Supplementation with omega fatty acids increases the mRNA expression level of PLA2G4A in patients with gastric cancer

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Background: Many lines of evidence suggest that arachidonic acid (AA)-based eicosanoid signaling pathway involved in development and progression of human cancers. Cytosolic phospholipase A2-α (cPLA2α) encoded by the PLA2G4A gene acts as an upstream regulator of eicosanoid signaling pathway through providing intracellular AA. The current study aimed to evaluate the effect of omega fatty acids on mRNA expression level of PLA2G4A in patients with gastric cancer (GC) and to assess the possible relation between its expression and clinicopathological features.

Methods: According to treatment strategy, 34 chemotherapy-naive patients were randomly divided into two groups including, treatment group I (17 subjects received cisplatin alone) and treatment group II (17 individuals received cisplatin plus omega fatty acids) in a double-blind manner. The gastric biopsies specimens were taken from subjects before and after treatment and then mRNA expression level of PLA2G4A was evaluated by quantitative real-time PCR procedure.

Results: The expression of the PLA2G4A gene at the protein level in the gastric biopsies samples was also determined by immunohistochemistry. Our findings revealed a significantly up-regulated expression of PLA2G4A mRNA in treatment group II after receiving cisplatin plus omega fatty acid compared to before treatment (P=0.003). In treatment group I, there was no significant difference in mRNA expression levels of PLA2G4A before and after treatment (P=0.790). We also found that mRNA expression of PLA2G4A in treatment group II was significantly associated with tumor size (P=0.007) and familial history (P=0.006).

Conclusions: This study provides evidence that supplementation with omega fatty acids increases the mRNA expression level of PLA2G4A in patients with GC and may be crucial in guarding the cell from transformation and carcinogenesis.

Keywords: Phospholipase A2; PLA2G4A; omega fatty acids; gastric cancer (GC)

Introduction

Gastric cancer (GC) is the fourth commonly diagnosed malignancy in both sexes and is still the second leading cause of cancer-related mortality globally (1,2). According to annual statistics reporting from the American Cancer Society, 26,240 new cases of GC are expected to be diagnosed and 10,800 deaths will occur in the United State in 2018 (3). GC is a multifactorial disorder caused by the interplay of multiple genetic factors and other factors, such as lifestyle and environmental factors (4,5). Several etiologic factors, including *Helicobacter pylori* bacterial infection,

gastric polyps, mucosa-associated lymphoid tissue (MALT) lymphoma, obesity, high salt diets, ethnicity and smoking have already been identified for GC (6). However, many questions have been remained on the exact pathophysiologic mechanisms of GC.

It has been fairly well demonstrated that variation in endogenously synthesized eicosanoids level, which depends on the phospholipase enzymes activity may play a fundamental role in the development and progression of cancers (7,8). In the humans, there are four major classes of phospholipases which catalyze the hydrolysis of membrane glycerophospholipids, termed phospholipase A1 (PLA1), phospholipase A2 (PLA2), phospholipase C (PLC) and phospholipase D (PLD) (9). The PLA2 enzymes (EC3.1.4.4) catalyze the hydrolysis of the acyl ester bond of membrane phospholipids at the sn-2 position to release biologically active fatty acids and lysophospholipids (10). The superfamily of PLA2 currently consists of five distinct types of enzymes: cytosolic PLA2 (cPLA2), calcium-independent PLA2s (iPLA2), secreted PLA2 (sPLA2), platelet-activating factor-acetylhydrolases PLA2 (PAF-AHs) and lysosomal PLA2 (10). The cPLA2 family includes 4 subgroups in human: cPLA2α; cPLA2β; cPLA2γ and cPLA2δ (11). The cPLA2α, which is encoded by the PLA2G4A gene, represents a marked preference for hydrolysis of the membrane phospholipids which contain arachidonic acid (AA) (12). The AA released from the phospholipids is metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) enzyme-catalyzed pathways to produce eicosanoids such as prostaglandins (PG), thromboxanes (TX), hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT) (12). These AA-derived lipid mediators modulate a wide array of physiological processes, including cell proliferation, cell differentiation, apoptosis, cell motility, invasion and angiogenesis (12,13). Not surprisingly, dysregulation of eicosanoid metabolism pathway component(s) expression, especially cPLA2α, contributes to initiation and progression of the various cancer types (14-16). Zhang *et al.* reported that mRNA expression level of PLA2G4A is decreased in patients with GC (14). However, the studies conducted by Sundarraj *et al.* and Patel *et al.* in non-small cell lung cancer and prostate cancer have shown conflicting results (15,16). On the other hand, accumulating evidence during the past decades suggest that omega fatty acids exhibit substantial effects in preventing and treating of the malignancies (17-20). Hence, the current study was designed to determine the effect of omega fatty acids on mRNA expression level

of PLA2G4A in chemotherapy-naive patients with GC. The possible relation between mRNA expression level of PLA2G4A and clinicopathological parameters was also evaluated.

Methods

Participants

The biopsies samples (before and after treatment) from 34 chemotherapy-naive patients with histologically and cytologically confirmed GC were collected at the Liver and Gastrointestinal Diseases Research Center (LGDRC) and Hematology-Oncology Research Center, Tabriz, Iran, during 2013–2015. All obtained gastric biopsy specimens from patients were stored in RNAlater solution (Qiagen, Germany) at –80 ℃ until RNA extraction. According to treatment strategy, subjects were divided into two groups including treatment group I (17 individuals; mean age, 67.5±11.21 years) received daily cisplatin 75 mg/m^2 alone and treatment group II (17 individuals; mean age, 71.25±9.81 years) received daily cisplatin 75 mg/m^2 plus orally administered omega fatty acid supplements 3,600 mg per day for three-course. Patients with the following criteria were excluded from the study: cardiac obstruction, pyloric obstruction, diabetes, renal disorders, inflammatory diseases and individuals who were used omega fatty acids supplement for three months before sampling. The histological grade and tumor size of all obtained biopsy samples were determined by histopathological analysis. A structured questionnaire form was used to obtain demographic data from all participants and these data are shown in *Table 1*. This study was approved by the Ethics and Human Rights Committee of Tabriz University of Medical Sciences (ethical code number 92213) and written informed consent was obtained from all subjects after the purpose of study was explained.

RNA extraction and cDNA Synthesis

Total RNA from gastric biopsies samples was extracted using the total RNA extraction RNeasy kit (Qiagen, Valencia, CA) and treatment of isolated RNA with RNase-free DNase (MBI Fermentas, Canada) was also performed based on the manufacturer's instructions. The quantity and integrity of purified RNA were assessed photometrically using NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA) and observation of 28S and 18S ribosomal

Table 1 Demographic and clinical properties of two treatment groups

Characteristic	Total subjects (n=34)		P value
	Treatment group I (n=17)	Treatment group II (n=17)	
Age (years)	67.50±11.21	71.25±9.81	0.235
Gender			0.695
Male (n=19)	9	10	
Female (n=15)	8	7	
Tumor stage			0.089
I (n=7)	4	3	
II (n=12)	6	6	
III (n=8)	4	4	
IV (n=7)	3	4	
Tumor size			0.759
≤4 cm (n=16)	7	9	
>4 cm (n=18)	10	8	
Familial history of gastric cancer			0.098
Yes (n=19)	9	10	
No (n=15)	8	7	
Smoking status			0.085
Current smoker (n=12)	6	6	
Second smoker (n=11)	4	7	
Never-smoker (n=11)	7	4	
Systolic blood pressure (mmHg)	131.1±9.20	128.8±10.20	0.235
Diastolic blood pressure (mmHg)	85.1±7.1	79.2±7.9	0.985
Fasting glucose (mg/dL)	98.54±15.25	102.85±18.65	0.234
Cholesterol (mg/dL)	148.98±21.56	151.25±25.65	0.125
Triglyceride (mg/dL)	87.25±18.25	78.25±15.65	0.256

RNA bands after electrophoresis on 0.8% agarose gel, respectively. Finally, complementary DNA (cDNA) was synthesized from 1 µg of DNase I-treated total RNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) with random hexamer primer following the manufacturer's protocol and published method (21).

Quantitative real-time PCR

To evaluate the mRNA expression level of PLA2G4A in 34 patients diagnosed with GC (before and after treatment), we used quantitative real-time PCR (qPCR) procedure. The qPCR was performed in triplicate by a Rotor-Gene™ 6000 system (Corbett Research; Mortlake, New South Wales, Australia) using gene-specific primers and SYBR Green Master Mix (SYBR® Premix Ex Taq™) (TaKaRa, Japan) following the manufacturer's recommendations. The primer pairs for PLA2G4A F 5'-GACGTGCTGGGAAGGTACAC-3' and R 5'-AGCCCACTGTCCACTACA-3' and for GAPDH F 5'-GAAGGTGAAGGTCGGAGTC-3' and R 5'-GAAGATGGTGATGGGATTTC-3' were used in the amplification assays. The amplification reactions were carried out in a final volume of 25 µL with the following

components: 100 ng cDNA, SYBR Green Master Mix 2×, ROX dye 50×, 10 pmol of forward and reverse primers for PLA2G4A and GAPDH genes. Thermocycling conditions were as follows: 94 ℃ for 5 min, 50 cycles (for PLA2G4A) and 35 cycles (for GAPDH) of 94 ℃ for 10 s, annealing at 60 ℃ (for PLA2G4A) and 61 ℃ (for GAPDH) for 15 s, and extension at 72 ℃ for 20 s. Efficiency of qPCR was evaluated using serially diluted cDNA method and standard curves were plotted in each run. The mRNA expression level of PLA2G4A in the biopsy samples were normalized using the mean expression of the house keeping gene GAPDH. The relative fold change in the gene expression of PLA2G4A was determined by the $2^{-\Delta\Delta Ct}$ comparative method. We also used no template sample as a negative control.

Immunohistochemistry

The cPLA2α protein expression on 5 μm sections of biopsy samples was determined by immunohistochemistry (IHC) technique. The sections of biopsy samples were incubated with a monoclonal cPLA2α antibody that can be visualized through staining.

Statistical analysis

The distribution of mRNA expression levels of PLA2G4A around cutoff points were analyzed using Kolmogorov-Smirnov test. This analysis revealed that data were not normally distributed and therefore non-parametric statistical tests were used to compare the data. We used two-tailed Mann-Whitney U test to evaluate the statistically significant difference in mRNA expression levels of PLA2G4A before and after treatment in both groups. The possible relation between clinicopathological parameters and the mRNA expression levels of PLA2G4A was examined using one way ANOVA test. The correlation between mRNA expression level of PLA2G4A and clinicopathological features in both groups was analyzed by Pearson's correlation coefficient test. All statistical analysis was carried out using SPSS version 16.0 statistic software package (SPSS Inc., Chicago, IL, USA), and all continuous variables were expressed as mean ± standard deviation (SD). P values less than 0.05 were statistically considered as significant.

Results
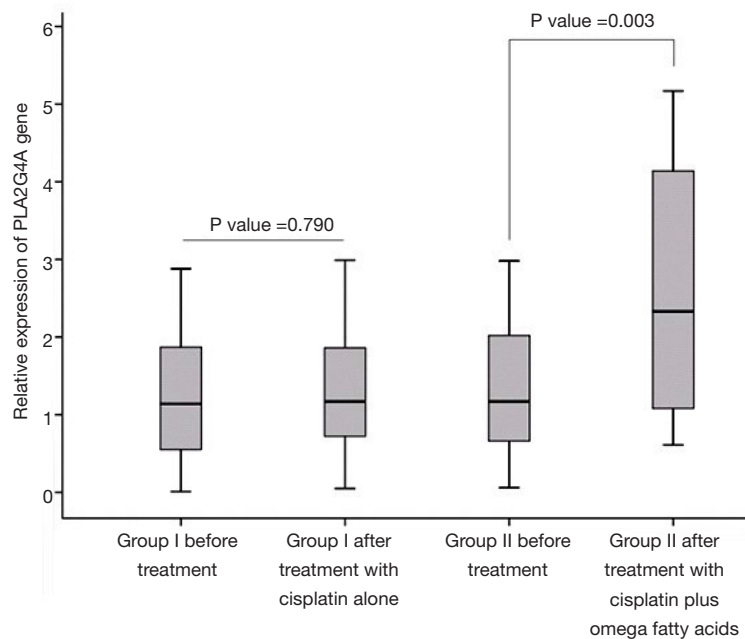
The mRNA expression level of PLA2G4A in 34 patients

with GC before and after treatment was quantified by qPCR method. Based on initial analysis of data using Kolmogorov-Smirnov test the mean of PLA2G4A mRNA expression level was chosen as a cut-off value. As shown in *Figure 1*, the mRNA expression level of PLA2G4A was significantly higher in treatment group II after treatment with cisplatin plus omega fatty acids, compared to before treatment (P=0.003) (*Figure 1*). No statistical difference was observed on mRNA expression level of PLA2G4A between before and after receiving cisplatin alone in group I (P=0.790) (*Figure 1*). The cPLA2α protein expression was also examined in sections of biopsy samples using IHC method. We found that PLA2G4A gene was expressed at protein level in gastric biopsies samples.

Analysis of the possible association between the mRNA expression levels of PLA2G4A and clinicopathological parameters was performed separately for both groups (*Table 2*). Our results showed that the mRNA expression levels of PLA2G4A were not significantly related to sex, histological stage, tumor size and family history of cancer in treatment group I (P>0.05) (*Table 2*). In treatment group II, higher levels of PLA2G4A mRNA was associated with sex (P=0.009), tumor size (P=0.007) and family history of cancer (P=0.006) (*Table 2*). No association was found between mRNA expression level of PLA2G4A and histological stage in treatment group II (P>0.05) (*Table 2*).

The correlation between the relative expression of PLA2G4A and clinicopathological parameters was analyzed by Pearson's correlation test (*Table 3*). The results showed that relative expression level of PLA2G4A in treatment group I were not significant correlated with age, sex, histological stage, tumor size and family history of cancer (P>0.05) (*Table 3*). However, we found that relative expression of PLA2G4A in treatment group II was significantly and positively correlated with histological stage (P=0.038) and tumor size (P=0.022) (*Table 3*).

Discussion

Over the past decades, results of cell culture, animal models, and epidemiological and clinical studies have provided evidence to support the anti-carcinogenic effects of omega fatty acids (18-20,22,23). Although the precise molecular mechanisms by which omega fatty acids inhibit the development and progression of cancers has not understood very well, several possible explanations have been suggested by earlier studies. Sun *et al.* previously demonstrated that the omega fatty acids can induce apoptosis in the human

Figure 1 Box plot graph depicting the mRNA expression level of PLA2G4A in two treatment groups before and after treatment.

Table 2 mRNA expression level of PLA2G4A in relation with clinicopathological parameters

Variables	The mean of PLA2G4A mRNA expression level					
	Treatment group I			Treatment group II		
	Before treatment	After treatment with cisplatin alone	P value[a]	Before treatment	After treatment with cisplatin plus omega fatty acids	P value[a]
Sex						
Male	1.39±0.79	1.47±0.91	0.838	1.18±0.79	3.04±1.71	0.009
Female	0.79±0.63	1.16±0.94	0.383	1.45±0.97	2.23±1.40	0.225
Histological stage						
I	1.07±1.24	0.96±0.12	0.886	0.57±0.37	0.92±0.26	0.630
II	1.26±0.88	1.70±0.78	0.386	1.43±1.18	3.03±1.57	0.074
III	1.09±1.07	1.54±0.98	0.560	1.41±0.58	3.42±1.75	0.075
IV	1.28±1.05	1.58±0.40	0.669	1.88±0.45	2.82±1.58	0.347
Tumor size						
≤4	1.07±1.00	1.32±1.03	0.659	0.73±0.84	1.95±1.61	0.102
>4	1.28±0.77	1.35±0.87	0.848	1.71±0.64	3.24±1.43	0.007
Family history						
Yes	1.14±0.79	1.60±0.83	0.237	1.52±0.57	3.28±1.59	0.006
No	0.96±0.93	1.25±0.97	0.568	1.07±1.10	1.89±1.30	0.210

[a], P value calculated by Student's t-test.

Table 3 Correlation between PLA2G4A mRNA expression levels with clinicopathological parameters

Variables	PLA2G4A mRNA expression levels	
	Treatment group I	Treatment group II
Age		
r*	0.008	0.225
P value	0.963	0.201
Sex		
r*	0.314	0.120
P value	0.071	0.497
Histological stage		
r*	0.188	0.357
P value	0.287	0.038*
Tumor size		
r*	0.072	0.392
P value	0.687	0.022*
Family history		
r*	0.153	0.283
P value	0.387	0.105

*, values represent Pearson's correlation coefficient.

breast cancer cell line MCF-7 through inhibition of MEK/Erk/Bad signaling pathway (24). In a similar study, Kang and co-workers reported the effect of docosahexaenoic acid (DHA) in induction of apoptotic cell death in human MCF-7 breast cancer cells via caspase 8 activation (25). Down-regulation of the Wnt/β-catenin signaling pathway is one of the mechanisms through which omega fatty acids exerts their anti-proliferative activity in human pancreatic cancer cells (SW1990 and PANC-1) (26). Incubation of the human breast cancer cell line MDA-MB-231 with omega fatty acids leads to decrease cell proliferation possibly by decreasing signal transduction through the Akt/NFκB cell survival pathway (27). A recent study demonstrated that the cell cycle arrest at G0/G1 phase and the down-regulation of the CDK2 and cyclin E expression triggered by omega fatty acids in human neuroblastoma LAN-1 cells (28). A study conducted by Abdi et al., revealed that supplementation or combination therapy with omega fatty acids in multiple myeloma (MM) chemotherapy could increase drug sensitivity to bortezomib and reduce chemoresistance (29). Furthermore, mRNA expression analysis results pointed

out that omega fatty acids modulate gene expression of proteins involved in Delta-Notch, Hedgehog (HHt) and Wnt signaling pathways (29). Treatment of PA-1, H1299, D54MG and SiHa cancer cell lines with omega fatty acids reduce cell viability and induce apoptosis which is mediated by mitogen-activated protein kinase (MAPK) activation (30). The potential effect of omega fatty acids supplementation in treatment of lung cancer has been investigated by Kikawa et al. (31). They reported that omega fatty acids can inhibit tumor cell growth by increasing oxidative stress in the A549 lung adenocarcinoma cell line (31). Alterations in fluidity, structure and/or function of lipid rafts have substantial consequences on plasma membrane organization and functionalities such as signal transduction, cell growth and migration (32,33). Because wide arrays of membrane-signaling proteins are located within the lipid rafts, change in lipid composition of membrane rafts can alter activity of these signaling proteins (32). The effects of omega fatty acids on the lipid composition of lipid rafts and epidermal growth factor receptor (EGFR) raft localization in MDA-MB-231 human breast cancer cells has been previously studied (34). Omega fatty acids may exert their growth inhibitory effects on cancer cells by modifying the lipid composition of membrane rafts and EGFR signaling (34). Similarly, Corsetto et al. documented that omega fatty acids reduce the cell proliferation in MCF-7 and MDA-MB-231 breast cancer cell lines probably through change the biophysical properties of lipid rafts decreasing the content of cholesterol and probably the distribution of key proteins (35). Hence, omega fatty acids, as precursors for the endogenous biosynthesis of bioactive lipid mediators, have been considered as potential chemopreventive and chemotherapeutic agents in treatment of malignancies. Meanwhile, the cPLA2α enzyme, as an important upstream regulator, clearly plays a key role in production of these lipid mediators. Therefore, we investigated the effect of omega fatty acids on mRNA expression level of PLA2G4A in patients with GC. It is worth noting that the current study is the first report evaluating the effect of omega fatty acids on mRNA expression level of PLA2G4A in patients with GC, to the authors' knowledge. We found that the expression of PLA2G4A at mRNA level is significantly increased in treatment group II after receiving cisplatin plus omega fatty acid compared to before treatment (P<0.05) (*Figure 1*). A low mean expression level of PLA2G4A gene was observed in patients before supplementation with omega fatty acids (*Figure 1*). These data are consistent with other studies (14,36). A significantly reduced expression of

PLA2G4A in GC has been reported by Zhang *et al.* (14). The results of this study suggest that the reduced expression of PLA2G4A is associated with unfavorable outcome for patients with GC (14). A study using immunohistochemical technique clearly revealed that the expression of PLA2G4A at protein level is markedly decreased in human colorectal tumors (36). Homozygous and heterozygous deletion of cPLA2 increases the azoxymethane-induced colon carcinogenesis in mice (37). It therefore seems likely that down-regulated expression of PLA2G4A may contribute to transformation and tumor progression. On the contrary, over expression of PLA2G4A has been reported in prostate cancer (16), non-small cell lung cancer (15), and squamous cell carcinomas (38). No reasons were given for these contradictories. It is argued that cPLA2α probably plays a dual role in carcinogenesis, depending on the tumor type.

In conclusion, our results suggested that omega fatty acids supplementation up-regulate the mRNA expression level of PLA2G4A in patients with GC. These data provide additional evidence to support the hypothesis that omega fatty acids, as potent antineoplastic agents, may improve the outcomes of chemotherapy. However, further studies are necessary to disclose the exact molecular mechanisms by which omega fatty acids regulate PLA2G4A gene expression.

Acknowledgements

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The current study was approved by the Ethics and Human Rights Committee of Tabriz University of Medical Sciences (No. 92213) and informed consent was taken from all the patients.

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