The effect of ω-fatty acids on the expression of phospholipase A₂ group 2A in human gastric cancer patients

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Background: Studies show that polyunsaturated fatty acids (PUFAs) may have an inhibitory role in carcinogenesis. It was previously shown that PLA2 group 2A (PLA2G2A) messenger RNA (mRNA) expression is associated with less frequent metastasis and longer survival in gastric adenocarcinoma. This study intends to investigate the effect of PUFAs on the expression of PLA2G2A in patients with gastric cancer. **Materials and Methods:** Thirty-four patients with gastric cancer (GC) were randomly divided into two groups. The first group received cisplatin medication. The second group received cisplatin medication and supplements of ω-fatty acids for three courses. The total RNA was extracted from the tissues and cDNA was synthesized. The gene expression of PLA2G2A was evaluated by the real-time polymerase chain reaction (PCR) method. To confirm the changes in gene expression, frozen section was utilized. The frozen tissue samples were sectioned and stained using the immunohistochemistry technique. **Results:** After chemotherapy and chemotherapy plus supplement, the relative mean of PLA2G2A gene expression increased 1.5 ± 0.5-fold and 7.4 ± 2.6-fold, respectively ($P = 0.006$). The relative mean of gene expression in patients who received cisplatin and ω-fatty acids supplement increased more significantly (7.5 ± 3.3-fold) than in patients who received only cisplatin ($P = 0.016$). **Conclusion:** It was found that PUFAs increased the gene and protein expression of PLA2G2A in gastric cancer. Concerning the fact that studies reveal protective function of PLA2G2A in gastric cancer, it is suggested that increased expression of PLA2G2A is helpful. Furthermore, PUFAs can be considered as a useful therapeutic supplement for patients with gastric cancer.

Key words: Gastric cancer (GC), ω-fatty acids, phospholipase A₂ (PLA2)

INTRODUCTION

Gastric cancer (GC) is considered to be the fourth frequent human malignancy and the second cause of deaths from cancer throughout the world.[1,2] More than 0.93 million new cases are diagnosed yearly.[3] The pathogenesis of GC is not exactly known. Genetic, nutritional, and microbial factors have been proposed, which act in a multifactorial and multistep process.[4] Surgery is the most significant treatment approach for GC.[5,6] However, additional supplementary treatment approaches such as chemotherapy and radiotherapy improve the opportunity of treatment.[5] Despite considerable progressions, GC is still a difficult disease to manage.[2]

Studies suggest that polyunsaturated fatty acids (PUFAs), particularly n-3 PUFA, may have a leading inhibitory role in carcinogenesis.[7] Previous studies showed that essential fatty acids (EFAs), including α-linolenic acid (ALA, 18:3, ω-3) and cis-linoleic acid (LA, 18:2, ω-6), have a considerable inhibitory role in the growth of tumor cells.[2]

Phospholipase A₂ (PLA2) contains a big family of lipases[8] catalyzing the hydrolysis of the fatty acyl ester

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bond at the sn-2 position of phospholipids and produce free fatty acids and lysophospholipids.[1,9-11] About 30 enzymes have been identified that exhibit PLA2 or relevant activity in mammals. PLA2 subdivided into several classes including the secreted PLA2 (sPLA2), cytosolic PLA2 (cPLA2), Ca2$^+$-independent PLA2 (iPLA2), lysosomal PLA2s, and adipose-specific PLA2.[12]

For sPLA2, 11 isoforms have been identified, one of which is group 2A [PLA2 group 2A (PLA2G2A)].[13] The PLA2G2A protein is expressed in normal intestinal mucosa and several disease states, which include atherosclerosis, inflammation, and cancer.[1]

Because of the association between PLA2G2A expression and increased survival, and less frequent metastasis in human gastric cancer, a tumor- inhibitory action of PLA2G2A was introduced.[10] Further, it was described that PLA2G2A expression had decreased in metastatic carcinomas compared to primary carcinomas.[14] In gastric cancer, PLA2G2A expression had a negative relationship with the depth of invasion, lymph node metastasis, and tumor-node-metastasis stage. Patients with positive PLA2G2A expression exhibited a higher 5-year survival in total.[9]

Although there are many studies showing the anticancer effect of PUFAs[2,15] and there are some investigations about the protective effect of PLA2G2A on gastric cancer,[1,9,10] the effect of PUFAs on gene and protein expression of PLA2G2A in GC has not been examined. There is just one study showing that exogenous PUFAs increase the release of arachidonic acid [C$_{20:4}$ (*n*-6)], from human neutrophils *via* activation of both cPLA2 and sPLA2.[11] Also Khanaki K *et al*. showed that PUFAs notably increased the production of PLA2G2A level in ectopic endometrial cells which were sampled from endometriosis patients.[16]

So, this study is designed to investigate the effect of ω-fatty acids on gene and protein expression of PLA2G2A in patients with gastric cancer. The authors used omega fatty acid supplements as an adjuvant treatment for patients who received chemotherapy. Then, PLA2G2A has been measured both in gene expression and protein level.

MATERIALS AND METHODS

Patients
In this study, only patients with gastric adenocarcinoma were selected. Among these patients, those with diabetes, renal dysfunction, cardiac obstruction, pyloric obstruction, the underlying inflammatory disease, and those who had received omega supplementary 3 months before were excluded from this study because they could not receive the supplementary drug or their disease might interfere with the result of this study.

With local ethical approval and informed consent (ethical code number 92213), 34 patients with GC (referred to the endoscopy clinic of Tabriz University of Medical Science) were included in this study. The patients were randomly divided into two groups consisting 17 cases each (there were 34 balls in a bag — 17 balls contained "with supplement" label and 17 balls contained "without supplement" label; before chemotherapy, each patient took a ball and gave it to the oncologist). The biopsy samples of GC tumor tissues were taken and moved to a nitrogen tank until assayed. Then, the subjects were sent to an oncologist to begin treatment. The first group received cisplatin medication without supplements as the treatment while the second group (intervention group) received cisplatin medication with Natural Factors (USA) Ultimate Omega Factors capsules (USA), supplements of ω-3, ω-6, and ω-9 fatty acids with the formula of fish oil blend 400 mg, flax seed oil 400 mg, borage oil 400 mg, 3,600 mg daily (three 1,200 mg capsules) for three courses (each course, 3 weeks). Then, requiring medical follow-up, endoscopy was performed on the patient for the second time and the cancer treatment was completed by taking a stomach biopsy. The biopsy samples were moved to the nitrogen tank right away. The demographic data of the subjects are summarized in Table 1. Both the patient groups were compared statistically with *t*-test. The groups have been well-matched regarding parameters such as age, gender, tumor location, tumor grade, and other information (*P* value > 0.05). The flow of subjects through the trial is summarized in the consort diagram [Figure 1]. This study was registered in the Iranian Registry of Clinical Trials under No.IRCT2014031016922N1.

Identifying fatty acids pattern
The pattern of fatty acids in the "Natural Factors Ultimate-Omega Factors" capsules was determined in order to insure the amount of fatty acids in Capsules utilized in the study on the patients. For this purpose, the contents in the capsules were diluted in proportion to 1/100, and using the protocol of Bligh and Dyer,[17] the process of total lipid extraction was performed. Then, the percentage of fatty acids was obtained by Gas Chromatograph (Buck Scientific, USA) compared to the corresponding standard.

RNA isolation and cDNA synthesis
Total RNA was extracted from the tissues using EZ-10 Spin Column Total RNA Mini-Perps Kit according to the manufacturer's instructions. Total RNA was treated with DNase and next, complementary DNA (cDNA) was synthesized with the use of RevertAid™ First Strand cDNA Synthesis Kit (Takara) based on the manufacturer's protocol. The cDNA samples were stored at −80°C before real-time polymerase chain reaction (PCR).

Table 1: Demographic data of the patients in the study group

Groups clinical and pathologic factors	Control (n = 17)	Case (n = 17)	P value
Age (years) (mean±SD)	67.5±11.21	71.25±9.81	0.235
Gender			
Male (*n*=19)	9	10	0.695
Female (*n*=15)	8	7	
Tumor size			
<4 cm (*n*=16)	7	9	0.759
>4 cm (*n*=18)	10	8	
Tumor primary location			
Upper (*n*=11)	5	6	0.714
Median (*n*=13)	6	7	0.790
Lower (*n*=10)	6	4	0.452
Stage classification of malignant tumors (*TNM*)			
I (*n*=7)	4	3	0.089
II (*n*=11)	6	5	
II (*n*=9)	4	5	
IV (*n*=7)	3	4	
Systolic blood pressure (mmHg)	131.1±9.2	128.8±10.2	0.235
Diastolic blood pressure (mmHg)	85.1±7.1	79.2±7.9	0.985
Cigarette smoking			
Current smoking (*n*=12)	7	7	1
Nonsmoker (*n*=11)	6	5	0.714
Ex-Smoker (*n*=11)	4	5	0.697
Fasting blood sugar (mg/dL)	98.54±15.25	102.85±18.65	0.235
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
History of family			
Yes (*n*=19)	10	9	0.73
No (*n*=15)	7	8	

Real-time polymerase chain reaction

Real-time quantitative PCR was performed using the RealQ Plus 2x Master Mix SYBR Green Kit (Ampliqun, Denmark), as described by the manufacturer. A dissociation curve was done at the end of each PCR reaction to confirm that one product was amplified. The amplification was run at 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 61°C for 60 s. The quantification of PLA2G2A was normalized to the endogenous gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using the $2^{-\Delta\Delta Ct}$ method. The primers used for PLA2G2A in this study are 5'-TGACGACAGGAAAGGAAGCC-3' as forward and 5'-GCCACTCGATGGTGAGGTAG-3' as reverse. The primers used for GAPDH are 5'-CCAGTGGACTCCACGACGTA-3' as forward and 5'-GCGAGATCCCTCCAAAATCA-3' as reverse.

Frozen section and immunohistochemistry staining

The frozen section was utilized to confirm the changes in gene expression of PLA2G2A. The frozen tissue samples were sectioned and stained by the immunohistochemistry method. First, 4-μm thick sections were provided from the tissues, and then the samples were located in the citrate buffer for 15 min. The samples were blocked by skim milk to prevent nonspecific binding and were incubated with primary rabbit monoclonal antibody against PLA2G2A overnight at 4°C. After three washes in buffer tris-buffered saline with Tween 20 (TBST), the corresponding secondary antibody was added and incubated for 1 h. To have the emergence of proteins, 3,3'-Diaminobenzidine (DAB) was utilized as a substrate and hematoxylin as counterstain.

Statistical analyzing

The obtained computed tomography (CT) values for PLA2G2A gene expression were calculated by 2^{-DDCT} formula.[18] Then, the mean was calculated in each group and these were compared using Mann-Whitney *U* test. The semi-quantitative results for PLA2G2A protein were expressed in percentages and were then compared using the Mann-Whitney *U* test and Wilcoxon signed-rank test. When *P* value was less than 0.05, the tests were considered to be significant. Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS) software (version 16). All the data were represented as mean ± standard error of the mean (SE).

RESULTS

Determination fatty acids pattern in Omega-3, 6, and 9 capsules.

In "Natural Factors Ultimate Omega Factors" capsules, the percentage of omega-3 fatty acids, including alpha-linolenic acid and eicosapentaenoic acid, was 22.76%. The percentage of omega-6 fatty acids, including arachidonic acid and linoleic acid, was 28.71%. Furthermore, the percentage of omega-9 fatty acids, including oleic acid, was 16.04%.

Comparison PLA2G2A gene expression in the studied groups.

As presented in Figure 2a, in patients who received chemotherapy (control group) the relative mean of PLA2G2A gene expression increased (1.5 ± 0.5-fold higher than the relative mean before receiving chemotherapy). Furthermore, relative mean of PLA2G2A gene expression in patients who received chemotherapy and supplement (case group) significantly increased (7.4 ± 2.6-fold higher than the relative mean before receiving chemotherapy and supplement). In addition, the relative mean of PLA2G2A gene expression in patients who received cisplatin and ω-fatty acid supplement increased more significantly (7.5 ± 3.3-fold) than in patients who received only cisplatin (*P* value = 0.016, Relative Expression Software Tool (REST), Germany) [Figure 2b].
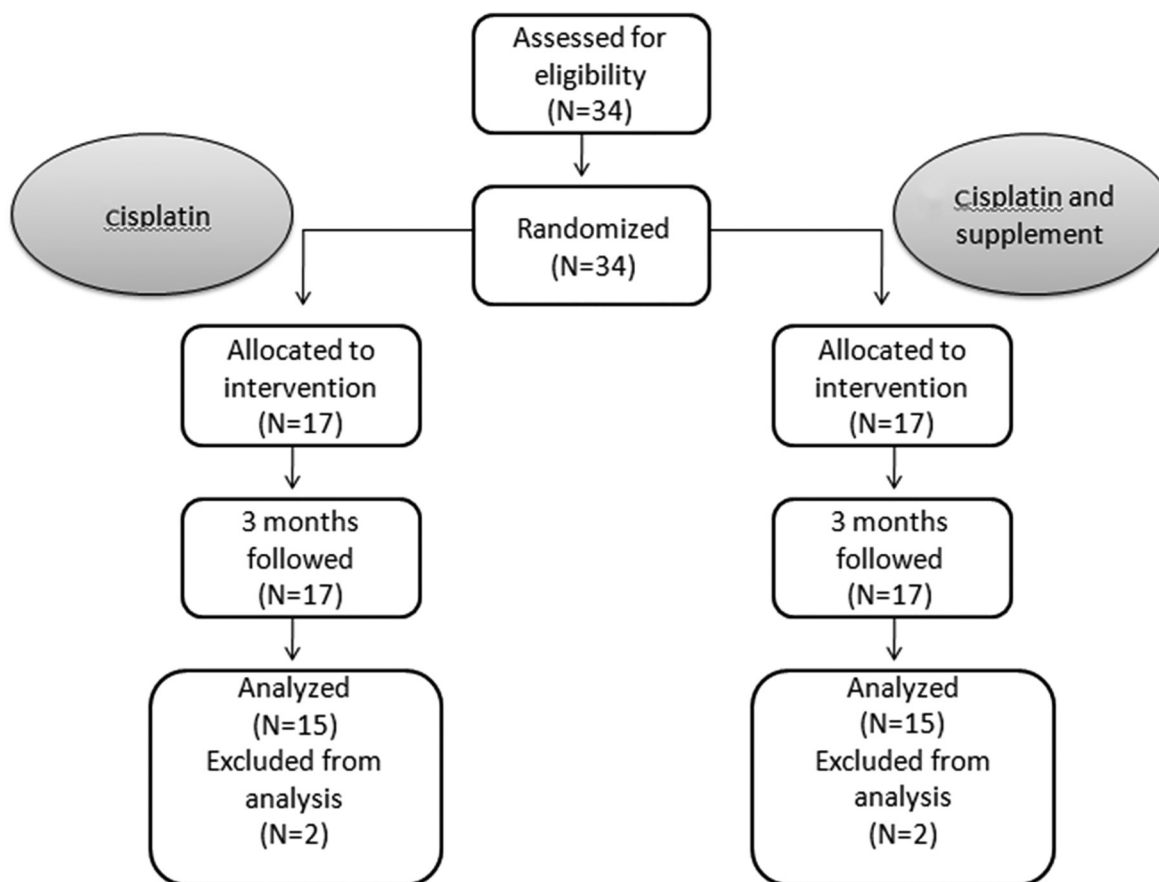
Figure 1: Consort diagram. The flow of subjects through the trial is summarized in the diagram

Comparison of PLA2G2A protein in GC tissues in studied groups.

According to Figure 3a, the results of PLA2G2A protein were from negative (−) to +4 (++++ positive). The semi-quantitative results were presented in percentages and then, using Mann-Whitney *U* test and Wilcoxon signed-rank test, they were compared together. As presented in Figure 3b PLA2G2A protein percentage increased after chemotherapy (*P* value = 0.001). Furthermore, PLA2G2A protein percentage after chemotherapy plus supplement significantly increased (*P* value = 0.001) [Figure 3c]. The percentages of PLA2G2A protein in the two groups were compared too. In the case group, PLA2G2A protein percentage increased more significantly than in the control group (*P* value = 0.011) [Figure 3d]. The abovementioned results of the level of changes in the PLA2G2A protein may verify the level of changes in gene expression of this protein.

The statistical data of results are summarized in Table 2.

DISCUSSION

This is the first study investigating the effect of PUFAs on gene and protein expression of PLA2G2A in GC. It was evaluated the expression of PLA2G2A affected by ω-fatty acids supplement in GC tissue using Real-time PCR, Frozen section and immunohistochemistry staining. The results revealed that the gene and protein expression of PLA2G2A increased in patients who received chemotherapy. Furthermore, the gene and protein expression of PLA2G2A in patients who received chemotherapy and the supplement significantly increased. In addition, the gene and protein expression of PLA2G2A in patients who received cisplatin and ω-fatty acid supplement increased more significantly than in patients who received only cisplatin.

Several studies have been performed to assess the cytotoxic effect of PUFAs on tumor cells. The effect of PUFAs on the PLA2G2A expression in various diseases has been poorly studied. PUFAs increased the production of PLA2G2A in ectopic endometrial cells sampled from endometriosis patients.[16]

There are few clinical experiments about the PLA2G2A expression in GC. The expression of PLA2G2A messenger RNA (mRNA) was associated with less frequent metastasis and longer survival in patients with gastric adenocarcinoma.[10] Wang *et al*. strongly suggested that PLA2G2A expression is protective for patients with GC.[1] But the effect of PUFAs on the PLA2G2A expression in GC has not been examined.

The findings of this study are consistent with Khanaki *et al.*, which showed that PUFAs exposure results in an increase in the PLA2G2A level within the ectopic endometrial cell. PUFAs and especially high ω-3:ω-6 notably enhanced the production of PLA2G2A level in ectopic endometrial cells sampled from their endometriosis patients.[16] Hughes-

Fulford *et al.* showed that exogenous arachidonic acid increased the mRNA and protein expression of cPLA2 in a human prostate cancer cell line in a dose-dependent manner.[19] In the HCT116, CaCo2, and SNU-C5 colon cancer cells and SK-BR3 and MCF7 breast cancer cells, treatment with phorbol 12-myristate 13-acetate (PMA) markedly increased phospholipase D1 (PLD1) mRNA and protein expression.[20]

Previous studies reported that interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) induce PLA2G2A expression and secretion over a long period in different animal cell systems.[21,22] IL-6 has been reported to stimulate gene expression and synthesis of PLA2G2A in HepG2 liver hepatoma cells.[23] Exposure to proinflammatory cytokines can stimulate PLA2G2A transcription and secretion of intracellular storage or *de novo* synthesized PLA2G2A in a temporary or sustained form.[24]

Studies have suggested that PLA2G2A expression may have a protective effect versus the progression of carcinomas.[10] Chen *et al.* found a reciprocal relationship between PLA2G2A expression and vascular invasion in hepatocellular carcinoma.[25] Expression of PLA2G2A has also been shown to be significantly associated with longer survival after surgery in pancreatic cancer.[26] These results each support the hypothesis that PLA2G2A plays a direct role in suppressing GC progression. Leung *et al.* indicated that PLA2G2A mRNA expression was associated with less frequent metastasis and longer survival in patients with gastric adenocarcinoma.[10] The results of a large cohort study strongly suggested that the expression of PLA2G2A is protective for patients with GC.[1] PLA2G2A catalyzes the release of arachidonic acid from membrane phospholipids. Arachidonic acid can induce apoptosis in diverse cells including human colon cancer cells. Nonsteroidal anti-inflammatory drugs that inhibit the conversion of arachidonic acid to prostaglandins by cyclooxygenases apparently confer significantly lower risk of both colorectal cancers and GCs. Elevated expression of PLA2G2A might similarly act as an inhibitor of the progression of GC *via* increased release of arachidonic acid.[10] Ganesan
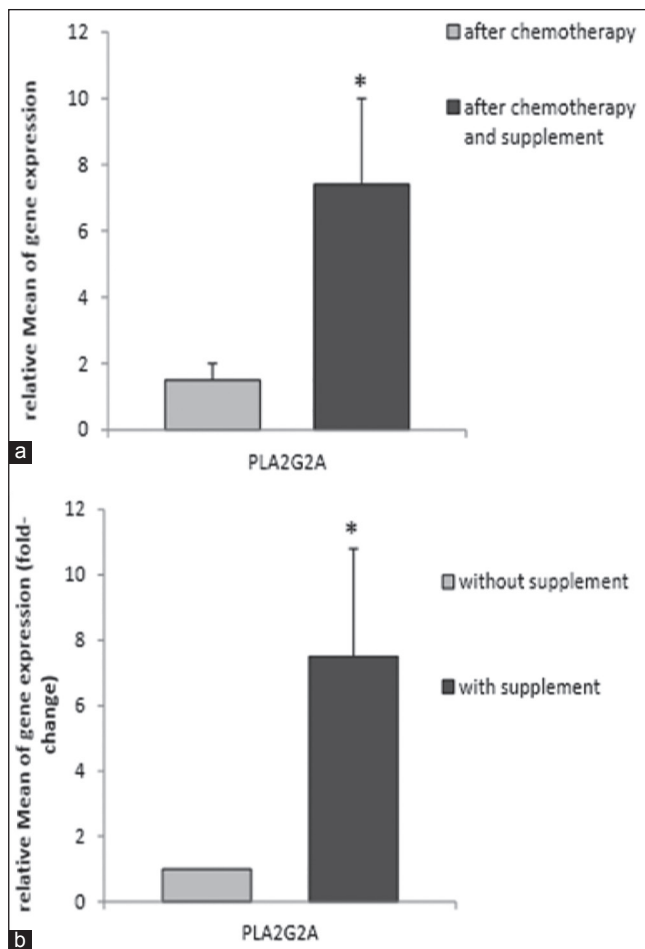
Figure 2: (a) Relative expression of PLA2G2A in patients who received chemotherapy (cisplatin) increased (1.5 ± 0.5-fold higher than before receiving chemotherapy). In patients who received chemotherapy plus ω-fatty acids supplement, relative expression significantly increased (7.4 ± 2.6-fold bigger than before receiving chemotherapy plus supplement) (*P = 0.006) (b) Relative expression of PLA2G2A significantly increased in patients who received chemotherapy plus ω-fatty acids supplement (7.5 ± 3.3-fold more than in patients who received chemotherapy) (*P = 0.016)

Table 2: The statistical data of results

PLA2G2A Gene Expression	Minimum ($2^{-\Delta\Delta CT}$)	Maximum ($2^{-\Delta\Delta CT}$)	Median ($2^{-\Delta\Delta CT}$)	Mean	SE	*P* value
After chemotherapy	0.05	8.0	0.8	1.5	0.5	[1]0.006
After chemotherapy and supplement	0.2	31.8	4	7.4	2.6	
PLA2G2A Protein	Minimum (%)	Maximum (%)	Median (%)	Mean	SE	P value
Before chemotherapy	0	25	12.5	10	2	[2]0.001
After chemotherapy	25	50	50	40	3	
Before chemotherapy and supplement	0	25	12.5	12	2	[2]0.001
After chemotherapy and supplement	25	100	50	60	5	
After chemotherapy	25	50	50	40	3	[1]0.011
After chemotherapy and supplement	25	100	50	60	5	

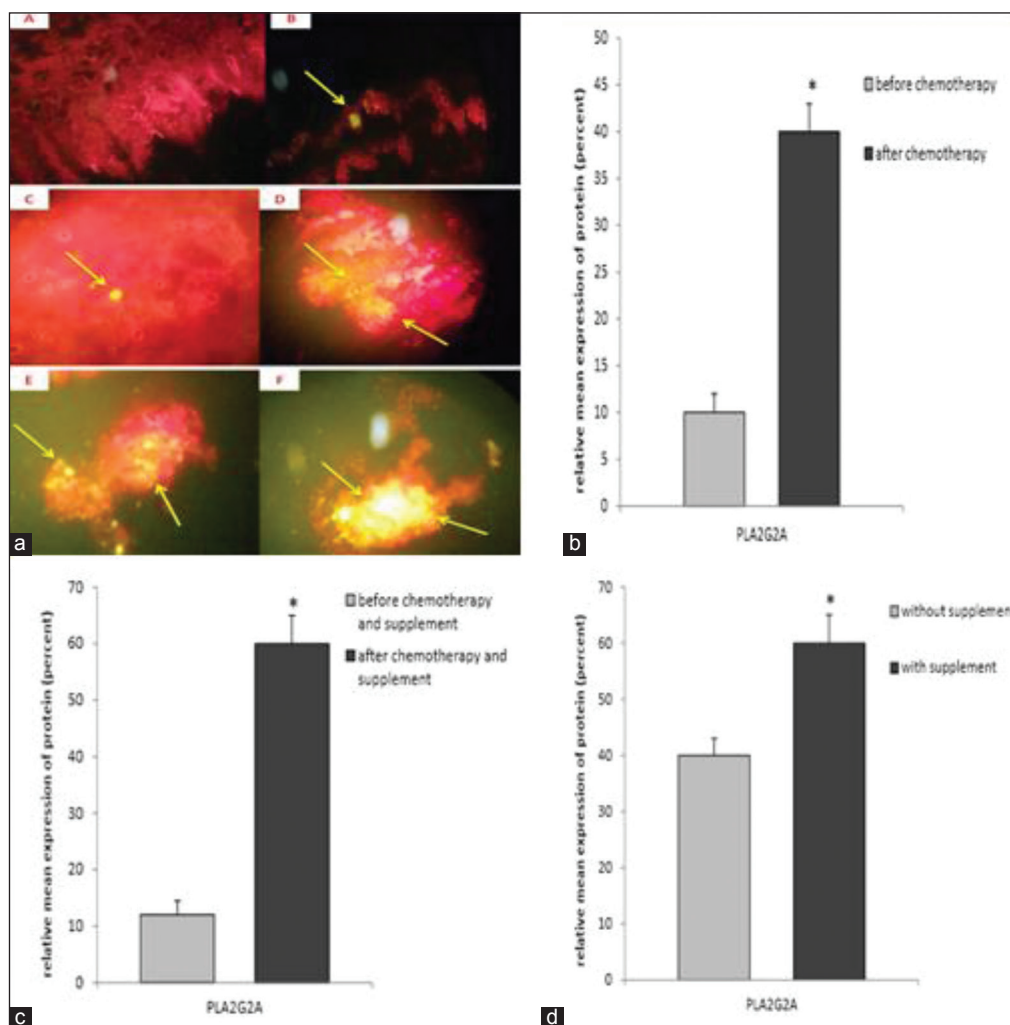[1]Mann–Whitney *U* test, [2]Wilcoxon signed-rank test

Figure 3: (a) Results of immunohistochemistry staining of frozen sections. A) Negative, B) +/-, C) +, D) 2+, E) 3+, and F) 4+ (b) PLA2G2A protein increased in patients after chemotherapy (40% ± 3%) (*P = 0.001) (c) PLA2G2A protein increased in patients after chemotherapy plus supplement (60% ± 5%) (*P = 0.001) (d) Comparing PLA2G2A protein in patients who received chemotherapy plus ω-fatty acids supplement with patients who received only chemotherapy (*P = 0.011)

et al. suggest that under the regulation of Wnt/β-catenin signaling, PLA2G2A inhibits the invasion and metastasis of GC cells.[27]

In this study, it was found that PUFAs can increase the expression of PLA2G2A. What are the causes of these results? Khanaki *et al.* have been proposed:

1. ω-6 and particularly high ω-3 PUFA ratios exposure can induce the production of certain cytokines or growth factors leading to increased PLA2G2A level and
2. ω-6 and especially high ω-3 PUFA can also have effects on other mechanisms that regulate the PLA2G2A level.[16]

Hughes *et al.* provided evidence that arachidonic acid regulates gene expression and protein synthesis.[19]

Studies show that rat PLA2G2A promoter contains peroxisome proliferator responsive elements (PPREs),[28,29] suggesting that peroxisome proliferator-activated receptor

(PPAR) activation in rat mesangial cells may be involved in the induction of PLA2G2A by exogenously added sPLA2s, and also by rat PLA2G2A endogenously produced after cytokine treatment. Considering this and because PPARs may be activated by PLA2 lipid products, Beck *et al.* postulated that PPARs might be involved in the upregulation of PLA2G2A expression by sPLA2s. Also they showed that docosahexaenoic acid (DHA) or linoleic acid (LA) as potential PPARα agonists have strong stimulatory effects on the rat's PLA2G2A promoter activity in mesangial cells. On testing specific PPARα activators for PLA2G2A induction, they found that PPARα activators such as unsaturated long chain fatty acids had a potentiating effect.[30]

According to these findings and theories, it has been proposed that PUFAs as potential activators of PPARα may have strong stimulatory effects on the human PLA2G2A promoter activity. This suggestion should be examined closely in future studies. In addition, PUFAs may induce

production of certain cytokines, resulting in increased PLA2G2A expression. There is a possibility that some special PUFAs such as arachidonic acid have an important role in PLA2G2A gene expression and protein synthesis regulation.

The exact mechanism of PUFAs on gene expression of PLA2G2A and the function of this enzyme in the pathogenesis and treatment of GC should be explored in future studies.

CONCLUSION

It was found that PUFAs increased the gene and protein expressions of PLA2G2A in GC. Regarding the fact that studies show a protective role of PLA2G2A in GC, it is suggested that increased expression of PLA2G2A helps to control GC. Furthermore, PUFAs can be considered as a useful therapeutic supplement for patients with GC.

Conflicts of interest
There are no conflicts of interest.

AUTHOR'S CONTRIBUTION

M Sh took part in the experiments and prepared the manuscript. MA participated in the design of the study, primer design, and manuscript preparation. AM participated in the design and coordination of the study. MHS identified the patients, performed endoscopy, and took GC tissues biopsy samples. HD participated in the design of the study, primer design, RNA extraction, and cDNA synthesis. All the authors have read and approved the content of the manuscript.

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