



## Determination of expression of regulatory cells of cell cycle S phase in patients with gastric adenocarcinoma with oral administration of PUFAs before and after chemotherapy

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### Abstract

**Background & Aims:** Gastric cancer is the 5th most common cancer and the third cause of death in the world. Studies have shown that gastric cancer is somewhat susceptible to chemotherapy, but the duration of tumour reduction is short, and patients have not had much success in survival, and in many cases, chemotherapy resistance has been observed. Therefore, the main objective of this study was to investigate the effect of omega-unsaturated fatty acids on the expression of Cyclin A2, and CDK2 germ cell cycle in patients with gastric adenocarcinoma under chemotherapy.

**Materials and Methods:** This is a double-blind, pre-and post-test clinical trial with the target population of patients with gastric adenocarcinoma that were first identified and subjected to chemotherapy. Twenty-four patients were selected randomly and randomly in control and control groups. In the control group, the treatment was routine with cisplatin plus placebo. In the case group, treatment with cisplatin plus a supplement of natural fatty acid supplementation capsules of Ultimate-Omega Factors with a dose of 1200 mg per day was 3,600 mg Three tablets of 1200 mg (for three courses) started on horizons three weeks. Three samples of stomach biopsy were taken from all patients before and after chemotherapy. Biopsy specimens were extracted from all tissue mRNAs and cDNA was synthesized from them, and then the expression of the genes was measured using Real-Time PCR. The results were analyzed by SPSS software version 24.

**Results:** The mean or average expression of Cyclin A2, CDK2 in the case group showed a significant decrease compared to the control group (P value was 0.021 and 0.026, respectively).

**Conclusion:** The results of this study showed that the use of omega-3, 6, 9 fatty acids with cisplatin can be useful in stopping the S-cell cycle in gastric cancer cells.

**Keywords:** Gastric Adenocarcinoma, PUFAs, Phase S Cell Cycle, CDK-2, Cyclin A2

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## Introduction

Gastric cancer is the 5th most common cancer in the world and the third most common cause of cancer deaths in both sexes around the world (723,000 deaths), 8.8% of the total). (More than 70% of cases (677,000 cases) are in Developing countries occur (456,000 in men, 221,000 in women). The incidence of men in men is about twice as high as women. (1) One of the most effective ways to treat cancer is chemotherapy (2) And the goal is to stop the cell cycle and eventually launch cell death (3). But one of the main obstacles to the success of chemotherapy is the increased resistance to chemotherapy drugs (4). According to published results laying gastric cancer cells fairly It is resistant to chemotherapy and only 30 to 40 per cent of cases of growth retardation is seen in tumour cells. (5).

Cisplatin is a chemotherapy drug that by binding to DNA and blocking transcription factors, activating mitotic inhibition Apoptotic signaling pathways, the blocking of transcription promoters of the genes involved in the cell cycle, eliminates the ability of the proliferation of cancer cells and degradation of the damaged DNA of the cell, which does not have the potential to rebuild and eliminate them (6). Recent studies have shown that cisplatin-resistant cancer cells escape apoptosis due to the activation of various protective mechanisms, and this is done by activating various pathways such as route activation (IP3K) -AKT. This pathway is one of the main and main pathways for anti-apoptotic mitochondrial and is known as one of the mechanisms for resistance to cisplatin. (5.7).

Although patients at the start of chemotherapy are good candidates for cisplatin, in some cases, their drug resistance is altered by cellular uptake and drug delivery from the cell, increased drug detoxification, apoptosis inhibition (8) Disturbances in cell cycle regulation are associated with various aspects of gastric cancer, including cellular proliferation of cancer. While in the cell cycle, by inhibiting a complex consisting of Cyclin-CDK, the cell can be stopped in a specific phase and subsequently induced apoptosis by inducing apoptotic signals such as activating the P53 protein (9).

On the other hand, Fatty acids are key nutrients that affect primary growth and development, as well as the prevention of chronic disease in later life (10). Among fatty acids, omega-3 fatty acids (n-3 PUFA) and unsaturated fatty acids Omega 6 (n-6 PUFA) have been proposed to reduce various diseases. The results from recent studies have shown that omega-3 fatty acids, omega-3 unsaturated fatty acids, omega-6 unsaturated fatty acids, and omega-9 unsaturated fatty acids inhibit the growth of cancer cells both in vitro and in (11) It has also been shown that omega-3 unsaturated fatty acids have beneficial effects on some of the chronic degenerative diseases, such as cardiovascular disease (12,13), rheumatoid arthritis (14), diabetes (15) Other autoimmune diseases (16) and cancer (17).

Chuck and Das (18), Trump Beta (19), in their study, showed that unsaturated fatty acids by inhibiting cell cycle in cancer cells, Suppresses these cells. In addition to these studies, Lee et al. And Barasch et al. (20,21) in recent years have shown that omega-3 fatty acids can exert their anti-tumour effects on various cancer cells from The method of stopping the induction of a cell cycle has been shown to indicate that this fatty acid has stopped the cell cycle in cancer cells of the hepatocyte class MHCC97L and metastatic melanoma cells SK-M el-110.

Therefore, the main purpose of this study was to evaluate the effect of oral administration of unsaturated fatty acids of omega-6, 3 and 9 with cisplatin on the expression of genes (Cyclin A2, CDK2), which identified key genes of cell-cycle S-phase regulation in patients with gastric adenocarcinoma To evaluate the effect of omega-3 fatty acids on cell cycle stopping by examining the expression of these genes.

## Materials & Methods

### Study Type and Sample Size Determination:

This study was a pre-and post-interventional (pre and post interventional) double-blind clinical trial with no oncologists and no patients diagnosed with type of treatment and prevented from possible errors. Among patients referring to Endoscopy Clinic of Tabriz University of Medical Sciences with the diagnosis of

gastric cancer, according to the goals and conditions of this study, we selected 24 people and divided into two groups of 12 people. To calculate the sample size according to the type of study, the formula:  $N \geq S^2 (\Sigma \alpha / 2 + \Sigma \beta) / \delta^2$ . In this formula, taking into account the 95% confidence factor and 85% power, according to the previous studies. (Tab.1) Also, this clinical trial has been registered in the registry centre for clinical trials of the ministry of health and medical education with no. IRCT201612216922N2 and confirmation no. IR.TB MED.REC.1395.677

#### Sampling method:

After the definitive diagnosis of gastric cancer, 3 biopsy samples from gastric tumours were removed and distal to then-tanks. Subsequently, these patients were referred to an oncology specialist and the chemotherapy of these patients under the supervision of this physician Beginning in the first two groups, the first group received 75 mg / m<sup>2</sup> cisplatin treatment with a non-prescriptive intravenous injection every other week, and the second group received cisplatin treatment plus Natural Factors Ultimate-Omega Factors 1200 mg Supplements Omega-3, Omega-6 and Omega-9 Fatty Acids with Formulation: Fish Oil Blend 400 mg,

Flaxseed Oil 400 mg, Borage Oil 400 mg, A daily dose of 3,600 mg (three tablets 1200 mg) was started for 3 courses (three-week courses). After this period, the endoscopy was followed up with compulsory follow-up therapy and the treatment of a tumour was followed by a gastric biopsy. Again, samples taken from patients were immediately transferred to the N-tank.

#### RNA extraction and Real-Time PCR

RNA extraction was used according to the RNA extraction kit protocol from EURX Poland. Also, for the production of cDNA, the Gene All code used for the First-strand Synthesis Hyper script kit was used. The primers of the Cyclin A2 and CDK2 genes were already designed and blotted, and the GAPDH gene was used as an internal control.

#### Design and selection of primers:

According to the above, primers of the studied genes were designed using Primer3 software and then checked with oligo7 software and checked for specificity in NCBI / primer blast and sequences designed to synthesize Sinaclon Company was sent. (Tab.1) In this study, the Real-Time PCR reaction was used for the Real-Time PCR Mic and the SYBR-Green Diagnostic Compound of the Sina Colon Company.

**Table 1.** Information about the sequence of primers designed for the genes studied

CDK-2 primer	Sequence (5'→3')	NCBI Ref. Sequence
Forward primer	GGACGGAGCTTGTATCGGT	NC_000012.12
Reverse primer	GCCCAGATTTACAGGGTCTCA	
Cyclin-A2 primer	Sequence (5'→3')	NCBI Ref. Sequence
Forward primer	GCTTTCCAAGGAGGAACGGT	NC_000004.12
Reverse primer	GCAAAGGCCAACCCATAAG	
GAPDH Primer	Sequence (5'→3')	NCBI Ref. Sequence
Forward primer	GAAGGTGAAGGTCGGAGTC	NC_000012.12
Reverse primer	GAAGATGGTGATGGGATTTC	

#### Statistical analysis:

Statistical analysis was performed using SPSS software version 24.0 (SPSS Inc). The difference between groups in tissue samples was investigated by the non-parametric Mann-Whitney test. Relative

measurement of CDK2, Cyclin A2 expression by  $2^{-\Delta\Delta ct}$  (22):

$$(\Delta CT = \text{Cyclin A2 (CT)} - \text{GAPDH (CT)}; \Delta\Delta CT = \Delta CT (\text{tumor}) - \Delta CT (\text{Normal}))$$

$$(\Delta CT = \text{CDK1 (CT)} - \text{GAPDH (CT)}; \Delta\Delta CT = \Delta CT (\text{tumor}) - \Delta CT (\text{Normal}))$$

And the mean  $\pm$  SEM data were used to assess the survival rate of the patient (OS) and to create surviving curves based on the low levels and the level of Cyclin A2 (as the definition of the ROC curve), using the Kaplan-Meier and Log-rank tests. Because of the independence of the studied groups, the mean and median of the results were calculated by SPSS software version 24 in each group, and the distribution of the normal results was examined by Shapiro Wilks test. The results of normal distribution were compared between the two groups by Independent Sample t-Test and the

results with normal distribution were compared by non-parametric Mann Whitney test.

## Results

### Comparison of demographic data of patients in the studied groups:

In Table 2, demographic data of both groups of patients have been shown to be statistically compared and, as can be seen, the studied groups are well-matched with regard to age, sex, tumour location, tumour grade, and other information. In all cases,  $p > 0.05$ .(23)

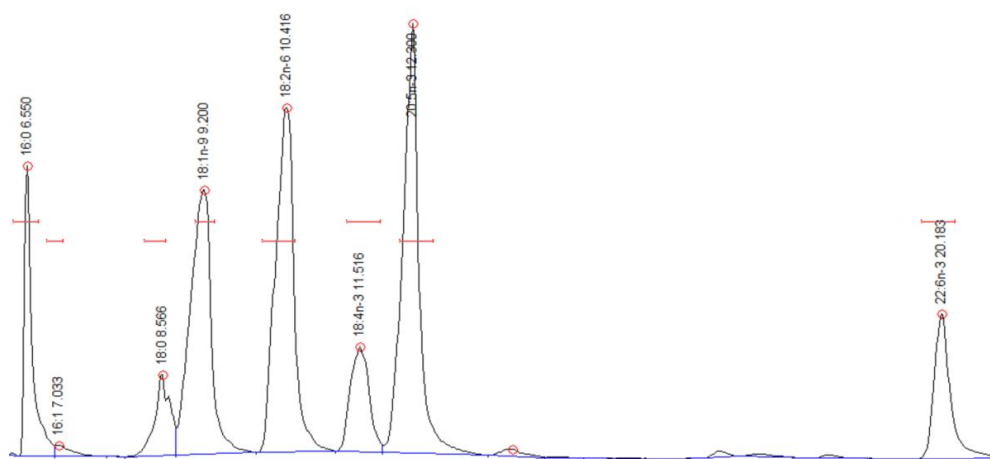
**Table 2.** Demographic information of the patients in study groups

Groups	Control (n=12)	Case (n=12)	p-Value
Clinical and Pathologic Factors			
Age(Years)(Means $\pm$ SD)	67.5 $\pm$ 11.21	71.25 $\pm$ 9.81	0.235
Gender			
Male (n=15)	7	8	0.695
Female (n=9)	5	4	
Tumor Size			
<4 cm (n=11)	4	7	0.759
>4 cm (n=13)	8	5	
Tumor Primary Location			
Upper (n=7)	3	4	0.714
Median (n=11)	5	6	0.790
Lower (n=6)	4	2	0.452
Stage Classification of Malignant Tumors (TNM)			
I (n=4)	2	2	0.089
II (n=7)	4	3	
III (n=5)	2	3	
IV (n=8)	4	4	
Systolic Blood Pressure (mmHg)	131.1 $\pm$ 9.2	128.8 $\pm$ 10.2	0.235
Diastolic Blood Pressure (mmHg)	85.1 $\pm$ 7.1	79.2 $\pm$ 7.9	0.985
Cigarette Smoking			
Current Smoking (n=10)	5	5	1
Non-Smoker (n=7)	4	3	0.714
Ex-Smoker (n=7)	3	4	0.697
Fasting Blood Sugar (mg/dl)	98.54 $\pm$ 15.25	102.85 $\pm$ 18.65	0.235
Cholesterol (mg/dl)	148.98 $\pm$ 21.56	151.25 $\pm$ 25.65	0.125
Triglyceride (mg/dl)	87.25 $\pm$ 18.25	78.25 $\pm$ 15.65	0.256
History of Family			
Yes (n=13)	7	6	0.73
No (n=11)	5	6	

### Determination of the pattern of fatty acids in omega 6 and 9 capsules prescribed to patients:

To ensure the exact amount of fatty acids in the capsules used in this study, which was prescribed as Natural Factors Ultimate-Omega Factors, is a model of

the fatty acids present in these capsules. By the gas chromatography machine, the BUCK factory in the United States was determined in relation to the relevant standards. (Tab.3)(Fig.1)



**Fig 1.** Chromatogram of fatty acids in soft-gel capsule

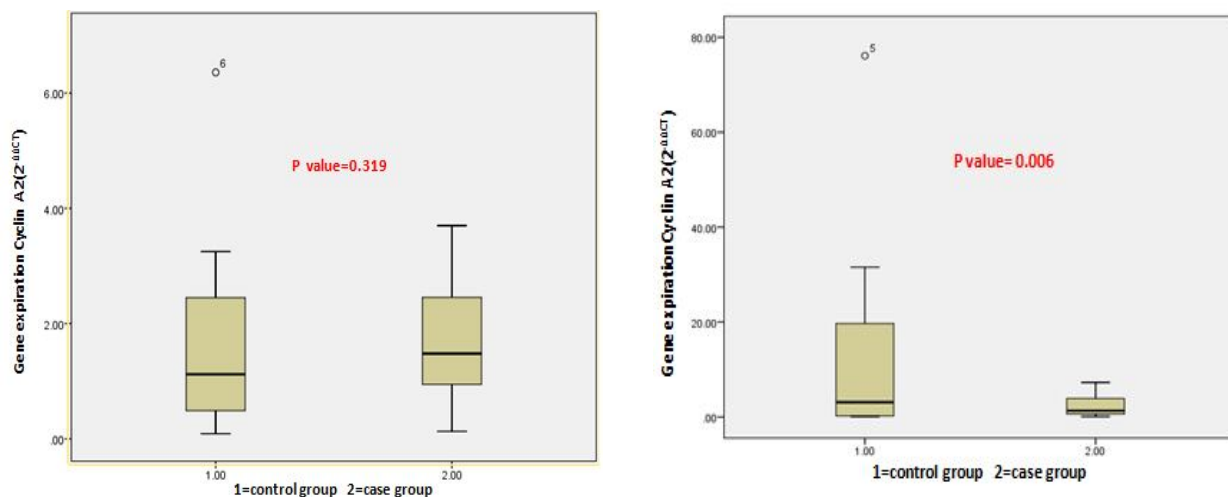
**Table 3.** The Percentage of polyunsaturated fatty acids in Natural Factors Ultimate-Omega 3, 6, 9

Unsaturated fatty acids	% of total
16:0	8.35
16:1n-7	0.65
18:0	4.49
18:1n-9	20.61
18:2n-6	23.88
18:4n-3	7.37
20:5n-3	25.70
22:6n-3	8.95

### Comparison of the expression of genes (Cyclin A2, CDK2) in the studied groups:

The level of expression of Cyclin A2 gene was compared in two groups. The expression level of Cyclin A2 in the post-chemotherapy group with supplementation of omega-3 fatty acids was significantly different from that of the control group, ie patients with chemotherapy without supplementation. The mean Cyclin A2 expression in the case group was

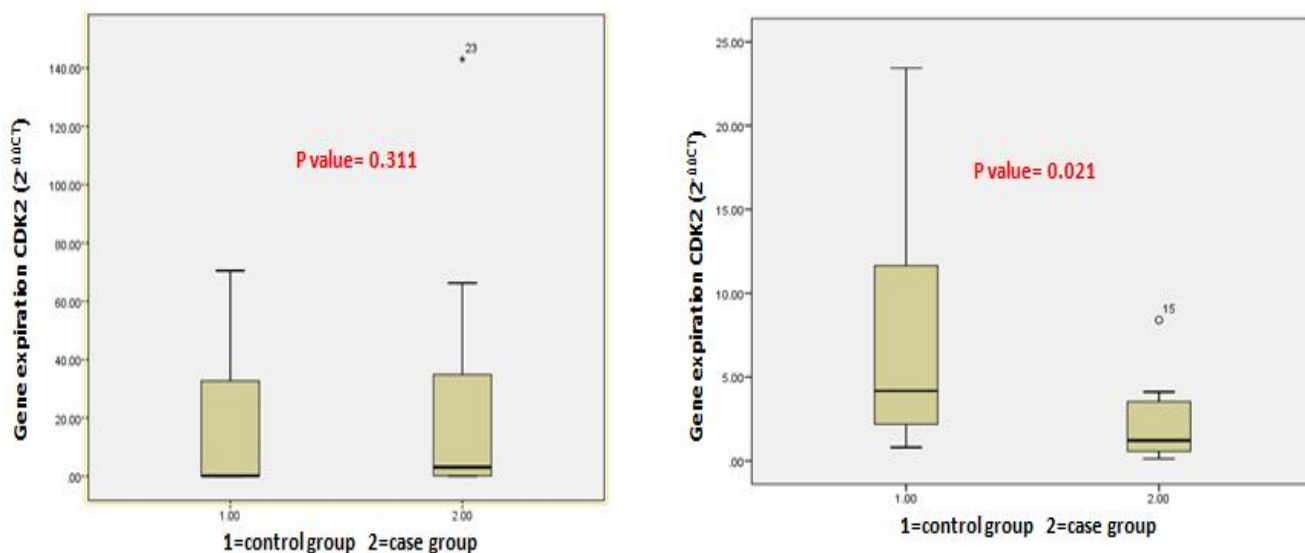
equal to  $2/41 \pm 2/2$  ( $2 - \Delta\Delta CT$ ) and in the control group is  $13/45 \pm 22/30$  ( $2 - \Delta\Delta CT$ ) and the value of p is 0/006. It should be noted that in the case group the minimum response was 0.7 ( $2 - \Delta\Delta CT$ ) and the maximum response was 76.7 ( $2 - \square\Delta CT$ ) and in the control group the minimum response was equal to 0.5 ( $2 - \Delta\Delta CT$ ) and the maximum response was 7.27 and the test coefficient was 9.205 (Fig-2).



**Fig.2-** The curve for comparing the mean of expression of Cyclin A2 gene expression in the gastric cancer before chemotherapy in the two groups studied. The curve for comparing the mean of expression of Cyclin A2 gene expression in the gastric cancer after chemotherapy in the two groups studied

The expression of CDK2 gene was compared in two groups. The expression of CDK2 expression in the post-chemotherapy group with supplementation of omega-3 fatty acids was significantly different from that of the control group, ie patients with chemotherapy without supplementation, and it was determined by non-parametric Mann-Whitney test that The mean CDK2 expression in the case group was 1.21 (2<sup>-ΔΔCT</sup>) and

in the control group it was 4.17 (2<sup>-ΔΔCT</sup>), and the p-value was 0.021. It should be noted that in the case group the minimum response was 0.13 (2<sup>-ΔΔCT</sup>) and the maximum response was 8.40 (2<sup>-ΔΔCT</sup>) and in the control group the minimum response was 0.8 (2<sup>-ΔΔCT</sup>) and the maximum response was 23.43 and the test coefficient was 32. (Fig-3)



**Fig.3-**The curve for comparing the mean of expression of CDK2 gene expression in the gastric cancer before chemotherapy in the two groups studied. The curve for comparing the mean CDK2 gene expression in the gastric cancer after chemotherapy in the two groups studied

## Discussion

Several methods for controlling and eliminating cancer cells are under consideration, but among these methods, the use of dietary factors, such as omega-fatty acids, is more likely to be seen due to their destructive effects on cancer cells. Stephenson. (24) Referred to basic mechanisms that show the anti-tumour effects of n-3 PUFA on cancer cells. They stated that:

1) Although the regulation of the epidermal growth factor receptor (EGFR), protein kinase (PKC), ras and NF- $\kappa$ B and growth factor of insulin (IGF), which are the most important cellular signalling agents that increase in cancer cells, are N -3 PUFA inhibits the growth signal transmission.

2) PUFA n-3 induces apoptosis in cancer cells by modulating the proliferative activating receptors (PPAR), the Bcl-2 family, and signalling the NF- $\kappa$ B cell.

3) n-3 PUFA reduces angiogenesis by suppressing the growth of blood vessels (VEGF) and suppressing the growth factor (PDGF), as well as reducing the stimulation of endothelial cells, reducing migration, increasing the inhibition of MMPs by producing nitric oxide, and Inhibition of signaling of NF- $\kappa$ B and  $\beta$ -catenin plays a role in cancer cells.

4) N-3 PUFA also reduces cell adhesion by regulating Rho-GTPase, which prevents stem cell reorganization and decreases intracellular adhesion (ICAM) 1 and reduces the expression of cellular adhesion factors (VCAM) 1.

Also, Cockbain (25) proposed four major anti-tumor actions for the n-3 PUFA:

1) COX-modulating , 2) change in membrane dynamics and cell surface receptor function, 3) elevated oxidative stress and, 4) derived anti-inflammatory lipid mediators.

1) N-3 PUFA can be replacing the COX-2 substrate instead of arachidonic acid leads to a decrease in the formation of PGE<sub>2</sub> in several cells, as well as the COX-2 substrate channel and inhibits COX-2 activity. 2) Adding N- 3 PUFA to cell membrane changes fluidity, structure, or function of lipid boats, and in particular, cell surface receptors, such as G protein (GPCRs)

receptors (TLRs) and Factor Receptor Receptors The epidermal growth (EGFR) in lipid raft, which seems to reduce the proliferation and apoptosis control signals to be effective. 3) N-3 PUFA (It may have an antinociceptive effect by changing the cellular reflux state. The n-3 PUFA can increase the reactive oxygen species (ROS) due to its oxidation, so P-3N-3 Can cause cancer cell apoptosis by increasing the level of intracellular ROS and 4) N-3 PUFA can produce new anti-inflammatory agents. The above studies, along with studies Lee (20), also showed that unsaturated fatty acids (PUFAs) were able to eliminate Tumor cells have cytotoxic effects.

Begin (26) and Horrobin (27,28) and Takeda (29) explain why cytotoxic effects of omega-acid acids express the susceptibility of cancerous cells to omega-3 fatty acids That healthy cells have more resistance to omega-3 fatty acids. The results of S.I. studies Grammatikos. (30) show that unsaturated fatty acids cannot be converted to end products of oxidation. Studies by Dunbar & Bailey et al. (31) and Cheeseman et al. (32) Iturralde. (33) Marra. (34) Maeda. (35) Robert and colleagues (36) showed Naval. (37) Omega fatty acid precursor (Doschores, 8, 7, 6, and 4  $\Delta$ ) has been heavily reduced in cancer cells. Horrobin. (27,28) showed that the cause of cytotoxic effects and the sensitivity of cancer cells to omega-3 fatty acids caused the lack of production of these fatty acids in cells due to the lack of desaturase enzymes, especially the absence of  $\delta 6$  desaturase Which cells cannot easily synthesize EFA-hepatitis, and this may be due to the sensitivity of cancer cells to EPA and DHA. . In research conducted by Dai. (38). Das. (18) found that the toxic effects of these fatty acids on cancer cells only affect cancer cells and have no effect on healthy cells. However, fatty acids can be used for doses higher than 40 micrograms Per ml to affect healthy body cells.

Observing the equilibrium in EFA use for healthy and natural growth is essential. If the diet contains omega-EPA (and DHA) fatty acids, part of these fatty acids will be converted to omega-6 fatty acids, especially AA, in the membranes of all Cells, especially in the membrane of platelets, erythrocytes, neutrophils,

monocytes and liver cells, but the combination of cell membranes is largely dependent on food intake and it is not possible to make them genetically for the body because the enzyme Due to the increase in the amount of omega-6 fatty acids in the diet, the eicosanoid metabolic products of AA, B Specific prostaglandins, thrombocytes, leukotrienes, hydroxyacetic fatty acids, and lipoxins are more likely to form omega-3 fatty acids (39). Studies by Ge and Kang (40) of the need for Supports Omega-6 / Omega-3 Balance. In these studies, it has been clearly shown that omega-3 fatty acid dischargers extracted from *C. Elegans* worms in both normal cells of cardiomyocytes and human breast cancer cells in the culture medium have the ability to convert all of the acid-Omega-6 fatty acids have omega-3 fatty acids. Omega 3 discharazes effectively and quickly converted omega-6 fatty acids, which convert omega-3 fatty acids, so that LA-omega-6 was converted to ALA-omega-3 and AA-EPAs, so that in equilibrium, the omega-6 ratio Omega-3 PUFA is close to 1: 1, and studies have shown that cells that destroy the omega-3 fatty acid production discourses are destroyed by apoptosis, while in cells with high fatty acid ratios Cellular omega-6 proliferation continues.

Studies in India show that a higher ratio of 18: 2 ω6 to 18: 3ω3 of 20-1 increases the incidence of non-insulin-dependent diabetes mellitus (NIDDM) in the population, while a diet with a ratio 1-1.6 leads to its reduction. James and Caldwell (41) reported beneficial effects in patients with rheumatoid arthritis, and Broughton. (42) have shown beneficial effects in asthma patients with dietary changes. These studies show that the use of omega-3 fatty acids along with the use of medications and dietary choices that increase omega-3 fatty acids and reduce omega-6 fatty acids can lead to On the other hand, Wai Wing So. (43), has shown that the expression of CDK2 and Cyclin E in LA-N-1 cells treated with DHA or EPA is affected, and this may suggest That the inhibitory effect of DHA and EPA growth on the cell wall may be due to inhibition of cell cycle stasis in the G0-G1 stage. The results of this study are also consistent with the results of the present study. Istfan NW. (44) showed that the diet A diet containing

omega-3 fatty acids prolongs the cell cycle and increases the doubling time of breast cancer cells in mice, which results in As the CDK-2 gene declines, the formation of the Cyclin A2-CDK-2 complex is postponed and the transition from phase S to the G2 phase becomes longer. In addition, studies by Albino AP (45) showed that DHA cells It stops the malignant in step S and prevents the progression of the S-cell cycle in human HT-29 cell colon cells. These observations have shown that DHA can apply its anticancer effects by preventing cell cycle progression. These results It is also consistent with the results of this study because, as previously mentioned, delaying the expression of the CDK-2 gene in the formation of the Cyclin A2-CDK2 complex is delayed and the transition from phase S to the G2 phase is prolonged. Carolyne-killed (46), using cellular MHCC97L cells of human hepatocarcinoma cells, showed that DHA can stop cell cycle by inhibiting DNA synthesis and agonizing apoptosis. They also showed that the duration of S phase significantly increased when omega-3 fatty acids were consumed. It was also observed in this study that the number of cells in the cell-cycle S cell cycle in cells treated with DHA was lower than the number of cells than control cells.

Concerned about the specific effects of DHA on inhibiting the progression of the G1-S phase in cancerous liver cells, researchers agree that DHA is a malignant cell in phase (S) of breast cancer cells (47) passing from the G0 to G1 phase of cancer cells It inhibits the FM3A class and also prevents the progression of the G1-S phase in human HT-29 colon cells (48) and also inhibits the human cellular Jurkat cell leukaemia cell cycle (49). which is consistent with the results obtained in this study. Akihisa Suzuki. (48) found that chemotherapy-resistant endometrial cancer endocrine tissue with higher cyclin A2 levels than other types of tissue has been shown to increase the expression of endogenous cyclin A2 protein in cell resistance against cisplatin in carcinoma cells Endometrium, and the expression of cyclin A2 in endometrial carcinoma of HHUA cells not only increases growth activity but also increases resistance to cisplatin in vitro and in vivo. These results clearly showed that expression of cyclin



A2 was resistant to cisplatin In endometrial cancer cells, it imposes the results of the research with the results Vashan there. The level of expression of Cyclin A2 gene in the control group given cisplatin alone is still high, while the rate of expression of the Cyclin A2 gene in the case group, which is saturated with the cisplatin of omega fatty acid, has been reduced. Has found.

## Conclusion

According to the results of the present study, it seems that the use of PUFAs with Cisplatin medicine in the patients with stomach adenocarcinoma can contribute to stopping S phase of the cell cycle in stomach cancerous cells. It can be one promising finding for controlling the cancerous cells in stomach adenocarcinoma.

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