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# Dietary polyunsaturated fatty acids affect PPARγ promoter methylation status and regulate the PPARγ/COX2 pathway in some colorectal cancer cell lines

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## Abstract

**Background** Promoter methylation silencing of peroxisome proliferator-activated receptor gamma (PPARγ) and dysregulation of the PPARγ/COX2 axis contribute to colorectal cancer (CRC) pathogenesis. This study investigated for the first time the effects of dietary polyunsaturated fatty acids (PUFAs) on promoter methylation of PPARγ and the PPARγ/COX2 axis in five CRC cell lines.

**Methods** Five CRC cell lines (SW742, HCT116, Caco2, LS180, and HT29/219) were treated with 100 µM of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) or linoleic acid (LA). The methylation patterns of the four regions within the PPARγ promoter were determined using methylation-specific PCR (MSP). Additionally, the mRNA expression levels of PPARγ and COX2 were examined using RT-qPCR.

**Results** Our results showed that M3 segment within the PPARγ promoter was hemimethylated in SW742 cells, whereas other cell lines remained unmethylated in this region. The M4 region was hemimethylated in all the CRC cell lines. Of all PUFAs, DHA demethylated the M3 region of the PPARγ promoter in SW742 cells and the M4 region in Caco2 cells. Functionally, these changes were accompanied by significant upregulation of PPARγ in SW742 (9.22-fold; $p=0.01$) and Caco2 cells (8.87-fold; $p=0.04$). Additionally, COX2 expression was significantly downregulated in all CRC cell lines after exposure to PUFAs ($p<0.05$).

**Conclusions** This study demonstrated that PUFAs, particularly DHA, altered PPARγ promoter methylation and expression, as well as modulated the PPARγ/COX2 axis in CRC cells in a cell type-dependent manner. DHA was more effective than the other PUFAs in regulating PPARγ promoter methylation. Our results highlight the potential clinical use of PUFAs in CRC treatment.

**Keywords** Colorectal cancer, PPARγ, Promoter methylation, PUFAs, COX2

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## Introduction

Colorectal cancer (CRC) ranks third among the most common cancers and continues to be a major cause of cancer-related mortality worldwide, with the second highest global mortality and incidence rates [1]. Despite advances in diagnostic and therapeutic approaches, patients experience a poor prognosis, which is reflected by a low 5-year survival rate [2]. CRC tumors exhibit a high level of heterogeneity, which presents a challenge in the selection of appropriate diagnostic and therapeutic options [3]. Therefore, it is necessary to gain more insights into the molecular pathways underlying CRC development, and the search for novel antitumor agents is essential.

CRC tumorigenesis is triggered by gradual accumulation of both genetic and epigenetic changes in the genome [4]. Among these changes, aberrant DNA methylation plays a critical role in the early stages of CRC [5]. Changes in both global and regional promoter methylation patterns have been frequently reported in CRC, leading to chromosomal instability, silencing of tumor suppressor genes, and activation of oncogenes [5, 6]. Peroxisome proliferator-activated receptor gamma (PPARγ) is one the genes have been reported to be dysregulated in CRC by epigenetic modifications [7]. This gene encodes a ligand-dependent transcription factor that is involved in cell differentiation, adipogenesis, vascular homeostasis, insulin sensitivity, lipid and glucose metabolism, and inflammation [7, 8]. Numerous in vivo and in vitro studies have highlighted the tumor-suppressive activities of PPARγ in various human cancers including CRC [9–12]. For instance, PPARγ has been shown to inhibit inflammation-induced CRC carcinogenesis by inhibiting nuclear factor kappa B (NF-κB), resulting in the reduction of inflammatory mediators like COX2 and TNF-α [9]. Previous studies have shown that the downregulation of COX2 by PPARγ activation considerably inhibits CRC growth and metastasis [13, 14]. PPARγ contains CpG islands within its promoter region, which is known to be a target for DNA methylation [10, 15]. It has been demonstrated that promoter DNA hypermethylation results in PPARγ downregulation in CRC [10, 11]. Likewise, much evidence is available regarding promoter DNA methylation silencing of PPARγ in various diseases, such as diabetes, glioblastoma multiform, and lung fibrosis [16–18]. Therefore, regulation of PPARγ expression by targeting promoter DNA methylation may be an effective strategy against CRC.

Dietary factors are widely believed to contribute to tumorigenesis and progression of CRC [19, 20]. In 2019, dietary risk-associated deaths accounted for 32% of all CRC cases, emphasizing the significance of improving dietary habits to decrease CRC risk [20]. Over the years, polyunsaturated fatty acids (PUFAs) have garnered great interest because of their beneficial effects on cancer and cardiovascular and metabolic diseases. The use of marine oil-derived ω-3 PUFAs, including eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), has been shown to exhibit preventive and therapeutic effects against CRC [21]. The precise mechanisms that underlie the anticancer effects of ω-3 PUFAs have not been fully elucidated. Previous researches have demonstrated that ω-3 PUFAs can promote programmed cell death, known as apoptosis, and inhibit inflammation, angiogenesis, and cell proliferation in CRC [22]. Furthermore, recent studies have suggested that ω-3 PUFAs can modulate altered global and gene-specific methylation patterns in CRC [23, 24]. However, the effects of PUFAs on PPARγ promoter methylation patterns and the PPARγ/COX2 axis as contributing factors in CRC carcinogenesis remain to be elucidated. In this study, we for the first time evaluated the effects of DHA, EPA, and linoleic acid (LA) on the DNA methylation status of four regions within the PPARγ promoter in five CRC cell lines. Next, we determined PPARγ expression levels in these cell lines after exposure to PUFAs and analyzed the association between PPARγ gene expression and its promoter methylation status. Finally, we determined COX2 expression in CRC cell lines exposed to PUFAs.

## Materials and methods

### Chemical and reagents

The analytical reagents and chemicals used in this study were obtained from Sigma Aldrich (Gillingham, United Kingdom) and Gibco-Invitrogen (Paisley, United Kingdom).

### Supplementing with PUFAs

In this study, bovine serum albumin (BSA) was used as PUFAs carrier [25]. To treat CRC cell lines with PUFAs, we used a conjugate containing BSA and PUFA, according to the protocol described by Svedberg et al. [25]. In summary, a stock solution was obtained by diluting each PUFA (ω-3 DHA, ω-3 EPA, and ω-6 LA) in ethanol (50%, v/v) and kept in the dark at − 20 °C until use. Before beginning the experiments, PUFAs were freshly made from the prepared stock solution by dissolving them in cell culture media consisting of fatty acid-free BSA (10 μM) in a 10:1 proportion to act as a carrier. Finally, to conjugate the PUFAs to BSA, the prepared mixture containing 0.1% (v/v) ethanol was incubated at 37 °C with shaking (2 h).

### Cell culture

This study included five CRC cell lines purchased from the National Cell Bank of Iran (NCBI; Pasteur Institute, Tehran, Iran). The SW742, HCT116, and HT29/219 cell lines were incubated in RPMI 1640, whereas LS180 and

Caco2 cells were cultivated in DMEM containing fetal bovine serum (10%), glutamine (2 mM), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a moisturised incubator with 5% $CO_2$. To conduct the experiments, $3.0 \times 10^4$ cells were dispensed into each well of six-well plates and incubated for 24 h to enable attachment. Subsequently, cells were exposed to 100 µM BSA-PUFAs for 24 h. Media containing only BSA were used as the reference group. Cell viability was determined using the trypan blue excluding test.

## Genomic DNA isolation and PPARγ promoter methylation analysis

Genomic DNA was extracted from treated and untreated cells using a well-established proteinase K digestion procedure, followed by phenol-chloroform isolation, as previously reported [23].

Methylation-specific PCR (MSP) was used to assess the methylation status of four regions (M1-M4) of the PPARγ promoter. In summary, genomic DNA extracted from treated and untreated cells was subjected to bisulfite treatment by incubation with sodium bisulfite (2 M) and hydroquinone (0.1 M) for 16 h at 55 °C. Subsequently, it was subjected to MSP using eight sets of primers targeting either the methylated (M) or unmethylated (U) regions of the PPARγ gene promoter. The Methyl Primer Express software version 1, provided by Applied Biosystems, was employed to design the primers for MSP (Table 1). CpG island was identified using Methyl Primer Express software version 1 with a PPARγ sequence obtained from the Gene database (NC_000003.12; Fig. 1). To validate the MSP reactions, unmethylated control DNA from normal human leukocytes and the Universal Methylated DNA standard methylated from Zymo Research Company (Freiburg, Germany) were utilized as negative and positive controls, correspondingly.

## Reverse transcription-quantitative real-time PCR (RT-qPCR)

TriPure isolation reagent (Roche Applied Science, Germany) was used to isolate total RNA from the cancer cell lines. Isolated RNA samples were treated with DNase I to eliminate DNA contamination (Yekta Tajhiz Azma, Iran). To verify the structural integrity of the extracted RNA, electrophoresis analysis was performed on an agarose gel (1.5%) containing formaldehyde (2%), after which the isolated RNA was maintained at − 80 °C. The purity and concentration of the isolated RNA were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) before to the synthesis of complementary DNA (cDNA) using M-MLVRT (MBI, Fermentas, Lithuania). Relative expression levels of PPARγ and COX2 were determined by RT-qPCR using BioFACT™ 2× real-time PCR Master Mix (Biofact, Korea). Reactions were replicated three times using a magnetic induction cycler (MIC) PCR instrument (Bio Molecular Systems, Brisbane, Queensland, Australia). A list of primers for the target genes and the housekeeping gene (GAPDH) is provided in Table 2. Primer sequences were obtained from previous studies (Table 2).

### Statistical analysis

Results were expressed as average values ± standard deviation (SD) and analyzed using GraphPad Prism (version 9.0.2, GraphPad Software Inc., USA), employing one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Statistical significance was assigned to differences with a p-value less than 0.05.

**Table 1** Sequence, annealing temperature, and product size of primers used for methylation-specific PCR (MSP) reactions

| Region | Primer | Sequence | Position | Annealing T (°C) | Product size (bp) |
|---|---|---|---|---|---|
| | PPARγ M1F | 5′-GTGGGTTTTATTGTGCGC-3′ | -123 to + 49 | 58 | 171 |
| | PPARγ M1R | 5′-AACCGAATCGAACCGAAC-3′ | | | |
| M1 | PPARγ U1F | 5′-GTGGGTTTTATTGTGTGT-3′ | -123 to + 49 | 50 | 171 |
| | PPARγ U1R | 5′-AACCAAATCAAACCAAAC-3′ | | | |
| | PPARγ M2F | 5′-CGGGGGTATTTTTTTAAATTTC-3′ | -151 to -270 | 54 | 119 |
| | PPARγ M2R | 5′-ACTCTCTACCCCGCGACA-3′ | | | |
| M2 | PPARγ U2F | 5′-GTTGGGGGTATTTTTTTAAATTTT-3′ | -151 to -272 | 54 | 121 |
| | PPARγ U2R | 5′-CCACTCTCTACCCCACAACA-3′ | | | |
| | PPARγM3F | 5′-AAGACGGTTTGGTCGATC-3′ | -235 to -359 | 52 | 124 |
| | PPARγ M3R | 5′-CGAAAAAAAATCCGAAATTTAA-3′ | | | |
| M3 | PPARγ U3F | 5′-GGGAAGATGGTTTGGTTGATT-3′ | -235 to -362 | 55 | 127 |
| | PPARγ U3R | 5′-TTCCAAAAAAAAATCCAAAATTTAA-3′ | | | |
| | PPARγ M4F | 5′-GAGATTAGCGGTTTTTTGAAC-3′ | -616 to -746 | 52 | 130 |
| M4 | PPARγ M4R | 5′-AAACGTAAAACACGAAAAACA-3′ | | | |
| | PPARγ U4F | 5′-TAGGAGATTAGTGGTTTTTTGAAT-3′ | -615 to -749 | 52 | 134 |
| | PPARγ U4R | 5′-AAAAACATAAAACACAAAAAACAA-3′ | | | |

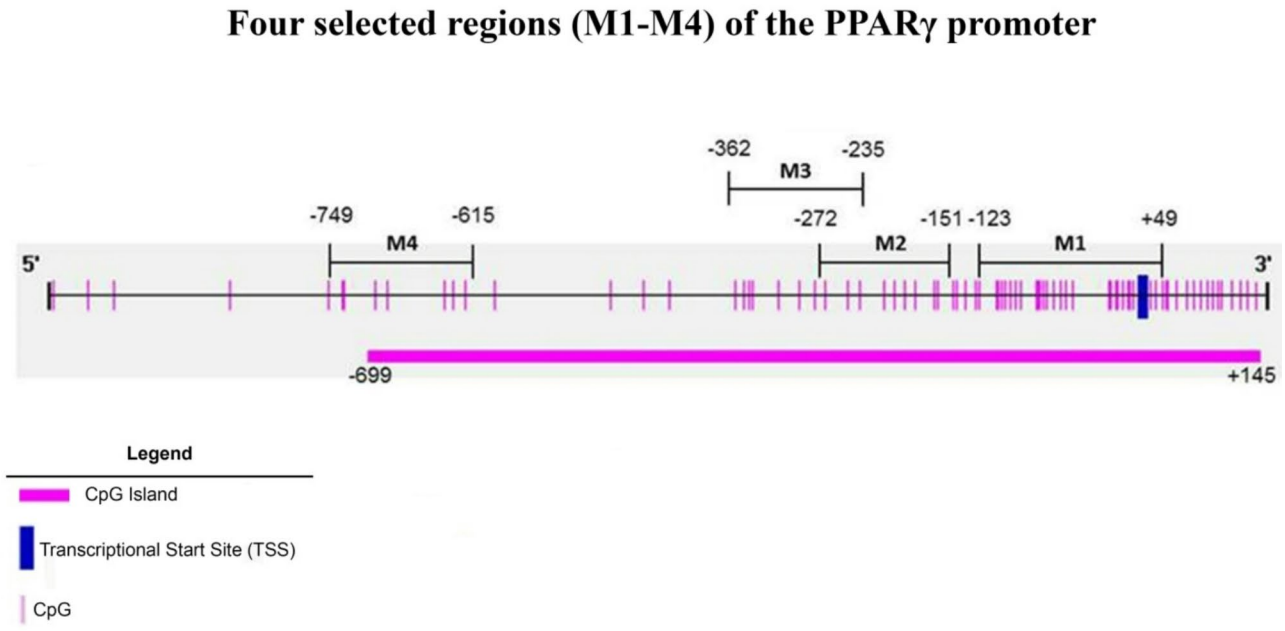


**Fig. 1** Schematic illustration of the PPARγ promoter and four selected regions in its promoter (M1-M4). Methyl Primer Express Software v1.0. was used to identify the CpG site, CpG island, and transcription start site. The PPARγ gene sequence was obtained from the Gene database (NC_000003.12)

**Table 2** Sequence and product size of primers used for RT-qPCR

| Gene | Primer position | Primer (5′ → 3′) | Product length (bp) | Ref. |
|---|---|---|---|---|
| GAPDH | Forward | GGTCGGAGTCAACGGATTTGG | 194 | [54] |
| | Reverse | TGATGACAAGCTTCCCGTTCT | | |
| COX2 | Forward | CCGGGTACAATCGCACTTAT | 103 | [55] |
| | Reverse | GGCGCTCAGCCATACAG | | |
| PPARγ | Forward | GTCACGGAACACGTGCAGC | 72 | |
| | Reverse | CAGGAGCGGGTGAAGACTCA | | [56] |

## Results

### Effects of PUFAs on the methylation profile of PPARγ promoter in CRC cell lines

Examination of the PPARγ promoter identified a CpG island spanning from − 699 to + 145, which could potentially serve as a site for DNA methylation. Another potential site for DNA methylation is a CpG-rich segment from − 793 to − 580, which undergoes persistent methylation and is associated with the early stages of metabolic syndrome [26]. In this study, we determined the methylation patterns of these regions in CRC cell lines and investigated whether treatment with PUFAs affects their methylation profiles. MSP was performed on four segments in the PPARγ promoter spanning from + 49 to − 123 (M1), − 151 to − 272 (M2), − 235 to − 362 (M3), and − 615 to − 749 (M4). Our results showed that five CRC cell lines exposed to BSA exhibited an unmethylated state in the M1 and M2 segments (Fig. 2A and B). For the M3 segment, the SW742 cell line exposed to BSA showed a hemimethylated state; however, the other cell lines remained unmethylated in this region (Fig. 2A). Across all BSA-treated cell lines analyzed, the M4 site was hemimethylated (Fig. 2A).

Treatment of CRC cell lines with EPA affected the methylation state of the M1 site only in HT29/219 cells compared to untreated cells, changing the methylation state from unmethylated to semi-methylated (Fig. 2A and B). However, in other CRC cell lines, there were no changes in the methylation state of the M1 segment after exposure to PUFAs. Interestingly, exposure to DHA altered the methylation state of the M3 segment only in SW742 cells compared to that in BSA-treated SW742 cells, shifting semi-methylation to unmethylation (Fig. 2A and B). Conversely, PUFAs treatment did not alter the methylation status of the M2 and M3 regions in other CRC cell lines. Intriguingly, DHA-treated Caco2 cells showed a changed methylation state for the M4 site compared to untreated cells, that is, it was changed to an unmethylated state (Fig. 2A and B). However, no changes were detected in the M4 methylation phenotype following PUFAs exposure in other CRC cell lines. It was observed that LA did not have any effect on any cell line. Overall, these findings suggest that DHA is more effective than other PUFAs in reducing promoter methylation, as manifested by the transformation of the methylation states of the M3 segment in SW742 cells and of the M4 region in Caco2 cells into an unmethylated phenotype.

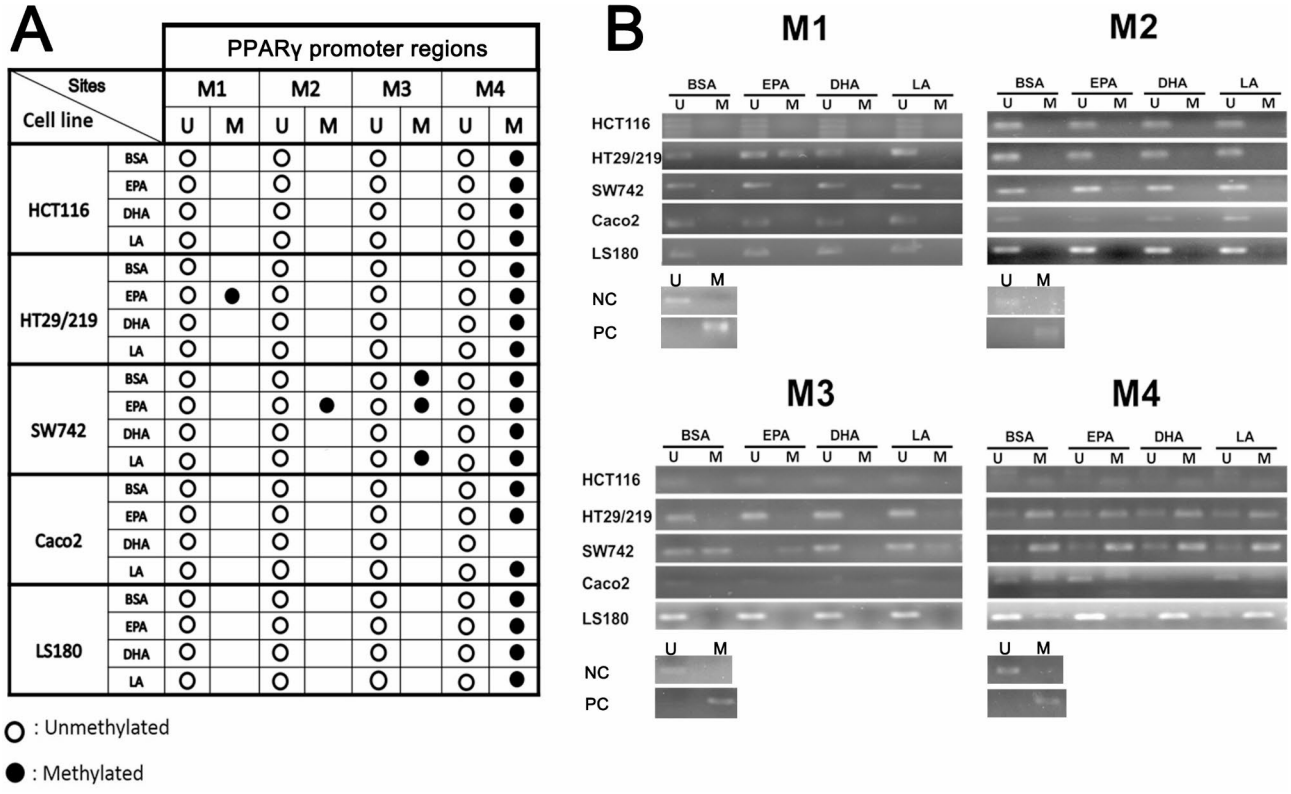


**Fig. 2** Analysis of methylation patterns in four regions of the PPARγ promoter (M1-M4) in various colorectal cancer (CRC) cell lines treated with BSA alone as a control, and in those treated with polyunsaturated fatty acids (PUFAs). (**A**) DNA methylation profiles of four regions within the PPARγ promoter were identified using the MSP method. Cell lines showing both methylated (●) and unmethylated (○) variants are referred to as hemimethylated, in which methylation occurs in only one of two strands of DNA within the promoter region. (**B**) Representation of MSP products from various regions of the PPARγ promoter in CRC cell lines using agarose gel electrophoresis. C; control, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid, LA; linoleic acid

### Effects of PUFAs on PPARγ gene expression levels in CRC cell lines treated with PUFAs

To evaluate the effects of PUFAs on PPARγ gene expression, we determined PPARγ expression in five CRC cell lines treated with PUFAs using RT-qPCR (Fig. 3). Our findings demonstrated that treatment with LA resulted in a significant increase (15.08-fold) in PPARγ expression in HCT116 cells ($p = 0.003$; Fig. 3A). However, no significant changes in PPARγ expression were detected with other PUFAs in HCT116 cells ($p > 0.05$; Fig. 3A). Similarly, following PUFAs exposure, there were no significant alterations in PPARγ expression in HT-29/219 cells ($p > 0.05$; Fig. 3A). Interestingly, DHA-treated SW742 and Caco2 cells showed significantly higher PPARγ expression (9.22-fold and 8.87-fold, respectively) than BSA-treated cells ($p = 0.01$ and 0.04, respectively; Fig. 3A). In LS180 cell line treated with various PUFAs, only LA significantly promoted PPARγ expression (2.34-fold) compared with BSA-treated cells ($p = 0.01$; Fig. 3A).

### Effects of promoter methylation pattern on PPARγ gene expression levels in CRC cell lines treated with PUFAs

According to our findings, DHA-treated SW742 cells exhibited considerable upregulation of PPARγ expression (9.22-fold; $p = 0.01$; Fig. 3A), which was accompanied by a shift in the methylation pattern of the M3 region from semi-methylated to unmethylated phenotype (Fig. 3A and B). Furthermore, the M4 region in DAH-exposed Caco2 cells with increased PPARγ expression (8.87-fold; $p = 0.04$; Fig. 3A) displayed a modified methylation pattern, changing from a semi-methylated to an unmethylated phenotype (Fig. 3A and B). Conversely, LA treatment enhanced PPARγ expression in HCT116 and LS180 cells without affecting the promoter methylation profile (Fig. 3A and B). Among PUFAs, only DHA increased PPARγ expression by affecting promoter methylation in SW742 and Caco2 cells, suggesting that PPARγ promoter methylation pattern may control its production in these cells.

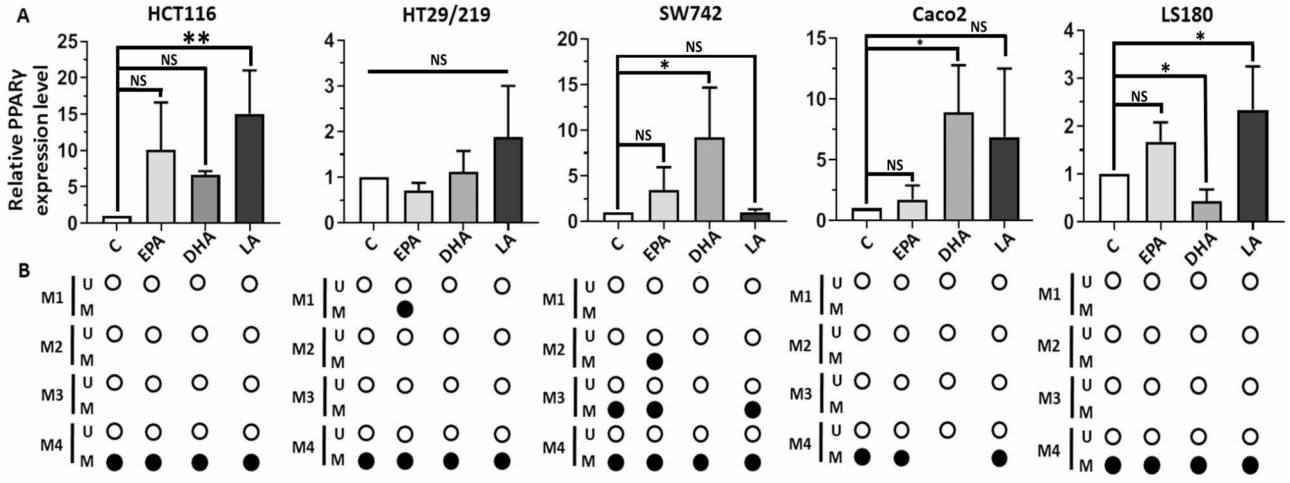


**Fig. 3** Effects of polyunsaturated fatty acids (PUFAs) on PPARγ expression in various colorectal cancer (CRC) cell lines. (**A**) PPARγ gene expression levels in CRC cells treated with BSA alone as a control, and in those treated with PUFAs. (**B**) Methylation status of four regions within the PPARγ promoter in BSA- and PUFAs-treated CRC cell lines. Cell lines showing both methylated (●) and unmethylated (○) variants are referred to as hemimethylated, in which methylation occurs in only one of two strands of DNA within the promoter region. Bars represent average values ± standard deviation (SD). *$p < 0.05$ and **$p < 0.01$. C, control; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; NS, not significant

### Effects of PUFAs on COX2 gene expression in CRC cell lines treated with PUFAs

In this study, we determined the expression levels of COX2 in five CRC cell lines after exposure to PUFAs using RT-qPCR (Fig. 4). Incubation of CRC cell lines, including HT29/219, SW742, Caco2, and LS180, with EPA, DHA, and LA significantly downregulated COX2 expression after 24 h ($p < 0.05$; Fig. 4).

## Discussion

Accumulated evidence from various studies points to DNA methylation silencing of PPARγ in the pathogenesis of various diseases, including CRC [7, 10, 15]. Strong evidence from experimental and clinical data support the protective effects of marine oils against CRC and other cancers by altering the gene-specific promoter DNA methylation phenotype [21, 27–29]. In this study, we exposed five CRC cell lines to three types of PUFAs and determined their effects on PPARγ promoter methylation patterns and expression as well as on the PPARγ/COX2 axis. Our results showed that exposure to PUFAs, especially DHA, affected PPARγ promoter methylation and regulated the PPARγ/COX2 axis in CRC cells.

Changes in DNA methylation are observed early during CRC development, potentially influencing both disease onset and progression [30]. CRC is characterized by considerable promoter hypermethylation of genes involved in key pathways in cancer biology [31]. Such hypermethylation often targets tumor suppressor genes, silencing them and thereby promoting tumorigenesis [11, 31]. PPARγ has been implicated in cancerous and noncancerous diseases, and several studies have shown a strong correlation between abnormal promoter hypermethylation and decreased PPARγ expression [11, 15, 16, 32]. For instance, Motawi et al. [10] reported PPARγ promoter hypermethylation in peripheral mononuclear cells and its silencing in CRC patients. Similarly, we found methylation of the PPARγ promoter in all CRC cell lines studied. Several in vitro and in vivo studies have suggested that PUFAs influence global and gene-specific DNA methylation patterns [28, 33]. However, no study has thoroughly examined their effects on the methylation status of various regions of the PPARγ promoter in colon cancer cells.

The results showed that DHA was more effective in reducing PPARγ promoter methylation than other PUFAs, as it changed the methylation status of the M3 segment of the PPARγ promoter to an unmethylated state in SW742 cells and shifted the M4 region to an unmethylated state in Caco2 cells. Interestingly, DHA-mediated changes in promoter methylation were accompanied by a significant increase in PPARγ expression. The mechanisms by which PUFAs alter DNA methylation have not been fully elucidated in this study. We speculated that the role of PUFAs in reducing PPARγ promoter methylation might be mediated by UHRF1 and DNMTs inhibition. UHRF1 has been recognized as a mediator of PPARγ silencing in CRC via promoter hypermethylation by recruiting MeCP2, EZH2, and DNMT3b [7, 11]. Previous studies have shown that PUFAs reduce DNMTs expression [23]. Additionally, PUFAs diminish DNMTs activity by removing the methyl groups of S-adenosyl methionine (SAM) and combining them with phosphatidylethanolamine [34].

We observed that various CRC cell lines exhibited different responses to PUFAs. Among the promoter

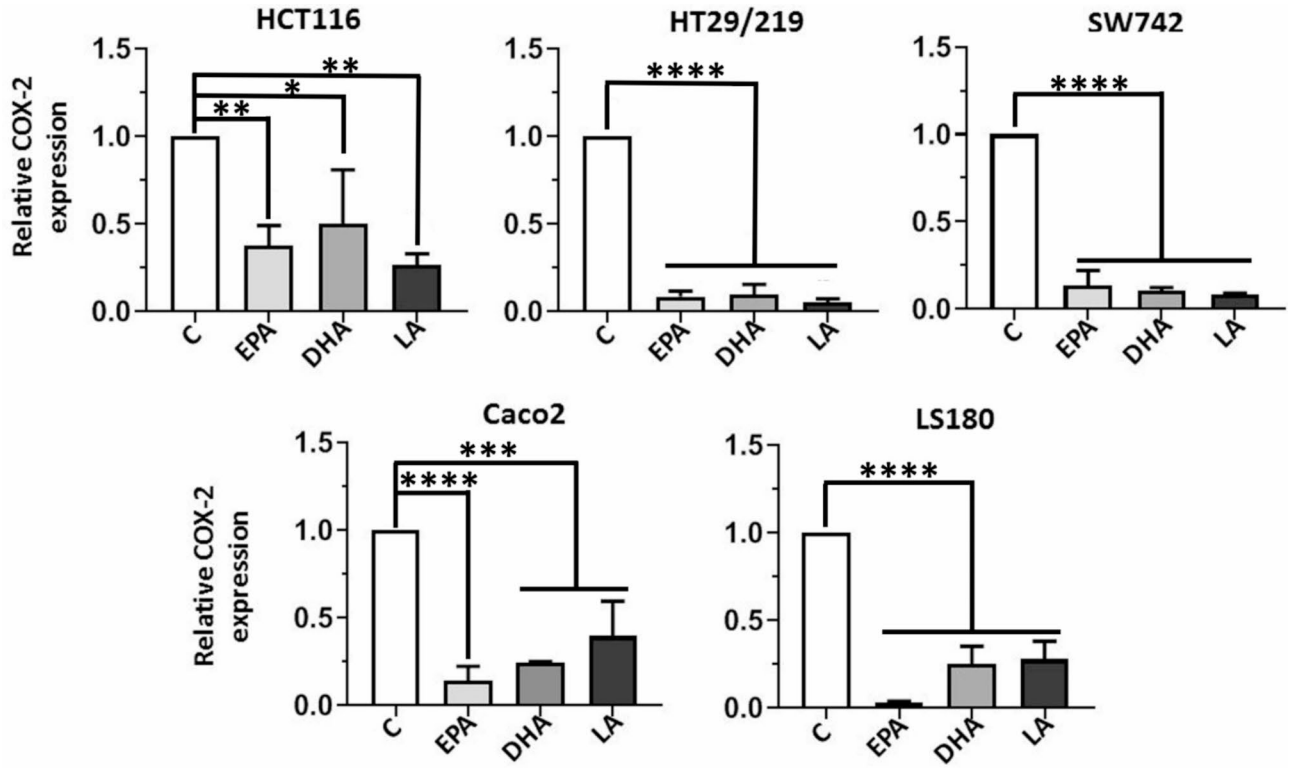


**Fig. 4** Effects of polyunsaturated fatty acids (PUFAs) on PPARγ expression in various colorectal cancer (CRC) cell lines. Bars represent average values ± standard deviation (SD).*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. C, control; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; NS, not significant

segments studied in different cell lines, DHA demethylated M3 segment in SW742 cells and the M4 segment in Caco2 cells. In contrast, the M2 region of the PPARγ promoter was semi-methylated by EPA in Caco2 cells. PUFAs appear to selectively influence methylation of the PPARγ promoter in various cell lines. However, this study did not reveal the mechanisms underlying the cell-specific effects of PUFAs on PPARγ promoter methylation. Similar results have been previously observed in CRC cells exposed to various demethylating compounds [35, 36]. A possible factor that might contribute to the different epigenetic responses to PUFAs is variation in the DNA sequence, as previously demonstrated that epigenetic responses to dietary fat may be affected by DNA sequence differences [37]. Interestingly, the CRC cell lines examined in current study show both genetic and phenotypic heterogeneity [38]. Caco2, SW742, and HT29/219 cell lines are characterized by microsatellite stability (MSS), whereas HCT116 and LS180 cell lines are defined by microsatellite instability (MSI) [38, 39]. The CRC cell lines used in this study also exhibit epigenetic variations. ARID1A is a tumor suppressor gene involved in chromatin remodeling. Promoter hypermethylation of ARID1A contributes to its silencing in SW742 cells; however, promoter hypomethylation results in high ARID1A expression in HT29/219 and HCT116 cells [40]. Despite Caco2 cells originate from human CRC, they can undergo differentiation and exhibit many transporters and enzymes analogous to those found in normal enterocytes in the human intestinal epithelium [41]. PUFAs have been shown to alter DNA methylation in a dose-dependent manner [42]. Investigating the dose-dependent effects of PUFAs may help to explain the variation in the responses of CRC cell lines to PUFAs. We used 100 μM EPA and DHA, which fell within the normal plasma range, and this concentration was below the normal level for LA [43].

Our study highlights the complex interplay between DNA methylation and PPARγ expression, which varies across cell lines and PUFAs treatments. Similar to previous studies [40, 44, 45], we found that DNA methylation did not have a straightforward inhibitory effect on gene expression. For instance, in HCT116 and HT29/219 cells, PPARγ expression levels increased considerably after LA exposure without altering promoter methylation, suggesting that other regulatory mechanisms such as histone modification, transcription factor binding, or non-coding RNAs may play a role in PPARγ upregulation, as previously reported [10, 46, 47]. Despite having the same promoter methylation status in PUFA-treated HCT116 and HT29/219 cells, these cells exhibited different PPARγ expression levels. Cell-specific availability or activity of

transcription factors may lead to different gene expression outcomes despite identical methylation patterns [48]. In contrast, DHA treatment of SW742 and Caco2 cells led to significant PPARγ upregulation, accompanied by methylation changes in specific promoter regions (M3 in SW742 cells and M4 in Caco2 cells), suggesting that the effects of DHA on PPARγ expression are mediated through epigenetic modifications. The differences in the promoter regions involved may reflect cell type-specific regulatory mechanisms affecting transcription binding and chromatin accessibility. However, in HT29/129 cells, although changes in methylation were observed, no corresponding decrease in PPARγ expression was observed. This indicates that changes in methylation alone may not always be sufficient for detectable gene inactivation [49], highlighting the importance of additional regulatory factors in gene expression control. Overall, our findings underscore the cell-specific nature of PPARγ regulation and suggest that PUFAs, particularly DHA, can modulate PPARγ expression through promoter methylation or other regulatory mechanisms.

Dysregulation of PPARγ and COX2 signaling contributes to CRC pathogenesis; lower PPARγ levels correlate with increased COX2/PGE2 activity, accelerating cancer progression, and presenting a promising target for prevention and treatment [50–52]. To evaluate the potential of PUFAs in regulating PPARγ and COX2 expression, CRC cells were exposed to PUFAs. COX2 expression was significantly downregulated in all studied cell lines after exposure to PUFAs, whereas PPARγ expression was significantly increased. Our results are consistent with those of previous studies showing that PPARγ upregulation inhibits CRC proliferation by suppressing COX2 [14, 51]. Although we did not clarify how PUFAs-induced PPARγ upregulation inhibited COX2 expression in CRC cells, several mechanisms have been reported in previous studies. PPARγ interacts with the nuclear retinoid X receptor (RXR) to form a PPARγ-RXR heterodimer. Binding of the PPAR-RXR heterodimer to the peroxisome proliferator responsive element (PPRE) sequence within the COX2 promoter is likely responsible for COX2 suppression [51]. Additionally, PPARγ can inhibit NF-κB, thereby suppressing its downstream genes, such as COX2 [9, 52]. In fact, PPARγ attaches to the p65 subunit and exports it from the nucleus to the cytoplasm, blocking NF-κB transcriptional regulation [9]. Moreover, PPARγ activation inhibits IκB degradation and prevents translocation of NF-κB from the cytoplasm to the nucleus [9].

The precise mechanisms by which epigenetic modifications silence PPARγ have been previously reported in CRC [7, 10, 11]; however, the roles of natural compounds, such as PUFAs, with more physiological relevance in reversing these epigenetic modifications, remain unclear. Several attempts have been made to activate PPARγ using synthetic agonists in CRC without considering epigenetic modifications [13, 14, 53]. Our study provides new insights into the epigenetic regulation of PPARγ in CRC by demonstrating that PUFAs alter PPARγ promoter methylation in some CRC cell lines, thereby increasing PPARγ expression. Therefore, the present study may expand the current understanding of the effects of PUFAs against CRC, suggesting new dietary interventions for CRC treatment and providing a basis for future research on diet-gene interactions in cancer therapy. The present study has some limitations. We evaluated the epigenetic effects of PUFAs on CRC in vitro; however, further in vivo investigations are required to confirm these findings. Furthermore, the MSP method used in this study did not provide quantitative information on promoter methylation. Determining the methylation patterns of different segments within PPARγ promoters in PUFAs-treated CRC cells using quantitative methods with high efficiency is highly recommended in future studies.

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**Author contributions**
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by E.B., P.K., and M.M.S. The first draft of the manuscript was written by E.B. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability**
No datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate**
This study was conducted according to the ethical protocols of the Lorestan University of Medical Sciences, Lorestan, Iran (ethics code: IR.LUMS.REC.1397.147).

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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