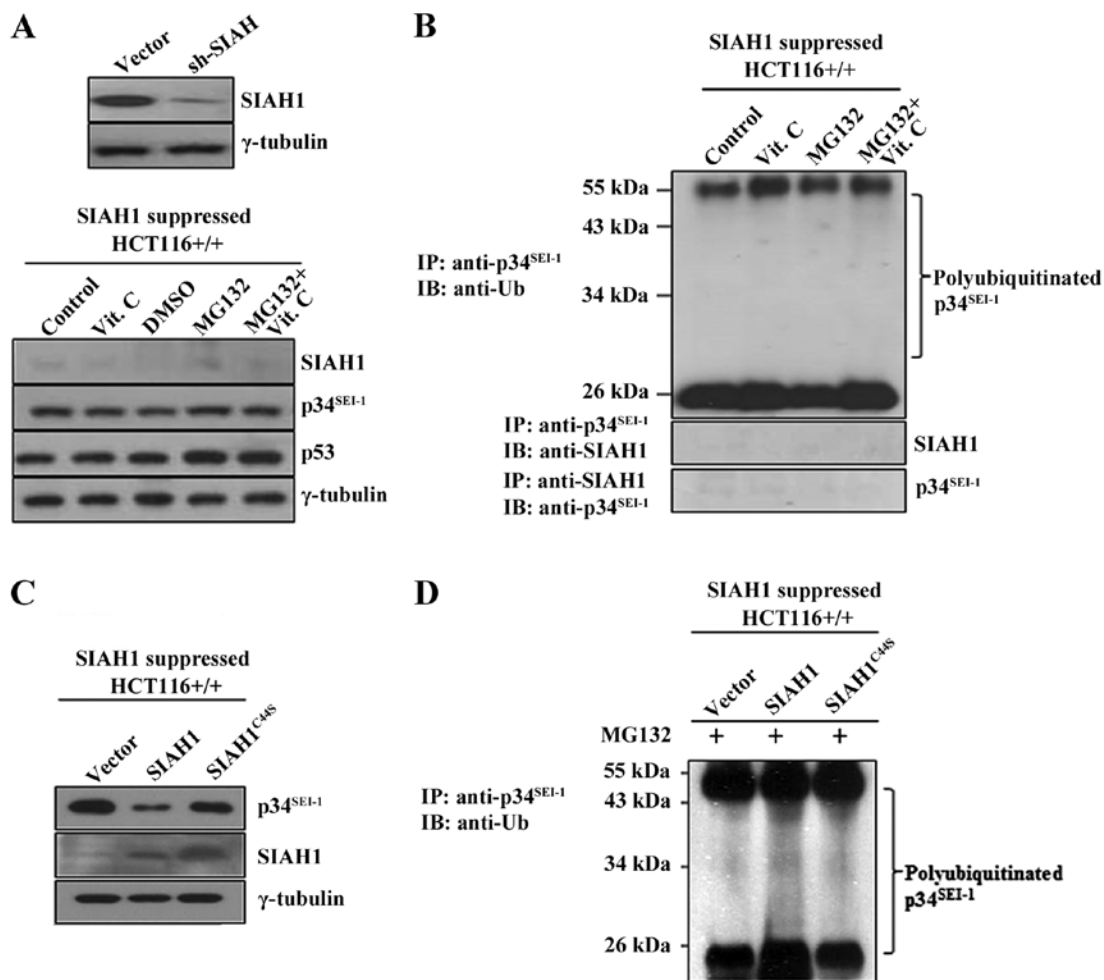


Figure 4. Effect of SIAH1 on the polyubiquitination/degradation of $p34^{SEI-1}$. (A) $HCT116^{+/+}$ cells were transfected with pLKO.1 control or pLKO.1/SIAH1-shRNA (sh-SIAH1) vector. SIAH1 suppressed $HCT116^{+/+}$ cells were pre-treated with or without 20 $\mu$M of MG132 for 1 h prior to treatment with 2 mM of vitamin C for 8 h. (B) $HCT116^{+/+}$ cells were transfected with a SIAH1 suppressing pLKO.1/SIAH1-shRNA vector and then treated with or without 2 mM of vitamin C in absence or presence of 20 $\mu$M MG132. $p34^{SEI-1}$ ubiquitination was examined by using immunoprecipitation (IP) with anti-$p34^{SEI-1}$ and immunoblot analysis (IB) with anti-ubiquitin antibody. Expression levels of SIAH1 and $p34^{SEI-1}$ were checked by western blotting (WB). (C) $HCT116^{+/+}$ cells were transfected with wild-type SIAH1 expressing pCMV-SPORT6/SIAH1 or ligase deficient mutant-type $SIAH1^{C44S}$ expressing pCMV-SPORT6/$SIAH1^{C44S}$ plasmid and the SIAH1 and $p34^{SEI-1}$ protein levels were checked by using western blotting. (D) The $HCT116^{+/+}$ cells were transfected with pCMV-SPORT6/SIAH1 or pCMV-SPORT6/$SIAH1^{C44S}$ plasmid in the presence of 1 $\mu$M of MG132 for 24 h. $p34^{SEI-1}$ ubiquitination was examined using immunoprecipitation (IP) with anti-$p34^{SEI-1}$ and immunoblot analysis (IB) with anti-ubiquitin antibody.

in response to the vitamin C. To further elucidate whether the proteasome-dependent degradation of $p34^{SEI-1}$ is dependent on p53 under vitamin C treated condition, immunoprecipitation was employed to examine whether vitamin C can induce $34^{SEI-1}$ polyubiquitination depending on p53. As shown in Fig. 3B, the vitamin C treatment significantly induced polyubiquitination of the endogenous $p34^{SEI-1}$ in the $HCT116^{+/+}$ and p53 overexpressing $HCT116^{-/-}$ cells compared with the control cells. However, this effect was not detected in the $HCT116^{-/-}$ and p53 silenced $HCT116^{+/+}$ cells (Fig. 3B). Our immunoprecipitation data also revealed that SIAH1 directly interacts with $p34^{SEI-1}$ (Fig. 3B). Collectively, these results suggest that p53 induce the direct interaction of SIAH1 with $p34^{SEI-1}$ and subsequent $p34^{SEI-1}$ ubiquitination/degradation under the conditions of a vitamin C treatment. In addition, we performed similar experiment in a wild-type p53 expressing MCF7 breast cancer cell line. $p34^{SEI-1}$ was also polyubiquitinated and degraded by the vitamin C in a wild-type p53 expressing MCF7 cells, but not in the p53 silenced MCF7 cells (data not shown). Altogether, our results strongly suggest that p53 induces the $p34^{SEI-1}$ polyubiquitination and subsequent degradation under the conditions of vitamin C treatment.

*SIAH1 is responsible for polyubiquitination/degradation of $p34^{SEI-1}$*. In order to confirm the SIAH1 requirement for the p53-dependent $p34^{SEI-1}$ polyubiquitination/degradation, the effect of SIAH1 on the expression and polyubiquitination of $p34^{SEI-1}$ was analyzed in the SIAH1 suppressed $HCT116^{+/+}$ cells after the vitamin C treatment. The vitamin C treatment did not induce the downregulation and polyubiquitination of $p34^{SEI-1}$ in SIAH1 deficient $HCT116^{+/+}$ cells even in the presence of the wild-type p53 (Fig. 4A and B). This result strongly suggests that SIAH1 is critically required for p53-mediated $p34^{SEI-1}$ polyubiquitination/degradation. In an extended study, $p34^{SEI-1}$ protein level was decreased in wild-type SIAH1 expressing $HCT116^{+/+}$ cells, but not in ligase deficient mutant-type $SIAH1^{C44S}$ expressing cells (Fig. 4C). Our data also show that $p34^{SEI-1}$ polyubiquitination was more increased in wild-type

SIAH1 expressing cells compared to control and $SIAH1^{C44S}$ expressing $HCT116^{+/+}$ cells (Fig. 4D). Taken together, these results strongly suggest that vitamin C-mediated $p34^{SEI-1}$ polyubiquitination/degradation is achieved in a SIAH1-dependent manner.

## Discussion

We previously reported that p53 makes cancer cells more sensitive to vitamin C treatment and therefore increases its cytotoxicity (13). According to our data, vitamin C induces the MDM2 polyubiquitination/degradation and consequently stabilizes p53 (13). It is in turn considered to modify the expression levels of different target proteins that are involved in many other signaling pathways to render cancer cells more susceptible to vitamin C. In our present study, our collective data support the view that p53 induces SIAH1-mediated polyubiquitination/degradation of the $p34^{SEI-1}$ oncogenic protein upon vitamin C treatment. However, there are controversial studies on the dependence of SIAH1 expression on p53. For example, tumor suppressor HIPK2 stability is regulated by SIAH1-mediated polyubiquitination/degradation under stress conditions, in which SIAH1 expression level is increased by p53 (34). On the contrary, Frew *et al* proposed that p53 overexpression has no effect on the expression of SIAH1 genes in a p53-null mouse erythroleukemic cell line (47). We considered that different stimuli or different cell types may evoke these discrepant results.

It has been reported that oxidative stress inducing compounds, sodium nitroprusside or cucurbitacin B can activate p53, which inhibits the cell cycle at the G2 phase (48-50). High dose of vitamin C also causes oxidative stress to cancer cells and functions as anticancer therapeutic agent (7,11). Our current data showed that pharmacological concentration of vitamin C induces cell cycle arrest at the G2 phase as well as apoptosis in p53-dependent manner. $p34^{SEI-1}$ is well-known as a positive regulator of the cell cycle (15) and SIAH1 expression is also closely related to G2 arrest (30). These data imply that vitamin C inducible G2 arrest might be at least partly influenced by SIAH1-mediated $p34^{SEI-1}$ degradation in the presence of p53.

In conclusion, our previous and current data suggest that wild-type p53 is the prerequisite factor for stronger anticancer effects of vitamin C, in which vitamin C cytotoxicity appears to be achieved at least partly through the downregulation of the $p34^{SEI-1}$ in a SIAH1-dependent manner. Therefore, $p34^{SEI-1}$ can be developed as a new target protein for an efficient therapeutic agent against various cancers.

## Acknowledgements

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