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Sulforaphane down-regulates SKP2 to stabilize p27^{KIP1} for inducing antiproliferation in human colon adenocarcinoma cells

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Sulforaphane is a cruciferous vegetable-derived isothiocyanate with promising chemopreventive and therapeutic activities. Induction of proliferation arrest and apoptosis principally contribute to sulforaphane's anticancer activity, but the precise molecular mechanisms remain elusive. The oncoprotein SKP2 is a key component of the SKP1-CULLIN1-F-box (SCF) E3 ligase complex and is responsible for directing SCF-mediated degradation of cyclin-dependent kinase inhibitor p27^{KIP1} to promote cell proliferation. We herein provide the first evidence supporting the critical involvement of the SKP2–p27^{KIP1} axis in sulforaphane-induced antiproliferation in various human colon adenocarcinoma cell lines. Specifically, sulforaphane markedly suppressed the levels of bromodeoxyuridine (BrdU) incorporation and clonogenicity in all tested cell lines, illustrating the antiproliferative effect of sulforaphane. Of note, sulforaphane-induced antiproliferation was accompanied with down-regulation of SKP2, leading to the stabilization and thus up-regulation of p27^{KIP1}. Additionally, sulforaphane was found to down-regulate SKP2 mainly through transcriptional repression, as sulforaphane lowered *SKP2* mRNA expression and the *SKP2* promoter activity. Furthermore, sulforaphane treatment led to the activation of both AKT and ERK, thus ruling out the possibility that sulforaphane down-regulates SKP2 by inhibiting AKT or ERK. Notably, sulforaphane-elicited suppression of BrdU incorporation and clonogenicity were significantly rescued in the context of SKP2 overexpression or p27^{KIP1} depletion, therefore highlighting the important role of SKP2 down-regulation and the ensuing stabilization of p27^{KIP1} in sulforaphane-induced antiproliferation. Collectively, these data expand our molecular understanding about how sulforaphane elicits proliferation arrest, but also implicate the application of sulforaphane in therapeutic modalities targeting SKP2.

[**Key words:** Sulforaphane; Antiproliferation; SKP2; p27^{KIP1}; Colon adenocarcinoma]

Colon cancer is the second cause of cancer death worldwide (1). In the western world, colon cancer is the third most common cancer in men and the second most common in women after breast cancer (2). It is noted that colon cancer is often first diagnosed at advanced stage (3). Chemptherapy remains as the primary option for the treatment of patients with advanced colon cancer, but the efficacy of current treatment modalities is limited. In light of this, chemoprevention is proposed as an alternative approach to manage colon cancer (4). Agents showing promising chemopreventive effect on colon cancer include asprin, selective COX-2 inhibitors and dietary phytochemicals including isothiocyanates (5).

Sulforaphane is an isothiocyanate derived from cruciferous vegetables and is particularly enriched in broccoli and broccoli sprouts (6). Sulforaphane has been received great attention due to its promising chemopreventive and therapeutic effects established in a variety of human cancer cell lines and in preclinical cancer models, including colon cancer (5). Nrf2-dependent antioxidative and anti-inflammatory defense mechanisms play important roles in sulforaphane-mediated chemoprevention (7), while induction of proliferation arrest and apoptotic death primarily contribute to the anticancer mechanisms against post-initiated/transformed cells (5,8,9). Intriguingly, recent discovery has identified sulforaphane as an agent targeting cancer stem cells (CSCs) (10), adding another level to the anticancer mode of action of sulforaphane. However, the precise molecular mechanisms of sulforaphane-elicited anticancer effect are not thoroughly elucidated.

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S-phase kinase-associated protein 2 (SKP2) is an integral component of the SKP1-CULLIN1-F-box (SCF) ubiquitin E3 ligase complex. SKP2 acts by using its F-box to recruit substrates for polyubiquitination by the SCF E3 ligase, leading to proteasome-mediated degradation of substrate proteins (11). SKP2 promotes cell proliferation mainly by directing the degradation of $p27^{KIP1}$, a key inhibitor of cyclin-dependent kinase (CDK); consistent with this notion, an inverse correlation between SKP2 and $p27^{KIP1}$ expression levels is generally present in multiple human malignancies (12). Of note, mounting lines of evidence have unraveled SKP2 as a potent oncoprotein involving in many human cancers, including colon cancer (13). Moreover, SKP2 is mostly overexpressed in tumor cells, and SKP2 overexpression in general indicates poor prognosis (12,14). The oncogenic action of SKP2 has been implicated in uncontrolled cell proliferation, apoptosis evasion, CSC stemness maintenance, metastases and drug resistance (11,15,16). Along this line, SKP2 has been regarded as a promising therapeutic target for various human cancers (14,17–19).

In this study we aimed to elucidate the role of the SKP2–$p27^{KIP1}$ axis in the antiproliferative effect of sulforaphane using an array of human colon adenocarcinoma cell lines as the model system. We revealed that sulforaphane induces transcriptional repression of *SKP2* to facilitate $p27^{KIP1}$ stabilization, consequently leading to the inhibition of cell proliferation. Our study therefore provides the first evidence to establish the critical involvement of the SKP2–$p27^{KIP1}$ axis in sulforaphane-elicited antiproliferation.

MATERIALS AND METHODS

Chemicals Sulforaphane (S6317 Sigma) was purchased from Sigma–Aldrich (St. Louis, MO, USA), prepared as a 20 mM stock solution in DMSO, aliquoted and then kept at −20°C until use. The *de novo* protein synthesis inhibitor cycloheximide and the proteasome inhibitor MG132 were obtained from Sigma–Aldrich as well.

Cell culture Human colon adenocarcinoma cell lines DLD-1 (ATTC no. CCL-221), HCT116 (ATTC no. CCL-247) and LoVo (ATTC no. CCL-229) were all purchased from the Bioresource Collection and Research Center (BCRC) located in Hsinchu, TAIWAN. All cell lines were grown in the culture media as recommended by ATCC. The media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). All cell lines were cultured at 37°C and 5% CO_2.

Bromodeoxyuridine cell proliferation assay Cell proliferation evaluated by the levels of bromodeoxyuridine (BrdU) incorporation was performed according to manufacturer's instruction [Cell Proliferation ELISA, BrdU (colorimetric), Roche]. Briefly, 8×10^3 cells/well were seeded in 96-well plate and then subject to sulforaphane treatment for 24 h. Cells were then supplemented with 10 μl/well BrdU labeling solution (final concentration 10 μM) and incubated at 37°C for 2 h. BrdU labeling solution were removed, and cells were then incubated with 200 μl/well FixDenat at room temperature (RT) for 30 min, followed by the treatment with Anti-BrdU-POD working solution (100 μl/well) at RT for 1 h. Subsequently, cells were rinsed three times by 200 μl/well Wash solution, and then were incubated with Substrate solution (100 μl/well) until color development is sufficient for detection. 25 μl of 1 M H_2SO_4 were then added to each well and the absorbance at 450 nM was then measured at ELISA reader.

Colony formation assay Colony formation assay was performed in order to evaluate the effect of sulforaphane on the long-term cell proliferation of human colon adenocarcinoma cells. The assays were conducted according to our reported protocol (20). The same procedure was repeated for at least three times.

Cycloheximide chase analysis The stability of $p27^{KIP1}$ protein was determined by cycloheximide chase analysis. Cells were seeded onto 60 mm Petri dishes (5×10^5 cells/dish) and treated without or with sulforaphane (20 μM) for 18 h. Cells were then treated with cycloheximide (60 μg/mL) to block *de novo* protein synthesis for 0, 1, 3 and 6 h. The levels of $p27^{KIP1}$ following cycloheximide treatment were then determined by immunoblot analysis.

Real-time RT-PCR analysis Total RNA extraction, the 1st strand cDNA synthesis, and quantitative real-time PCR analysis were performed according to our established protocol (21). The primers used for *SKP2* mRNA PCR were 5′-GCTGCTAAAGGTCTCTGGTGT-3′ (forward) and 5′-AGGCTTAGATTCTGCAACTTG-3′ (reverse). All experiments were repeated for at least three times with triplicated samples in each experiment.

Luciferase reporter assay pSKP2-Luc, the luciferase reporter vector for the activity of the human *SKP2* promoter encompassing the region between −1148 and +20 relative to the translational start site, has been previously described (22). Luciferase activity assays by Dual-Luciferase Reporter assay kit (Promega, USA) were conducted by following our reported procedure (22). All experiments were repeated for at least three times with triplicated samples in each experiment.

SKP2 overexpression and RNAi interference for p27KIP1 pBabe-SKP2, the retroviral vector-based ectopic expression of SKP2, and pLKO-p27 shRNA, the lentiviral vector to express p27 shRNA (clone ID:TRCN0000039930), were described previously (22). The production and infection of pBabe.puro-derived retroviral particles and pLKO.1-derived lentiviral particles were conducted in accordance to our published protocol (22). Immunoblotting for SKP2 and $p27^{KIP1}$ were performed to validate the ectopic expression of SKP2 and the depletion of endogenous $p27^{KIP1}$.

Immunoblot analysis Immunoblotting was performed by following our established protocol (21). Antibodies against SKP2, poly (ADP-ribose) polymerase (PARP), total AKT, phospho-AKT (Ser473), total ERK, phospho-ERK (Thr202/Tyr204), total GSK-3β, phospho-GSK-3β (Ser9) were all purchased from Cell Signal Technology (Beverly, MA, USA). Antibody against $p27^{KIP1}$ was obtained from BD Transduction Laboratories (San Jose, CA, USA). β-Tubulin antibody was purchased from Sigma–Aldrich. GAPDH polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The signals were detected with an enhanced superSignal West Pico chemiluminescence (Pierce, USA).

Statistical analysis All data were expressed as means ± standard error of mean (SEM) from at least three individual experiments. Differences between groups were evaluated for statistical significance using Student's *t*-test. A p value below 0.05 was regarded as the minimum criteria for statistical significance.

RESULTS

Sulforaphane inhibits cell proliferation in multiple human colon cancer cell lines We started by examining the anti-proliferative effect of sulforaphane on an array of human colon adenocarcinoma cell lines, including DLD-1, HCT116 and LoVo. As shown in Fig. 1A, sulforaphane dose-dependently repressed the level of BrdU incorporation in all tested colon cancer cell lines, with an IC_{50} of 18.82 μM, 15.73 μM and 7.17 μM for DLD-1, HCT116 and LoVo, respectively. To further substantiate the anti-proliferative activity of sulforaphane, we probed the impact of sulforaphane on the long-term proliferative events by evaluating colony-forming capacity of sulforaphane-treated cells. It is noted that in all tested colon cancer cell lines, the level of colony formation was clearly reduced by sulforaphane in a dose-dependent manner (Fig. 1B). Specifically, sulforaphane at 20 μM lowered the colony-forming capacity to 3.67 ± 1.49%, 36.60 ± 3.78% and 23.57 ± 4.55% of the untreated controls in DLD-1, HCT116 and LoVo cells, respectively ($p < 0.001$). Altogether, these results evidently demonstrate the ability of sulforaphane to induce anti-proliferation in human colon cancer cells.

Sulforaphane down-regulates SKP2 to facilitate $p27^{KIP1}$ stabilization Next, we aimed to explore the mode of action underlying sulforaphane-induced antiproliferation in colon cancer cells. In particular, we focused on the effect of sulforaphane on the expression of SKP2, a key component of the SCF E3 ligase complex integral to drive cell proliferation mainly by targeting the cell cycle inhibitor $p27^{KIP1}$ for degradation. To this end, DLD-1, HCT116 and LoVo cells were treated with sulforaphane for 24 h, followed by immunoblotting for the level of SKP2. It is noteworthy that in all tested cell lines, sulforaphane markedly down-regulates SKP2, along with a dose-dependent increase in the level of $p27^{KIP1}$ (Fig. 2A). To further clarify whether $p27^{KIP1}$ up-regulation induced by sulforaphane results from increased $p27^{KIP1}$ protein stability, cycloheximide chase analysis was performed to evaluate the rate of $p27^{KIP1}$ degradation by monitoring the level of $p27^{KIP1}$ decrease at 1 h, 3 h and 6 h following cycloheximide treatment in sulforaphane-stimulated DLD-1, HCT116 and LoVo cells. We did not observe a noticeable difference in $p27^{KIP1}$ levels between 0 h and 1 h of cycloheximide treatment (data not shown). Notably, when *de novo* protein synthesis was blocked by cycloheximide, the rate of $p27^{KIP1}$ degradation was significantly attenuated upon sulforaphane stimulation (Fig. 2B). Collectively, these lines of evidence strongly argue that sulforaphane down-regulates SKP2