BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 23(4), 339-344 (2015)

Anti-Proliferative Effect of Naringenin through p38-Dependent Downregulation of Cyclin D1 in Human Colorectal Cancer Cells

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Abstract

Naringenin (NAR) as one of the flavonoids observed in grapefruit has been reported to exhibit an anti-cancer activity. However, more detailed mechanism by which NAR exerts anti-cancer properties still remains unanswered. Thus, in this study, we have shown that NAR down-regulates the level of cyclin D1 in human colorectal cancer cell lines, HCT116 and SW480. NAR inhibited the cell proliferation in HCT116 and SW480 cells and decreased the level of cyclin D1 protein. Inhibition of proteasomal degradation by MG132 blocked NAR-mediated cyclin D1 downregulation and the half-life of cyclin D1 was decreased in the cells treated with NAR. In addition, NAR increased the phosphorylation of cyclin D1 at threonine-286 and a point mutation of threonine-286 to alanine blocked cyclin D1 downregulation by NAR. p38 inactivation attenuated cyclin D1 downregulation by NAR. From these results, we suggest that NAR-mediated cyclin D1 downregulation may result from proteasomal degradation through p38 activation. The current study provides new mechanistic link between NAR, cyclin D1 downregulation and cell growth in human colorectal cancer cells.

Key Words: Naringenin, Cyclin D1, Cancer chemoprevention, Human colorectal cancer

INTRODUCTUIN

Cancer as a multifactorial heterogeneous disease is one of the major causes of mortality worldwide (Siegel *et al*., 2014). Although the development of anticancer therapies has been substantially progressed, the incidence of cancer is still increasing worldwide (Tanaka, 2013). Thus, the demand for the development of new anti-cancer agents has been raised (Ravishankar *et al*., 2013). Many anticancer agent has been established from the natural products (Ravishankar *et al*., 2013). Flavonoids have attracted attention due to their various pharmacological properties including anti-oxidant, anti-mutagenic, anti-bacterial, anti-angiogenic, anti-inflammatory, anti-allergic, modulators of enzymatic activities and anti-cancer activity (Kawai *et al*., 2007; Garcia-Lafuente *et al*., 2009; Cushnie and Lamb, 2011). Thus, flavonoids have been regarded as one of the potential cancer chemopreventive agents (Kuo, 1997).

Naringenin, a naturally occurring flavonoid in grapefruit, has been reported to exhibit various pharmacological effects such as anti-inflammatory, anti-mutagenic, anti-atherogenic, hepatoprotective and anti-cancer activity (Choi *et al*., 1994; Lee *et al*., 2001; Ekambaram *et al*., 2008; Amaro *et al*., 2009; Renugadevi and Prabu, 2010). Recently, there is growing evidence that naringenin induces apoptosis in human cancer cells, while it shows no toxic effect on normal cells at a similar dose (Kanno *et al*., 2003; Kanno *et al*., 2004; Kanno *et al*., 2006; Wang *et al*., 2006). In addition, naringenin suppresses both cell proliferation and motility through regulating the PI3K and MAPK pathway (Totta *et al*., 2004; Galluzzo *et al*., 2008; Bulzomi *et al*., 2012).

Cyclin D1 as one of the proto-oncogenes regulates G1 to S phase progression in many different cell types through phosphorylating and inactivating the retinoblastoma protein by complex with CDK4/6 (Kato *et al*., 1993). Cyclin D1 overexpression as a common event in cancer has been observed in 68.3% of human colorectal cancer, which indicates that deregulation of cyclin D1 is associated with colorectal tumorigenesis (Bahnassy *et al*., 2004). Therefore, it has been accepted that

Open Access **http://dx.doi.org/10.4062/biomolther.2015.024**

Received Mar 2, 2015 **Revised** Apr 15, 2015 **Accepted** Apr 22, 2015
Published online Jul 1, 2015

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the control of cyclin D1 level may provide a promising chemopreventive and therapeutic way for human colorectal cancer.

Phytochemicals exert their anti-proliferative effect through the regulation of various molecular targets associated with cell cycle. Based on the anti-proliferative effect of naringenin, the elucidation of additional potential mechanisms of NAR for anti-proliferative effect may be necessary. Thus, we hypothesize that this anti-proliferative effect may be mediated by its ability to down-regulate cyclin D1 in human colorectal cancer cells. To assess the effect of naringenin on the regulation of cyclin D1, we employed human colorectal cancer cell lines, HCT116 and SW480. In the present study, we investigated the effect of naringenin against cyclin D1-downregulatory mechanism. We found that NAR facilitated the proteasomal degradation of cyclin D1. Furthermore, p38 might be involved in cyclin D1 degradation.

MATERIALS AND METHODS

Reagents

Naringenin and 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). Antibodies against cyclin D1, phospho-cyclin D1 (Thr286), phospho-p38, total p38, HA-tag and β-actin were purchased from Cell Signaling (Bervely, MA, USA). SB203580 (p38 inhibitor) and PD98059 (ERK1/2 inhibitor) were purchased from Calbiochem (San Diego, CA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Cell culture and treatment

HCT116 and SW480 were purchased from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM/F-12 supplemented with 10% fatal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37°C under a humidified atmosphere of 5% CO_2. Naringenin (NAR) was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

Cell proliferation assay

HCT116 and SW480 cells were plated onto 96-well plated and grown overnight. The cells were treated with NAR at the indicated concentrations for 24 h. Then, the cells were incubated with 50 μl of MTT solution (1 mg/ml) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

After NAR treatment, total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and 1 μg of total RNA was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was performed using PCR Master Mix Kit (Promega, Madison, WI, USA) with human primers for cyclin D1 and GAPDH as followed : cyclin D1: forward 5'-aactacctggaccgcttcct-3' and reverse 5'-ccacttgagcttgttcacca-3', GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

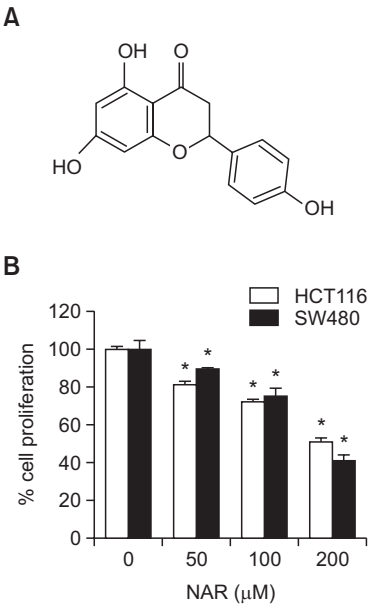

Fig. 1. Chemical structure (A) and effect of NAR on cell growth (B). HCT116 and SW480 cells were treated with NAR at the indicated concentrations for 24. Cell growth was measured using MTT assay system and expressed as % cell growth. *p<0.05 compared to cells without NAR.

SDS-PAGE and Western blot

After NAR treatment, cells were washed with 1×phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich), and centrifuged at 15,000×g for 10 min at 4°C. After determining protein concentration by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA), the proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with specific primary antibodies in 5% non-fat dry milk at 4°C overnight. After three washes with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized in Polaroid film.

Expression vectors

Wild type HA-tagged cyclin D1 and point mutation of T286A of HA-tagged cyclin D1 were provided from Addgene (Cambridge, MA, USA). Transient transfection of the vectors was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction.

Statistical analysis

All the data are shown as mean ± SEM (standard error of

mean). Statistical analysis was performed with one-way ANOVA followed by Dunnett's test. Differences with *p<0.05 were considered statistically significant.

RESULTS

Effect of NAR on the cell growth in human colorectal cancer cell lines, HCT116 and SW480

NAR has been reported to possesses three hydroxyl groups in aromatic rings and this structure is responsible for its potent antioxidant activity and various pharmacological property (Fig. 1A) (van Acker *et al*., 2000). To evaluate whether NAR affects the proliferation of human colorectal cancer cells, HCT116 and SW480 cells were treated with NAR at the indicated concentrations for 24 h and the cell proliferation was measured by MTT assay. As shown in Fig. 1B, NAR suppressed the cell proliferation by 19% and 11% at 50 μM, 28% and 25% at 100 μM, and 49% and 59% at 200 μM in HCT116 and SW480 cells, respectively.

Effect of NAR on the level of cyclin D1 in HCT116 and SW480 cells

To test whether NAR down-regulates the level of cyclin D1, HCT116 and SW480 cells were treated with 0, 50, 100 and 200 μM of NAR for 24 h and then Western blot was performed. As shown in Fig. 2A, NAR dose-dependently decreased the level of cyclin D1 in both HCT116 and SW480 cells. In time-course experiments (Fig. 2B), cyclin D1 started to decrease at 3 h after NAR treatment in HCT116 cells, while NAR decreased cyclin D1 level at 1 h after treatment in SW480 cells. To determine if downregulation of cyclin D1 by NAR is responsible for the transcriptional inhibition, we evaluated mRNA level of cyclin D1 in HCT116 and SW480 cells. As a result (Fig. 2C), 200 μM of NAR slightly attenuated the expression of cyclin D1 mRNA in HCT116 cells, which indicates that cyclin D1 downregulation by high dose (200 μM) of NAR may partially result from the transcriptional inhibition of cyclin D1. However, mRNA level of cyclin D1 was not affected by treatment of NAR in SW480 cells. From these results, NAR may decrease protein stability of cyclin D1 because downregulation of cyclin D1 protein level by NAR is more sensitive than that of mRNA level.

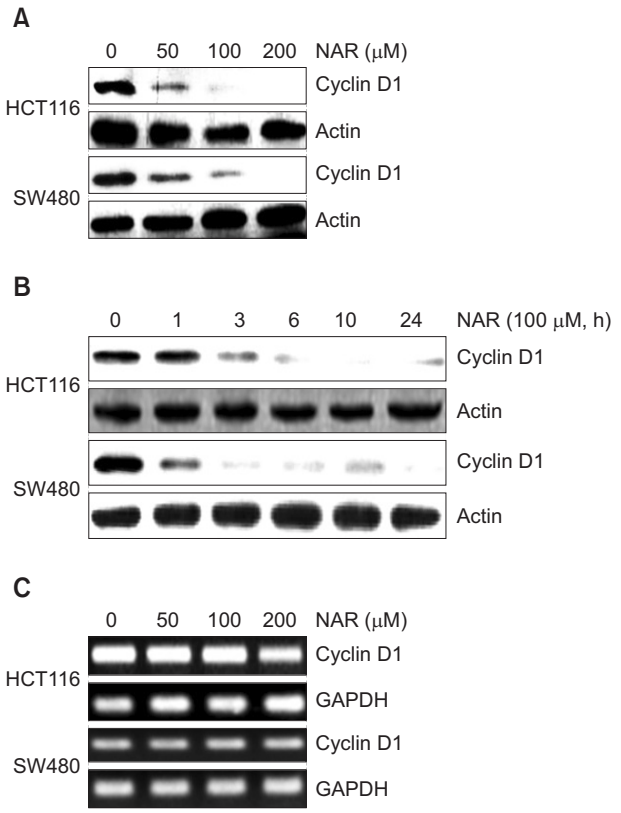

Fig. 2. Effect of NAR on cyclin D1 level in HCT116 and SW480 cells. (A) HCT116 and SW480 cells were plated overnight and then treated with NAR at the indicated concentrations for 24 h. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against cyclin D1 and actin. (B) HCT116 and SW480 cells were plated overnight and then treated with 100 μM of NAR for the indicated times. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against cyclin D1 and actin. (C) HCT116 and SW480 cells were plated overnight and then treated with NAR at the indicated concentrations for 24 h. For RT-PCR analysis of cyclin D1 gene expression, total RNA was prepared after NAR treatment for 24 h. Actin and GAPDH were used as internal control for Western blot analysis and RP-PCR, respectively.

Effect of NAR on cyclin D1 proteasomal degradation in HCT116 and SW480 cells

To evaluate whether NAR affects cyclin D1 proteasomal degradation, the cells were pretreated with the proteasome inhibitor (MG132) and then co-treated with NAR. As shown in Fig. 3A, pre-treatment of MG132 blocked NAR-mediated downregulation of cyclin D1 in HCT116 and SW480 cells. To verify these results, the cells were pre-treated with DMSO or NAR and then exposed to cycloheximide for indicated times. As shown in Fig. 3B, NAR treatment decreased half-life of cyclin D1 protein in HCT116 cells.

Effect of NAR on threonine-286 phosphorylation of cyclin D1 in HCT116 cells

To evaluate whether NAR affects cyclin D1 phosphorylation at threonine-286, HCT116 cells were treated with NAR for the indicated times. As shown in Fig. 4A, threonine-286 of cyclin D1 started to be phosphorylated at 1 h after NAR treatment. For the contribution of its phosphorylation to NAR-induced proteasomal degradation of cyclin D1, HCT116 cell were transfected with the expression vectors, HA-tagged wild type cyclin D1 or HA-tagged T286A cyclin D1 and then treated with NAR. As a result (Fig. 4B), NAR down-regulated HA-tag in the cells transfected with wild type cyclin D1, while the transfection with T286A cyclin D1 blocked NAR-induced down-regulation of HA-tag.

To elucidate the upstream kinases associated with NAR-induced proteasomal degradation of cyclin D1, HCT116 cells were pretreated with PD98059 (ERK1/2 inhibitor) or SB203580 (p38 inhibitor) and then co-treated with NAR. As shown in Fig. 4C, ERK1/2 inhibition by PD98059 did not inhibit NAR-induced cyclin D1 down-regulation compared to the cell treated with DMSO and NAR. But, cyclin D1 down-regulation by NAR was attenuated in SB203580-pretreated cells. Then, we tested if NAR activates p38 and observed that NAR induced p38 phosphorylation (Fig. 4D). In addition, we

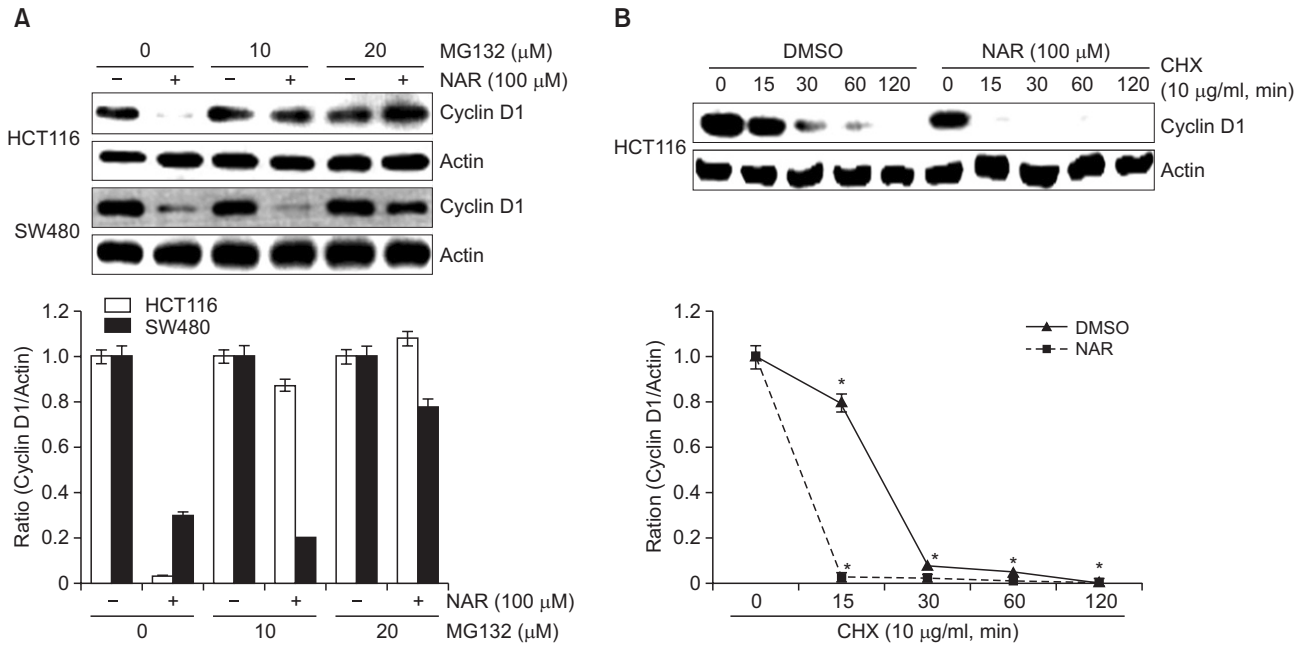

Fig. 3. Proteasomal degradation of cyclin D1 by NAR in HCT116 cells. (A) HCT116 cells were plated overnight. The cells were pretreated with MG132 for 2 h and then co-treated with NAR for the addition 3 h. (B) HCT116 cells were pretreated with DMSO for 100 μM of NAR for 3 h and then co-treated with 10 μg/ml of cycloheximide (CHX) for the indicated times. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against cyclin D1. Actin was used as internal control.

observed that SB203580 attenuated NAR-mediated cyclin D1 phosphorylation (Fig. 4E).

DISCUSSION

There is substantial evidence that flavonoids can inhibit carcinogenesis *in vitro* and *in vivo* (Ravishankar *et al*., 2013). Anticancer effect of flavonoids against colorectal cancer cells was also reported extensively (Imai *et al*., 2009; Ramos, 2007).

NAR as a flavonoid from grapefruit has been reported to exert an anti-cancer properties in human colorectal cancer cells (Chidambara Murthy *et al*., 2012; Yoon *et al*., 2013). However, more detailed mechanism by which naringenin (NAR) exerts anti-cancer properties still remains unanswered. Thus, in this study, we have shown that NAR down-regulates the level of cyclin D1 in human colorectal cancer cell lines, HCT116 and SW480.

The level of cyclin D1 can be regulated by multiple mechanisms. One is through transcriptional regulation. In this study, we observed that NAR decreased the protein level of cyclin D1 from the concentration of 50 μM in both HCT116 and SW480 cells. But, the concentration (50-100 μM of NAR) did not affect the mRNA level of cyclin D1. These data indicates that NAR may decrease protein stability of cyclin D1.

Another mechanism to regulating cyclin D1 level is through the activation of proteasomal degradation and deregulated cyclin D1 degradation appears to be responsible for the increased levels of cyclin D1 in several cancers (Alao, 2007). In this study, we showed that MG132-induced inhibition of proteasomal degradation attenuated cyclin D1 down-regulation by NAR and the half-life of cyclin D1 was attenuated in the cells treated with NAR. These data indicate that the primary mechanism of NAR for down-regulation of cyclin D1 may be the enhancement of proteasomal degradation. Since cyclin D1 proteasomal degradation has been regarded as one of important anti-cancer mechanisms (Spinella *et al*., 1999; Mukhopadhyay *et al*., 2002; Huang *et al*., 2005), cyclin D1 proteasomal degradation may be one of the molecular targets for the anti-cancer activity of NAR.

Threonine-286 phosphorylation is associated with cyclin D1 phosphorylation and its mutation is observed in human cancer cells (Benzeno *et al*., 2006). Thus, failure of phosphorylation-dependent degradation of cyclin D1 may contribute to the development of cancer. (Diehl *et al*., 1997). In this study, we demonstrated that NAR phosphorylates threonine-286 of cyclin D1, and the mutation of threonine-286 to alanine (T286A) failed to NAR I-mediated cyclin D1 proteasomal degradation. Therefore, cyclin D1 proteasomal degradation by NAR may result from its threonine-286 phosphorylation.

Phosphorylation-dependent degradation of cyclin D1 can be regulated by ERK1/2 and p38 (Okabe *et al*., 2006; Thoms *et al*., 2007). This study is the first to show that NAR activates p38 and the inactivation of p38 blocks NAR-mediated cyclin D1 degradation. Because p38 has been shown to directly phosphorylate cyclin D1 for proteasomal degradation (Casanovas *et al*., 2000), we suggest that p38 targets cyclin D1 directly in response to NAR.

Taken together, our findings demonstrate that NAR-induced proteasomal degradation of cyclin D1 might inhibit the cell proliferation in human colorectal cancer cells. Furthermore, this study provides information on molecular events for the anticancer activity of NAR.

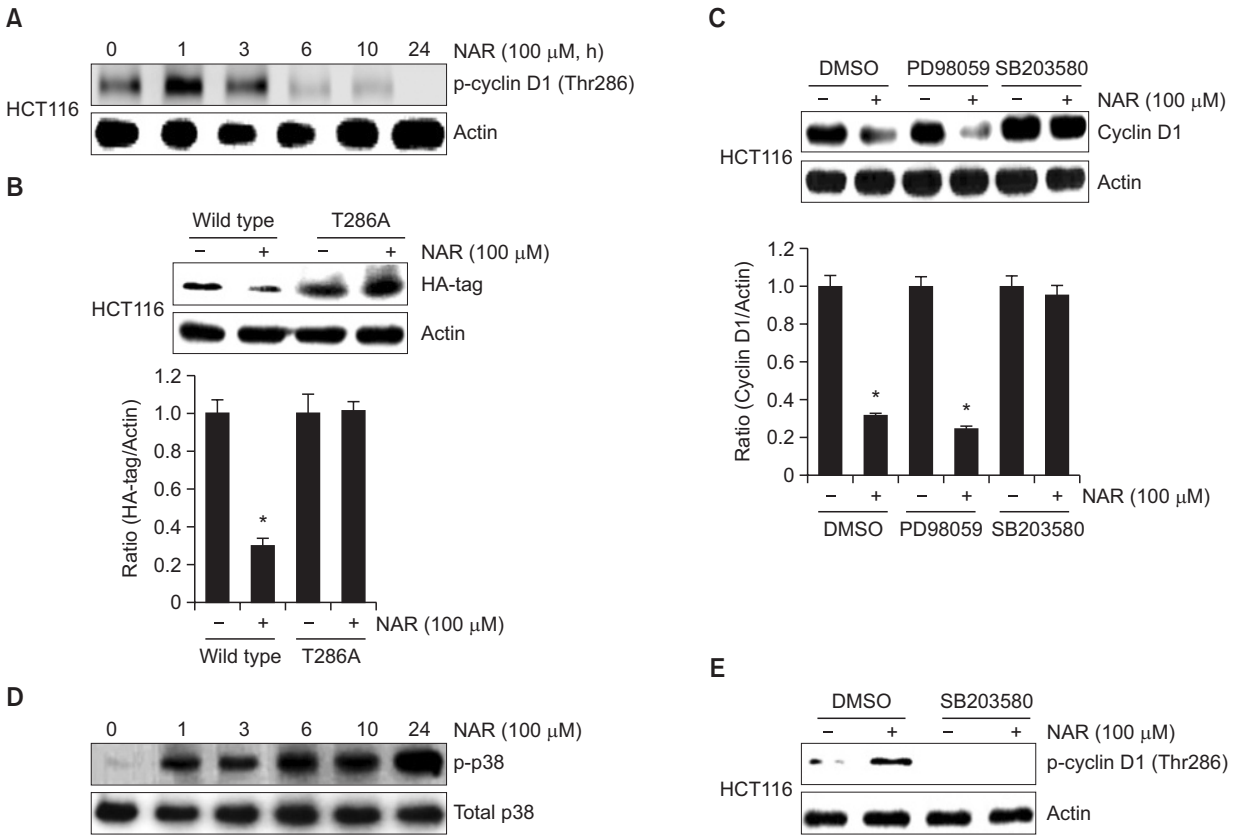

Fig. 4. Effect of NAR on threonine-286 phosphorylation and proteasomal degradation of cyclin D1 in HCT116 cells. (A) HCT116 cells were plated overnight and then treated with 100 μM of NAR for the indicated times. (B) HCT116 cells were transfected with wild type HA-tagged cyclin D1 or HA-tagged T286A cyclin D1 and then treated with 100 μM of NAR for 3 h. (C) HCT116 cells were pretreated with 50 μM of PD98059 (ERK1/2 inhibitor) or SB203580 (p38 inhibitor) for 2 h and then co-treated with 100 μM of NAR for 3 h. (D) HCT116 cells were treated with 100 μM of NAR for the indicated times. (E) HCT116 cells were pretreated with 50 μM of SB203580 (p38 inhibitor) for 2 h and then co-treated with 100 μM of NAR for 1 h. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against phospho-cyclin D1 (Thr286), HA-tag, cyclin D1, p-p38 or total p38. Actin was used as internal control.

ACKNOWLEDGMENTS

This work was supported by a grant from 2014 Research Fund of Andong National University (2014-0169), the BK21 PLUS program of Ministry of Education, and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1A1A2053448).

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