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ORIGINAL ARTICLE

Omega-3 PUFA Alters the Expression Level but Not the Methylation Pattern of the WIF1 Gene Promoter in a Pancreatic Cancer Cell Line (MIA PaCa-2)

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Abstract

Pancreatic cancer is the fourth leading cause of death in both males and females, with a 5-year relative survival rate of 8%. The Wnt signaling pathway has a significant role in the pathogenesis of many tumors, including those of pancreatic cancer. Hypermethylation of the Wnt inhibitory Factor-1 (WIF1) gene promoter have been detected in different types of cancer. In contrast, the anticancer effects of long-chain omega-3 PUFA (ALA) have been reported. Regarding its anticancer effects, in this study, we investigated the effects of various concentrations of omega-3 PUFA on expression level and promoter methylation of the WIF1 gene in MIA PaCa-2 cells in 24, 48, and 72 h after treatment. MIA PaCa-2 cells were treated with different concentrations of omega-3 PUFA (25, 50, 100, 250, 500, and 1000 μM). Cell viability assay was carried out followed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and methylation-specific PCR (MSP). This investigation suggested that dietary consumption of omega-3 PUFAs (250–1000 μM) has a significant effect on the proliferation and WIF1 gene expression of the MIA PaCa-2 cancer cell line but no effect on the promoter methylation of this gene. Changes in promoter methylation were not observed in any of the treatments.

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Introduction

Cancer is the most significant public health problem around the world and it is the second leading cause of death in the United States. Among the types of cancer, pancreatic cancer remains the fourth most lethal disease in both males and females (Siegel et al. 2016).

Wnt proteins are extracellular signaling molecules, and Wnt morphogens play prominent roles in embryonic development, as well as in adult stem cell biology. Conversely, in some cancers, particularly those of tissues for which Wnts normally stimulate self-renewal and repair, components of this signaling pathway are frequently mutated (Malinauskas and Jones 2014). Wnt inhibitory factor 1 (*WIF1*), encoded by the *WIF1* gene, binds Wnt ligands, blocks their signaling activities, and acts as a Wnt antagonist. This secreted protein is expressed in fish, amphibian, and mammalian cells, and it comprises a WIF domain and five epidermal growth factor (EGF)-like domains. It also takes part in mesoderm segmentation (Cruciat and Niehrs 2013). Methylation and histone acetylation are considered important epigenetic events in the DNA of eukaryotes. Promoter methylation is a well-known mechanism for controlling of transcription factors' binding to DNA (Hughes and Jones 2007).

Hypermethylation of CpG islands in the promoter region results in the transcriptional silencing of genes (Okino et al. 2007), while global or gene-specific hypomethylation events can induce gene expression (Sato et al. 2004). Promoter methylation of many genes has been shown in various human tumors. Among these are *WIF1*, which is hypermethylated in many human cancers, including non–small cell lung (Lee et al. 2013), colorectal (Roperch et al. 2013), breast (Ai et al. 2006), nasopharyngeal (Lin et al. 2006), gastrointestinal (Taniguchi et al. 2005), and hepatocellular carcinoma (Ding et al. 2009); Wilms' tumor 1 (*WT1*) is also hypermethylated in many cancers, such as primary breast tumors (Loeb et al. 2001), ovarian clear-cell adenocarcinoma (Kaneuchi et al. 2005), lung cancer (Oji et al. 2002), and colorectal cancer (Hiltunen et al. 1997).

The interactions between genetics and environment on the one hand and nature and nurture on the other are the fundamental determinants of future health or disease. It seems that nutrition is an important environmental factor influencing both gene expression and health (Simopoulos 2008). Essential fatty acids (EFAs) are important components in all types of cell membranes; in addition, they are essential for human survival. Moreover, they cannot be synthesized in the body; thus, they should be obtained from our diet (Das et al. 1988). Included among the naturally occurring EFAs in the body is the omega-3 series. The longer-chain metabolites of this PUFA are particularly important in regulating membrane function, and they are considered to have major importance in the brain, retina, liver, kidney, adrenal glands, and gonads (Das 2006). PUFAs act as second messengers in signaling pathway (Das 2002) and antibiotic-like agents (Das 2006); moreover, they engage in close interactions with nitric oxide (NO) synthase and cyclooxygenase (COX)

enzymes (Das 2005). Lower methylation of the tumor necrosis factor-alpha (TNFα) promoter in circulatory white blood cells (WBCs) has been reported in association with metabolic features and dietary fat intake.

Mechanisms by which PUFAs assert their anticancer effects are under research. For example, in the MCF-7 and T47D breast cancer cell lines for example, it was found that Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) can convert prosurvival estrogen signals to a proapoptotic effect. In contrast, pretreatment of MDA-MB-231 cells with DHA was shown to increase the anticancer effects of doxorubicin by increasing the membrane raft content of CD59 and FADD (Ewaschuk et al. 2012). In the Pc3 and LNCaP prostate cancer cell lines, growth suppression occurs due to inhibition of the Akt survival signaling pathway (Gu et al. 2013). Change of DNA expression through methylation and its effect on the stability, folding, positioning, and organization of DNA have been considered in different epigenetic studies. In line with these studies our recent works were focused on the effects of some anticancer agents such as calprotectin (S100A8 and S100A9) and omega-3 PUFA (ALA) (Najafi et al. 2017; Siddiqui et al. 2001). S100A9 can change expression level of OCLN gene and the methylation status of its promoter in A375 melanoma cell line (Najafi et al. 2017). Omega-3 PUFA (ALA) on WT1 gene of WNT signaling pathway in a pancreatic cancer cell line (MiaPaca-2) induced down-regulation but not promoter methylation alteration of this gene.

Following previous studies, we aimed to investigate the effects of an omega-3 PUFA (ALA) on the expression and promoter methylation of the *WIF1* gene at concentrations of 25, 50, 100, 250, 500, and 1000 μM in a time-dependent manner in a pancreatic cancer cell line (MIA PaCa-2).

Materials and Methods

Omega-3 Treatments and MTT Assay

The human pancreatic cancer cell line MIA PaCa-2 (ATCC® CRM-CRL-1420™) was purchased from the Pasteur Institute of Iran and cultured in RPMI1640 (Glutamax, Biosera, France) medium containing 10% fetal bovine serum (FBS; Gibco, USA), 1% antibiotic (100 U/ml of penicillin and 10 mg/ml of streptomycin) at 37 °C under a humidified atmosphere of 5% CO_2. To check for anchorage-dependent cell growth, a colorimetric [3-(4, 5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide] tetrazolium reduction assay (MTT) was performed. MTT assay is based on the principle that the viable cell number is directly proportional to the purple formazan color of the reduced MTT dye, which can be quantitatively measured by spectrophotometry.

In 96-well flat-bottomed tissue-culture plates, 2500–3000 MIA PaCa-2 cells were seeded and incubated for 24 h and then treated with 25, 50, 100, 250, 500, and 1000 μM concentrations of omega-3 PUFA (ALA). After 24, 48, and 72 h of treatment, cells were re-fed with fresh media and allowed to grow for 1 population doubling time (PDT); they were then washed with phosphate-buffered saline (PBS), and MTT (Sigma) solution was added at a concentration of 0.05 mg/ml diluted in PBS.

Cells were incubated at 37 °C for 4 h to allow the formation of purple formazan crystals due to mitochondrial dehydrogenase activity. About 100 µl of detergent solution (10% NP-40 with 4 mM HCl in isopropanol) was added to each well and incubated for 30 min at 37 °C. The color intensity was then measured by recording changes in absorbance at 570 nm using an absorbance microplate reader ELx800TM spectrophotometer (BioTek, USA) and analyzed with Gen5 2.01 software.

Cell Culture and Treatments

Cells (80,000/well) were seeded in six-well tissue-culture plates (JET BIOFIL, USA) and left overnight in serum-deprived (0.5%) medium. Then, the culture medium was replaced with serum-free medium together with the indicated reagents. Control cells were treated with ethanol 0.5%, which is not toxic to cells (Siddiqui et al. 2001). Cells were exposed to omega-3 PUFA (Lot. L2376, Sigma, USA) with a constant concentration of 25, 50, 100, 250, 500, or 1000 µM for 24, 48, and 72 h.

Cells were harvested 24, 48, and 72 h after treatment with PUFA at different concentrations, and DNA was extracted using GeneAll, Exgene™ Cell SV kit, Korean. The DNA concentration was then assessed using NanoDrop (ND-1000, USA). The final concentration was adjusted for each sample in 5–10 ng/µl.

Via bisulfite treatment, all unmethylated cytosines convert to uracil/thymine, while methylated cytosines remain the same (Shukeir et al. 2006). Bisulfite treatment was performed using an Imprint® DNA Modification Kit (Sigma) following the manufacturer's instructions. Ultimately, the clean-up procedure for modified DNA was performed as recommended in the kit instructions.

Primers and PCR Reactions for Assessing the Promoter Methylation Alteration of the WIF1 Gene

The primers used for analyzing the methylated and unmethylated promoters of the *WIF1* gene are described below. The sequences of the methylation-specific and unmethylation-specific reverse primers were 5′-ACGCGAACGAAATAC GAACG-3′ and 5′-CCCACAAAACCTAAACAACCA-3′, respectively. The forward primer (5′-ATTGGGYGTATTGTATTGTGAATG-3′) was designed to anneal equally well to methylated and unmethylated DNA (28). Primers were purchased from Macrogene (Korea). PCR product size for methylated and unmethylated was 135 bp (65121535–65121670) and 105 bp (65121562–65121670) respectively.

Human placental DNA was purchased (Sigma), then treated in vitro with SssI methyltransferase (New England Biolabs). This was used as a positive control for the methylated MSP. The DNA from normal donors' whole blood was used as a control for the unmethylated reaction. For the negative PCR control, we used water in both the methylated and unmethylated reactions.

The methylated and unmethylated MSP reactions were carried out in a total volume of 25 µl containing: 12.5 µl of 2× PCR EpiTect MSP Kit (100) EpiTect MSP Master Mix (59305, Qiagen), 1.5 µl of primer mix, 4 µl of the sample, and 7 µl of H_20. The PCR reaction was carried out as follows: 95 °C for 10 min, followed by 35

cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 40 s, and a final step of 72 °C for 5 min. The PCR results were run in 1.5% gel agarose and visualized with GelRed (GelRed Nucleic Acid Stain, Biotium) via real-time electrophoresis (runVIEW, Cleaver Scientific, UK).

RNA Extraction, cDNA Synthesis, and RT-PCR

Cells were harvested after 24, 48, and 72 h of exposure to PUFA at the concentrations mentioned above. Total RNA was isolated from control cells and treated cells using a total RNA extraction kit (GeneAll Ribospin™, Korea), and reverse transcription was performed with 2 µg of total RNA using a cDNA synthesis kit (Revert A-L, RT Reagent AmpliSens®, Russia) according to the manufacturer's recommendations.

Reactions for determining *WIF1* gene (gcf-000001405.33) were performed using RealQ Plus 2x Master Mix Green, Low ROX™. Arbitrary PCR units of each gene were defined as the mRNA levels normalized to the *GAPDH* expression in each sample. Specificity was verified by melting curve analysis. The forward and reverse primer sequences used were 5′-CCGAAATGGAGGCTTTTGTA-3′ and 5′-TGG TTGAGCAGTTTGCTTTG-3′, respectively (Kim et al. 2013). The forward and reverse primer sequences used for the *GAPDH* gene were 5′-CTGCACCACCAA CTGCTTAG-3′ and 5′-AGGTCCACCACTGACACGTT-3′, respectively (Ai et al. 2006). Real-time RT-PCR was carried out (Rotor-Gene Q, USA), and the analysis was conducted using Rest 2009 software.

Statistical Analysis

Calculations of the mean, standard deviation, and standard error and Statistical analysis for the comparison of each set of experimental means were performed using Graph Pad Prism 7. A two-tailed Student's t test was used, where $p < 0.05$ was accepted as statistically significant. For gene expression analysis used REST 2009 software.

Results

In MIA PaCa-2, viability decreased at omega-3 concentrations of 100, 250, 500, and 1000 µM ($p < 0.05$), with IC_{50} values of 250 µM after 24, 48, and 72 h of treatment. Concentrations of 25 and 50 µM omega-3 had no significant anti-proliferative effects on the MIAPaCa-2 cell line ($p > 0.05$). These data demonstrate that omega-3 PUFA has anti-proliferative activity in a pancreatic cancer cell line (Fig. 1). In addition, these effects are clearly time-dependent.

Data obtained from an expression analysis of the *WIF1* gene showed significantly increased *WIF1* gene levels with increasing concentration and time for 100, 250, 500, and 1000 µM but not 25 and 50 µM concentrations of omega-3 PUFA (Fig. 2).

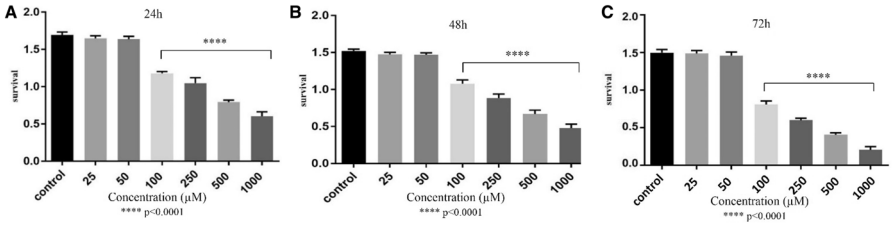

Fig. 1 Anti-proliferative effects of omega-3 PUFA (ALA), MTT assay on MiaPaca2 cell line. In compare with control, the concentrations of 25 and 50 μM had no significant effect on cell proliferation ($p > 0.05$) but 100, 250, 500, and 1000 μM concentrations of omega-3 PUFA had significant anti-proliferative effects at all three timepoints ($p < 0.05$). As shown here, these effects were most intense at 72 h (**c**). The half maximal inhibitory concentration (IC_{50}) value was 250 μM, at the three time intervals of treatment, namely 24, 48, and 72 h. **a** 24 h, **b** 48 h, **c** 72 h cell proliferation results after concerned concentrations

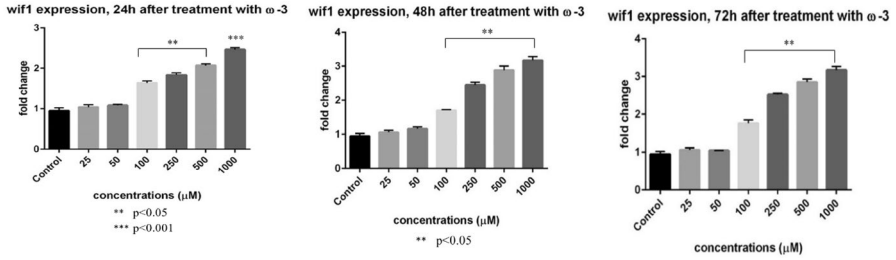

Fig. 2 Expression fold of omega-3 treated MIA PaCa-2 cells. The 25 and 50 μM concentrations of omega-3 polyunsaturated fatty acid (PUFA) had no significant effect on *WIF1* gene expression ($p > 0.05$) at the three time points. The concentrations of 100, 250, 500, and 1000 μM upregulated the gene expression up to three-fold ($p < 0.05$) at 24, 48, and 72 h after treatment. **a** 24 h, **b** 48 h, and **c** 72 h *WIF1* gene expression alterations after different ALA treatments

As shown in Fig. 3, methylation- and unmethylation-specific PCRs were carried out for the *WIF1* gene promoters. Clear bands were observed for the methylated and unmethylated *WIF1* gene promoters. The coexistence of bonds revealed the hemimethylation status of the two promoters in both the control and omega-3-treated MIA PaCa-2 cell line. (Only the MSP for 48 h is shown here).

Discussion

This study illustrated the decreasing cell viability of the MIA PaCa-2 cell line after treatment with different concentrations of omega-3 PUFA where IC_{50} values of 500, 500, and 250 μM were obtained after 24, 48, and 72 h, respectively. These results show that omega-3 asserts its effects in a time-dependent manner. Epidemiological studies have shown that the reduced consumption of omega-3 PUFAs in the modern diet is an indication of risk factors for the increased prevalence of chronic noncommunicable diseases, including obesity, diabetes, and cardiovascular disease

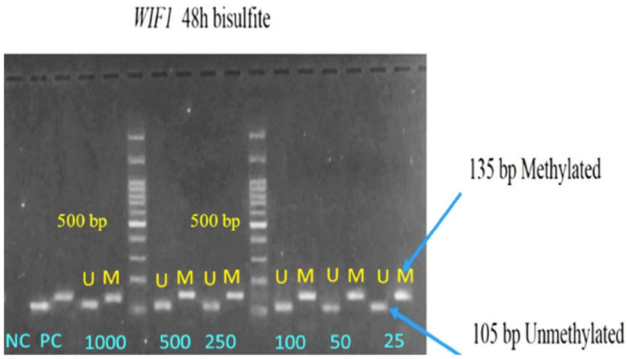

Fig. 3 *WIF1* promoter methylation-specific polymerase chain reaction (MSP) after 48 h. No significant effect on methylation pattern of examined region of promoter was seen after 48 h treatment with 25–1000 μM (data for 24 and 72 h are not shown). *NC* negative control, *PC* positive control (placental for methylated and normal human for unmethylated status), *U* unmethylated 105 bp, *M* methylated 135 bp

(Simopoulos 2006). More recently, nutrients and other environmental factors have been shown to induce epigenetic modifications, such as CpG island methylation and histone modification, which determine gene silencing or expression, again leading to susceptibility to many noncommunicable chronic diseases (Dolinoy et al. 2007; Waterland 2009). Some studies have proposed that fat quality probably affects DNA methylation patterns on a large genomic scale (Voisin et al. 2015).

Omega-3 PUFAs can affect chronic diseases like cancer (Laviano et al. 2013). Apoptosis can be induced via omega-3 PUFAs in a dose- and time-dependent manner both in vitro and in vivo (D'Eliseo and Velotti 2016). In vitro apoptosis induction has been shown in ovarian cancer (Sharma et al. 2005), lung cancer (Yao et al. 2014), melanoma (Albino et al. 2000), and many other cancers. As reported in previous investigations 10 g of daily consumption of Linoleic acid (omega 6) decreased 1% on the risk of breast cancer (Jandacek 2017) and the α-Linolenic acid (Omega 3) resulted to prevention and control of breast cancer through decrease cell proliferation and induction of apoptosis with probable alteration in lipid content of cytoplasmic membrane and intracellular transcription pathways (Liu and Ma 2014). As presented in Fig. 1 the anti-proliferative effects of omega-3 PUFA (ALA) on MIA PaCa-2 cell line in the concentration range of 100–1000 μM confirm the above mentioned investigations. The concentrations of 25 and 50 μM had no significant effect on cell proliferation.

WNTs have critical roles in the regulation of various processes, such as cell proliferation, survival, migration and polarity, the specification of cell destiny, and self-renewal in stem cells. The frequent mutations of the WNT pathway in many different cancers highlights the key role of WNT-CTNNB1 signaling in carcinogenesis (Anastas and Moon 2013); thus, this study sought to consider the *WIF1* gene to survey the expression and methylation of promoters after treatment with omega-3 PUFA in a pancreatic cancer cell line (MIA PaCa-2), representing the research has been done for the first time. In the previous study n-3 PUFAs with anti-proliferative and

apoptosis induction properties induced its inhibitory effects on Wnt/β-catenin signaling through reduction in β-catenin protein level and the down-regulation of certain downstream genes in the Wnt/β-catenin pathway in breast cancer 4T1 cells in vivo and in vitro (Xue et al. 2014). As illustrated in Fig. 2, we found that *WIF1* as the other key components of the Wnt signaling was significantly expressed in Maia paca II cell line after treatment with omega-3 PUFA in a concentration (100–1000 μM) and time-dependent manner (24, 48, and 72 h). Wnt/β-catenin signaling pathway activation has been suggested as a crucial role in human tumor genesis (Polakis 1999; Behrens 2000). Considering *WT1* as a proto oncogene, it has been reported in our previous study that the cell viability and level of *WT1* mRNA was decreased during omega-3 ALA treatments for 100, 250, 500, and 1000 μM concentrations (Rahmani et al. 2018) Thus, the results of this study emphasized to inhibitory effects of n-3 fatty acid on the Wnt/β-catenin signaling through up-regulation of WIF 1 as a tumor suppressor gene.

Long-term consumption of PUFAs can alter cells' gene expression profiles (Bouwens et al. 2009). As mentioned above, although many studies have reported on the anticancer effects of omega-3 PUFA, well-documented data on the methylation-specific action of this long-chain fatty acid have not yet been produced. We hypothesized that the different expression rates of the *WIF1* gene may be related to its promoter methylation status after treatment with the mentioned concentrations of omega-3 PUFA. Therefore, the epigenetic modification of the *WIF1* gene promoter was assessed using MSP, and the resulting data are depicted in Fig. 3 for 48-h time interval (data for 24 and 72 h are not shown). There was no observable change in the methylation pattern of this gene promoter.

In overall from this study the cell proliferation of MIA PaCa-2 cell line was significantly decreased at 100, 250, 500, and 1000 μM ($p < 0.0001$), and the expression level of WIF*1* as a tumor suppressor gene was increased in these concentrations ($p < 0.01$), a clue for Wnt signaling pathway inhibition. In MSP-PCR no effect was seen on promoter methylation of examined region. The *WIF1* gene promoter was hemimethylated in the MIA PaCa-2 cell line; thus, it is probably regulated by a different mechanism.

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