



Linoleic Acid Modulates the Expression of Metastatic and Angiogenic Markers MMP-2 and Talin-2 in Gastric Cancer Cell Line MKN-45

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Abstract

Background: Linoleic acid (LA) has modulatory effects on gastric cancer cell lines. This study aimed to investigate the effects of linoleic acid on the expression of metastatic and angiogenic molecular markers in gastric cancer cell line MKN-45.

Methods: In this study performed in Tabriz, Iran in 2021, MKN-45 cells were treated with LA in the presence or absence of docetaxel. Total RNA was extracted, and cDNA synthesized from the cells before and after treatment. The expression levels of Talin-2 and MMP-2 genes and mir-20, mir-30, mir-126, and mir-194, were determined by quantitative real-time PCR.

Results: LA treatment reduced the expression levels of mir-126, mir-194, mir-30, and MMP-2, while increased the expression levels of Talin-2 mRNA. Docetaxel treatment could decrease the expression levels of mir-20, Talin-2, and MMP-2 mRNA levels while increasing the expression levels of mir-126, mir-194, and mir-30. Additionally, the combined treatment of MKN-45 cells with LA and docetaxel could reduce the expression levels of mir-20 and mir-126 and increased the expression levels of mir-194, mir-30, Talin-2, and MMP-2 mRNAs.

Conclusion: Modulation of the expression levels of gastric cancer involved microRNAs, Talin-2, and MMP-2 may be a mechanism through which LA may exert its biological effects on GC cell line MKN-45. LA may have an antimetastatic effect by reducing the MMP-2 expression and pro-angiogenic effect through increasing Talin-2 expression levels.

Keywords: Gastric cancer; Linoleic acid; Talin-2



Introduction

Linoleic Acid (18:2 ν 6; cis, cis-9,12-octadecadienoic acid), is a polyunsaturated fatty acid (PUFA) that is highly consumed in the human diet (1). Conjugated linoleic acid (CLA) consists of a variety of positional and geometrical isomers of LA determined by conjugated cis/trans double bonds located mostly at sites 9 and 11 or 10 and 12 (2).

The association of CLA (either conjugated form or isolated isomers) has been investigated in the pathology of several cancers in human and animal models (3, 4). LA has been reported to inhibit the growth of normal gastric cells (GES1) and a variety of gastric cancer cell lines (5). Additionally, several mechanisms of action including lipid peroxidation have been suggested through which LA exerts inhibitory effects on these cell lines (5). The inhibitory effects of conjugated LA on SGC-7901 cells and the expression of several cyclins were reported by Liu and colleagues. They concluded that LA might block the physiologic process of the cells including growth and proliferation by decreasing the expression of cyclin A, B1, and D1 (6). The results revealed that c9, t11-CLA may inhibit metastasis in SGC-7901 cells (a human gastric cancer cell line) by hampering the cell-matrix component interaction, reducing the activity of metalloproteinase, and induction of the expression of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP2 mRNA (2, 7, 8).

MKN-45 cells line is a human poorly differentiated adenocarcinoma cell line that is widely used in gastric cancer research (9, 10). Talin-2 acts as an adaptor protein that regulates the integrin: integrin receptor interactions on both the cells and exosomes (11). Additionally, talin-2^{-/-} T-cell exosomes were less efficiently taken up by MAD-CAM-1 and ICAM-1 expressing endothelial cells (11-13).

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases (14, 15) of which MMP-2 and MMP-9 use collagen IV and V, laminin, and chondroitin sulfate proteoglycan as substrates through which mediate a variety of bio-

functions either physiologic (such as wound healing), (8, 16) or pathologic (such as inducing tumor vascularization and promoting tumor invasiveness) (14).

Assessment of the expression levels of microRNA has gained attention in diagnosis of cancers including GC and their role in suppression or development of GC has been investigated (17). In this regard, miR-27b interferes with the proliferation of tumor cells in vivo and in vitro therefore, acts as a tumor suppressor in GC (18). miR-126 was shown to suppress the proliferation and invasion of GC cells by targeting the 3'-UTR of insulin-like growth factor 1 receptor (IGF1R) and suppressing its expression (19). Additionally, the downregulation of miR-126 and Crk protein up-regulation has been reported to have a synergistic effect on the progression of GC (20).

Upregulation of miR-30 has been shown to enhance the proliferation and inhibits apoptosis and interestingly, the downregulation of miR-30 was found to increase P53 expression (21). MicroRNAs may contribute to the development or metastasis in GC but there is inconsistency in the literature. As an example, miR-194 has been reported to activate the Wnt/ β -catenin pathway that results in promotion of gastric carcinogenesis (22). However, miR-194 was already reported considerably down-regulated in GC tissues and cell lines and that overexpression of miR-194 inhibits GC cell proliferation, migration, and invasion in vitro (23).

We investigated the synergistic effects of docetaxel (not only efficient as monotherapy but promising when applied in triplet chemotherapy in the treatment of GC (24, 25)) and LA on the expression of the markers.

Materials and Methods

Cell culture and cell viability assay

For this in vitro study conducted at Tabriz University of Medical Sciences in 2021, MKN-45 cells were cultured in RPMI1640 supplemented

with 10% FBS, 2 mM glutamine, and penicillin/streptomycin (each 100 U/mL) at 37 °C and 5% CO₂. To determine the optimal doses of docetaxel, MTT assay was performed for determining IC₅₀ of docetaxel, as described elsewhere (26, 27).

LA treatment

LA was prepared as 30mM solution in ethanol, kept at -80 °C, and was added to wells when it was diluted with FBS (previously diluted in PBS). The final concentration of LA for each well was 50µM. We used another group of cells without LA treatment containing LA-free buffer as the control group. The cells in the test and control groups were harvested using trypsin-EDTA after 24h.

RNA Extraction and cDNA Synthesis

RNA was extracted from the cells using the TRIzol RNA extraction kit (RiboEX, GeneAll biotechnology- South Korea). The concentration of the extracted RNA was measured by NanoDrop™ spectrophotometer (Thermo Fisher-USA) and the quality of the extracted RNA was tested using 3% agarose gel electrophoresis. The samples were kept at -80 °C until analysis. cDNA synthesis was performed using a random hexamer primer (Bio fact- South Korea). The normalization step was done before cDNA synthesis and the RNA concentration was set at 1.1-5 µg/µl. To perform cDNA synthesis, we added 0.5µg of random hexamer and oligo dt, each to a microtube. We then added 10µl of RT premix and added distilled water to reach a final volume of 20µl for each sample. The samples were loaded into thermocycler for 5min at 60 °C followed by 40 min at 50 °C and 5 min at 95 °C. After finalizing the process, the synthesized cDNA samples were kept at -20 °C.

Quantitative real-time PCR (qRT-PCR)

We used qRT-PCR (Light cycler, Roche-Germany) to amplify cDNA samples. The primers were purchased from Sinaclone, Iran. We confirmed our results obtained from SYBR Green in qRT-PCR with melting curve analysis. Additionally, we performed electrophoresis to

check the length of the PCR product. To perform the qRT-PCR test, 0.5 µl cDNA, 0.5 µl primer, 5µl master mix 1x, and 4µl nuclease-free water (diethylpyrocarbonate (DEPC)) were added, mixed, and reached to a final volume of 10 µl. Time and temperature corresponding to each step of qRT-PCR were as the followings: hold step (10 min/95 °C), denaturation (10 sec/94 °C), annealing (40 sec/56 °C), and extension (20 sec/72 °C). GAPDH gene was used as the internal control (4). To assess the gene expression of miRs, the cDNAs were diluted 1:5 and mixed with 4 µl of the diluted specimen and added to 1 µl PCR primer mix, and 5 µl of PCR Master mix to reach a final volume of 10 µl. Samples were analyzed using LightCycler® 96 System, Roche-Switzerland. Time and temperature corresponding to each step of qRT-PCR were as the followings: polymerase activation /denaturation (10 min/95 °C), amplification (10 sec/ 95 °C), cycling (40 cycles) 60 sec/60 °C. U6 gene was used as the internal control.

Assessment of the Talin-2 and MMP-2 gene expression

To investigate the Talin-2 and MMP-2 gene expression in MKN-45 cells before and after LA and docetaxel exposure, the cells were cultured in RPMI1640 supplemented with 10% FBS, 2mM glutamine, and penicillin/streptomycin (each 100 U/mL) at 37 °C and 5% CO₂ and MTT assay was performed as described above. The RNA extraction and cDNA synthesis were performed and the expression of Talin-2 and MMP-2 mRNAs in the presence of beta-actin (internal control) was assessed. The following equation was used to calculate the quantitative expression of each mRNA:

$$\text{Fold difference} = 2^{-\Delta\Delta\text{Ct}}$$
$$\Delta\text{Ct sample} - \Delta\text{Ct calibrator} = \Delta\Delta\text{Ct}$$

Assessment of the mir-30, mir-126, and mir-194

Like the protocol described in section 2-5, the microRNA content was extracted from MKN-45 cells before and after exposure to LA and docetaxel using U6 as the internal control, and cDNAs were synthesized. To calculate the quantitative

expression of each microRNA the equation in section 2-5 was used.

Statistical analysis

The data regarding the effect of LA in the presence or absence of docetaxel on the expression of mir-30, mir-126, and mir-194 was analyzed using SPSS Ver. 21 (IBM Corp., Armonk, NY, USA). To analyze the qualitative variables, we used independent t-test and one-way ANOVA tests, and $P < 0.05$ was statistically significant.

Ethical approval

This study was approved by the ethics committee of Tabriz medical university (EC59528). No human sample was used in this study.

Results

Altered expression levels of microRNAs before and after treatment with LA and docetaxel

After treatment of MKN-45 cells with either LA or docetaxel alone or in combination, the expression of miR-20 was investigated. The expression of miR-20 decreased by 2.5-fold ($P < 0.0001$) in docetaxel-treated MKN-45 cells when compared to untreated MKN-45 cells. Treatment of MKN-45 cells with LA could increase the expression of mir-20 by 1.12-fold ($P = 0.02$) when compared to the control cells. The combined treatment of LA and docetaxel could reduce the expression of miR-20 by 5-fold ($P < 0.0001$) (Fig. 1a).

The expression of miR-126 increased by 1.92-fold ($P = 0.001$) in docetaxel-treated MKN-45 cells when compared to untreated MKN-45 cells. Moreover, the treatment of MKN-45 cells with LA could decrease the expression of mir-126 by 2.5-fold ($P < 0.0001$) when compared to the control cells. The combined treatment of LA and docetaxel could decrease the expression of miR-126 by 1.5-fold ($P < 0.0001$) (Fig. 1b).

Additionally, the expression of miR-194 increased by 2.43-fold ($P < 0.0001$) in docetaxel-treated MKN-45 cells when compared to untreated MKN-45 cells. Additionally, the treatment of MKN-45 cells with LA could decrease the expression of mir-194 by 2-fold ($P < 0.0001$) when compared to the control cells. The combined treatment of LA and docetaxel could increase the expression of miR-194 by 1.29-fold ($P = 0.003$) (Fig. 1c).

The expression of miR-30 increased by 1.92-fold ($P = 0.026$) in docetaxel-treated MKN-45 cells when compared to untreated MKN-45 cells. Additionally, the treatment of MKN-45 cells with LA could decrease the expression of mir-30 by 2-fold ($P < 0.0001$) when compared to the control cells. The combined treatment of LA and docetaxel could increase the expression of miR-30 by 1.30-fold ($P = 0.003$) (Fig. 1d).

Altered expression of Talin-2 and MMP-2 genes before and after treatment with LA and docetaxel

The expression of Talin-2 was decreased by 1.5-fold ($P = 0.0083$) in docetaxel-treated MKN-45 cells when compared to untreated MKN-45 cells. Additionally, the treatment of MKN-45 cells with LA could increase the expression of Talin-2 by 20.35-fold ($P = 0.0002$) when compared to the control cells. The combined treatment of LA and docetaxel could increase the expression of Talin-2 by 50.97-fold ($P = 0.0004$) (Fig. 1e).

The expression of MMP-2 was decreased by 4-fold ($P < 0.0001$) in docetaxel-treated MKN-45 cells when compared to untreated MKN-45 cells. Additionally, the treatment of MKN-45 cells with LA could decrease the expression of MMP-2 by 1.4-fold ($P = 0.01$) when compared to the control cells. The combined treatment of LA and docetaxel could increase the expression of MMP-2 by 1.68-fold ($P = 0.02$) (Fig. 1f).

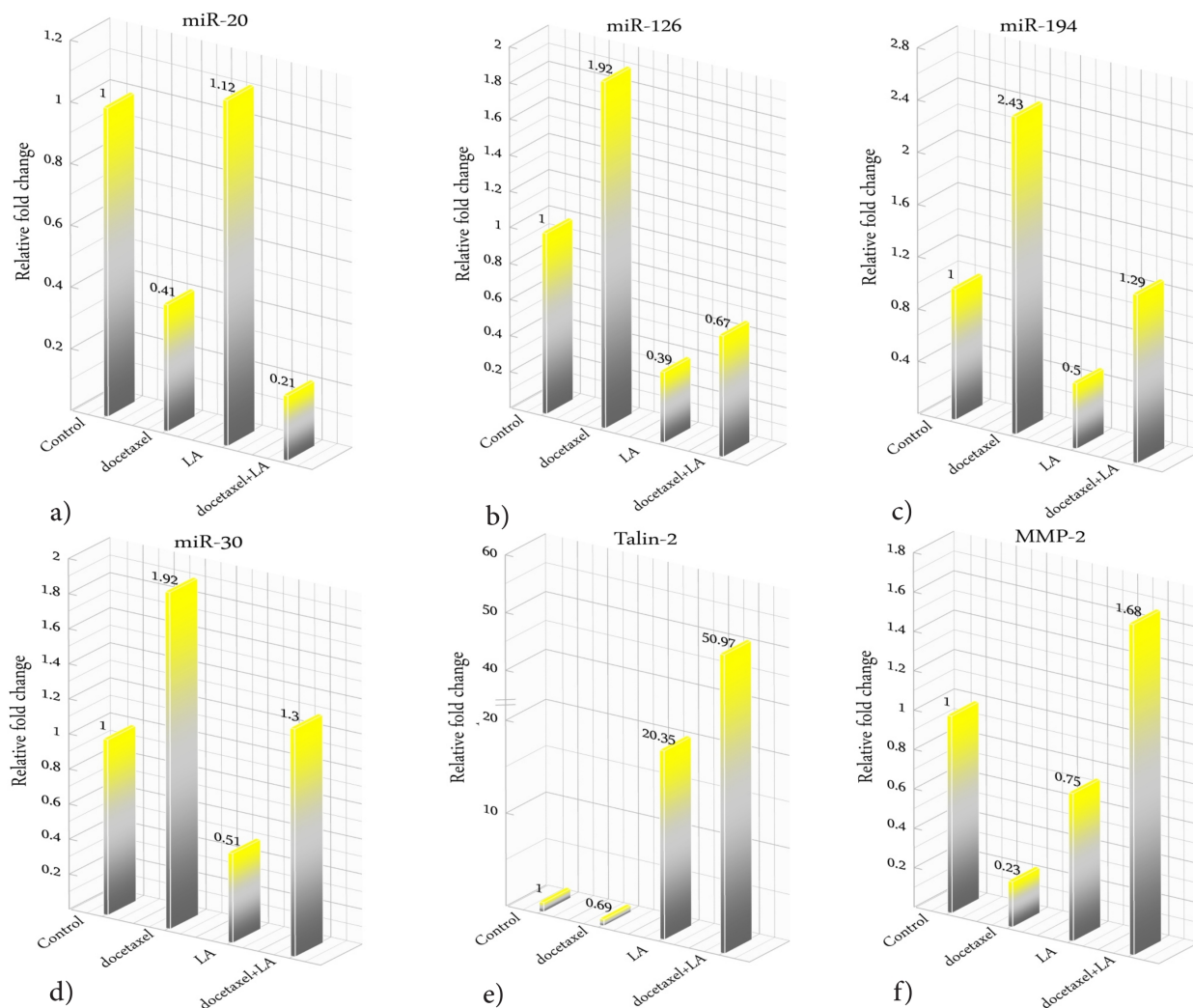


Fig. 1: The effect of MKN-45 cells treatment with either LA or docetaxel alone or in combination on the expression levels of a) miR-20, b) miR-126, c) miR-194, d) miR-30, e) Talin-2, and f) MMP-2

Discussion

The effects of LA on the progression of several cancers have been investigated. While some papers reported beneficial effects and recommended LA to suppress tumor growth (28-30), several other papers provided evidence that LA may support the metastasis and invasiveness of the studied tumors in human and animal models (31, 32). The effects of LA on the progression of GC have been studied in several papers. The main results of these studies and the suggested mecha-

nism of action through which LA plays a role in the pathogenesis of GC have been listed in Table 1. MicroRNAs (miRNAs) are non-coding endogenous RNAs that act as gene regulators mechanistically through translational inhibition or exonucleolytic mRNA decay (40, 41). Their participation has been well-documented in the physiologic process of the cells mainly cell proliferation, cell cycle, apoptosis, angiogenesis, and even pathologic processes including invasion, and metastasis (40).

Table 1: The main results of the previous studies on the effects of LA on the pathology of GC.

<i>Main results and highlights</i>	<i>Cell line</i>	<i>Ref</i>
Cis-9, trans-11-conjugated LA treatment decreased the expression levels of cyclin A and cyclin B1 through which the cell cycle was blocked and cell proliferation was affected.	SGC-7901	(6)
c9, t11-conjugated LA increased the levels of E-cadherin and α -catenin, and decreased the intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression. LA may affect the adhesion of SGC-7901 cells to laminin and fibronectin.	SGC-7901	(2)
LA treatment resulted in the suppression of invasion of the treated cells. The mechanism of action includes decreasing the activity of type IV collagenase and induced TIMP-1 and TIMP-2 mRNA expression	SGC-7901	(33)
LA treatment resulted in the activation of caspases 3, 8, and 9 and induced the apoptosis of treated cells through Fas/Fas-ligand interaction. Additionally, LA induced the degradation of poly (ADP-ribose) polymerase and phospholipase C-1 protein.	AGS	(34)
The production of COX-2 and prostaglandin-E2 (PGE2) was suppressed in LA-treated cells. Moreover, the expression of hTERT and telomerase activity was suppressed. LA could suppress the proliferation of the treated cells.	AGS	(35)
LA increases the permeability of the cell membrane of MKN28 cells by activating connexin hemichannels.	MKN28	(36)
LA could suppress the metastasis and decrease the production of TGF- α and epidermal growth factor receptor (EGFR).	MKN28	(37)
LA treatment suppresses the invasion and angiogenesis through enhancing plasminogen activator inhibitor-1 (PAI-1) mRNA and suppressing angiotatin respectively.	OCUM-2MD3	(38)
LA may stimulate the invasion and peritoneal metastasis through COX-catalyzed metabolism and activation of ERK in grafted OCUM-2MD3 cells on the stomach wall of LA-fed mice.	OCUM-2MD3	(39)

The omega-6 including LA influence the expression of miRNAs (42). Previous studies provided a link between the bio-function of miRNAs and the pathology of GC. In this regard, miR-30 has been reported as an oncomiR by regulating the P53-mediated mitochondrial apoptotic pathway (21). The downregulation of serum miR-126 is associated with a poorer prognosis in individuals with GC and that measuring its serum levels may be a novel prognostic molecular biomarker in GC (43). Further studies revealed its molecular mechanism of action. miR-126 binds directly to 3'-UTR of serine-arginine protein kinase 1 (SRPK1) mRNA through which it inhibits the migration and invasion of GC cell lines including BGC-823 and MKN-28 cells in vitro (44). miR-126 is an endothelial-specific microRNA that can suppress tumor angiogenesis and progression of GC

through VEGF-A signaling (34). The results showed a negative association between the expression of miR-126 and MVD and VEGF-A in GC (34).

We sought to investigate if treatment with LA may affect the expression levels of GC-involved microRNAs. In this study, we investigated the effects of LA and docetaxel on the expression levels of metastasis-related genes including MMP-2, and Talin-2, and the expression of mir-30, mir-126, mir-194, and mir-20 in MKN-45 cell line. In this study, LA treatment could reduce the expression levels of mir-126, mir-194, mir-30, and MMP-2, while increasing the expression levels of Talin-2 mRNA. Moreover, we investigated the effects of docetaxel treatment on MKN-45 cell line.

Docetaxel may decrease the expression levels of mir-20, Talin-2, and MMP-2 mRNA levels while increasing the expression levels of mir-126, mir-194, and mir-30. Additionally, the combined treatment of MKN-45 cells with LA and docetaxel could reduce the expression levels of mir-20 and mir-126 and increased the expression levels of mir-194, mir30, Talin-2, and MMP-2 mRNA levels.

According to our results, modulation of microRNA expression level may be a possible mechanism through which LA may exert its effects on MKN-45 cell line. To have a better insight into the role of LA in modulation of cancer cells in GC, we suggest including more related microRNAs and using other GC cell lines.

Conclusion

Our results provided a line of evidence that modulation of the expression levels of gastric cancer involved microRNAs, Talin-2 and MMP-2 may be a mechanism through which LA may exert its biologic effects on GC cell line MKN-45. Moreover, the combination of LA and docetaxel may provide a beneficial approach to the treatment of GC. Finally, the present study highlighted a possible mechanism on how LA may exert its antimetastatic effect by reducing the MMP-2 expression and pro-angiogenic effect through increasing Talin-2 expression levels.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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