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Antiproliferative Effects of Short-chain Fatty Acids on Human Colorectal Cancer Cells *via* Gene Expression Inhibition

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Abstract. *Background/Aim: Short-chain fatty acids (SCFAs) inhibit human colorectal cancer cell growth and tumorigenicity. We investigated the mechanism of the anti-proliferative effects of SCFAs on human colorectal cancer cells by examining their effects on gene expression. Materials and Methods: The DLD-1 cell line was cultured with different SCFAs. Gene groups whose expression levels decreased to <50% or increased >50% compared to untreated cells and the signalling pathways responsible for DLD-1 cell growth inhibition were identified and analyzed. Results: Genes whose expression levels decreased to ≤50% (791 genes) showed remarkable changes in gene function compared to genes whose expression levels increased ≥50%. These genes encode proteins involved in DNA replication and cell cycle/proliferation that contribute to major pathways responsible for suppression of colorectal carcinogenesis pathways. Conclusion: SCFAs inhibited the expression of genes encoding proteins involved in DNA replication and cell cycle/proliferation of human colorectal cancer cells and exerted antiproliferative activity via different pathways.*

Functional lactic-acid bacterial foods, such as probiotics, drastically improve intestinal microbial flora. The consumption of probiotics has attracted attention for the maintenance of health and the prevention of diseases, such as colorectal cancer or hepatocellular carcinoma (1-3). However, the preventive effects of prebiotics and probiotics on disease and their mechanisms of action remain unknown. Short-chain fatty acids (SCFAs) produced by intestinal microbiome have antitumor effects (3-8). We previously found that SCFAs, such as butyric acid, isobutyric acid and acetic acid, inhibit the growth of cultured human colorectal cancer cells and that butyric acid is the strongest inhibitor (3). For example, butyric acid has been found to up-regulate the expression of toll-like receptor 4 (TLR4) and the phosphorylation of MAPK and NK-κB in colon cancer cells *in vitro* (9). Further, sodium butyrate has been shown to influence the adhesion and growth-regulating galectin network that controls the proliferation of human colon cancer cells *in vitro* (10). DNA microarray analysis has contributed to our knowledge of tumour biomarkers and their potential suitability as targets of anticancer therapy as revealed by bioinformatics analyses (11-13).

Herein, DNA microarray analysis was conducted to investigate the common mechanisms responsible for the antitumor effects of butyric acid, isobutyric acid and acetic acid on human colorectal carcinoma cells and identified candidate signal transduction pathways using the Ingenuity Pathway Analysis (IPA) software.

Materials and Methods

Cell culture and in vitro assessment of the inhibitory effects of butyric acid, isobutyric acid and acetic acid on the proliferation of a human colorectal carcinoma cell line. The human colorectal carcinoma cell line (DLD-1 cells) was purchased from the Riken BRC Cell Bank, Japan. DLD-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin. Cultures were maintained at 37˚C in a humidified atmosphere comprising 5% CO_2. The cells (2×10^6 cells per well) were added to the wells of a 96-well plate and incubated with each SCFA or DPBS used as a vehicle for the test substances. The pH values of the cell culture media comprising butyric acid, isobutyric acid and acetic acid were maintained between 7 and 8, even after the addition of high concentrations of the SCFAs. Further, there were no inter-test differences in the effects on pH determined using different concentrations (50-300 μM) of each SCFA. Following similar published protocols (14), DLD-1 cells were cultured in the presence of each SCFA or DPBS, and the IC_{50} values were determined.

The inhibition of cell proliferation was quantified using the WST-8 assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) by spectrophotometrically measuring the absorbance (450 nm) of the

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water-soluble formazan formed *via* reduction by intracellular dehydrogenases. Here, we used the WST-8 assay to measure mitochondrial dehydrogenase activity. The IC_{50} value was calculated from the dose-response curve of each SCFA.

RNA extraction. For RNA extraction, the cells were seeded into a 6-well plate and incubated for 24 h. The cells were then treated for 24 h with each SCFA (15). Control DLD-1 cells were treated with the vehicle alone (DMSO, 0.39% v/v). After treatment for 24 h, total RNA was extracted from DLD-1 cells and purified using a PureLink RNA Mini Kit (Catalog No. 12183018A; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantities and purities of the RNAs were verified using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) by measuring absorbance ratios at 260/280 nm and 260/230 nm. RNA quality and integrity were verified using agarose gel electrophoresis. Only RNA preparations with an absorbance ratio ≥2.0 were used for microarray and quantitative reverse transcription PCR (RT-qPCR) assays.

Microarray and pathway analyses. Total RNA was extracted from the DLD-1 cells and used for transcriptome profiling using an Applied Biosystem SurePrint G3 Human CGH Microarray Kit, 8x60K, 1-color method (Agilent Technologies Inc., Lexington, MA, USA).

This was followed by fluorescent labelling and clean-up of labelled genomic DNA and implementation of the microarray. After scanning and calculating several signals on each probe, the resulting data files were generated and transferred.

Gene expression data were analysed using Applied Biosystems Expression Console and Transcriptome Analysis Console software. After data mining, pathway enrichment analysis was performed using IPA software (http://www.ingenuity.com). The microarray data were validated by profiling the expression of genes through RT-qPCR assays.

The DNA microarray was used to search for a group of differentially expressed genes by determining and adjusting the total RNA concentration of cells treated with butyric acid, isobutyric acid and acetic acid at their IC_{50} values for 24 h. Gene groups with expression levels that were <50% or >50% of those of untreated cells were subjected to IPA analysis. Gene expression data were analysed using Transcriptome Analysis Console software (version 3.1) (Applied Biosystems). The genes were filtered using the microarray fold-change (MA_FC) values. The list of genes (MA_FC <0.5) was analysed using IPA software to identify canonical pathways, biological functions, gene networks and key processes that were enriched, modulated effectively or both by SCFAs in DLD-1 cells. The raw microarray data files were submitted to the Gene Expression Omnibus.

Statistical analysis. Data are presented as the mean of n experiments with the standard error (mean±SE). Data processing was performed using XLSTAT (http://WWW.xlstat.com) and the Student's *t*-test. *p*-Values <0.05 indicate statistically significant differences.

Results

The proliferation of DLD-1 cells was inhibited in the presence of acetic acid, isobutyric acid or butyric acid (Figure 1). The IC_{50} values of butyric acid, isobutyric acid and acetic acid were 2.89±0.29, 12.9±0.80 and 16.6±1.10, respectively [IC_{50} (mM, mean±SEM, n=4)].

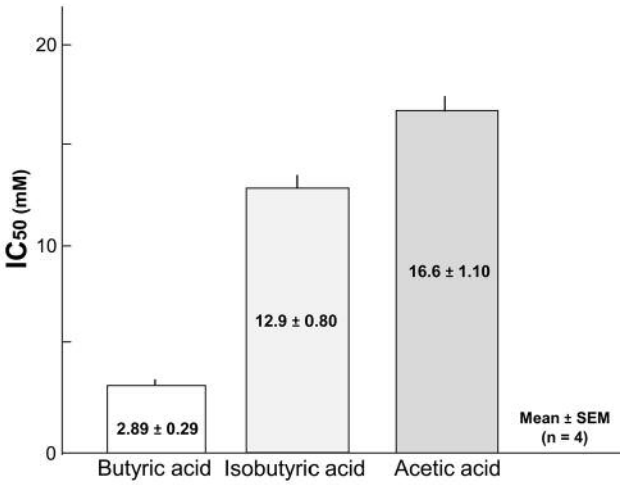

Figure 1. *Volume response curves of the cell growth-inhibitory activity in DLD-1 cell*.

According to the results of DNA microarray and pathway enrichment analysis by IPA, there was hardly any genetic function that had greatly changed in the group of genes whose expression levels increased to ≥50% (the gene group in which blue colour was increased to >50%). Conversely, the change in genetic function was remarkable in the group of genes whose expression levels decreased to ≤50% (the group of genes whose green colour was reduced to <50%). Therefore, genes whose expression levels were decreased to ≤50% after 24 h of treatment with SCFAs were examined in detail. The number of such genes was 791. These genes are involved in the cell cycle/proliferation and DNA replication of most colorectal cancer cells. Some of the main genes involved in cell cycle and DNA replication are as follows: E2F1, UHRF1, HIST2H3A, HIST1H4K, HIST1H4L, HIST1H3B, HIST1H3D, HIST1H3H, FOXM1, *etc*.

The effects of genes whose expression levels were reduced by ≤50% after butyric acid treatment on cell cycle/proliferation and DNA replication functions were examined, and antitumor pathway and the disease & function heat map analysis of butyric acid was performed. Similar analysis was performed for isobutyric acid and acetic acid. The results of pathway analysis and the disease & function heat map analysis of butyric acid, isobutyric acid, and acetic acid are shown in Figures 2, 3 and 4, respectively. The disease and function heat map analysis results showed that the antitumor effect of butyric acid was the strongest among the three SCFAs. The results also showed that SCFA hardly suppresses the expression of cancer-related genes and strongly suppresses tumour metabolic molecules such as tumour cell cycle, DNA replication, recombination and repair. This is a notable finding demonstrating the mechanism of antitumor activity of SCFAs.

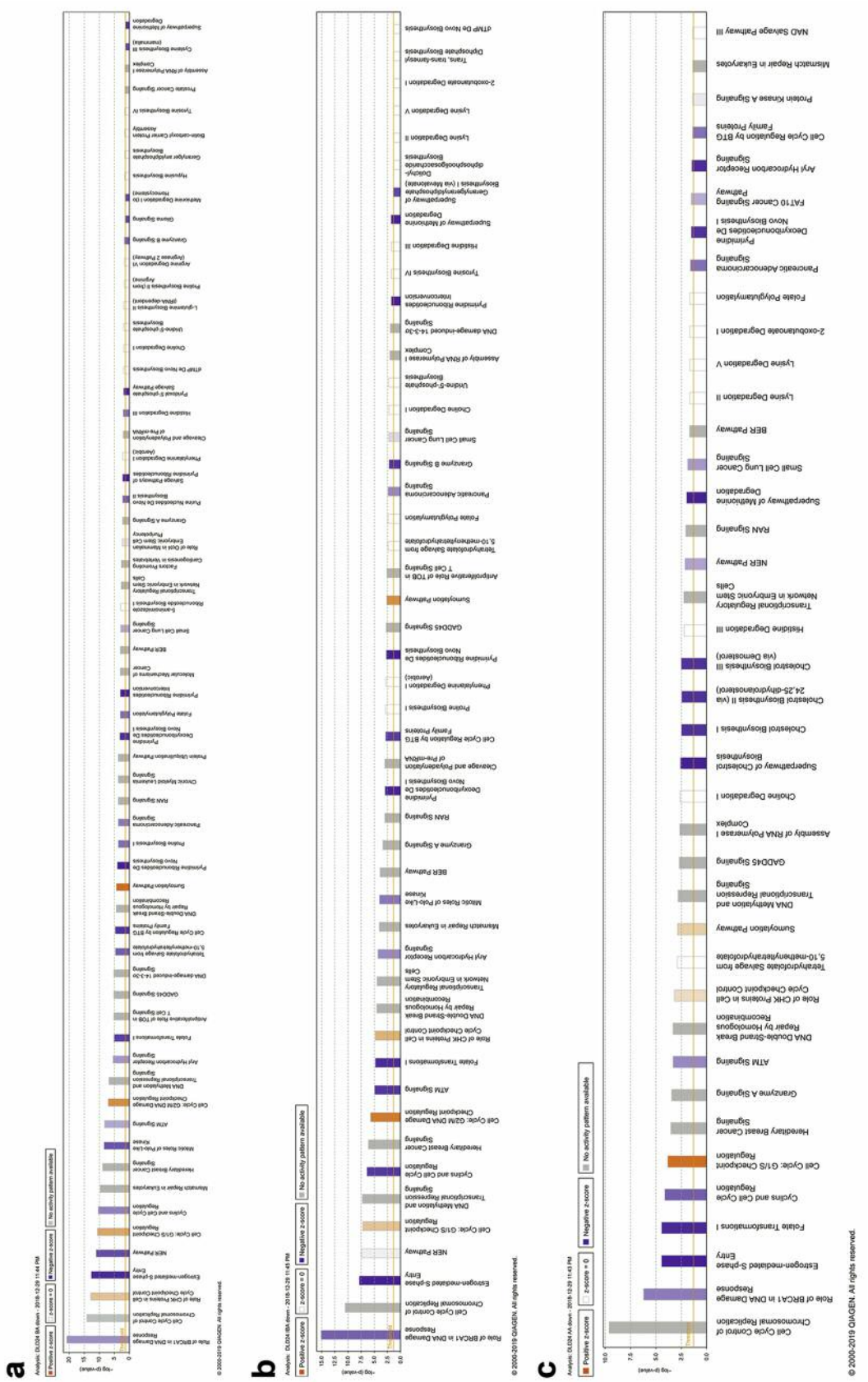

Figure 2. *Influence on the cell cycle/proliferation and DNA replication function of the group of genes whose expression levels decreased by ≤50% following treatment with butyric acid (a), isobutyric acid (b), and acetic acid (c).*

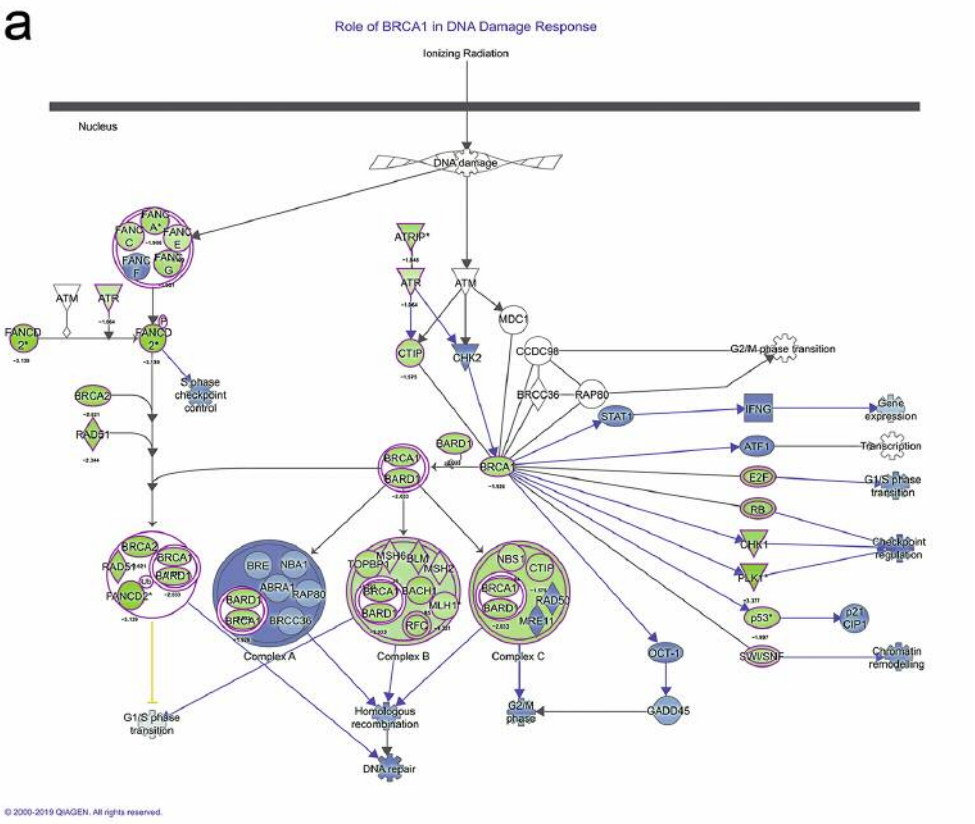

Figure 3. *Continued*

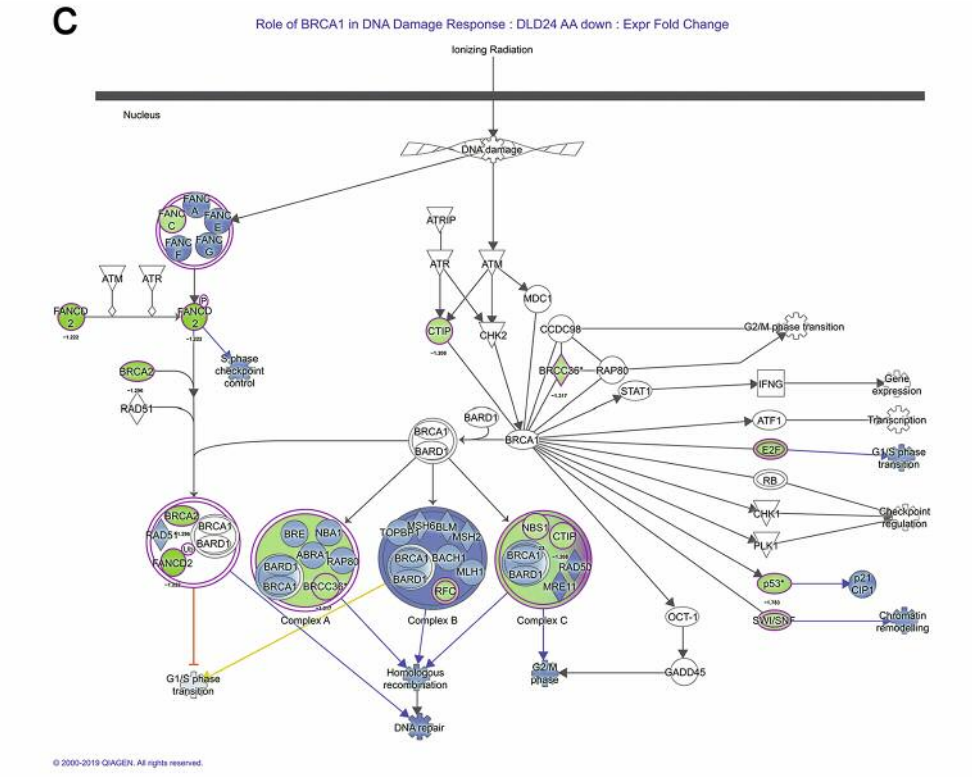

Figure 3. *Analysis of the antitumor pathway of butyric acid (a), isobutyric acid (b), and acetic acid (c).*

Discussion

The mechanism of the antitumor action of SCFAs has been studied in various systems. In this study, we revealed that butyric acid had the strongest antitumor activity among butyric acid, isobutyric acid and acetic acid SCFAs. Most reports on the antitumor activity of SCFAs are for butyric acid (6, 7) or isobutyric acid (16). This is the first report on the comprehensive analysis of the mechanism of the antitumor action of butyric acid, isobutyric acid, and acetic acid in human colon cancer cells. The Wnt/β-catenin, PI3K/Akt/mTOR and Ras/MEK/ERK signalling systems are known as oncogenic pathways of colorectal cancer (17-19).

The analysis revealed that the main mechanism of the antitumor activity of SCFAs was suppression of tumour growth and metabolic functions such as cell cycle, DNA replication, recombination and repair, and almost no suppression of cancer-related genes. Suppression of a group of molecules involved in tumour growth and metabolism leads to death of tumour cells. The mechanism of the antitumor effect of SCFAs obtained by this analysis has been considered to be a ground-breaking finding. In addition to conventional cancer research targeting cancer-related genes such as molecular targeted therapy, the one that targets the metabolic system of cancer has recently begun to develop. Satoh K has performed a metabolomic analysis of colorectal cancer tissue and reported that tumour cells were rich in S-adenyl methionine (SAM), which is a metabolite of methionine (20). The involvement of SCFA in the suppression of SAM formation in colorectal tumour cells is noteworthy and should be assessed in future research.

Although SCFAs such as butyric acid, isobutyric acid, and acetic acid have shown the possibility of an antitumor effect by suppressing the growth/metabolism system of colorectal cancer, probiotics have also been shown to efficiently produce SCFAs such as butyric acid, among others. Furthermore, functional foods such as probiotics and prebiotics have attracted attention for playing a role in preventing colon cancer development, with people taking these for their beneficial effect in preventing cancer. The results of this study suggest that colorectal carcinogenesis may be significantly suppressed by sustained intake of food such as probiotics or prebiotics. In contrast, *Fusobacterium nucleatum* has attracted attention as the cause of colorectal cancer (21-23). For example, FadA Adhesin, a fungal component of the nucleatum, degrades E-cadherin on the cell membrane and binds to β-catenin *via* p120 to activate oncogenic signalling *via* pathways such as Wnt/β-catenin (24). However, the presence of *F. nucleatum*-

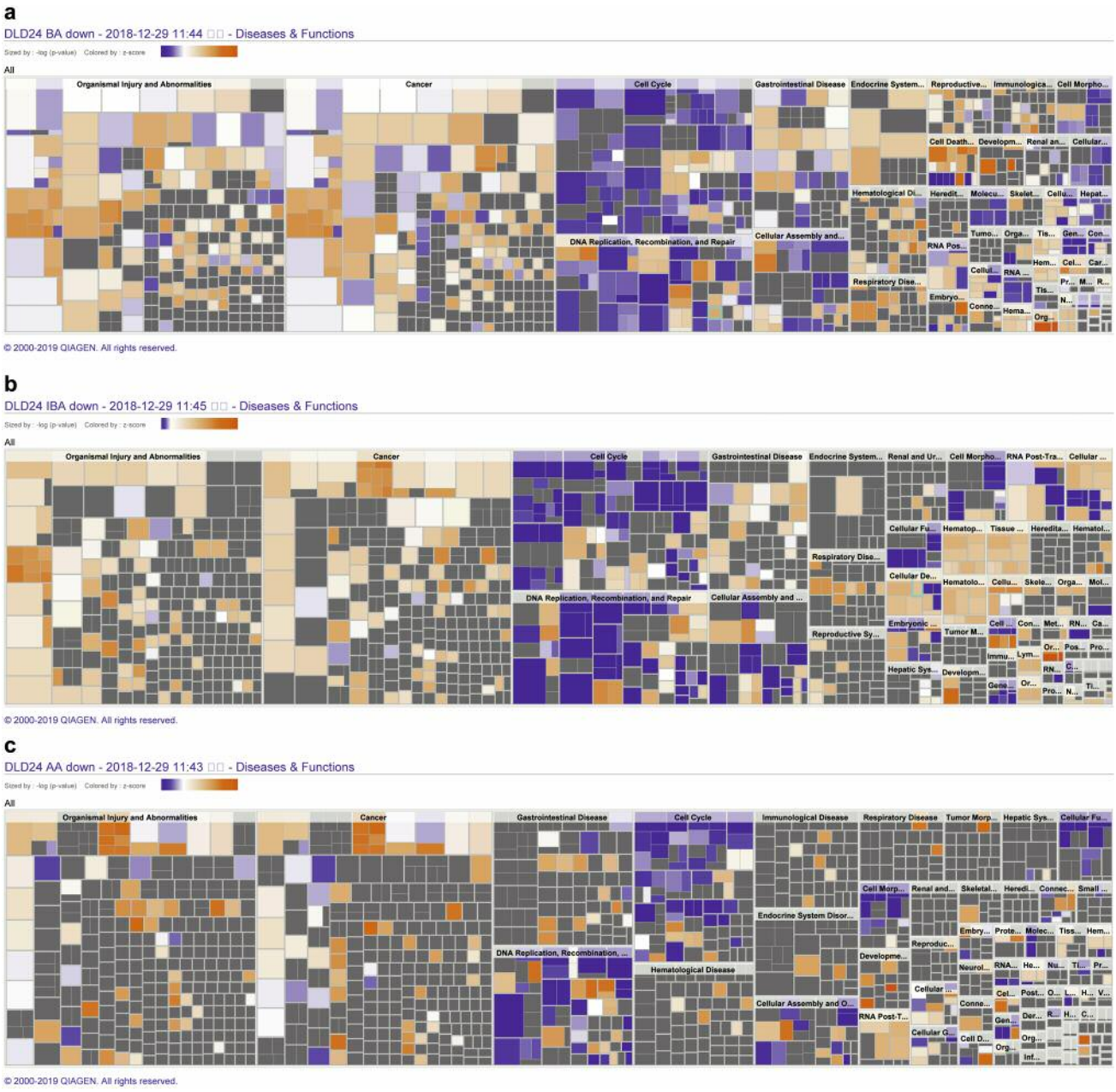

Figure 4. *The disease and function heat map of butyric acid (a), isobutyric acid (b), and acetic acid (c) following treatment of DLD-1 cells for24 h.*

negative colorectal cancer and colorectal cancer with low levels of *F. nucleatum* has also been reported (25). *F. nucleatum* aggressively acts on colorectal cancer lesions and has been shown to activate β-catenin signalling through the TLR4/P-PAK1/P-β-catenin S675 cascade (26). However, the role of *F. nucleatum* in colorectal carcinogenesis has not been yet completely elucidated. The relationship between *F. nucleatum* and antitumor activity of SCFAs is also an issue that needs to be clarified in the future.

Conflicts of Interest

The Authors declare that no competing interests exist regarding this study.

Authors' Contributions

Tadashi Ohara was in charge of the culture experiments and DNA microarray part, and Tsutomu Mori was in charge of all the data mining and IPA analysis of the results.

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