

Genome-wide transcriptome analysis reveals inactivation of Wnt/ β -catenin by 3,3'-diindolylmethane inhibiting proliferation of colon cancer cells

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Abstract. Multiple genetic and signaling pathway alterations underlie the development of colon cancer. We utilized genome-wide transcriptome analysis to identify important gene expression patterns following treatment with 3,3'-diindolylmethane (DIM), a natural compound derived from cruciferous vegetables, on colon cancer cells. Statistical analyses of gene expression data from DIM treated cells revealed that 692 genes were significantly upregulated, while 731 genes were downregulated. Putative gene networks showed that several oncogenes (β -catenin, Myc and FOS) were significantly suppressed by DIM treatment. Using clinical data from colon cancer patients, activation of β -catenin was found to be significantly associated with patient prognosis by Kaplan-Meier plot analysis. We validated the mRNA and protein expression levels of c-Myc, β -catenin, and cyclin D1, all of which were significantly suppressed after DIM treatment in DLD-1 and HCT116 cells. System level characterization of our findings suggests for the first time that β -catenin and c-Myc, which are major genes involved in colon carcinogenesis, were significantly downregulated by DIM treatment in colon cancer cells. Therefore, targeting Wnt/ β -catenin signaling by DIM may be an attractive strategy for the prevention and treatment of colon cancer.

Introduction

Colorectal cancer is the third leading cause of cancer-related deaths in the USA and has one of the highest rates of mortality among cancers worldwide. In addition, the incidence of

colorectal cancer is increasing dramatically in Asian countries including South Korea (1-6). In the USA alone, a total of 136,000 new colorectal cancer cases were diagnosed in 2014, and the American Cancer Society projects that more than 50,310 patients will die from this disease during 2014. Despite continual efforts to understand the biological mechanisms of colon cancer progression, the primary mechanisms underlying colon cancer pathogenesis remain largely unclear.

Wnt proteins were first identified as oncogenes in mouse breast cancers (7,8), and downstream targets of Wnt signaling are highly activated in human colon cancers (9-11). Dysregulation of the Wnt/ β -catenin signaling pathway plays a key role in colon carcinogenesis (11). Likewise, both abnormal activation of the canonical Wnt/ β -catenin pathway and upregulation of the β -catenin/T-cell factor (TCF) response to transcriptional signaling play critical roles in early colorectal carcinogenesis (12,13). Taken together, it is clear that abnormal regulation of the Wnt/ β -catenin signaling has an important role in colon carcinogenesis.

3,3'-Diindolylmethane (DIM) is a natural compound derived from cruciferous vegetables. Studies from our laboratory and others have shown that DIM has inhibitory effects on cancer cell growth and induces apoptotic cancer cell death (4,14-19). Although several studies have shown that DIM has anti-proliferative effects on many types of cancer (19), the biological mechanisms by which DIM induces apoptosis in colon cancer cells have not been fully elucidated. However, the inhibitory effects of DIM on colon cancer cells may be due to altered patterns of gene expression. To clarify the changes in gene expression patterns of DIM on colon cancer, we performed a microarray experiment, seeking to identify important changes in gene expression patterns following DIM treatment of colon cancer cell lines. In this study, we generated gene expression data from the HCT116 human colon cancer cell line. Statistical analyses on gene expression data revealed that the levels of a total of 1,424 genes were significantly altered, many of which were associated with regulation of cell growth, cell cycle, and apoptosis. Noteworthy, the Wnt signaling pathway network including β -catenin and c-Myc was significantly downregulated by DIM treatment in colon

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cancer cells. Therefore, our results suggest for the first time that DIM suppresses the proliferation of colon cancer cells via inactivation of β -catenin and targeting the Wnt/ β -catenin signaling pathway by DIM may represent a new approach for the treatment and prevention of colon cancer.

Materials and methods

Cell lines, culture conditions and reagents. The DLD-1 and HCT116 cell lines were obtained from the University of Texas, M.D. Anderson Cancer Center (Houston, TX, USA). The DLD-1 cell line was cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) and the HCT116 cell line was cultured in DMEM F12 medium (Gibco). The media contained 10% fetal bovine serum (Gibco), 100 mg/ml streptomycin, and 100 IU/ml penicillin. Cells were maintained under standard conditions at 37°C in a 5% CO₂ humidified atmosphere. DIM was purchased from LKT Laboratories (St. Paul, MN, USA). Antibodies were purchased from the following commercial sources: Cyclin D1, p- β -catenin, β -catenin (Cell Signaling Technology, Inc., Beverly, MA, USA); c-Myc, SOX4, GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and c-FOS (Cell Signaling Technology Inc., Danvers, MA, USA).

Cell viability assay. Cell proliferation was assessed as previously described (4). Briefly, DLD-1 and HCT116 cells were seeded in 96-well plates and allowed to grow overnight. The following day, cells were treated with varying doses of DIM. After 72 h, 50 μ l of MTT [3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide; 2 mg/ml] was added to each well. After incubation for 3 h at 37°C, the media were removed and DMSO was added to solubilize the formazan crystals for 30 min. Finally, the absorbance at 570 nm was determined on an Epoch microplate spectrophotometer (BioTeck, Winnoski, VT, USA).

Colony formation assay. Colony formation assay was performed as previously described (20). Briefly, cells (5x10⁴ cells) were seeded into 6-well plates and incubated for 2 weeks until the colonies were large enough to be clearly discerned. The cells were fixed with methanol and stained with crystal violet, and the number of colonies was counted manually under a microscope and photographed.

Microarray experiment. The microarray experiment was performed as previously described (4). Briefly, a mirVana™ miRNA isolation labeling kit (Ambion Inc., TX, USA) was used according to the manufacturer's protocol to isolate total RNA from colon cancer cells. An Illumina Human-12 BeadChip V.4 microarray (Illumina, San Diego, CA, USA) was used for sample hybridization. Gene expression data were extracted using Genome Studio (Illumina). Microarray analysis was performed by the Shared Research Equipment Assistance Program of the Korea Basic Science Institute, MEST.

Microarray data analysis. Data were normalized using the quantile normalization method with the linear models for microarray data (LIMMA) package in the R statistical environment (21,22). The class comparison tool, part of BRB Array Tools suite (Biometrics Research Branch, National

Cancer Institute, MD, USA), was used to perform multiple comparisons of t-statistics with estimation of false discovery rate (FDR) and gene expression differences were considered statistically significant at P-value <0.001. Cluster and Treeview programs were used to generate heat maps of gene expression (23). Gene networks were analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems Inc., CA, USA). Kaplan-Meier plots and the log-rank test were used to estimate patient prognosis.

Real-time PCR. Real-time reverse transcription PCR analysis was used to quantify levels of gene expression as previously described (24). Briefly, Total RNA (1 μ g) from each sample was reverse-transcribed using the PrimeScript™ RT reagent kit (Takara Bio Inc, Otsu, Shiga, Japan) according the manufacturer's protocol. The PCR program was initiated at 95°C for 30 sec and 95°C for 15 sec followed by 40 cycles and 60°C for 1 min. Primer sequences were as follows: β -catenin sense: 5'-GAGCCTGCCATCTGTGCTCT-3' and β -catenin antisense: 5'-ACGCAAAGGTGCATGATTTG-3'; c-Myc sense: 5'-CAGCTGCTTAGACGCTGGATT-3' and c-Myc antisense: 5'-GTAGAAATACGGCTGCACCGA-3'; cyclin D1 sense: 5'-AGGAACAGAAGTGCAGGAGG-3' and cyclin D1 antisense: 5'-GGATGGAGTTGTGCGGTGTAGATG-3'; c-FOS sense: 5'-GAGGACCTTATCTGTGCGTGAA-3' and c-FOS antisense: 5'-TGAGTCCACACATGGATGCTT-3'; SOX4 sense: 5'-CCCCCTGGGCCTGTACG-3' and SOX-4 antisense: 5'-CCGGGCTCGAAGTTAAAATC-3'; GAPDH sense: 5'-GTCTCCTCTGACTTCAACAGCG-3' and GAPDH antisense: 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Western blot analysis. DLD-1 and HCT116 colon cancer cells were plated and allowed to attach for 24 h. DIM was added to cell cultures at the indicated concentrations and incubated for 72 h. Cell lysates were prepared by suspending the cells in lysis buffer (Intron Biotechnology, Seoul, South Korea) and the resulting lysates were subjected to routine western blotting analysis as previously described (4,14).

Cell cycle analysis. Cell cycle analysis was performed by analyzing total DNA content with a FACstar flow cytometer (Becton-Dickinson, San Jose, CA, USA) using Becton Dickinson software (Lysis II and CellFit) as previously described (25).

Other statistical analysis. Statistically significant differences between experimental groups and controls were determined by one-way ANOVA and comparisons between groups were made with Student's t-test. Results are expressed as the mean \pm SEM. P-values <0.05 were considered to indicate statistical significance.

Results

DIM inhibits the growth of colon cancer cells. The effects of DIM on human colon cell growth were examined using DLD-1 and HCT116 cells following treatment at various concentrations for 72 h. As shown in Fig. 1, DIM inhibited cell proliferation of DLD-1 and HCT116 cells in a dose-dependent manner (Fig. 1A). Treatment of DIM resulted in a significant

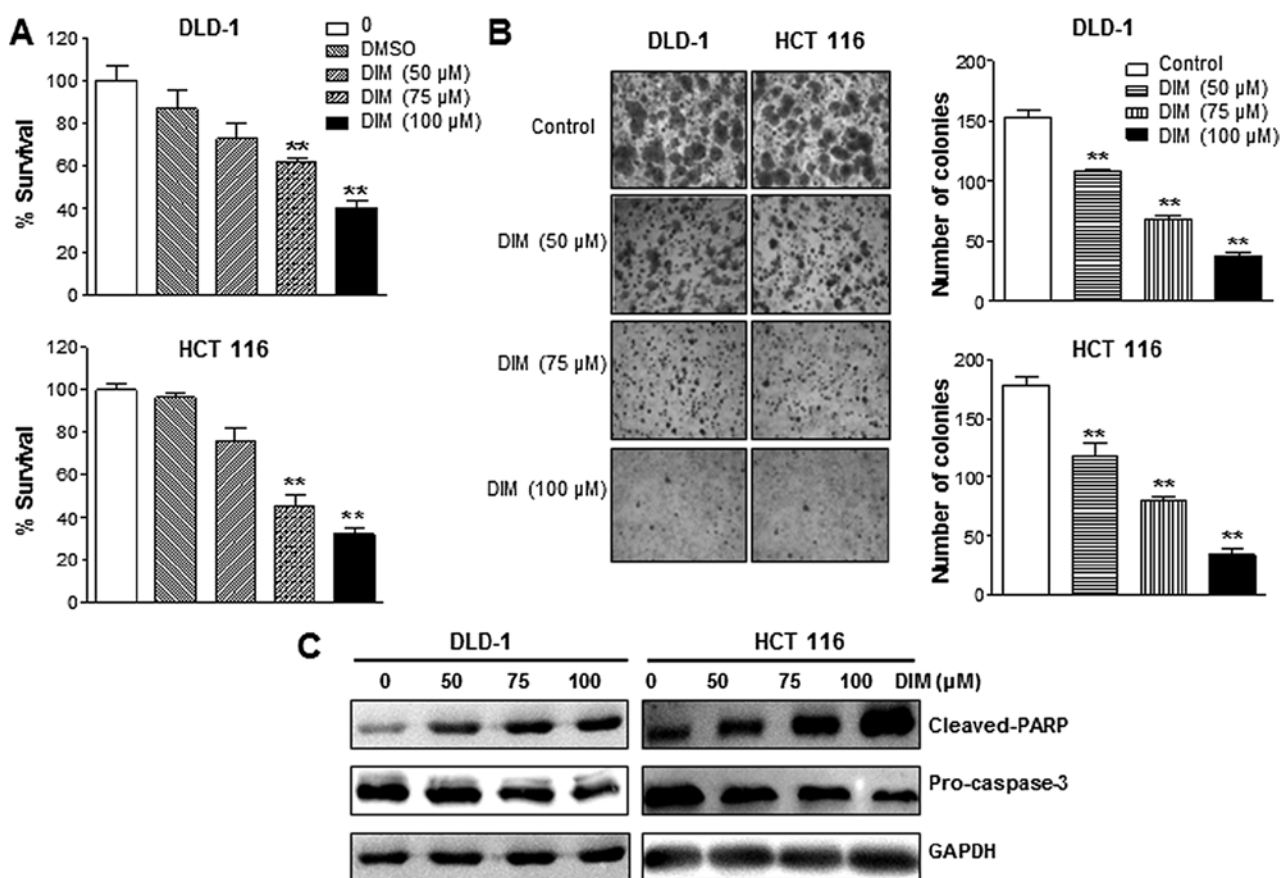


Figure 1. Effect of 3,3'-diindolylmethane (DIM) on the growth of human colon cancer cells. (A) Inhibition of cell growth was assessed by MTT assay. Human colon cancer cell lines (DLD-1 and HCT116) were treated with various concentrations of DIM for 72 h. (B) Colony formation assay. DIM significantly inhibited the colony formation ability in DLD-1 and HCT116 cells in a dose-dependent manner. (C) Effects of DIM on apoptosis in colon cancer cells. Western blot analysis of cleaved-PARP and pro-caspase-3 exposed to different concentrations of DIM. GAPDH was used as an internal control. Data are expressed as the percentage of control cells. Error bars express the mean (\pm SE) from three independent experiments. One-way ANOVA followed by Student's t-test was used for comparison among different doses of DIM (** $P < 0.01$ compared to the control).

inhibition of colony formation in DLD-1 and HCT116 cells in a dose-dependent manner (Fig. 1B). We also evaluated cell viability by quantifying levels of apoptotic proteins. We found that the DIM treatment resulted in a significant induction of apoptosis in DLD-1 and HCT116 cells. As shown in Fig. 1C, DIM significantly increased cleaved-PARP protein levels in a dose-dependent manner. Likewise, levels of pro-caspase-3 were significantly decreased by DIM treatment (Fig. 1C). Taken together, these data indicated that the DIM inhibits proliferation of human colon cancer cells.

Microarray analysis. To further investigate the effects of DIM on colon cancer cells, we performed gene expression profiling using an Illumina bead array platform. We identified a total of 1,424 genes significantly associated with the effect of DIM in HCT116 cells (Fig. 2A). Specifically, a total of 692 genes were significantly upregulated by DIM treatment while 731 genes were downregulated. Among these genes, *s100A16*, *VGF*, *FOSL*, *KRT15*, *CD68*, *FTHL1*, *CPA4* were the most highly upregulated while *SUSD2*, *MIR1974*, *CYP24A1*, *SOX4*, *FOS*, *CYR61*, *CNTNAP2*, *MYC*, *JAG1*, *EGR2*, *CCNE2*, *CTGF*, *MCM2* were the most significantly downregulated (Fig. 2B). The most significantly altered pathway was that of cell cycle control of chromosomal

replication (*CDC7*, *CDK2*, *CDT1*, *MCM2*, *MCM3*, *MCM4*, *MCM5*, *MCM6*, *MCM7*, *E2F2* and *E2F3*), which was significantly downregulated (Fig. 2C). Several oncogenes (*Myc*, *SOX4*, *CTGF*, *MCM2* and *FOS*) and cell cycle related genes (*cyclin D* and *cyclin E*) were significantly downregulated by DIM treatment in HCT116 cells (Fig. 2C). Gene set enrichment analysis also revealed highly presentation of cellular growth and proliferation involved in cell cycle regulation by DIM treatment (Fig. 2D). We next tried to identify the gene networks potentially involved in the DIM effects on colon cancer cells using Ingenuity™ Pathway Analysis (IPA). Our initial analysis identified a number of putative networks, and functional connectivity of the top network revealed significant downregulation of β -catenin (*CTNNB1*), *Myc*, and *FOS* genes (Fig. 3), suggesting suppression of β -catenin (*CTNNB1*), *Myc* and *FOS* activity by DIM treatment in colon cancer cells. Importantly, altered expression of these genes by DIM treatment affects other genes that are subsequently responsible for controlling colon cancer cell growth.

Activation of β -catenin is associated with poor prognosis in colon cancer patients. Given that Wnt proteins are regarded as oncogenes and activating mutations frequently convert the downstream target of Wnt signaling, β -catenin, to an oncogene

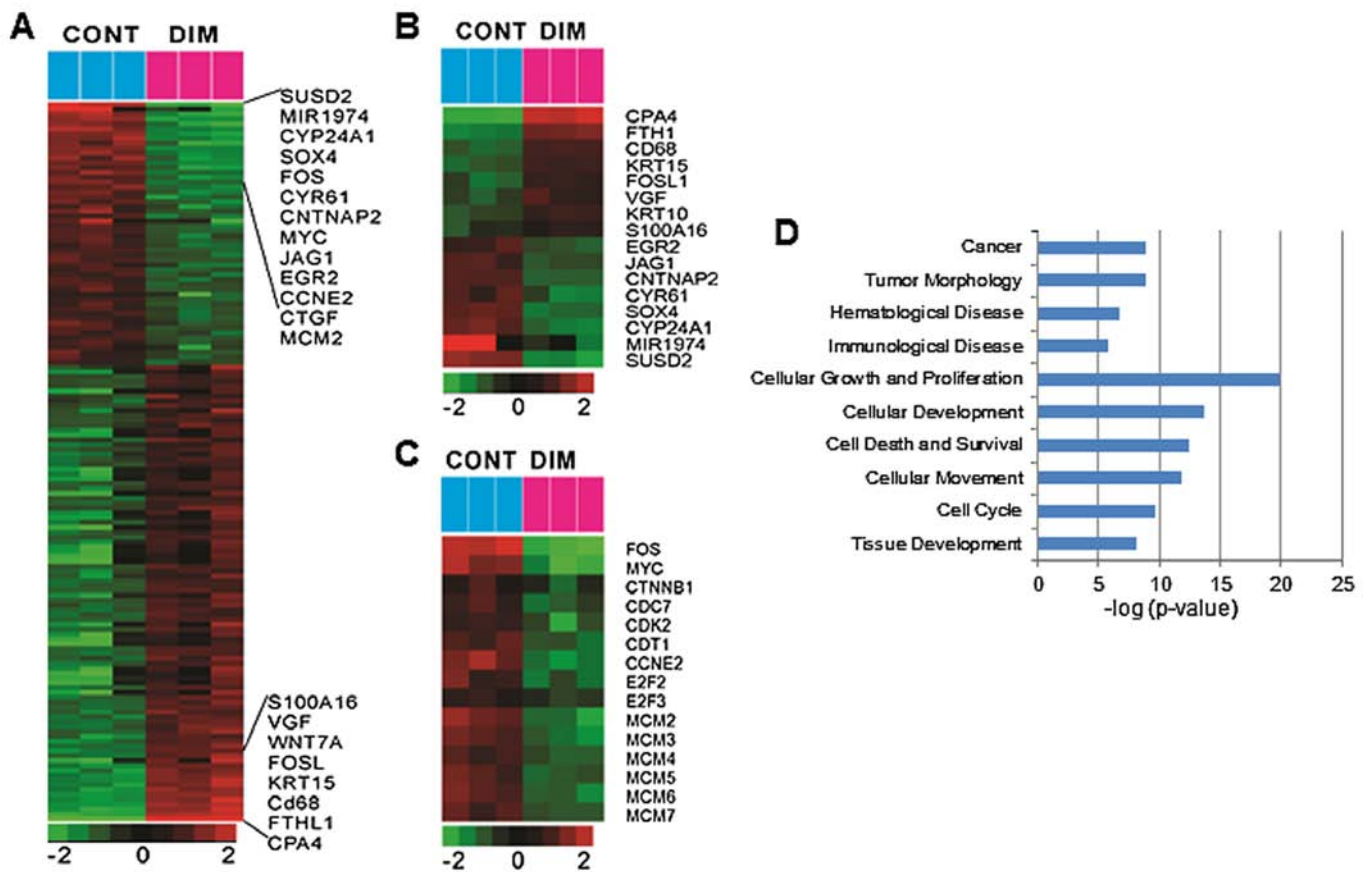


Figure 2. (A) Cross comparison of gene lists from two independent statistical tests from control and 3,3'-diindolylmethane (DIM)-treated HCT116 cells. Heat map summarizing the effects of DIM on the most significantly down- or up-regulated genes (B) and Wnt signaling pathway-related genes (C) in HCT116 cells. The data are presented in matrix format, with rows representing individual genes and columns representing each sample. Each cell in the matrix represents the expression level of a gene feature in an individual tissue. Red and green reflect high and low expression levels, respectively, as indicated in the scale bar (log₂ transformed scale). (D) Gene set enrichment analysis.

in human colon cancers, we next examined the association between the expression of β -catenin and clinical data of colon cancer patients. Publically available data from 226 colon cancer patients were downloaded from the National Center for Biotechnology Information GEO (GSE 14333). To estimate the β -catenin expression signature responsible for the clinical overall survival, we used receiver operating characteristic (ROC) curves from censored overall survival (OS) data using nearest neighbor estimation methods with a cut-off value of 7.057 intensities and under the curve (AUC) were calculated (AUC=0.635) (26–28). Associations between β -catenin and the patient survival rate were assessed using Kaplan-Meier plots and log-rank tests. Analysis of Kaplan-Meier plots revealed that patients with high expression of β -catenin (CTNNB1) had poorer overall survival compared with those of patients with low expression of β -catenin (CTNNB1), suggesting that activation of β -catenin (CTNNB1) is significantly associated with prognosis in colon cancer patients ($P=0.039$, Fig. 4). When comparing the microarray gene expression data, the expression levels of β -catenin (CTNNB1) were significantly different between low and high expression of β -catenin with colon cancer patients ($P<0.001$, Fig. 4).

Downregulation of β -catenin and c-Myc expression by DIM treatment in DLD-1 and HCT116 cells. Since β -catenin can

control expression of c-Myc and cyclin D1 and induce cell proliferation in colon cancer cells, we next tested whether β -catenin and c-Myc genes could be altered by DIM treatment in DLD-1 and HCT116 cells using qPCR and western blot analysis. qPCR showed that β -catenin and c-Myc were significantly decreased in both cell lines by treatment with 100 μ M DIM (Fig. 5A). Protein levels of β -catenin and c-Myc were also downregulated, while that of p- β -catenin, the inactive form of β -catenin, was upregulated in a dose-dependent manner after DIM treatment (Fig. 5B). These results suggested that DIM inhibited activation of β -catenin and c-Myc in colon cancer cells, indicating that DIM induces colon cancer cell death by inactivation of the Wnt signaling pathway, which normally promotes proliferation of cells in the colon.

DIM induces cell cycle arrest in DLD-1 and HCT116 cells. FACs analysis was performed to determine whether DIM regulates cell cycle progression in human colon cancer cells. DIM treatment resulted in a significant, time-dependent increase in the proportion of the cell population in the G1 phase in both DLD-1 and HCT116 human colon cancer cells, suggesting that DIM induced G1 cell cycle arrest (Fig. 5C). We also found that protein level of cyclin D1 were downregulated in a time-dependent manner after DIM treatment in DLD-1 and HCT116 cells (Fig. 5D). Taken together, these results

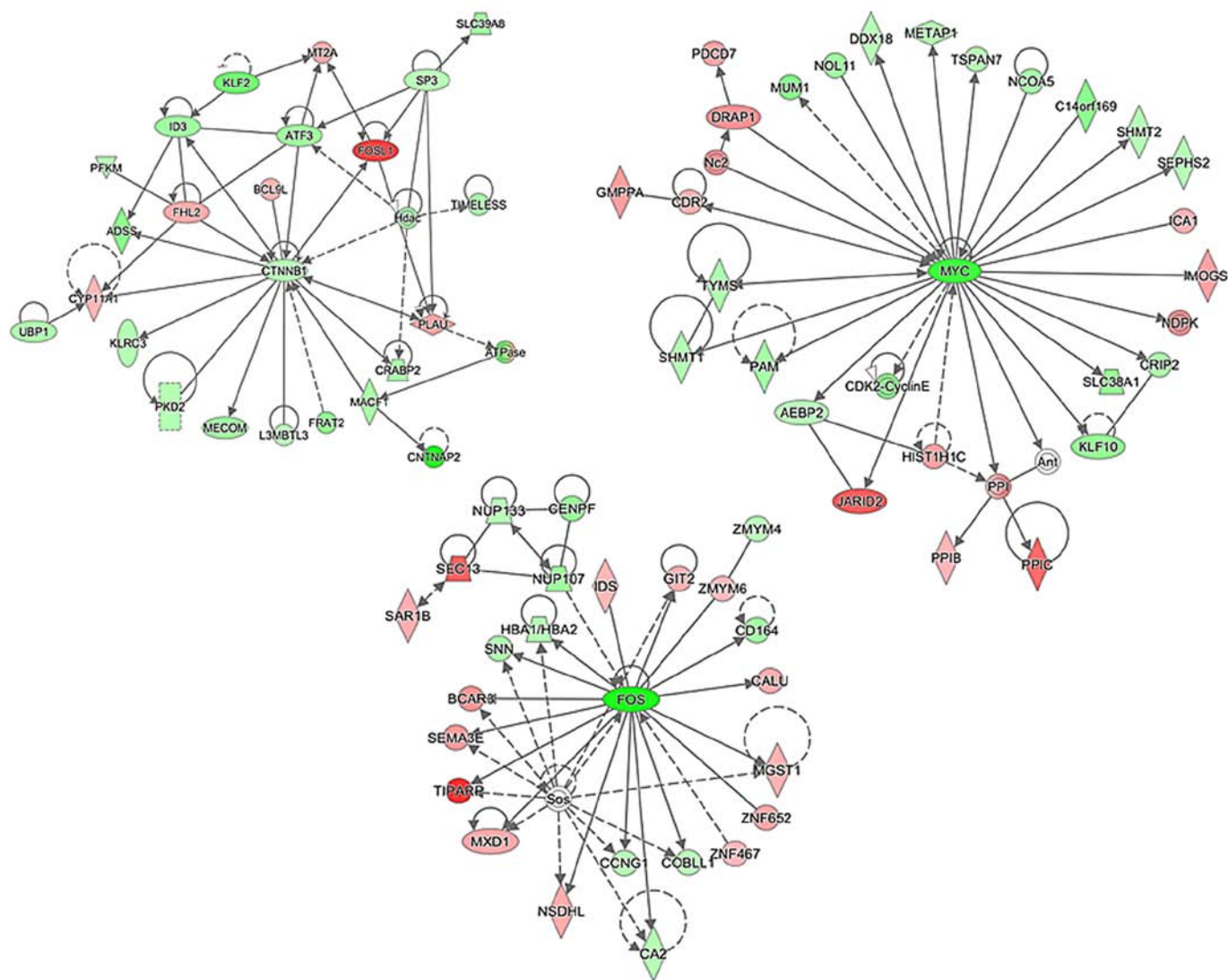


Figure 3. Ingenuity™ pathway analysis of β -catenin, Myc and FOS gene networks. Global networks showing interconnections among genes and their expression. Red and green colors in each shape indicate up- or down-regulation of gene expression after DIM treatment, respectively. Each line and arrow represents functional and physical interactions.

indicated that DIM induced G1 phase arrest in human colon cancer cells through downregulation of cyclin D1.

Downregulation of FOS and SOX4 expression by DIM treatment in DLD-1 and HCT116 cells. Protein and mRNA levels of c-FOS and SOX-4 were significantly decreased in both DLD-1 and HCT116 cell lines by treatment with 100 μ M DIM (Fig. 6A and B). These results suggested that DIM inhibited activation of c-FOS and SOX-4 in colon cancer cells, suggesting that DIM also suppressed onco-proteins in colon cancer cells.

Discussion

In the present study, we profiled gene expression following DIM treatment of colon cancer cells. We found that DIM significantly inhibited proliferation of colon cancer cells mediated by altered expression of genes involved in cell cycle progression and Wnt signaling. Our results showed for the first

time that β -catenin and c-Myc, which are major genes involved in colon carcinogenesis, are significantly downregulated by DIM treatment in colon cancer cells.

Gene expression profiling is a powerful tool that is useful for uncovering the molecular mechanisms underlying cellular functions in human cancers. Since DIM is a nontoxic, natural compound isolated from cruciferous vegetables, its anti-cancer effects have been actively explored by many researchers for cancer prevention and treatment (19,29-31). Wide ranging pleiotropic anti-tumor signals elicited by DIM have been previously shown to be orchestrated through the Ah receptor, Akt and NF κ B pathways impinging on cell cycle arrest and altering angiogenesis, invasion, and metastasis of a variety of cancer cells (19,29,31-34). DIM has been reported to induce apoptosis in colon cancer cells by several mechanisms involving cell cycle arrest, inactivation of Akt, induction of proteasomal degradation of class I histone deacetylases, and downregulation of survivin (4,35-37). However, a comprehensive biological mechanism by which

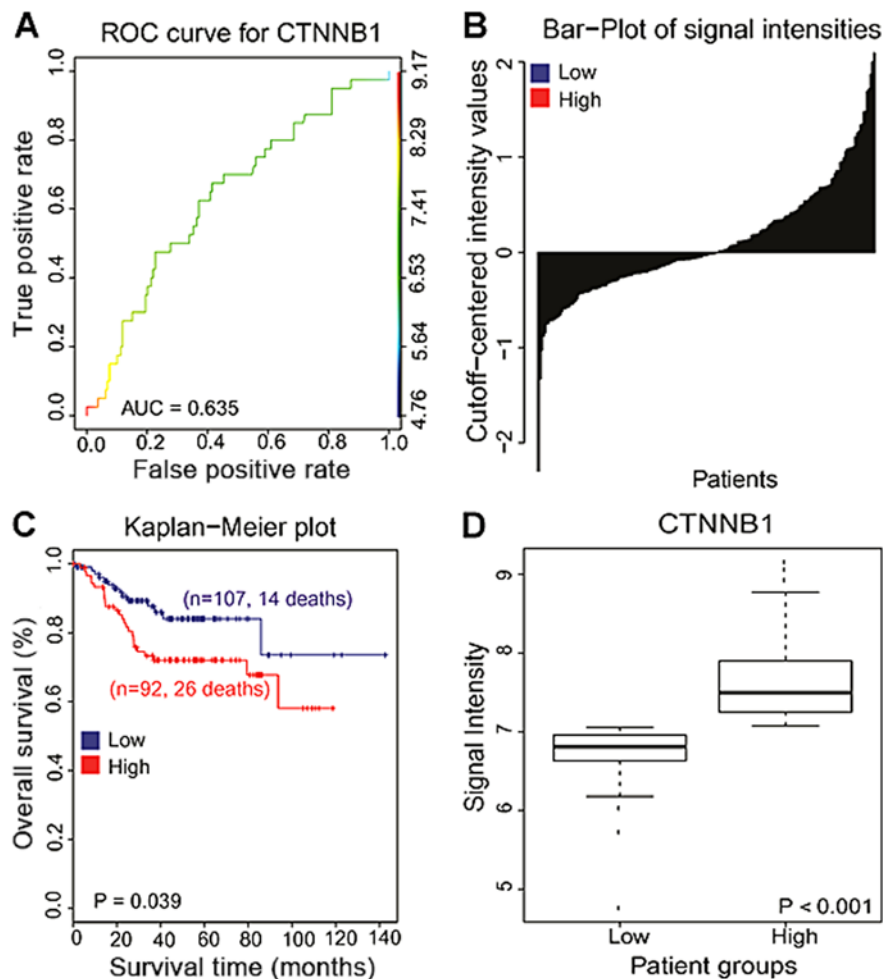


Figure 4. Data from 226 colon cancer patients were downloaded from NCBI GEO (GSE 14333). (A) Receiver operating characteristic (ROC) curve was constructed by using gene expression data from GSE 14333. (B) Intensity values based on the β -catenin (CTNNB1) signature of each patient (each bar represents the signal intensity of an individual patient). The intensity value was used to dichotomize patients into high- or low- β -catenin (CTNNB1) expression groups, with the 50th percentile as the cut-off value. (C) Kaplan-Meier plot of overall survival of colon cancer patients grouped on the basis of gene expression profiling. A log-rank test was used to evaluate differences between groups ($P < 0.05$ compared with high and low expression of β -catenin levels). (D) Comparison of expression levels of β -catenin (CTNNB1) in 226 colon cancer patients. The expression levels of β -catenin (CTNNB1) were significantly different in colon cancer patients ($P < 0.001$).

DIM inhibits colon cancer cell growth and induces apoptosis remains elusive.

We used gene expression profiling to determine how exposure to DIM alters the transcriptome of colon cancer cells. We found that DIM significantly altered the expression of more than 1000 genes. The top canonical pathway modified by DIM treatment in colon cancer cells was cell cycle control of chromosomal replication. Specifically, we observed significantly decreased expression of numerous genes involved in cell cycle control of chromosomal replication (CDC7, CDK2, CDT1, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, E2F2 and E2F3). Gene set enrichment analysis also showed high presentation of cellular growth and proliferation involved in cell cycle regulation by DIM treatment. In addition, several oncogenes (Myc, SOX4, CTGF, β -catenin and FOS) were significantly downregulated by DIM treatment. IPA showed that DIM significantly altered putative gene networks surrounding β -catenin (CTNNB1), Myc and FOS. Specifically, the expression of many downstream target genes of β -catenin (CTNNB1) and Myc were downregulated, as were β -catenin

and Myc themselves, suggesting that transcriptional activity of β -catenin and Myc was suppressed by DIM. This down-regulation might be responsible for the inhibition of colon cancer cell growth. Alterations in gene expression profiles by DIM exposure of breast cancer cells have been reported (38); however, to the best of our knowledge, this is the first report of a gene expression profiling study on colon cancer cells treated with DIM.

Since the downstream target of Wnt signaling, β -catenin, is an oncogene in human colon cancer, we investigated the relationship between the expression of β -catenin and clinical outcomes in colon cancer. Publicly available data from 226 colon cancer patients was used to determine whether there is an association between β -catenin activation and patient survival. Kaplan-Meier plot analysis and log-rank test analysis showed that patients with high expression levels of β -catenin had poorer overall survival when compared with patients who had low levels of β -catenin expression, suggesting that β -catenin is significantly associated with prognosis in colon cancer patients. Our results strongly indicated that the Wnt/ β -catenin signaling

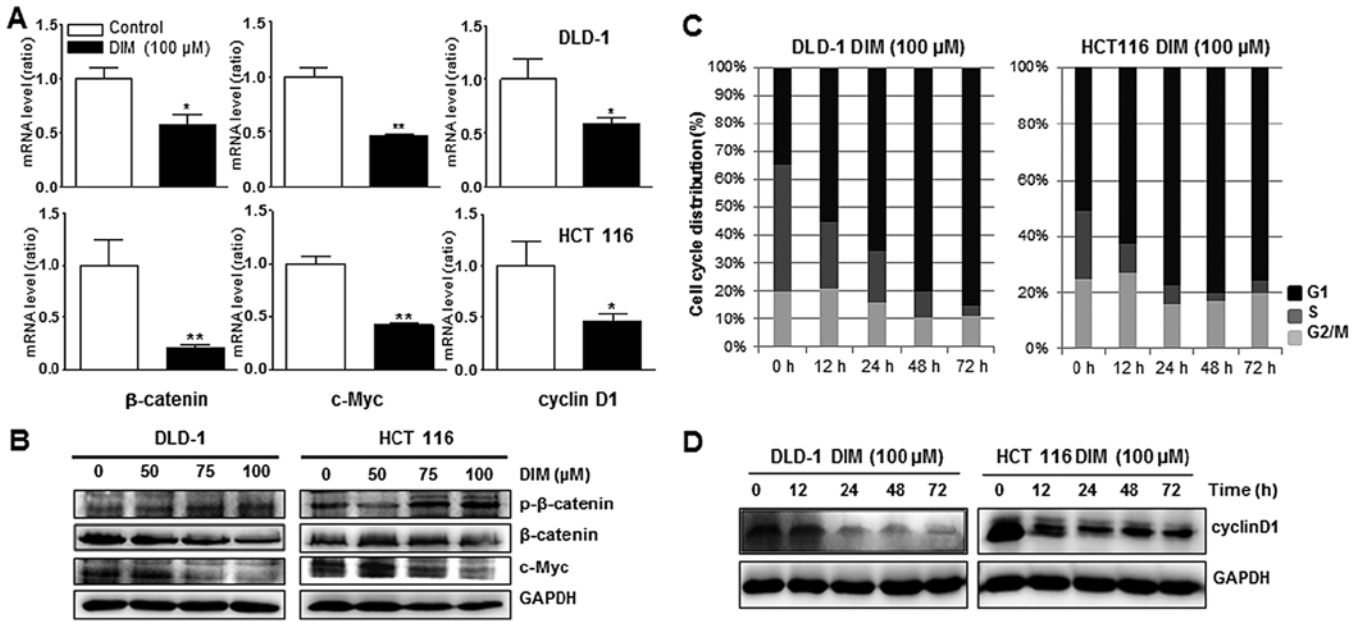


Figure 5. Effects of 3,3'-diindolylmethane (DIM) on β -catenin and its target genes. (A) Regulation of mRNA expression by DIM treatment at 72 h in colon cancer cells. Data are shown as the mean (\pm SE) of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared to the control. (B) Western blot analysis of β -catenin, p- β -catenin and c-Myc in cells treated with varying doses of DIM. (C) Effects of DIM on cell cycle distribution in colon cancer cells. Cell cycle analysis was performed in DLD-1 and HCT116 cells after DIM treatment at 12, 24, 48 and 72 h. Cell cycle distribution was calculated as the percentage of cells in the G1, S or G2/M phase. All experiments were performed at least four times. (D) Effects of DIM on cell cycle-related proteins in DLD-1 and HCT116 cells. Cyclin D level was measured by western blotting. GAPDH was used as an internal control.

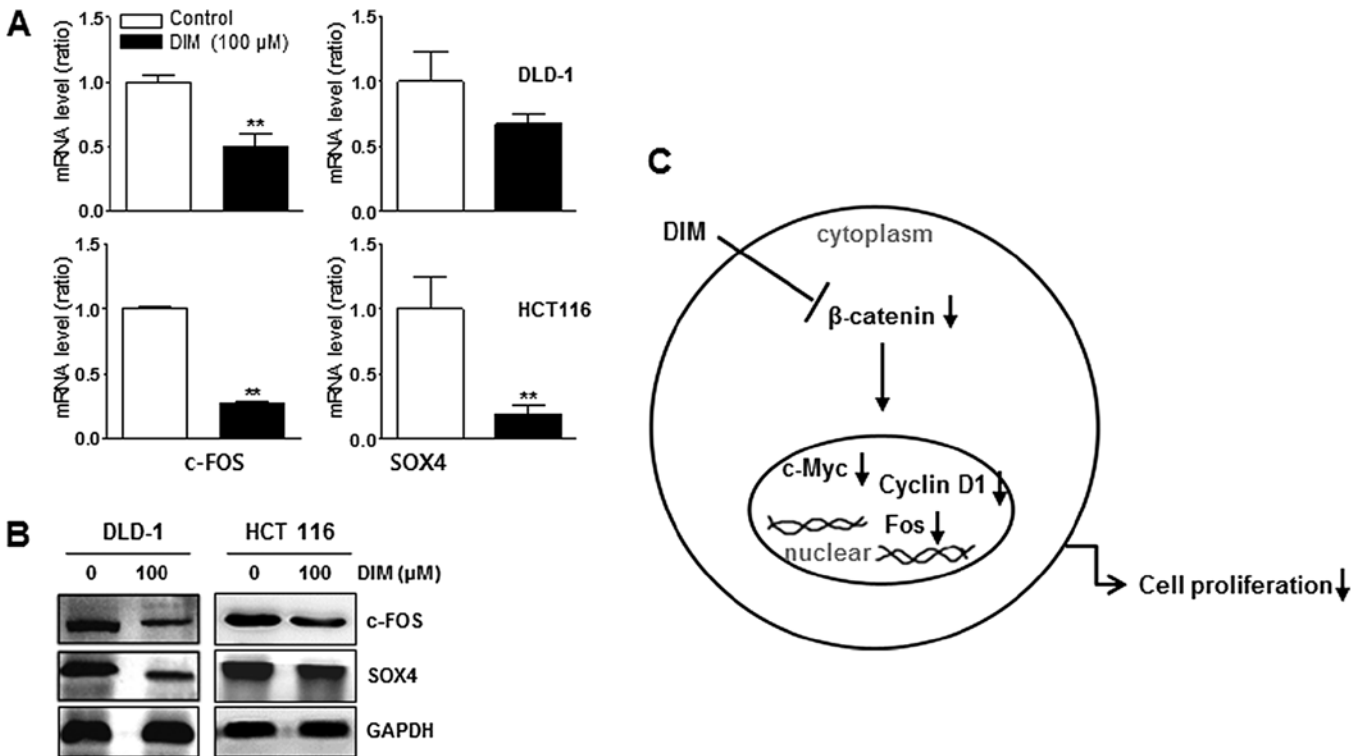


Figure 6. Effects of 3,3'-diindolylmethane (DIM) on c-FOS and SOX4. (A) Regulation of mRNA expression by DIM treatment at 72 h in colon cancer cells. Data are shown as the mean (\pm SE) of three independent experiments. ** $P < 0.01$ compared to the control. (B) Western blot analysis of c-FOS and SOX4 in cells treated with 100 μ M of DIM. GAPDH was used as an internal control. (C) Schematic representation of how DIM negatively regulates proliferation of colon cancer cells via suppression of c-Myc, FOS and cyclin D1 through inhibition of β -catenin translocation to the nucleus.

pathway plays a crucial role in carcinogenesis and progression of colon cancer.

The β -catenin and Tcf genes regulate expression of c-Myc and cyclin D1 and regulate cell proliferation (39,40). In the

present study, we found that DIM significantly suppressed the mRNA expression levels of β -catenin and c-Myc in two different colon cancer cell lines (DLD-1 and HCT116). We also found that protein levels of β -catenin and c-Myc were significantly downregulated after DIM treatment while level of phosphorylated β -catenin, the inactive form of β -catenin that is degraded by a ubiquitin-proteasome mechanism, was increased. We also showed that DIM induced a significant increase in the proportion of the cell population in the G1 phase of the cell cycle in a time-dependent manner in both DLD-1 and HCT116 cells. We confirmed that protein levels of c-Myc, FOS, and Cyclin D1 were also inhibited in a time-dependent manner after DIM treatment in DLD-1 and HCT116 cells. Together, these findings suggested that DIM induced G1 phase arrest in human colon cancer cells by altering the expression of c-Myc, FOS and cyclin D1, which may be mediated through suppression of β -catenin. Therefore, the anti-proliferative effects of DIM in colon cancer cells may be associated with inhibition of β -catenin, which in turn may induce cell cycle arrest by regulating expression of cyclin D1, FOS and c-Myc (Fig. 6C). However, further studies are needed to elucidate how DIM specifically regulates target genes downstream of β -catenin in the cytosol and nucleus.

In conclusion, to the best of our knowledge, this is the first study to show that DIM inhibits the Wnt/ β -catenin pathway in colon cancer cells. We believe that targeting Wnt/ β -catenin signaling with DIM may be an attractive strategy for the prevention and treatment of colon cancer.

Acknowledgements

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