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Original Article

Microarray analysis of anti-cancer effects of docosahexaenoic acid on human colon cancer model in nude mice

Shitao Zou[1*], Xingjun Meng[1*], You Meng[2*], Jin Liu[1], Biao Liu[3], Shuyu Zhang[4], Weiqun Ding[5], Jinchang Wu[6], Jundong Zhou[1,6]

[1]Suzhou Cancer Center Core Laboratory, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou 215001, Jiangsu, P. R. China; [2]Department of Surgical Oncology, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou 215001, Jiangsu, P. R. China; [3]Department of Pathology, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou 215001, Jiangsu, P. R. China; [4]School of Radiation Medicine and Protection, Medical College of Soochow University, Suzhou 215001, Jiangsu, P. R. China; [5]Department of Pathology, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma, USA; [6]Department of Radio-Oncology, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou 215001, Jiangsu, P. R. China. [*]Equal contributors.

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Abstract: Docosahexaenoic acid (DHA), a derivative of ω3- polyunsaturated fatty acids present in fish oil, is well known to have anticancer activity on colon cancer cells, but the molecular and cellular mechanisms remain to be further clarified. In this study, anti-cancer effects of DHA on colon cancer cells were observed in a nude mouse HCT-15 xenograft model. And then, the different genes expression and signal pathways involved in this process were screened and identified using cDNA microarray analysis. Results of genes expression profiles indicated a reprogramming pattern of previously known and unknown genes and transcription factors associated with the action of DHA on colon cancer cells. And several genes related to tumor growth and metastasis including COX2, HIF-1α, VEGF-A, COMP, MMP-1, MMP-9, SCP2, SDC3, which were down-regulated by DHA, were further confirmed in HCT-15 cell line using RT-PCR method. In summary, our data might provide novel information for anti-cancer mechanism of DHA in colon cancer model.

Keywords: DHA, colon cancer, microarray analysis, in vivo, nude mice

Introduction

The pathogenesis of colon cancer is a long and multifactorial process involved alterations in gene expression which are induced by non-genotoxic and epigenetic mechanisms [1]. Inflammation of bowel significantly increases the risk of developing malignancy in the colon [2].

Dietary omega-3 polyunsaturated fatty acids (ω3-PUFAs) contain more than one carbon double bond and have a variety of anti-inflammatory and immune-modulating effects. These ω3-PUFAs are able to regulate eicosanoid production [3], formation of lipid peroxidation products [4], genes transcription [5], Wnt/β-catenin signal pathway [6], and cell autophagy [7]. Epidemiological studies have showed that high intake of saturated fat increases the risk of colon cancer, and that diets rich in ω3-PUFAs reduce the risk of colon cancer development [8], and there is an inverse association of consumption of fish oil (ω3-PUFAs) with colon cancer.

Docosahexaenoic acid (DHA), a derivative of ω3-PUFAs, has been demonstrated to have beneficial effects on several autoimmune and inflammation disorders [9] and have anticancer properties both in vitro and in vivo [10]. DHA enriched diets can exert anti-inflammatory properties, thus suppressing colon cancer development [11]. Importantly, DHA is cytotoxic to tumor cells, with little or no effects on normal cells. However, the mechanism of how DHA sup-

Table 1. PCR primer sequences

Genes	Primer sequences	Products (bp)
COX2	F: 5'-AACATCTCAGACGCTCAGGAAATAG-3' R: 5'-GCCGTAGTCGGTGTACTCGTAG-3'	183
HIF-1α	F: 5'-CACTGCACAGGCCACATTCA-3' R: 5'-GGTTCACAAATCAGCACCAAG-3'	101
VEGF-A	F: 5'-AGGAGGGCAGAATCATCACGA-3' R: 5'-AGGATGGCTTGAAGATGTACTCG-3'	134
COMP	F: 5'-GCCTTCAATGGCGTGGACTT-3' R: 5'-CACATGACCACGTAGAAGCTGG-3'	116
MMP1	F: 5'-GCACATGACTTTCCTGGAATTG-3' R: 5'-TTTCCTGCAGTTGAACCAGCTA-3'	159
MMP9	F: 5'-GAGGTGGACCGGATGTTCC-3' R: 5'-GCACTGCAGGATGTCATAGGTC-3'	168
SCP2	F: 5'-GTTTGAGAAGATGAGTAAGGGAAGC-3' R: 5'-ATCTGAGGAGCAACTGGGTGAG-3'	126
SDC3	F: 5'-TGCTCGTAGCTGTGATTGTGGG-3' R: 5'-GTATGTGACGCTCGCCTGCTT-3'	140
GAPDH	F: 5'-GAAGGTGAAGGTCGGAGTC-3' R: 5'-GAAGATGGTGATGGGATTTC-3'	224

presses tumor growth has not been firmly established.

In this study, we constructed a xenograft nude mouse model of colon cancer to investigate the effect of DHA on colon cancer growth in vivo. Furthermore, we explored the molecular mechanism of DHA inhibiting colon cancer progression using cDNA microarray analysis.

Materials and methods

Cell culture

Human colon cancer cell line HCT-15 was obtained from Shanghai Cell Bank (Shanghai, China). Cells were maintained in DMEM medium (Hyclone, Logan, Utah, USA) containing 10% FBS (Hyclone, Logan, Utah, USA) and cultured in a humidified 5% CO_2 atmosphere at 37°C.

Tumor formation assay in a nude mouse model

Two-Three weeks old female BALB/c nude mice were obtained from Laboratory Animal Center of Soochow University (Suzhou, China) and maintained under specific pathogen-free conditions temperature (23-25°C) and humidity (40-50%). HCT-15 cells were harvested from subconfluent cell culture plates, washed with PBS and resuspended with physiological saline at a concentration of 1×10^5 cells/μl. A 0.1 ml of HCT-15 suspended cells was subcutaneously injected into the right flank of each mouse aged 4 weeks. Mice were randomly assigned to two groups (12 mice for each group), one group mice were fed a 7.5% fish oil-based diet (Teklad Diets, Madison WI, high in DHA), and the other one were fed a 7.5% corn oil-based diet (no DHA) beginning three days prior to injection. After transplantation, the mouse weight and growth of the subcutaneous tumors were assessed every two days. Xenograft tumor size was monitored by measuring the width (W) and length (L) with callipers, and volumes were calculated with the formula: $(W^2 \times L)/2$. Eight weeks after injection, the mice were euthanized, and the volumes of subcutaneous tumors were recorded. The tumors will be dissected, sectioned and preserved in liquid nitrogen.

RNA isolation and microarray analysis

Total RNA from the tumors of each group was extracted by TRIzol (Invitrogen, California, USA). Double-stranded cDNA was synthesized from total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, California, USA) according to the manufacturer's instructions. cDNA was labeled overnight using the NimbleGen One-Color DNA Labeling Kit (Roche, Basel, Swiss) and washed with NimbleGen wash buffer kit. Hybridization was carried out by NimbleGen Hybridization System according to the manufacturer's instructions. Microarray slides were scanned using an Axon GenePix 4000 B scanner (Axon Instruments, Foster City, CA, USA).

RT-PCR analysis

RT-PCR analysis was carried out using DHA-treated and untreated cDNA in HCT-15 cells. Amplification was performed over 28 cycles consisting of 95°C for 3 min, 57°C for 30 sec and 72°C for 30 sec. The sequences for primers were listed on the **Table 1**.

PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The relative intensity of each band was normalized against the intensity of the GAPDH band amplified from the same sample.

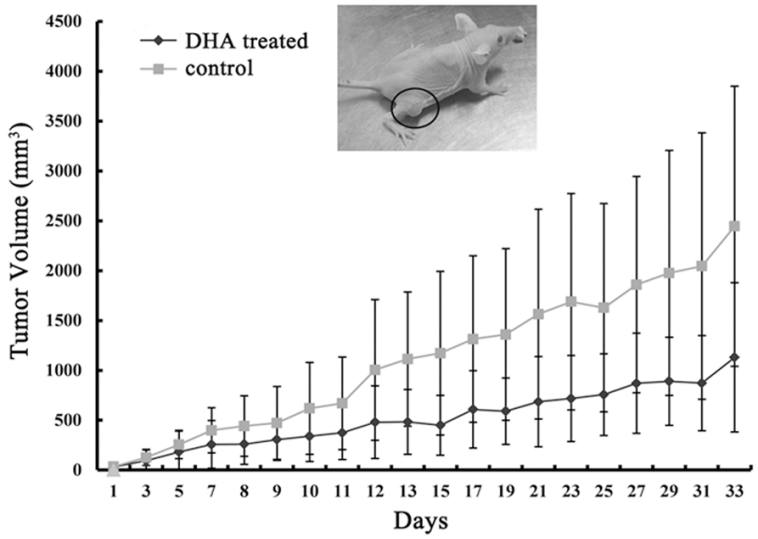

Figure 1. Fish oil diet suppresses colon tumor growth in nude mice. HCT-1 cells (1×10^5 in 0.1 ml PBS) were injected into the right flank of each mouse aged 4 weeks. Tumor volume was calculated with the formula: $(W^2 \times L)/2$ and expressed as cubic millimeters.

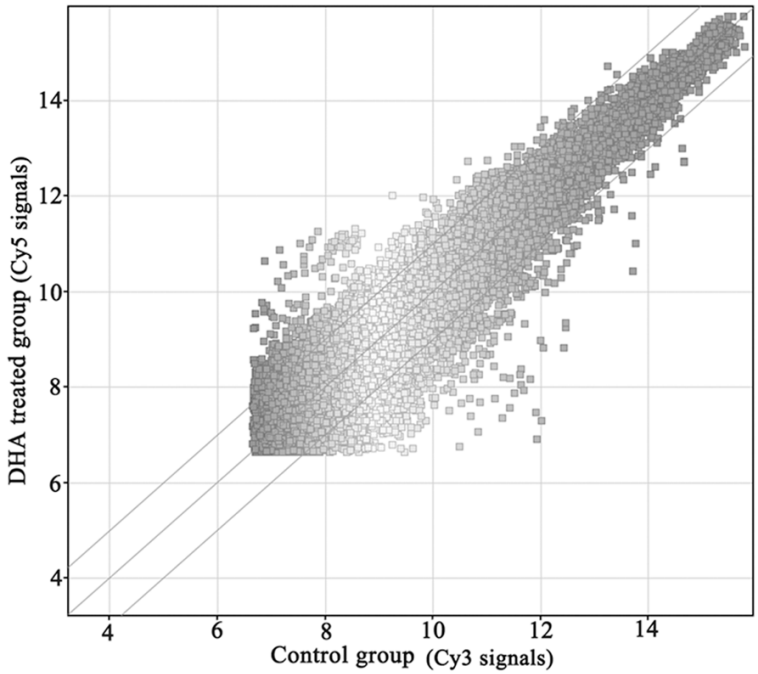

Figure 2. Scatter plot view of gene expression. Expression intensity Cy5: Cy3 ratios of DHA-treated versus untreated HCT-15 cells. The ratios (Cy-5: Cy-3) of genes that have 2-fold expression were considered up-regulated, and those with 0.5-fold expression were considered down-regulated. Approximately 2073 differentially expressed genes were detected in DHA treated.

Statistical analysis

Statistical analyses were calculated using SPSS 17.0. The results shown were the means ± SD. A. *P-value* < 0.05 was considered to indicate a statistically significant difference.

Results

DHA inhibits tumor growth in vivo

HCT-15 cells were subcutaneously injected into nude mice which were randomly assigned to two groups. The volumes of the tumors were recorded every two days, and xenograft tumors were harvested at the end of experiment. Tumor volumes from DHA treated mice were remarkably smaller than those from the control mice. Efficacy of DHA treatment became much significant with time (**Figure 1**, $P < 0.05$). Clearly, the in vivo results indicated that DHA could inhibit colon cancer cell growth.

DHA induced differential gene expression pattern in xenograft tumors

In this study, gene expression analysis was done for two group samples: DHA treated xenograft tumors and control group. Measurements on the intensities of the expressed genes were represented in **Figure 2**, as simple bivariate scatter plots comparing the profiles of DHA treated xenograft tumors to the vehicle-treated xenograft tumors. The X axis represented the control signal values and the Y axis meant DHA treated signal values. Changes in the gene expression pattern were observed as indicated by the shifts of the data points in the scatter plot. To obtain an overall gene expression pattern on a specific cluster of genes in DHA treated xenograft, an in-depth analysis of microarray data was carried out. As **Table 2**

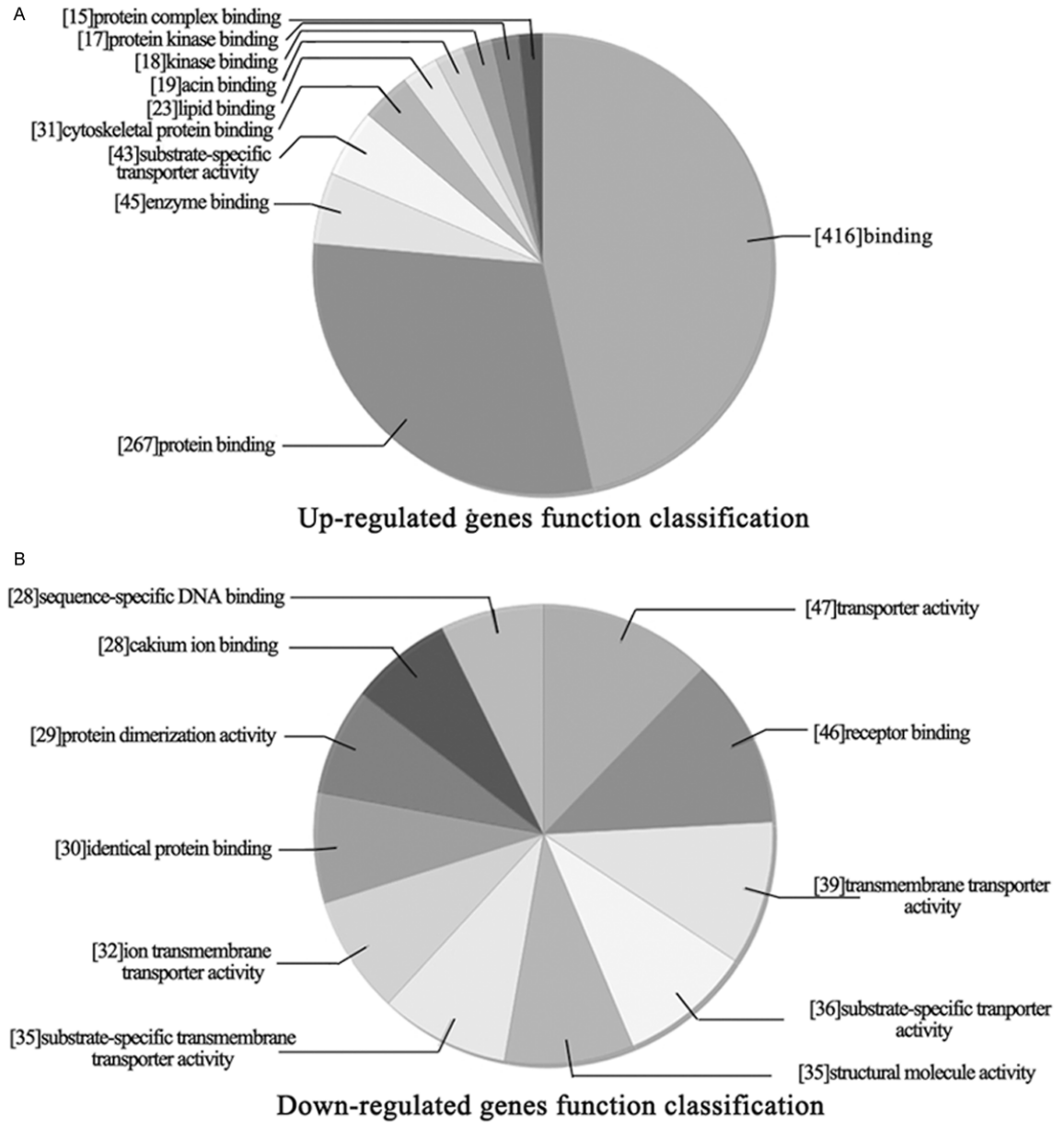

Figure 3. Molecular function classification of differentially expressed genes in DHA treated. A. Up-regulated genes function classification. B. Down-regulated genes function classification.

showed (Top 20-fold), after treated with DHA, there was a total of 2073 differentially expression genes (fold change ≥ 2) including 967 up-regulated genes and 1106 down-regulated genes.

DHA induced differential gene ontology analysis

To obtain gene ontology on a specific cluster of genes in DHA treated xenograft, an in-depth analysis of microarray data was carried out. As shown in **Figure 3**, up-regulated genes were divided into several groups according to the category of ontology, including protein complex binding, protein kinase binding, kinase binding, actin binding, lipid binding, cytoskeletal protein binding, substrate-specific transporter activity, enzyme binding, protein binding and binding molecular. Meanwhile, down-regulated genes were divided into: sequence-specific DNA binding, cakium ion binding, protein dimerization activity, identical protein binding, ion trans-

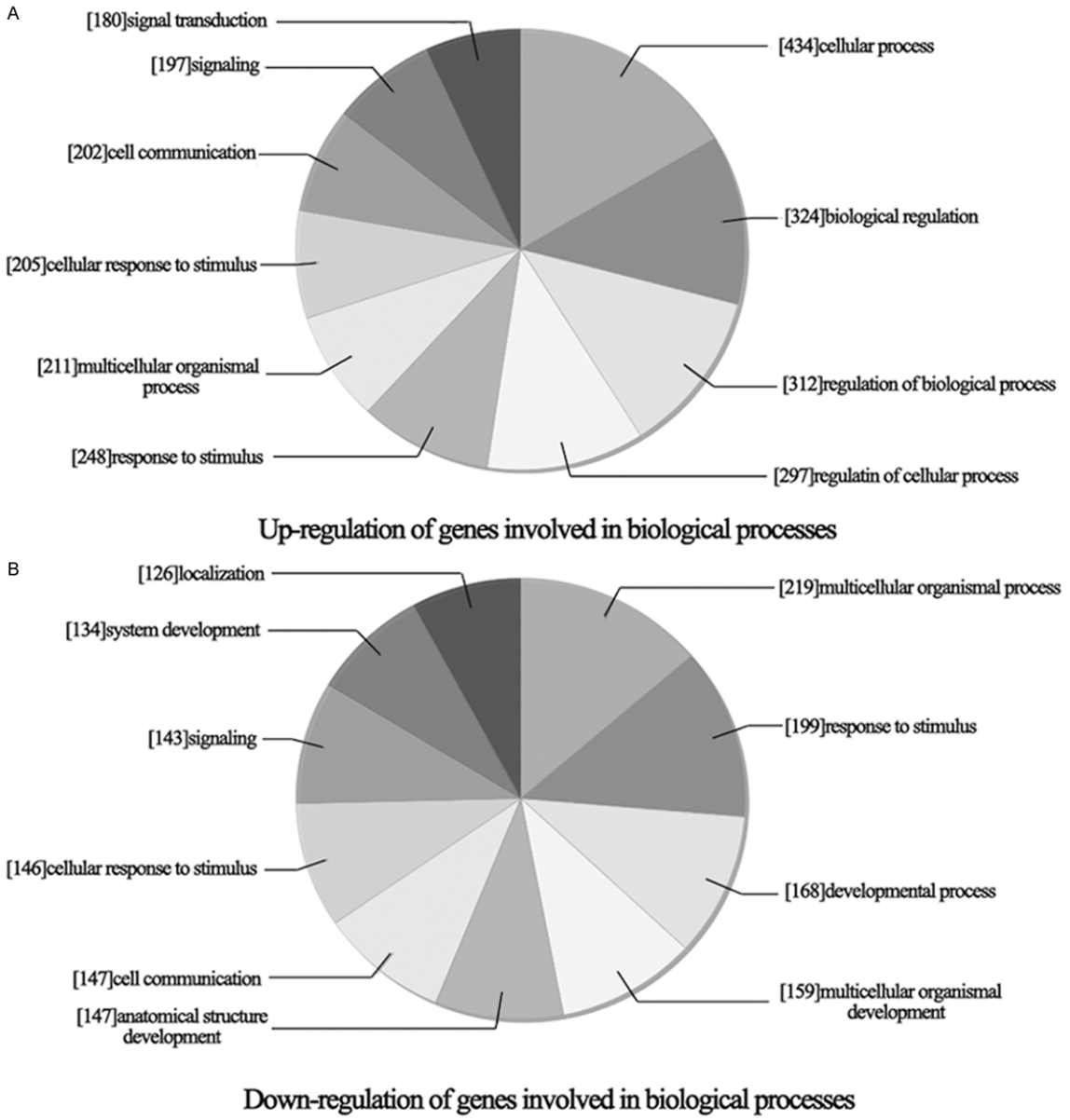

Figure 4. Biology process classification of differentially expressed genes in DHA treated. A. Up-regulation of genes involved in biological process. B. Down-regulation of genes involved in biological process.

membrane transporter activity, substrate-specific transmembrane transporter activity, transporter activity, receptor binding, transmembrane transporter activity, substrate-specific transporter activity and structural molecule activity molecular.

DHA induced differential genes involved in biological process

Furthermore, analyze the effect of DHA on tumor cell biological process. As shown in **Figure 4**, up-regulated genes (**Figure 4A**) mainly involved in signal transduction, signaling, cell communication, cellular response to stimulus, multicellular organismal, response to stimulus, cellular process, biological regulation, regulation of biological process and regulation of cellular process. Down-regulated genes (**Figure 4B**) mainly involved in localization, system development, signaling, cellular response to stimulus, cell communication, anatomical structure development, multicellular organismal process, response to stimulus, developmental process and multicellular organismal development.

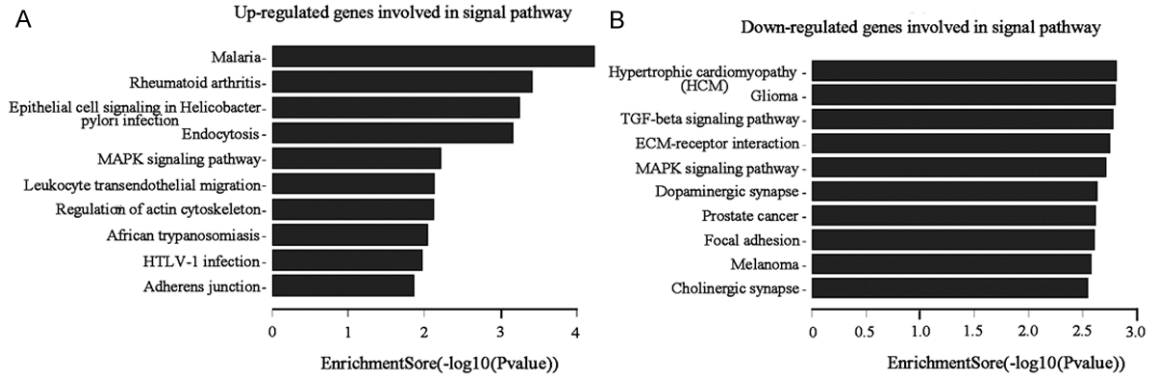

Figure 5. Pathway analysis of genes differentially expressed in DHA treated. A. Up-regulated genes involved in signal pathways. B. Down-regulated genes involved in signal pathways.

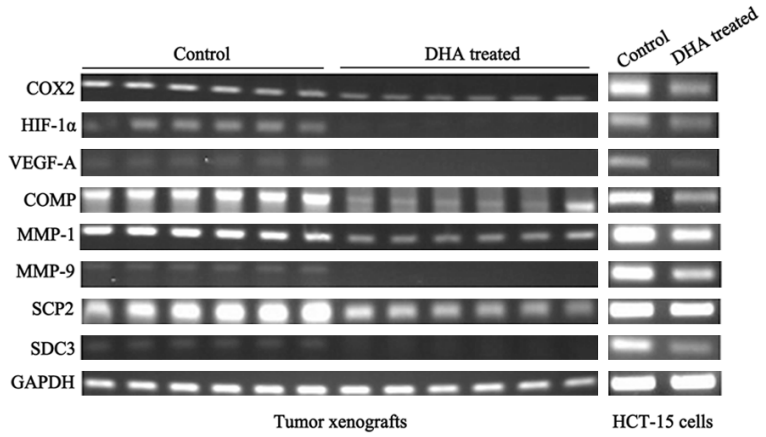

Figure 6. mRNA levels expression of genes differentially expressed in DHA treated: RT-PCR analysis was conducted using sequence specific primers for selected expressed genes to confirmation the expression changes detected by the cDNA microarrays.

DHA induced differential gene pathway analysis

Next, analyze the signal pathway involved in differential expression genes induced by DHA. As shown in **Figure 5**, up-regulated genes (**Figure 5A**) got primary involved in malaria, rheumatoid arthritis, epithelial cell signaling in Helicobacter pylori infection, endocytosis, MAPK signaling pathway, leukocyte transendothelial migration, Regulation of actin cytoskeleton, African trypanosomiasis, HTLV-1 infection and adherens junction. Down-regulated genes (**Figure 5B**) got mostly involved in hypertrophic cardiomyopathy, glioma, TGF-beta signaling pathway, ECM-receptor interaction, MAPK signaling pathway, dopaminergic synapse, prostate cancer, focal adhesion, melanoma and cholinergic synapse.

RT-PCR detects genes expression in xenograft tumors and colon cancer cells HCT-15

As microarray assays showed that, the mRNA level expression of COX2, HIF-1α, VEGF-A, COMP, MMP-1, MMP-9, SCP2 and SDC3 in DHA treated tumors were down-regulated by 2-3 folds. Then, RT-PCR was used to verify the 8 genes expression, and to explore the roles of them in DHA anti-cancer effect. As shown in **Figure 6A**, the expression of COX2, HIF-1α, VEGF-A, COMP, MMP-1, MMP-9, SCP2 and SDC3 were significantly reduced in DHA treated mice tumors. Those results indicated that COX2, HIF-1α/VEGF-A and MMPs signal pathways might mediate DHA inhibited colon cancer growth. Furthermore, we detected the effect of DHA on COX2, HIF-1α, VEGF-A, COMP, MMP-1, MMP-9, SCP2 and SDC3 expression in cultured colon cancer cells HCT-15. After 48 h treatment with DHA, COX2, HIF-1α, VEGF-A, COMP, MMP-1, MMP-9, SCP2 and SDC3 expression were obviously decreased. Collectively, this result was completely consistent with the results in vivo.

Discussion

In this study, we demonstrated that DHA could inhibit colon xenograft tumor formation and growth in vivo. Then, cDNA microarray analysis and RT-PCR analysis were used to screened

Table 2. Differentially expressed genes in colorectal tumors treated with DHA or not (Top 20-fold)

Up-regulated genes	Fold	Chromosome	Gene description
MGP	13.865016	chr12	matrix Gla protein
MGP	13.296371	chr12	matrix Gla protein
IGKC	11.253024	chr2	immunoglobulin kappa constant
IGKV1-5	10.592265	chr2	immunoglobulin kappa variable 1-5
IGKC	10.222312	chr2	immunoglobulin kappa constant
N/A	10.1450815	chr2	Homo sapiens cDNA clone MGC: 40426 IMAGE: 5178085, complete cds.
LOC651928	9.441747	chr2	similar to Ig kappa chain V-II region RPMI 6410 precursor
N/A	8.916328	chr2	Homo sapiens cDNA clone MGC: 12418 IMAGE: 3934658, complete cds.
IGKV1-5	8.710333	chr2	immunoglobulin kappa variable 1-5
IGKV1-5	8.467994	chr2	immunoglobulin kappa variable 1-5
AGC1	8.335031	chr15	aggrecan 1 (chondroitin sulfate proteoglycan 1, large aggregating proteoglycan, antigen identified by monoclonal antibody A0122)
IGKC	7.951564	chr2	immunoglobulin kappa constant
N/A	7.855886	chr2	Homo sapiens cDNA clone MGC: 23888 IMAGE: 4704496, complete cds.
AZGP1	7.7640123	chr7	alpha-2-glycoprotein 1, zinc
N/A	7.670388	chr2	Homo sapiens cDNA clone MGC: 32764 IMAGE: 4618950, complete cds.
N/A	7.6073294	chr2	Homo sapiens cDNA clone MGC: 27376 IMAGE: 4688477, complete cds.
C11orf43	7.5508437	chr11	chromosome 11 open reading frame 43
IGKC	7.5209823	chr2	immunoglobulin kappa constant
N/A	7.502064	chr2	Homo sapiens cDNA clone MGC: 71990 IMAGE: 30353269, complete cds.
IGKC	7.418584	chr2	immunoglobulin kappa constant
PDLIM3	31.80185	chr4	PDZ and LIM domain 3
LOC391749	26.017834	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC402207	21.524765	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC391763	15.055618	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC646066	14.325004	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC391766	13.561946	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC402199	13.3026	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
CLIC2	13.166692	chrX	chloride intracellular channel 2
REG3A	12.277953	chr2	regenerating islet-derived 3 alpha
LOC285563	12.225561	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC391761	12.132503	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
REG3A	11.841921	chr2	regenerating islet-derived 3 alpha
LOC391745	10.417792	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC285697	10.091853	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
LOC643211	9.717468	chr2	hypothetical protein LOC643211
LOC653442	9.707283	chr4	similar to deubiquitinating enzyme 3
REG3A	9.436449	chr2	regenerating islet-derived 3 alpha
LOC649159	9.336311	chr16	similar to CG7467-PA, isoform A
LOC391742	9.3053875	chr5	similar to Transcription initiation factor TFIID subunit 11 (Transcription initiation factor TFIID 28 kDa subunit) (TAF(II)28) (TAFII-28) (TAFII28) (TFIID subunit p30-beta)
UNC5B	9.165422	chr10	unc-5 homolog B (C. elegans)

and identified the different genes and signal pathways involved in this process. We found that DHA inhibited colon cancer growth was a complex process of multi factors and multi plane interconnected, containing molecular interaction, receptor activity, membrane recep-

tor trafficking and so on. In addition, this study confirmed that DHA down-regulated several genes expression related to tumor growth and metastasis including COX2, HIF-1α, VEGF-A, COMP, MMP-1, MMP-9, SCP2, SDC3.

Cyclooxygenase (COX), known as prostaglandin (PG) H2 synthase, is the rate-limiting enzyme in the conversion of arachidonic acid into PGs. COX has two kinds of isoenzymes COX1 and COX2. As an inducible enzyme, overexpression of COX2 has been frequently observed in melanoma, colon cancer, breast cancer, liver cancer, cervical cancer, esophageal cancer, pancreatic cancer, gastric cancer, but rarely examined in normal tissues [12, 13]. Many studies have implicated that PGE2, the metabolite of COX2 enzyme, can promote the extracellular matrix degradation and induce cancer cell invasion and metastasis [14]. When COX2 is targeted through either gene knockouts or COX2-specific inhibitors, a significant reduction of number of tumors is observed suggesting that COX2 plays a key role in colon tumorigenesis [15]. DHA can be used as a competitive inhibitor of COX2 and block PGs (mainly PGE2) synthesis, thus inhibit tumor cell proliferation [16, 17].

In addition, studies have shown that COX2/PGE2 is closely related to inflammation, cell cycle progression and angiogenesis [18]. Angiogenesis is the precondition of tumor growth, invasion and metastasis. Several growth factors involve in endothelial cell proliferation, migration and angiogenesis, and VEGF which mediates tumor vascularization and subsequently initiates the formation of metastases [19] is the most prominent and the most important factor for tumor angiogenesis. Study shows that over-expression of COX2 can promote VEGF expression through up-regulating HIF-1α expression, thus inducing tumor angiogenesis [20]. ω-3 PUFA suppresses colon cancer cell proliferation, angiogenesis and metastasis by inhibiting COX2 expression in vitro [14]. Rao et al reported that fish oil diet could reduce the activity of COX2 and suppress tumor growth in xenograft rat model of colon cancer [21]. In this study, we confirmed that DHA inhibition of colon cancer had a high correlation with down-regulation of COX2-HIF-1 α-VEGF pathway both in vitro and in vivo.

Cartilage oligomeric matrix protein (COMP) is a member of the thrombospondin family of extracellular glycoproteins. The function of COMP remains unclear, but it may have a structural role in endochondral ossification and in the assembly and stabilization of the extracellular matrix by its interaction with collagen fibrils and matrix components [22]. Matrix metalloproteinases (MMPs) are a family of multidomain Ca^{2+}-dependent and Zn^{2+}-containing endopeptidases, which can degrade almost all components of the extracellular matrix (ECM) [23]. Recent evidence has implicated MMPs in the regulation cell proliferation, migration, differentiation, angiogenesis, inflammation and signaling [24, 25]. In this study, we detected DHA could inhibit COMP, MMP1 and MMP9 expression both in vivo and in vitro, indicating that COMP, MMP1 and MMP9 might mediate the inhibition of DHA on colon cancer.

Sterol carrier protein 2 (SCP2) is a soluble alkaline protein, which play an important role in cholesterol biosynthesis, transport, transformation [26]. Syndecan 3 (SDC3) is a kind of cell surface transmembrane proteoglycan protein, which belongs to the syndecan family proteins. Syndecans are capable of mediating a broad range of functions including cell-cell and cell-extracellular matrix adhesion as well as acting as co-receptors in growth factor binding. In this study, we found that SCP2 and SDC3 expressions were down-regulated after colon cancer cells or colon xenograft tumor models were treated with DHA.

In summary, we firstly used cDNA microarrays of colon xenograft tumor to screen a vast array of DHA-responsive signaling genes and molecules representing several signaling pathways involved in colon cancer growth. The modulation of colon cancer cell growth by DHA is apparently mediated through the inhibition of COX2, HIF-1α, VEGF-A, COMP, MMP-1, MMP-9, SCP2 and SDC3 expression. However, the detailed molecular mechanisms of DHA inhibited those genes expression are not clear and remain to be elucidated.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jundong Zhou, Department of Radio-oncology, Nanjing Medical University Affiliated Suzhou Hospital, 16 West Baita Road, Suzhou 215001, Jiangsu, P. R. China. E-mail: zhoujundong330@163.com

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