Expression of cyclin B1, D1 and K in non-small cell lung cancer H1299 cells following treatment with sulforaphane

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Abstract. Sulforaphane (SFN) was first isolated from broccoli sprout and it is present at high concentrations in plants belonging to the Cruciferae family. The chemotherapeutic and anti-cancerogenic capacities of SFN have been demonstrated by inhibition of cancer cell proliferation in several cancer cell lines. The aim of the present study was to evaluate the effect of SFN on apoptosis, cell cycle and expression of selected cell cycle-associated proteins: Cyclin B1, cyclin D1 and cyclin K in the H1299 cell line. The non-small cell lung cancer cell line H1299 was treated with increasing concentrations of SFN (5, 10 and 15 μM) for two days. After incubation, the percentage of cells in the individual cell cycle phases, as well as the percentage of necrotic and apoptotic cells, were estimated using flow cytometry. The expression of cyclins was examined by immunofluorescence staining, flow cytometry, western blot analysis and qRT-PCR. Cyclin K was characterized by nuclear localization and increased expression after treatment with SFN. The expression data were confirmed by qRT-PCR. SFN-induced cell cycle arrest was associated with a decrease in cyclin B1 expression. Cells treated with SFN were also characterized by higher cyclin D1 and cyclin K expression. These data suggest the involvement of cyclin K in response to SFN. Moreover, we investigated the prognostic value of cyclin K, CDK12 and CDK13 in adenocarcinoma patients using 'The Kaplan-Meier plotter' (KM plotter) database. It was shown that high expression of CDK12 and CDK13 but no cyclin K proteins is associated with worse overall survival among adenocarcinoma patients.

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Introduction

Sulforaphane (SFN) was first isolated from broccoli sprout and is present at high concentrations in plants which belong to the Cruciferae family (1). SFN is a chemopreventive agent which displays functions, including inhibition of carcinogen-activating enzymes, such as the cytochrome p450 isoenzyme 2E1, induction of conjugating enzymes, such as glutathione S-transferases, and reduction of the DNA binding ability of nuclear factor-κB (NF-κB) (2-4). Furthermore, SFN was found to exert antiproliferative effects on various cancer cell lines *in vitro* and *in vivo* (5-7). According to Choi and Singh, SFN treatment induces Bax and Bak protein expression and conformational change and mitochondrial translocation of Bax to trigger the release of apoptogenic molecules from the mitochondria to the cytosol causing activation of caspases and cell death. Their research showed that both Bax and Bak are crucial for SFN-induced cell death (8). SFN acts as an indirect antioxidant and inducer of antioxidant response element (ARE) genes, and Moreover, the exposure to SFN results in a transient reactive oxygen species (ROS) burst, for which the duration and magnitude both depend on the SFN concentration and exposure period (9,10). Cyclin K plays a dual role by regulating CDKs and transcription.

Cyclin K was first discovered in a yeast screen, which was based on its ability to restore cell cycle progression and rescue *Saccharomyces cerevisiae* cells from lethality caused by deletion of G1 cyclin (11). Cyclin K triggers Cdk9 activity by creating a stable protein complex with Cdk9. The Cdk9/cyclin K complex phosphorylates the carboxyl-terminal domain (CTD) of RNA polymerase II (RNAP II). This reaction is said to be one of the most significant steps in the transcription of many genes (12,13). Moreover, the role of cyclin K and Cdk9 has been implied in the pathways that provide genomic stability in response to replication stress. Cyclin K also binds Cdk12 and Cdk13 to form two different

complexes (cyclin K/Cdk12 or cyclin K/Cdk13) in human cells. Phosphorylation of Ser2 in the C-terminal domain of RNAP II and expression of a small subset of human genes are regulated by the cyclin K/Cdk12 complex as revealed in expression microarrays (14). Decreased expression in mainly long genes with a high number of exons is a result of depletion of cyclin K/Cdk12. Human cells without cyclin K/Cdk12 generate spontaneous DNA damage and are sensitive to a variety of DNA damage agents. Moreover, cyclin K/Cdk12 protects them from genomic instability (14). The regulation of Cdk13 activity is currently not well understood as the expression levels of the corresponding cyclin subunit, cyclin K, are rather stable. This is similar to other cyclins controlling transcriptional CDKs, e.g., cyclin T1, but different from cell cycle-regulating cyclins such as cyclin A (15). Cyclin D1 regulates the progression of cells from the G1 phase to the S phase of the cell cycle. It is involved in complexes with cyclin-dependent kinases 4 and 6 (Cdk4/6) (16).

Complexes of cyclin D1 with CDKs 4 or 6 are essential to preserve cell homeostasis when impairments in cyclin D1 expression can trigger tumorigenesis. Moreover, changes in cyclin D1 expression can significantly affect cellular response to drug treatment (17-19). Cyclin B1 and Cdk1 form a complex called 'mitotic promoting factor' (MPF) that is crucial for G2/M transition (20). Therefore, insufficient levels of cyclin B/Cdk1 complexes, e.g. as a consequence of the attenuation of cyclin B1 promoter by p53, are related to cell cycle arrest at G2 phase (21). Cyclin B1 and Cdk1 are expressed in late S and G2 phases of the mammalian cell cycle and are active at late G2 phase. After cyclin B1 is produced in the cytoplasm during S phase it is transported to the nucleus at the late G2 phase and then finally removed during anaphase via a ubiquitin-related pathway (22). The redistribution of cyclin B1/Cdk1 to the mitochondrial matrix shows a unique mechanism which coordinates the mitotic events in other cellular compartment and mitochondrial activity for G2/M progression. In spite of the fact than cyclin B1/Cdk1 is detected in the mitochondria of asynchronous cells in different cell cycle phases, the timing of mitochondrial influx of cyclin B1/Cdk1 is consistent with the accumulation of G2/M cells. What is important, the kinase activity of Cdk1 is also maximized at the G2/M which indicates that cyclin B1/Cdk1-mediated mitochondrial bioenergetics is integrated into the overall process of G2/M transition (23). There are reports suggesting that changes in the regulation of cyclin expression may be connected not only with unrestrained cell growth and malignant transformation, but also in tumor suppressor mechanisms (24).

The aim of the present study was to evaluate cyclin B1, cyclin D1 and cyclin K expression in H1299 cells treated with SFN as well as to investigate a potential involvement of these cyclins in the therapeutic outcome of SFN treatment.

Materials and methods

Cell culture and SFN treatment. The human non-small cell lung carcinoma cell line H1299 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in monolayers at 37˚C in a humidified CO_2 incubator (5% CO_2) in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc) and 50 μg/ml of gentamycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Twenty-four hours after seeding, the cells were treated with SFN (Sigma-Aldrich; Merck KGaA) (5, 10 and 15 μM) for 48 h, and the following experimental procedures were performed.

MTT assay. Twenty-four hours prior to SFN treatment, the H1299 cells were seeded in 12-well plates. The cells were then treated with appropriate SFN concentrations (5, 10 and 15 μM) for 24 and 48 h. Following the treatment, cells were washed with phosphate-buffered saline (PBS) and 1 ml of DMEM without phenol red and 100 μl of the thiazolyl blue tetrazolium bromide (MTT) working solution (Sigma-Aldrich; Merck KGaA; 5 mg/ml in PBS) were added to each well and incubated for 3 h. Formazan crystals were diluted in isopropanol and the absorbance was read at 570 nm in a spectrophotometer (Spectra Academy, K-MAC, Daejeon, Korea).

Annexin V/propidium iodide (PI) binding assay. To assess the mode of cell death, the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions. In short, after the SFN treatment, the cells were collected from 6-well plates using trypsin-EDTA solution, centrifuged at 300 x g for 8 min, resuspended in ABB (Annexin binding buffer) and incubated with Annexin V Alexa Fluor® 488 and propidium iodide (PI) at room temperature in the dark for 20 min. The cells were examined using a Guava 6HT-2L Cytometer (Merck KGaA). The data were analyzed by InCyte software (version 3.2; Merck KGaA) and expressed as the percentage of cells in each population (viable, Annexin V^-/PI^-; early apoptotic, Annexin V^+/PI^-; late apoptotic, Annexin V^+/PI^+; necrotic, Annexin V^-/PI^+).

DNA content analysis. For DNA content analysis, the Guava Cell Cycle reagent (Merck KGaA) was used according to the manufacturer's instructions. Briefly, the cells were harvested from 6-well plates by trypsinization, rinsed with PBS, fixed in ice-cold 70% ethanol at 4˚C and maintained at -25˚C overnight. The cells were then centrifuged at 650 x g for 5 min at room temperature (RT) and washed with PBS. After centrifugation at 500 x g for 7 min, the cells were resuspended in Guava Cell Cycle reagent. Following a 30-min incubation at RT in the dark, the cells were analyzed using Guava 6HT-2L cytometer (Merck KGaA), and the percentage of cells in each phase of the cell cycle was determined using InCyte software (version 4.03; De Novo Software, Piscataway, NJ, USA).

Flow cytometric analysis of cyclins B1, D1 and K. Cells grown in 6-well plates were harvested, washed with PBS, centrifuged (for 5 min, at 300 x g) and incubated with eBioscience Fixable Viability Dye eFluor 660 (Thermo Fisher Scientific, Inc.) for 30 min at 4˚C to exclude dead cells. Then, the cells were fixed with Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA, USA). After incubation on ice (for 15 min in the dark) and subsequent centrifugation (for 5 min, at 300 x g), the cells in pellets were permeabilized by the addition of 1 ml of ice-cold

80% (v/v) methanol (POCH) overnight at -20˚C, washed with cold Perm/Wash solution (BD Biosciences) and resuspended in 3% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA). For intracellular staining, cells were incubated with the appropriate antibody diluted in Perm/Wash solution. Following a 30-min incubation at room temperature (RT) in the dark and washing with Perm/Wash solution, the cells were centrifuged for 5 min, at 500 x g to wash off excess antibody. Cells stained with cyclin B1 and cyclin K antibody were incubated with Alexa Fluor 488 for 30 min at RT. Cells were resuspended in 300 μl of PBS for flow cytometric analysis on Guava 6HT-2L Cytometer. InCyte software was used to calculate the mean fluorescence intensity. The antibodies used in the experiment were the following: Mouse monoclonal anti-cyclin B1 antibody (dilution 1:100; cat. no. MA5-14319; Thermo Fisher Scientific, Inc.), FITC conjugated mouse cyclin D1 antibody (cat. no. 554109; BD Biosciences), or mouse monoclonal anti-cyclin K antibody (dilution 1:50; cat. no. sc-376371; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

RT PCR analysis of cyclin B1, D1 and cyclin K expression. Total RNA from H1299 cells was isolated using a Total RNA kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instruction. The concentration and purity of *RNA* were determined spectrophotometrically (BioSpectrometer Basic; Eppendorf, Hamburg, Germany). The reverse transcription and quantitative PCR reactions were performed in a single 20-μl LightCycler capillary (Roche Applied Science, Mannheim, Germany) as a one-step real-time qRT-PCR with using LightCycler RNA Master SYBR-Green I (Roche Applied Science). For each target gene, the reactions were carried out in a 20-μl volume containing 100 ng of RNA and 0.2 μM of each primer in addition to LightCycler RNA Master SYBR-Green I kit components. The sequences of the primers were as follows: *CCNK* forward, 5'-ACCCAAAGG AGGAAGTAATGG-3' and *CCNK* reverse, 5'-GAACTGGTA TGGATGTTCTACCT-3'; *CCND1* forward, 5'-TGAGGCGGT AGTAGGACAGG-3' and *CCND1* reverse, 5'-GACCTTCGT TGCCCTCTGT-3; *CCNB1* forward, 5'-TTTCGCCTGAGC CTATTTTG-3' and *CCNB1* reverse, 5'-GCACATCCAGAT GTTTCCATT-3'. The thermocycling conditions used for the qRT-PCR were as follows: One cycle of reverse transcription for 20 min at 61˚C, one cycle of denaturation for 1 min at 95˚C, and 45 cycles of denaturation for 5 sec at 95˚C, followed by annealing and extension for 20 sec at 57-61˚C (depending on the melting temperature of the primers) and 5 sec at 72˚C, respectively. The samples were run in at least triplicate on the LightCycler 2.0 Instrument (Roche Applied Science) and evaluated with LightCycler Software (version 4.0; Roche Applied Science). The expression of the target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) internal control and assessed using the ΔΔCq method ($2^{-\Delta\Delta Cq}$ method) (25).

Cyclin B1, cyclin D1 and cyclin K immunofluorescence. H1299 cells growing on coverslips were briefly washed with PBS, fixed in 4% paraformaldehyde (for 15 min at RT) and then washed with PBS (3x5 min). After that, the cells were incubated in permeabilization solution (0.1% Triton X-100 in PBS) and blocked with 3% BSA. After permeabilization, the cells were incubated with mouse monoclonal anti-cyclin B1 antibody (dilution 1:100; cat. no. MA5-14319; Thermo Fisher Scientific, Inc.) or mouse monoclonal anti-cyclin D1 antibody (cat. no. C7464; Sigma-Aldrich; Merck KGaA) or mouse monoclonal anti-cyclin K antibody (dilution 1:50; cat. no. sc-376371; Santa Cruz Biotechnology, Inc.), respectively (60 min at RT), washed three times with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen; Molecular Probes) (60 min, in the dark). Nuclear staining was performed with DAPI (Sigma-Aldrich; Merck KGaA). Contract labeling with Alexa Fluor 594 phalloidin (Thermo Fisher Scientific, Inc.) was used additionally. After incubation, the cells were washed with PBS and then mounted on microscope slides in Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA, USA). The cells were examined using a C1 laser-scanning confocal microscopy system (Nikon, Tokyo, Japan) with a 100x oil immersion objective. Fluorescence images were obtained and analyzed with Nikon EZ-C1 software (ver3.80; Nikon Instruments, Melville, NY, USA).

Western blot assay. For semi-quantitative protein expression measurement, western blot analysis was conducted. Whole-cell lysates were obtained from lysis in RIPA buffer (Merck KGaA). Following normalization of the protein concentration using the BCA protein assay kit (Thermo Fisher Scientific, Inc.), equal amounts of protein (25 μg of total protein per lane) were separated using 4-12 or 16% NuPAGE Bis-Tris Gel (Novex/Life Technologies; Thermo Fisher Scientific, Inc.) and transferred onto nitrocellulose membranes using the iBlot dry western blotting system (Invitrogen; Thermo Fisher Scientific, Inc.). The membrane was processed in iBind Flex Western Blot system (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Bands were detected using 1-Step™ Ultra TMB-Blotting solution (Thermo Fisher Scientific, Inc.).

Gene expression analysis (KM plotter). Kaplan-Meier database (http://kmplot.com/analysis/) was conducted to evaluate the impact of CCNK, CDK12 and the CDK13 expression on the outcome of adenocarcinoma patients (26). The following probeset was used (ID Affymetrix): CCNK (219273_at), CDK12 (225690_at), CDK13 (228991_at).

Statistical analysis. The analysis was performed using statistical software (ver. 6.0; GraphPad Prism, San Diego, CA, USA). The data were compared with the non-parametric Mann-Whitney U test or non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test, and the changes were considered statistically significant at the level of $P<0.05$. To analyze Kaplan-Meier survival curves, hazard ratio and log-rank test were calculated on the KM plotter web page.

Results

Annexin V and MTT assay. Following treatment with 5, 10 and 15 μM of SFN used in this study, the percentage of proliferating cells was decreased in a dose-dependent manner at both 24 and 48 h and the difference was significant at 10 and 15 μM of SFN ($P<0.05$) when compared with

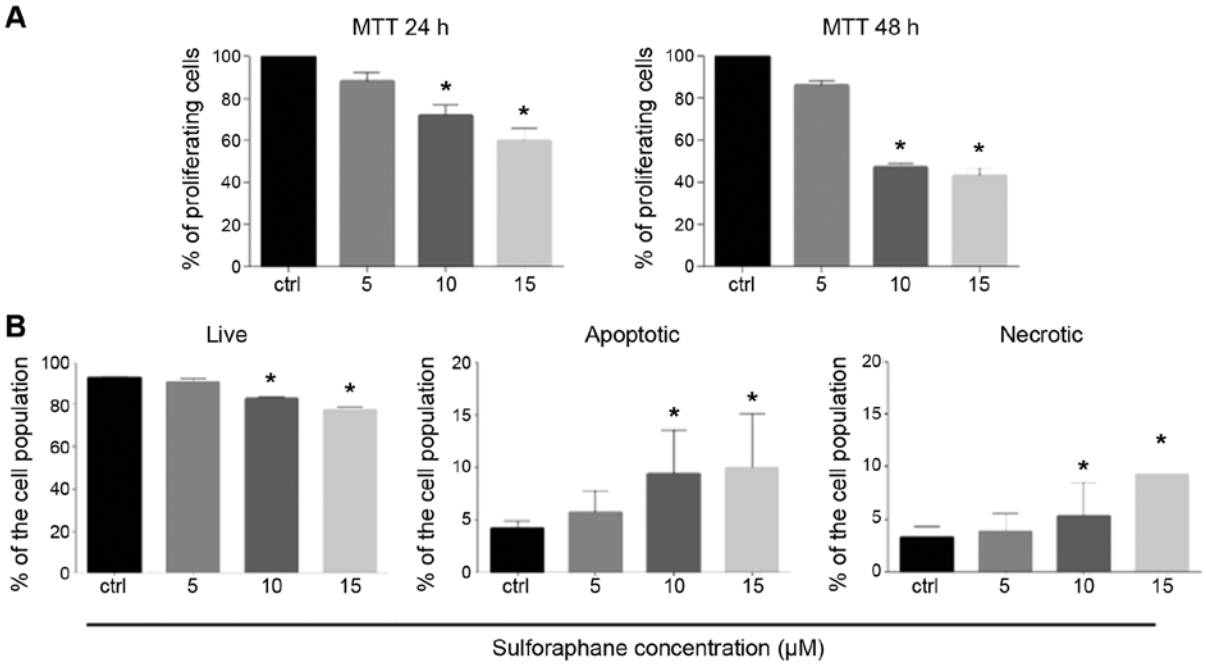

Figure 1. (A) Percentage of proliferating H1299 cells at 24 and 48 h following treatment with 5, 10 and 15 μM sulforaphane as determined by MTT assay. (B) Percentage of live, apoptotic and necrotic cells after treatment with 5, 10 and 15 μM sulforaphane as determined by the Annexin V/propidium iodide (PI) binding assay. *P<0.05, statistically significant as compared to the control (ctrl) cells (Kruskal-Wallis). Results are representative of five independent experiments.

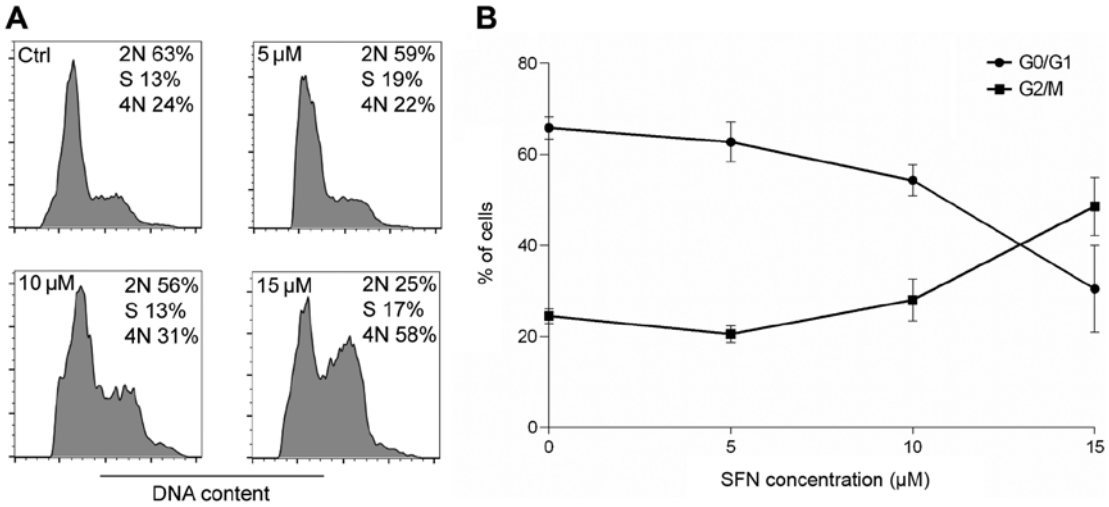

Figure 2. (A) Analysis of the cell cycle distribution in H1299 cells incubated with 5, 10 and 15 μM sulforaphane. (B) Percentage of H1299 cells in the G0/G1 and G2/M phases. No statistical significance compared to the control (ctrl) cells (Kruskal-Wallis). Results are representative of five independent experiments.

the control as determined by the MTT assay (Fig. 1A). In addition, as shown by the Annexin V/propidium iodide (PI) binding assay, the percentage of living cells was significantly decreased at 10 and 15 μM of SFN treatment (P<0.05). A dose-dependent increase in the percentage of apoptotic cells and a slight increase in the percentage of necrotic cells were observed and the differences were significant at 10 and 15 μM (Mann-Whitney U; P<0.05) (Fig. 1B). The results indicated that SFN induces both apoptosis and necrosis in a similar extent.

DNA content analysis. Flow cytometry was conducted to assess whether SFN treatment induced changes in cell cycle distribution in the H1299 cell line. Cell cycle analysis showed that along with the increase in the concentration of SFN, the percentage of cells arrested at the G2/M phase of the cell cycle was increased which was correlated with a decrease in cells in the G0/G1 phase. These results indicated that SFN suppressed cell cycle progression in the H1299 cell line (Fig. 2).

Expression of cyclin B1. Following SFN treatment, expression of cyclin B1 was decreased. qRT-PCR analysis showed that treatment with 5, 10 and 15 μM concentrations of SFN induced a significant decrease in cyclin B1 mRNA (Mann-Whitney

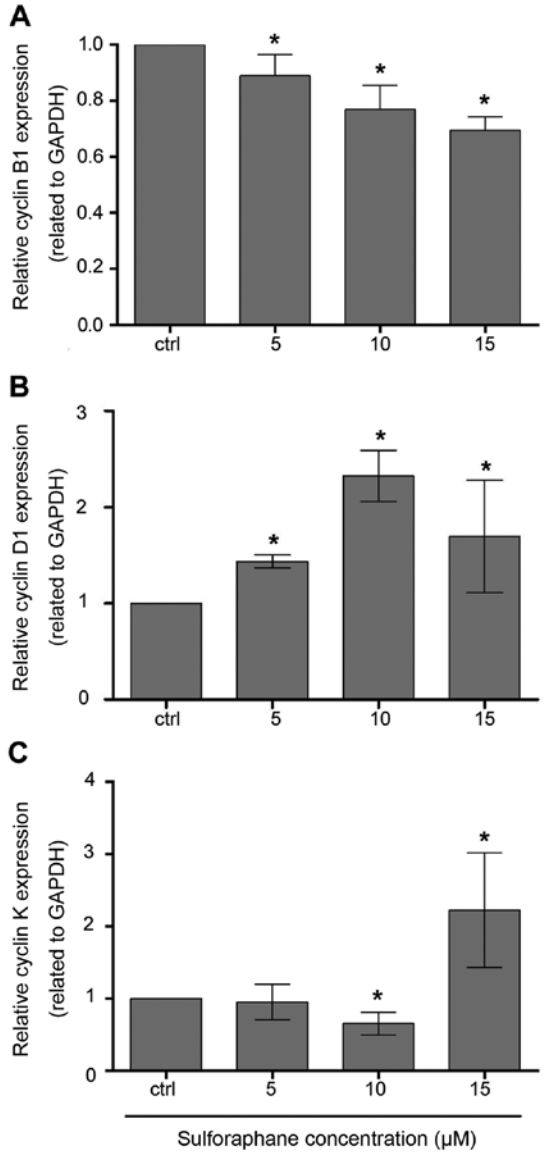

Figure 3. Real-time qRT-PCR analysis of (A) cyclin B1, (B) cyclin D1 and (C) cyclin K genes in H1299 cells after sulforaphane (SFN) treatment. The cells were treated with 5, 10 and 15 μM SFN for 48 h. *P<0.05, statistically significant difference between control (ctrl) and treated sample (Kruskal-Wallis with Dunn's post hoc test).

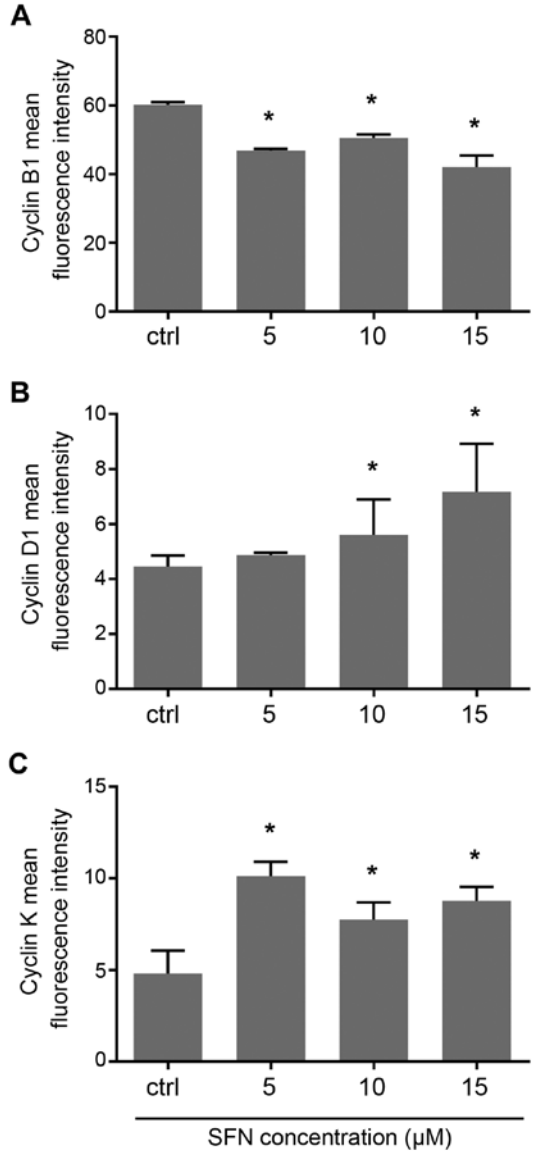

Figure 4. Flow cytometric analysis of of (A) cyclin B1, (B) cyclin D1 and (C) cyclin K expression in H1299 cells after sulforaphane (SFN) treatment. *P<0.05, statistically significant difference between control (ctrl) and treated sample (Kruskal-Wallis with Dunn's post hoc test).

U; P<0.05) (Fig. 3A), similarly to cytometric analysis which revealed a slight decrease at all doses of SFN (Fig. 4A). Immunofluorescent labeling of cyclin B1 in SFN-treated cells showed a trend similar to that noted in the flow cytometric and qRT-PCR results (Fig. 5). Western blot analysis showed a decrease in cyclin B1 expression (Fig. 6).

Expression of cyclin D1. The qRT-PCR experiment showed a significant increase in cyclin D1 mRNA after treatment with 10 μM SFN. Slightly increased levels resulted from the treatment with other doses, i.e. 5 and 15 μM SFN, when compared with the control (Fig. 3B). Flow cytometric measurements indicated that the fluorescence intensity of cyclin D1 protein was increased at all doses of SFN treatment with significant increases at 10 and 15 μM doses (Fig. 4B). Fluorescence microscopic examination of cyclin D1 in H1299 control and SFN-treated cells revealed the highest induction of this protein at 10 and 15 μM SFN (Fig. 7). Western blot analysis showed a slight decrease at the protein level following treatment with SFN, which suggests post-transcriptional inhibition of cyclin D1 (Fig. 6).

Expression of cyclin K. qRT-PCR analysis displayed that treatment of SFN at a 15 μM concentration induced a significant increase in cyclin K mRNA (Mann-Whitney U, P<0.05) (Fig. 3C). Flow cytometric measurements demonstrated that the fluorescence intensity of cyclin K protein was significantly higher comparing to the control cells (Fig. 4C). Immunofluorescent labeling of cyclin K in SFN-treated cells showed a trend similar to that noted in the qRT-PCR results (Fig. 8). Importantly, we observed down-regulation of cyclin K after treatment with the lowest dose of SFN. However, to date, not enough data exist to explain this change in expression profile. We will try to investigate this

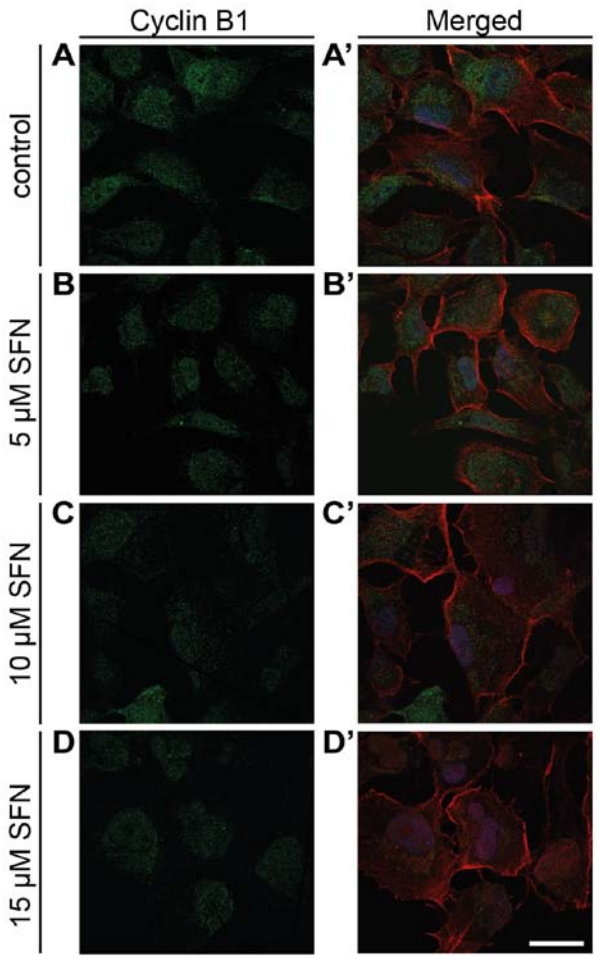

Figure 5. Confocal fluorescence microscopic examination of the localization of cyclin B1 in H1299 cells treated with sulforaphane (SFN). The cells were treated with 5, 10 and 15 μM of SFN and immunolabeled for the presence of cyclin B1 (A-D). Cell nuclei were stained with DAPI (A'-D' merged).

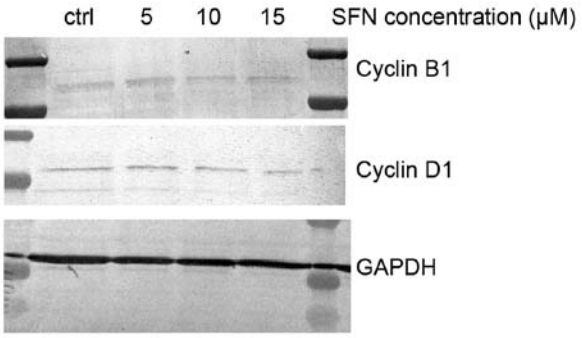

Figure 6. Western blot analysis of cyclin B1 and D1 expression in the H1299 cell line after treatment with increasing doses of sulforaphane (SFN).

phenomenon in the future and we hope that we can provide a more detailed mechanism which is responsible for changes in cyclin K expression after the treatment with a variety of drug and compounds. Additionally, due to the very low cyclin K expression in the H1299 cell line, we failed to perform western blot analysis.

Prognostic value of cyclin K, CDK12 and CDK13. Overall survival analysis of the patients with adenocarcinoma

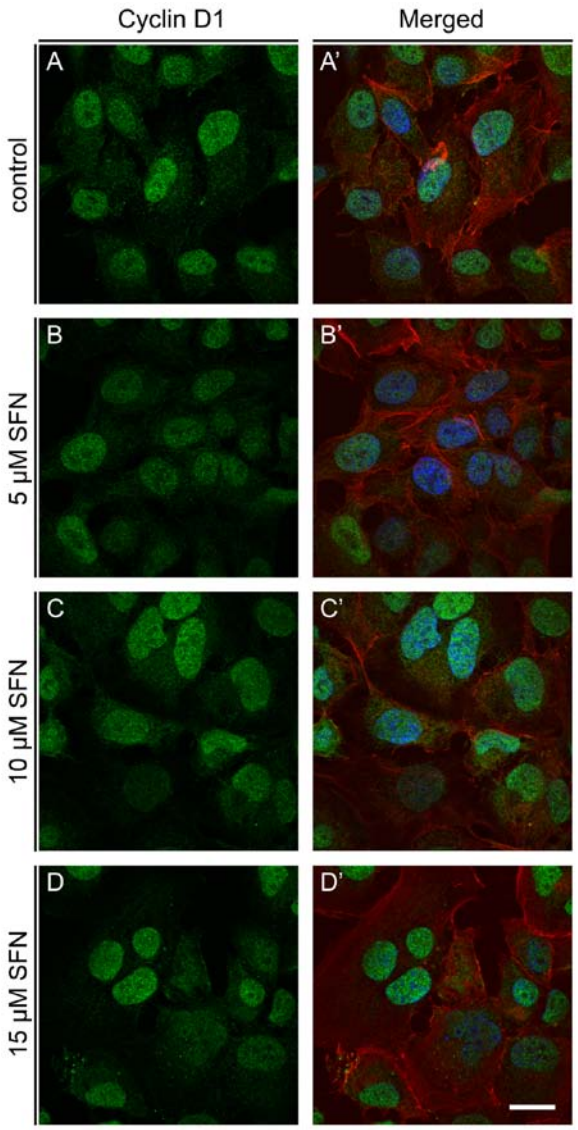

Figure 7. Confocal fluorescence microscopic examination of the localization of cyclin D1 in H1299 cells treated with sulforaphane (SFN). The cells were treated with 5, 10 and 15 μM of SFN and immunolabeled for the presence of cyclin D1 (A-D). Cell nuclei were stained with DAPI (A'-D' merged).

revealed that high expression of CDK12 and CDK13 but no cyclin K expression (CCNK) is associated with a worse prognosis (Fig. 9).

Discussion

Despite the significant advance in diagnosis and treatment, cancer remains a major cause of death worldwide. One of the deadliest cancers, which leads to a high death rate for both women and men, is lung cancer. With a 5-year survival rate below 20%, it is still a challenge for all those involved in cancer research (27). Phytochemicals are a potent source of anticancer agents, which can act unassisted or synergize with traditional drugs. Sulforaphane (SFN) is one of the most promising natural compound which exerts anticancer activities. This broccoli sprout-derived isothiocyanate acts through different pathways, which are not yet fully understood. Tumor cells are often characterized by imbalanced processes

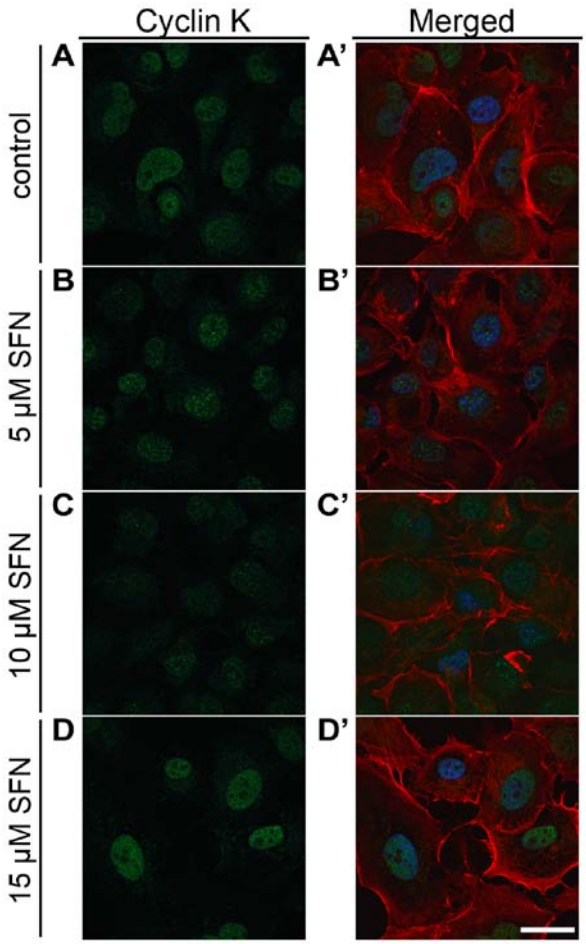

Figure 8. Confocal fluorescence microscopic examination of the localization of cyclin K in H1299 cells treated with sulforaphane (SFN). The cells were treated with 5, 10 and 15 μM of SFN and immunolabeled for the presence of cyclin K (A-D). Cell nuclei were stained with DAPI (A'-D' merged).

responsible for maintaining homeostasis, regulation of cell cycle and cell death. Uncontrolled divisions hold the danger of genetic impairments which directly lead to malignant transformation. In the present study, we demonstrated that SFN induces apoptosis, cell cycle arrest and changes in the expression of proliferation markers. What is important, the chosen doses can be attained by a normal consumption of broccoli. Approximately 50 grams of this vegetable delivers a sufficient amount of SFN to achieve a 20-μM concentration in urine (28). The ability of SFN to induce apoptosis has been demonstrated in several models *in vivo* and *in vitro*. In the highly metastatic melanoma cell line B16F-10 apoptosis was associated with morphological changes involving membrane blebbing and presence of apoptotic bodies. On the molecular level, activation of caspases-3 and -9, Bax and p53 with a simultaneous decrease in Bcl-2, caspase-8, Bid and NF-κB was observed (29). In U251MG glioblastoma cells, SFN induced apoptosis associated with expression of Bad, Bax and cytochrome *c* and decreased levels of survivin and Bcl-2. Moreover, cells treated with SFN were characterized by reduced invasion capabilities connected with increased expression of E-cadherin and lower expression of MMP-2, MMP-9 and galectin-3 (30). Mutations in the *TP53* gene have been observed in most human cancers and p53 protein responses to low or constitutive stress through cell cycle arrest. When the stress drives to severe DNA damage, p53 triggers apoptosis. The H1299 cell line is characterized by p53 deficiency and only a few studies have shown details for the p53-independent response following SFN treatment. Ferreira de Oliveira *et al* (31) found that SFN induced DNA damage and increased the number of nuclear and mitotic abnormalities. In another study by Ferreira de Oliveira *et al* (32), the authors presented a model of SFN involvement in ROS generation and cytotoxicity in the p53 null osteosarcoma MG-63 cell line. SFN treatment was found to induce a significant increase in ROS generation and subsequent changes in mitochondrial membrane potential and eventually execution of apoptosis in a p53-independent manner. Higher concentrations of SFN were found to lead to inhibition of ROS-scavenging enzymes such as SOD, Cat and GPx and contribute to lowered glutathione regeneration and impaired antioxidative potential. Cancer cells have a lower ratio of oxidized glutathione to reduced glutathione which confers drug resistance (33,34). Targeting the glutathione system can be utilized to increase the effectiveness of chemotherapy. We showed that SFN alone has the potential to inhibit proliferation of the H1299 cell line, thus it is possible to use SFN as a sensitizer in the combinational therapy of tumors with dysfunctional p53 protein.

In the present study, the antiproliferative effect of SFN was manifested by G2/M phase arrest associated with downregulation of cyclin B1 which is a potent cell cycle regulator. Its overexpression is observed in many different cancer types, including lung, breast and gastric cancer (35-37). Cyclin B1 silencing can exert antiproliferative effects on cancer cells, thus cyclin B1 can be considered as a potential therapeutic target. Kedinger *et al* downregulated cyclin B1 expression using modified siRNAs and achieved a reduction in the growth of melanoma xenografts and inhibition of formation and dissemination of melanoma lung metastases (38). In cervical cancer, G2/M arrest induced by SFN was also associated with downregulation of cyclin B1. Moreover, the cellular response involved upregulation of GADD45β, known as cyclin B1/Cdk1 inhibitor. The results suggest that SFN has the potential to inhibit cancer growth via the cyclin B/GADD45β pathway (39). It is noteworthy that downregulation of cyclin B1 is not unequivocally beneficial for cancer patients. In colorectal cancer patients, negative or low cyclin B1 staining was inversely correlated with lymph node metastatic potential. Tumors with low cyclin B1 expression more frequently present with lymphatic permeation or vessel invasion (40). Fang *et al* showed that cyclin B inhibits lymph node metastasis via E-cadherin both *in vivo* and *in vitro*. Cyclin B1 silencing resulted in decreased E-cadherin expression, which is one of the molecular hallmarks associated with EMT. Downregulation of cyclin B1 suppressed $p53^{+/+}$ HCT116 and $p53^{-/-}$ HCT116 colorectal cancer cell lines. The same significant effect was observed in the SW480 cell line and in a xenograft model (41). Correspondingly with a previously described model, Wang *et al* showed that SFN suppressed EMT and metastasis in human lung cancer cell lines through miR-616-5p-mediated GSK3β/β-catenin signaling pathways. SFN treatment was found to alter the expression of EMT-related proteins such as β-catenin, N-cadherin, vimentin and E-cadherin (42). These data show that the impact of cyclin B1 depends on the cellular environment and it is important to determine the factors

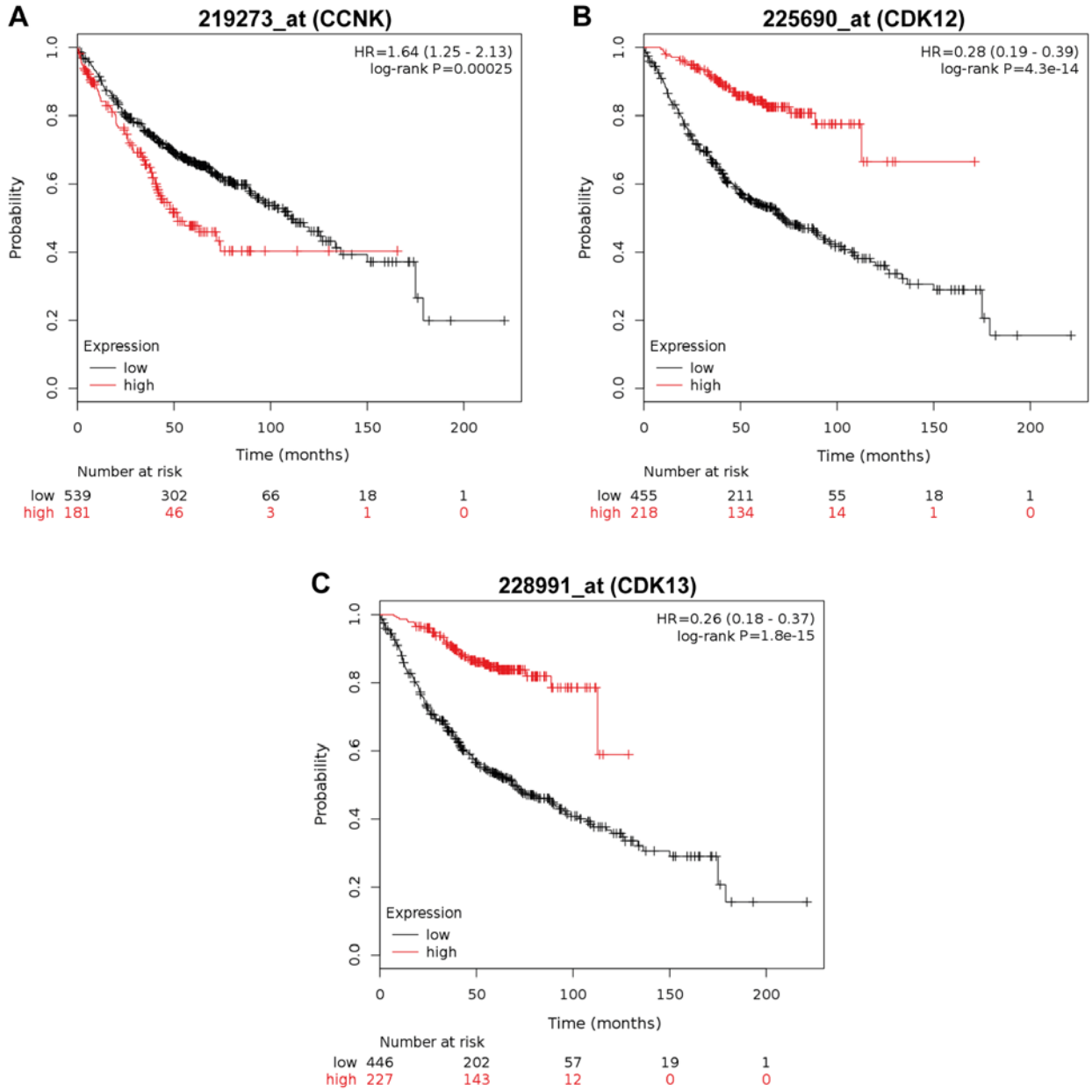

Figure 9. The prognostic values of (A) cyclin K (CCNK), (B) CDK12 and (C) CDK13 in adenocarcinoma patients with high expression of sulforaphane (SFN) or low expression of SFN. The log-rank P-value and hazard ratio (HR) values were evaluated by K-M Plotter. Patients with high and low expression for SFN were divided using auto selected best cut-offs in K-M Plotter.

which promote the antimetastatic effect of cyclin B1 and when cyclin B1 enhances EMT events.

Cyclin D1 is frequently overexpressed in many cancer types. After treatment with SFN, we observe an increase in cyclin D1 mRNA and protein expression. It is possible that cyclin D1 overexpression in the H1299 cell line is responsible for moderate response to drug intervention. Our previous study showed that cyclin D1 overexpression in the A549 cell line prevented SFN-induced apoptosis (43). Moreover, we also observed an increase in cyclin D1 after treatment with the lowest dose of SFN (30 μM). It is possible that at lower doses, SFN inhibits cyclin D1 association with cyclin-dependent kinases and this is the cause of the increase in cyclin fluorescence. Hagemann *et al* showed that SFN can upregulate MDM-2 protein expression which is a p53 suppressor but also can act independently of p53. MDM-2 as an oncoprotein promotes the survival of cancer cells and contributes to drug resistance. The protective effect of SFN can be blocked with MDM-2 inhibitor, Nutlin-3 (44). Moreover, silencing of cyclin D1 enhances the cytostatic effect of Nutlin-3 and makes different cancer cell lines more susceptible to cell death (45). It is feasible that the axis cyclin D1-MDM-2 significantly contributes to the cellular response to SFN treatment. Treating the H1299 cell line with another cytostatic drug, actinomycin D induced an increase in MDM2 expression. Silencing of MDM2 sensitized cells to actinomycin D and increase the number of cells undergoing apoptosis (46). The interaction between cyclin D1 and MDM-2 in the H1299 cell line warrants further investigations.

Cyclin-dependent kinases 12 and 13 with its regulatory subunit cyclin K, take part in gene transcription regulation

through interaction with RNA polymerase II and phosphorylation of C-terminal domain which is an important step in the generation of mature mRNA (47,48). The activity and oncogenic status of cyclin K/Cdk12 and cyclin K/Cdk13 warrant further elucidation. To investigate how the expression of CDK12 and CDK13 mRNA affects adenocarcinoma and the squamous cell carcinoma outcome we employed 'The Kaplan-Meier plotter' (KM plotter) database and discovered that high expression of Cdk12 and Cdk13 mRNA is correlated with worse overall survival. To date, limited and inconsistent data have been published, showing both a suppressive and a promoting effect on different cancer types. The loss of function mutations in high-grade serous ovarian cancer probably result in deregulated expression of DDR genes and contribute to genomic instability (49,50). Moreover, patients bearing mutations in the CCNK gene also present with abnormalities in BRCA1 and BRCA2 genes, well-known cancer suppressors (51). Amplification of the CDK12 mRNA increased protein levels, and elevated phosphorylation was observed in HER-2 driven breast cancer samples (52). Involvement in DNA repair leads to believe that targeting of Cdk12/cyclin K complex can significantly alter the drug response and can be considered as a new strategy in therapeutic development. The cyclin K gene was found to sensitize cancer cells to camptothecin which is a topoisomerase inhibitor inducing DNA damage (53). The loss of function mutations in the CDK12 gene leads to increased cisplatin sensitivity and downregulation of CDK12 induces spontaneous cell death and decreases resistance to different DNA-damaging agents such as etoposide, mitomycin C and cisplatin (14,54).

In turn, the impact of CDK13 activity on cancer development and disease outcome is still an uncharted territory. The CKD13 gene is frequently amplified in many different cancer cell lines. Clonogenic and invasion assays on the NIH3T3 cell line revealed the high oncogenic potential of the CDK13 product and showed that stable expression of CDK13 resulted in the stronger colony forming ability and an intermediate degree of migration activity. Additionally, cells overexpressing CDK13 exhibited increased resistance to 5-fluorouracil and doxorubicin and a simultaneous increase in tamoxifen susceptibility (55). These data suggest a significant role of CDK12 and CDK13 in human cancer and response to a treatment. The role of cyclin K in cancer development is unclear. Cyclin K plays important roles in transcriptional regulation and cell cycle control, but we know little about how cyclin K expression affects drug response. Schecher *et al* demonstrated the potential role of cyclin K in prostate cancer. Depletion of cyclin K in prostate cancer cell lines led to an increase in the number of multinucleated cells, mitotic catastrophe and eventually reduction of proliferation or apoptotic cell death. Moreover, prostate cancer patients with high expression of cyclin K, treated with an adjuvant therapy had worse biochemical recurrence-free survival rates compared to patients with low cyclin K expression. Silencing of cyclin K resulted in a decrease in Aurora B mRNA and protein expression. Aurora B is crucial for the formation of chromosomes and spindle assembly and its depletion is probably caused by the reduction of CDK12 activity rather than cyclin K itself (56). In this study, the increase in cyclin K expression could be a part of drug response. SFN induces DNA damage and activates various genes associated with DNA repair. The results suggest that high expression of cyclin K and CDK12 and CDK13 kinases should be considered as a potential obstacle in the successful therapy of tumors. It was shown that inhibitors of Cdk12 and Cdk13 can exert an antiproliferative effect on cancer cells thus it is reasonable to conduct further studies to investigate CDK12, CDK13 and cyclin K as targets for cancer treatment (57,58).

In conclusion, this study revealed that SFN induced cell cycle arrest and apoptosis in the H1299 cell line. Cell cycle arrest was associated with downregulation of cyclin B1. Significant changes in the expression of cyclin D1 and K suggest the role of these proteins in response to SFN treatment. Moreover, our *in silico* analysis showed that upregulated mRNA expression of the cyclin K catalytic partners CDK12 and CDK13 is associated with lower overall survival rates in adenocarcinoma patients. The present study shows the urgent need for elucidating the role of cyclin K/CDK12/CDK13 complexes in development and progression of lung cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

AŻ conceived and designed the study. AK and AŻ were responsible for the DNA content analysis and the DNA content analysis for cytometric analysis, the immunofluorescent labeling and the western blot analysis, they jointly designed the figures and they wrote the study. AK was responsible for the Annexin V and MTT assay and for the prognostic value of cyclin K, CDK12 and CDK13. AKW was responsible for the qRT-PCR analysis. DG and AG performed the statistical analysis; AZ, AK, AG, AKW and DG reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments have been approved by the Bioethics Committee of the Nicolaus Copernicus University in Toruń functioning at Collegium Medicum in Bydgoszcz.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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