

## Omega-3 PUFAs induce apoptosis of gastric cancer cells via ADORA1

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## 1. ABSTRACT

Omega-3 polyunsaturated fatty acids (Omega-3 PUFAs), including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been suggested to have anti-cancer effects by epidemiological and clinical studies. However, their underlying anti-cancer mechanisms are still unclear. In this study, we examined the influence of two Omega-3 PUFAs (DHA and EPA) on the proliferation and apoptosis of gastric cancer (GC) cells, and found that DHA and EPA reduced the viability of GC cells and induced apoptosis by activating caspase-3. Moreover, we screened the expression profile of apoptosis-related genes in GC cells upon the treatment of DHA and/or EPA, and discovered that ADORA1, one subtype of adenosine receptor functionally involved in cell death, was up-regulated in response to DHA and EPA. Importantly, when GC cells were treated with a selective ADORA1 antagonist, DPCPX, the DHA/EPA-induced apoptosis was substantially reduced. Taken together, our results suggest that the anti-cancer effect of Omega-3 PUFAs on gastric cancer is at least partly dependent on activating the ADORA1-mediated apoptosis pathway.

## 2. INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer worldwide. More than 42% of GC patients are found in China (1). In recent years, despite the great progress in the treatment of gastric cancer, the survival rate for gastric cancer remains poor. Therefore, in addition to conventional surgery and chemotherapy, an effective and non-toxic treatment is urgently needed to improve the prognosis.

Apoptosis, or programmed cell death, is an essential component of cell number regulation and a crucial mechanism to prevent damaged or mutated cells from surviving and dividing, hence contributing to carcinogenesis. Apoptosis is triggered by extrinsic or intrinsic signals and regulated by many different molecular pathways (2). Studies have demonstrated that  $\omega$ -3 polyunsaturated fatty acids ( $\omega$ -3 PUFAs) can induce apoptosis in human colon cancer (3,4), breast cancer (5,6), pancreatic cancer (7,8), prostatic cancer (9,10), and leukemia (11). In addition, mounting evidence suggests that  $\omega$ -3 PUFAs can reduce cancer risk, inhibit tumor growth and potentially improve the cancer prognosis.

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are known as the most important members of ω-3 PUFAs and found abundant in fish oil. Epidemiological studies suggest that diets rich in DHA and EPA seem to be associated with reduced cancer risk (12). Consistently, experimental data from animal models and cell lines demonstrate that DHA/EPA can inhibit tumor growth in a variety of cancer models (13,14). Moreover, clinical data suggest that DHA/EPA can improve the prognosis, resulting in a reduction in morbidity, infectious complications and overall hospital stay (15,16). However, the anti-cancer mechanisms of DHA and EPA are still poorly understood.

In this study, we attempted to explore the influence and working mechanism of ω-3 PUFAs (DHA and EPA) on gastric cancer (GC) cells. Our results showed that DHA and EPA inhibit proliferation of GC cells, and induce apoptosis of GC cells through the ADORA-1 signaling pathway. Our findings suggest that ω-3 PUFAs have similar antagonistic effects on gastric cancer as previously reported in other types cancers, and that one specific mechanism of ω-3 PUFAs' anti-cancer effects is to activate the ADORA apoptosis pathway.

### **3. MATERIALS AND METHODS**

#### **3.1. Cell lines and culture condition**

The human gastric cancer cell line, MKN-45 was originally obtained from Institute of Biochemistry and Cell Biology, Shanghai Chinese Academe of Science. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Gibco, Invitrogen) in 5% CO<sub>2</sub> cell culture incubator at 37°C. Results from all studies were confirmed in at least three independent experiments.

#### **3.2. Reagents**

EPA, DHA were all purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Fatty acids stock solutions were prepared in 99% ethanol as described in the instruction manual. The stock solutions were then stored under nitrogen gas in opaque microcentrifuge tubes at -80°C to prevent their degradation. The control cells were treated with the same amount of vehicle alone. Stock solution of DPCPX (Sigma-Aldrich, St. Louis, MO) was prepared in DMSO (Sigma-Aldrich, St. Louis, MO) and stored at -20°C. MKN45 cells were treated with DPCPX (3mg/ml) for 1h at first, and then treated with DHA, EPA for 24h and prepared for experimental detection.

#### **3.3. Cell proliferation assay**

Gastric cancer cells ( $5 \times 10^3$ /well) were seeded in 96 well plates in serum-containing medium and treated with EPA, DHA in different concentrations for 24h, 48h and 72h, respectively. A 20μl cell counting kit-8 (Dojindo, Kumamoto, Japan) was added in each well. After 3h incubation at 37°C, the coloring reaction was quantified by an automatic plate reader (Tecan, Swiss) at 450 nm.

#### **3.4. Apoptosis assay**

Hoechst 33342 staining: Nuclear DNA in treated cells contained in 24-well plates was visualized by staining

with the DNA-specific dye Hoechst 33342 (KeyGen BioTech, Nanjing, China) at a final concentration of 5μg/ml. Cells were observed immediately with filters for blue fluorescence.

Flow cytometry analysis: Cells treated with gradient concentrations of EPA and DHA were harvested and washed twice with ice-cold buffered saline (PBS, Invitrogen, UK). Staining according to the Annexin V/PI procedure was performed as instructed by the Annexin V Apoptosis Detection Kit (BD). Measurements were obtained using the FACScan Flow Cytometer (Epics Altra, Beckman Coulter, Brea, CA, USA), and the results were analysed by EXPO32 ADC analysis software (Coulter, Krefeld, Germany).

#### **3.5. Microarray gene expression profiling**

MKN45 cell lines were treated with 50μg/ml EPA, 75μg/ml DHA or with ethanol alone (control) for 24h. Total-RNA from each sample was extracted and purified using Trizol reagent (Invitrogen, UK), according to the manufacturer's instructions. Total RNA from each sample was quantified using the NanoDrop ND-1000 and the RNA integrity was assessed by standard denaturing agarose gel electrophoresis. For microarray analysis, Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. Briefly, 1 μg of total RNA from each sample was amplified and transcribed into fluorescent cRNA using the manufacturer's Agilent's Quick Amp Labeling protocol (version 5.7, Agilent Technologies). The labeled cRNAs were hybridized onto the Whole Human Genome Oligo Microarray (4x44K, Agilent Technologies). After washed the slides, the arrays were scanned by the Agilent Scanner G2505B. Agilent Feature Extraction software (version 10.7.3.1) was used to analyze acquired array images.

#### **3.6. Quantitative reverse transcription-PCR**

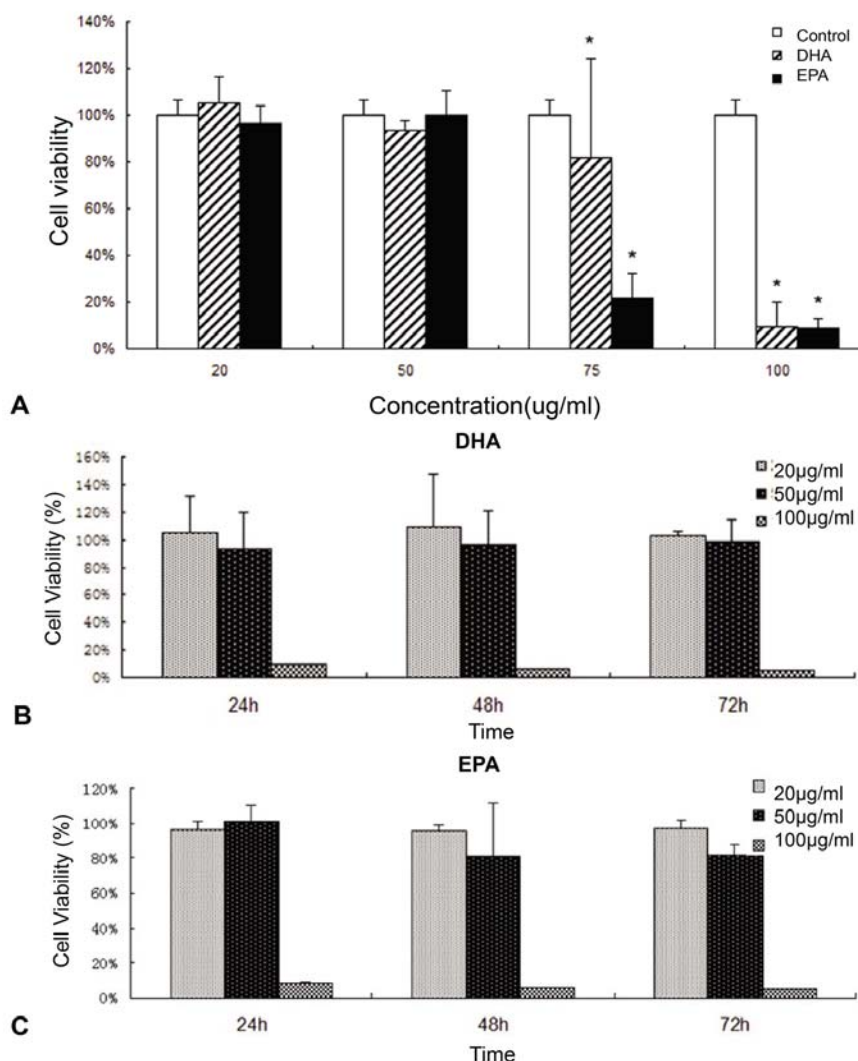
Total RNA was extracted using Trizol solution (Invitrogen, UK). Reverse transcription (RT) was performed in a 20 ml reaction system according to the manufacturer's recommendation (Promega, USA). Real-time PCR was performed with the follow program: initial denature at 95°C for 5min, followed by 40 cycles of 95°C for 15s, 60°C for 30s, and 72°C for 30s. The data were analyzed by the comparative *C<sub>t</sub>* method. The expression of BAX, BID, Caspase3, Caspase8, Bcl-2 and ADORA1 were detected by qPCR.

#### **3.7. Flow cytometry**

MKN45 cells ( $1 \times 10^5$ /well) were treated in 6-well plates as described the instruction manual. Cells suspensions were incubated with ADORA1 (adenosine A1 receptor) antibody for 3h at room temperature, washed three times with PBS, and incubated with FITC-labeled goat anti-human secondary antibodies for 2h at room temperature, washed three times with PBS, and fixed by 0.4% Paraformaldehyde. The expression levels of ADORA1 were determined by flow cytometry.

#### **3.8. Statistical analysis**

All data were statistically analyzed. Statistically significant differences were determined using one-way



**Figure 1.** The effect of DHA, EPA on the inhibition of proliferation in GC cells. A: The effect of the concentration of DHA, EPA on the inhibition of the proliferation in GC cells (\* $P < 0.05$ ). B: The effect of the treatment times of DHA on the inhibition of proliferation in gastric cancer cells; C: The effect of the treatment times of EPA on the inhibition of proliferation in gastric cancer cells.

analysis of variance (ANOVA) followed by Student *t*-test. Differences were considered significant at  $P < 0.05$ . All computations were performed using SPSS 15.0 software.

## 4. RESULTS

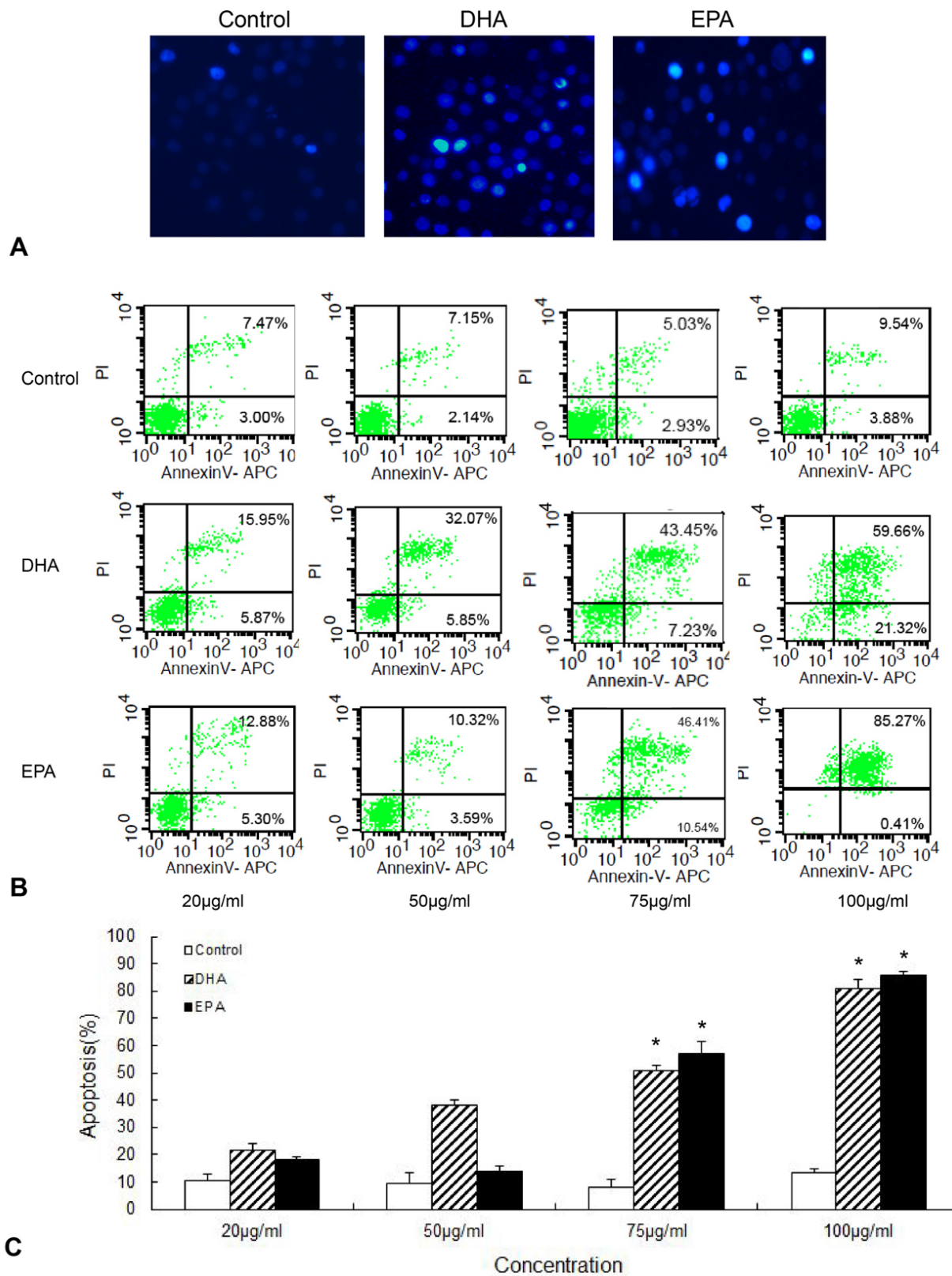
### 4.1. ω-3 PUFAs inhibit GC cells viability

We first evaluated the effect of DHA or EPA on the viability of MKN45 cells. As shown in Figure 1A, after treatment with different concentrations (particularly  $\geq 50$   $\mu\text{g/ml}$ ) of DHA and EPA for 24h, decreased viability was observed in a dose-dependent manner. Notably, there was no statistical difference of cell viability as the time increases (Figure 1B,1C).

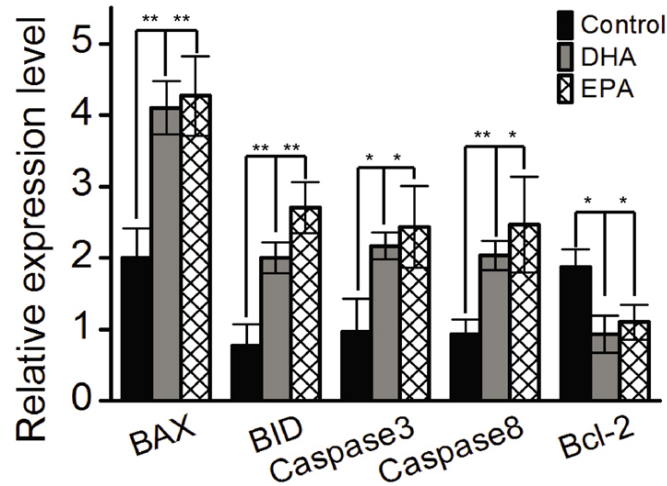
### 4.2. ω-3 PUFAs induce apoptosis of GC cells

The effect of ω-3 PUFAs on the apoptosis of

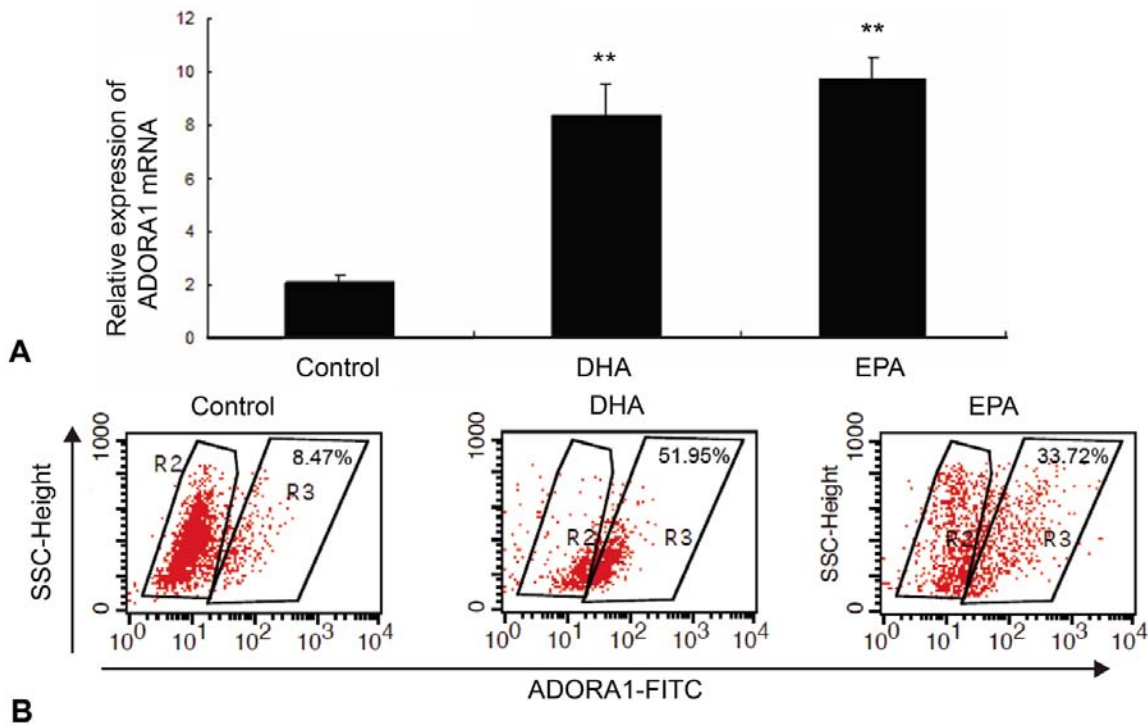
MKN45 cells was evaluated by Hoechst staining and AnnexinV-PI (propidium iodide). Apoptotic cells with nuclear condensation and DNA fragmentation can be detected by Hoechst 33342 staining and fluorescence microscopy. As illustrated in Figure 2A, MKN45 cells treated with DHA and EPA for 24h exhibited intense Hoechst-positive staining for condensed nuclei, indicative of apoptosis. Flow cytometry analysis of MKN45 cells after 24 hours of DHA or EPA treatment (20  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$ , 75  $\mu\text{g/ml}$ , 100  $\mu\text{g/ml}$ ) revealed that the percentage of AnnexinV and PI-positive cells increased with escalating concentrations of DHA or EPA (Figure 2B). For instance, the percentage of apoptotic cells increased from 21.82% to 80.98% with DHA supplement and from 18.18% to 85.68% with EPA supplement, respectively, as the concentrations of the drugs increased from 20 to 100  $\mu\text{g/ml}$ . The percentage of apoptotic MKN45 cells with DHA or EPA supplement



**Figure 2.** DHA, EPA induced the apoptosis of GC cells. A: Hoechst 33342 staining of MKN45 cells treated with DHA, EPA or control solution for 24h. Fragmented or condensed nuclei could observe at 200 $\times$  magnification; B: Apoptosis was assessed by AnnexinV-APC/PI; C: Statistical analysis of apoptosis rates (\* $P < 0.05$ ).



**Figure 3.** DHA, EPA altered the expression of apoptosis-related genes. Relative expression of apoptosis-related genes in DHA or EPA treated MKN45 cells were detected by qPCR (\* $P < 0.05$ , \*\* $P < 0.01$ ).



**Figure 4.** ω-3 PUFAs increased the expression of ADORA1 in GC cells. A: The mRNA expression of ADORA1 in MKN45 cells (\*\* $P < 0.01$ ), B: Representative expression profiles of ADORA1 on MKN45 cells surfaces.

was significantly highly compared to that of untreated MKN45 cells (Figure 2C).

#### 4.3. ω-3 PUFAs regulate the expression of apoptosis related genes

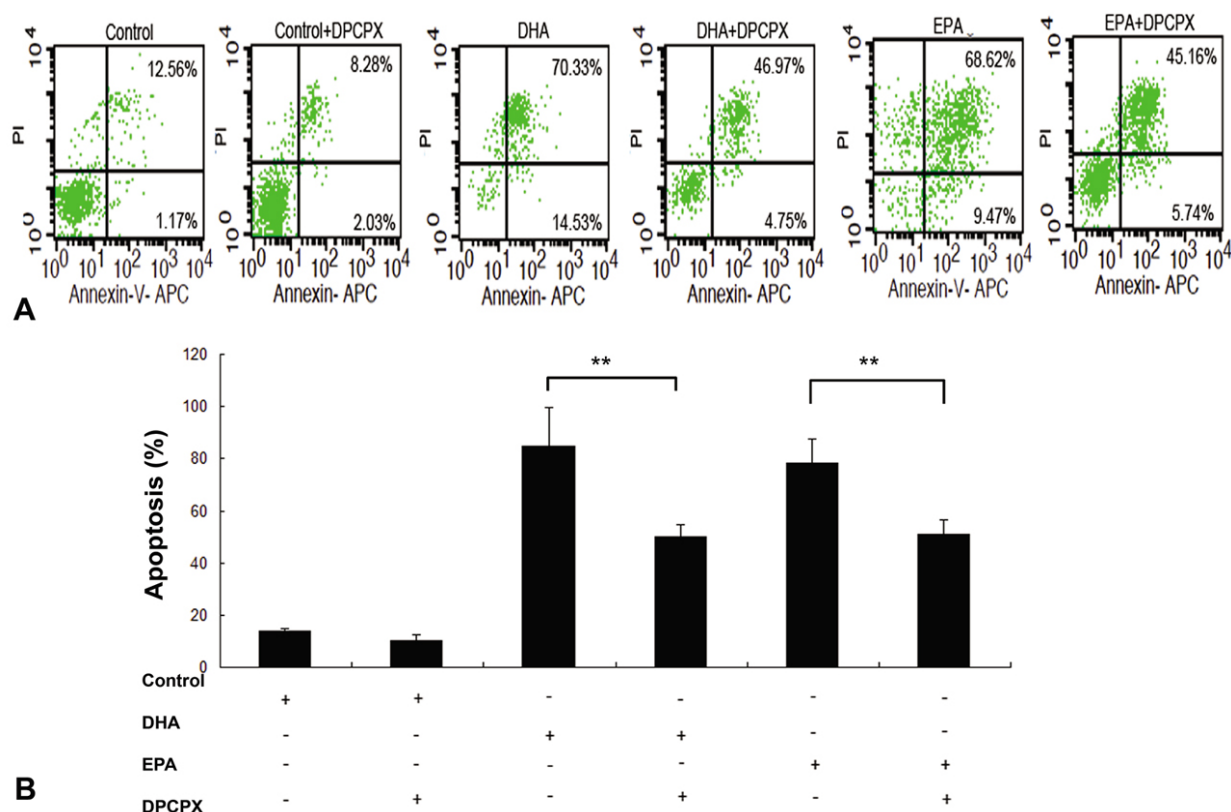
The observation that DHA and EPA induce apoptosis of MKN45 cells prompted us to exam the expression levels of some known apoptotic genes in MKN45 cells upon treatment, including major players in apoptosis such as Caspase 3, Caspase 8, Bax, Bid and Bcl-

2. As show in Figure 3, DHA and EPA treatment increased the expression of a series of apoptotic genes including Caspase 3. These results suggest that ω-3 PUFAs may inhibit the proliferation of GC cells by up-regulating the expression of apoptotic genes.

#### 4.4. ADORA1 is one of candidate genes regulated by ω-3 PUFAs

To identify possible targets of ω-3 PUFAs, we compared the gene expression profile of MKN 45 cells





**Figure 5.** The effect of ADORA1 on the apoptosis induced by  $\omega$ -3 PUFAs in GC cells. A: The representative profiles of cells apoptosis; B: The apoptosis rate in the different treated groups. (\*\*  $P < 0.01$ )

with or without  $\omega$ -3 PUFAs treatment using microarray. Compared with the control group, the expression levels of 54 apoptosis-associated genes were changed in the  $\omega$ -3 PUFAs (DHA and EPA) treated groups. Among these, 29 genes were up-regulated while 25 genes were down-regulated. Intriguingly, ADORA1 (A1 adenosine receptor), a gene involved in lipids metabolism, was first found to participate in  $\omega$ -3 PUFAs induced apoptosis in gastric cancer cells. Thus, we decided to conduct further studies on ADORA1.

For independent verification of the up-regulated ADORA1 expression in  $\omega$ -3 PUFAs-treated MKN45 cells, qRT-PCR and flow cytometry were performed. As expected, incubation of MKN45 cells with DHA and EPA increased ADORA1 mRNA expression (Figure 4A). We also observed a significant increase in surface ADORA1 expression in MKN45 cells treated with DHA and EPA (Figure 4B).

#### 4.5. $\omega$ -3 PUFAs may promote the apoptosis of GC cells via ADORA1

Although the expression of ADORA1 was significantly up-regulated by  $\omega$ -3 PUFAs treatment, but the molecular mechanism is not clear. To investigate whether the increased ADORA1 expression had a functional consequence, we incubated MKN45 cells with a selective ADORA1 antagonist, DPCPX (3 mg/ml) for 12h and

measured the percentage of apoptosis by AnnexinV/PI. As shown in Figure 5, DPCPX treatment of MKN45 cells resulted in significantly less DHA/EPA-induced apoptosis compared to cells without DPCPX treatment. These data indicated that ADORA1 mediates the apoptosis induced by  $\omega$ -3 PUFAs in MKN45 cells.

#### 5. DISCUSSION

$\omega$ -3 PUFAs, a component of immunonutrition, regulate immune function and inflammation reaction (17), which have been shown to result in improved outcome in patients undergoing gastrointestinal surgery for cancer (18,19). The supplement of  $\omega$ -3 PUFAs can improve the cachexia for cancer (20). However, the anticancer effect of  $\omega$ -3 PUFAs remains poorly understood. Apoptosis is one of process which is programmed cell death that can occur by a variety of internal or external stimuli. Apoptosis is also one of mechanism widely noted in oncotherapy.

Our study demonstrated that DHA and EPA inhibited MKN45 cell growth and this was associated with increased apoptosis. We showed a statistically significant inhibition of MKN45 cell proliferation in a dose-dependent manner, particularly, the effect enhanced when the concentration  $\geq 50$   $\mu$ g/ml. However, the suppression effect was no time-dependent. Similarly, we found that the apoptosis of MKN45 cells induced by DHA and EPA was

in a dose-dependent manner. Apoptosis induced by DHA and EPA was also consistent with the observed changes in morphology and consistent with those of other studies. Therefore, in order to investigate the underlying molecular mechanisms involved in the induction of apoptosis, we focus our research on the gene expression variation in ω-3 PUFAs stimulation.

We used cDNA microarray analysis to investigate the changes in expression of apoptosis related genes. We found that ADORA1 was one of up-regulated genes relative with apoptosis and lipid metabolism. ADORA1 is one subtype of adenosine receptors and a member of the G protein-coupled receptor (GPCR) superfamily. Adenosine receptors have their particular pharmacological characteristic, tissue distribution and coupling protein. Adenosine receptors have been actively studied as a potential drug target for the treatment of ischemic diseases (brain and heart), cancer, disorder of immune function and inflammation system (21). A variety of studies investigating the role of ADORA1 in tumor development have been performed with contrasting anti- and pro-tumoral effects. ADORA1 activation has been found to inhibit proliferation of different types of tumor cells including human LoVo, leukemia MOLT-4, breast tumors T47D, HS578T, and MCF-7 (22). Adenosine suppresses CW2 human colonic cancer growth by inducing apoptosis via A1 adenosine receptors (23). More interestingly, the ADORA1, endogenously expressed in melanoma cell line, has been demonstrated to increase the chemotaxis of tumor cells (24). Furthermore, ADORA1 increases both cell growth and cell proliferation in MDA-MB-468 human breast carcinoma cells (25). It is suggested that ADORA1 in different cell lines exerts different effects. In the present study, there was an increase expression of ADORA1 at both mRNA and protein levels in DHA and EPA treated MKN45 cells. Then we used DPCPX, an ADORA1 selective adenosine antagonist to identify if DHA and EPA-induced apoptosis is mediated by ADORA1. The results showed that DPCPX inhibits the effect of adenosine on ADORA1 by specifically binding to this receptor and reduced the apoptosis of MKN45 cells. This indicates that DHA and EPA can induce MKN45 cell apoptosis by modulating the expression of ADORA1. A number of studies revealed that ADORA1s are linked to Gi protein involving in inhibition of adenylatecyclase, raising the possibility that a decrease in intracellular cAMP is a critical factor to induce cell death for extrinsic pathway. cAMP can activate Caspase-9 followed by Caspase-3 on RCR-1 astrocytoma cell (26). Caspase-3 is a very important key of apoptosis and has been recognized as the crucial executioner caspase (27). In this study, DHA and EPA up-regulated the expression of Caspase-3. Nevertheless, how caspase-3 activated via an ADORA1 signaling pathway on gastric cancer cell remains to be explored. To address these questions, we are currently carrying out further experiments.

In conclusion, the results from our study have provided that ω-3 PUFAs, DHA and EPA can inhibit the proliferation of MKN45 cells and induce of apoptosis mediated via ADORA1. Increased expression of caspase-3, ADORA1 and resulting apoptosis may be a potential

mechanism underlying ω-3 PUFAs' s ability to induce inhibition of MKN45 cell growth. Although additional investigations are needed to prove the anticancer effects of ω-3 PUFAs, this study highlights the application potential of ω-3 PUFAs in gastric cancer therapy.

## **6. ACKNOWLEDGMENTS**

Dr Hong Sheng and Dr Pu Li equally contributed to this work. This study is supported by grants from the National Science Foundation of China (numbers 81372187 and 81300290).

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**Key Words:** ω-3 PUFAs, gastric cancer, DHA, EPA, ADORA1

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