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Study of the Effect of Linoleic Acid on the Expression Level of MicroRNA-106b and MicroRNA-20a and their Related Target MHC Class I Chain-related Protein A in Docetaxel-treated Gastric Cancer Cells

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Abstract

Background: MicroRNAs are involved in response to therapeutic agents and have the ability to regulate the expression level of the targets associated with cancer growth and progression. As a dangerous signal in tumor cells, increased expression level of MHC class I chain-related protein A (MICA) could activate the immune system and induce responses to tumor cells. We conducted the present research to study the effect of linoleic acid (LA) and docetaxel alone or in combination with miR-106b, miR-20a, and MICA expression level in metastatic gastric cancer (GC) cell line MKN-45.

Method: The study was an in vitro study using the gastric cancer cell line MKN-45, which was cultured and treated with docetaxel and LA. Subsequently, the expression level of miR-106b, miR-20a, and MICA were assessed with quantitative real-time polymerase chain reaction.

Results: MiR-106b decreased in LA and LA/docetaxel (P<0.0001 and P=0.002), and increased in docetaxel alone (P = 0.01). Meanwhile, miR-20a significantly decreased in docetaxel and LA/docetaxel (P<0.0001), increased in LA treatment (P=0.02). Regarding MICA, it significantly decreased in all the treated cells (P<0.0001, P<0.0001, and P=0.0002 for docetaxel, LA and docetaxel/LA, respectively) but with different reduction intensities.

Conclusion: Using LA or docetaxel alone had a different effect on miR-106b, miR-20a, and MICA expression level, yet in a simultaneous treatment, their positive effects were intensified. LA enhanced the effect of docetaxel concerning the expression level of miR-106b, miR-20a, and MICA and vice versa, which suggested that LA could be employed as an effective complementary agent in GC along with docetaxel.

Keywords: Linoleic acid, Docetaxel, miR-106b, miR-20a, MICA

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Introduction

Cancer, as one of the most important global health problems, annually leads to several new cases and deaths all around the world. Despite the efforts to find the best therapeutic strategy in terms of rapid response and minimal side-effects, researchers' efforts remain insufficient. Gastric cancer (GC) has a high rate of death, despite its lower prevalence compared with other most common cancers and patients do not respond well to the current therapies.[1] Among the available therapies, surgery is only appropriated in the early stages. As the disease progresses, the role of chemotherapy and the use of anticancer agents is highlighted.[2] Among the chemotherapy regiments with a herbal base, docetaxel has been shown to have noteworthy anticancer properties by triggering depolymerization of β-tubulins, inhibition of cell division, and induction of apoptosis.[3] A study on GC patients has shown that docetaxel combined chemotherapy along with cisplatin and fluorouracil, even in molecular levels, has a synergistic effect and improves the outcome of the therapy; accordingly, docetaxel is one of the main suggested drugs for the therapy of GC patients.[4] Despite all its benefits, the therapeutic effects of chemotherapy agents are not enough and combination therapies, such as radiotherapy, targeted therapy, and even nutritional therapies are needed.[5]

Cotreatment of dietary supplements, such as polyunsaturated fatty acids (PUFAs) along with other chemotherapy drugs has a positive effect on the treatment of cancer patients, including GC.[6] The two main categories of PUFAs include ω-3 and ω-6. Omega-3 fatty acids is mainly found in seafood while ω-6 is found in plants. Linoleic acid (LA, C18:2, ω-6) is an essential fatty acid, which is not made by the human body and its major sources are crop seeds and vegetable oils.[7] As the precursor of ω-6s, LA could be converted to a variety of products, including γ-linolenic acid (GLA), dihomo-γ-linolenic acid (DGLA), and arachidonic acid (AA), which have shown anticancer effects.[8] LA promotes apoptosis[9] and causes an inhibitory effect on the proliferation of tumor cells in GC.[10]

MicroRNAs (miRNAs) are among those factors that are changed during carcinogenesis in tumor cells and indicate a potential relation with cancer progression. Depending on whether they are upregulated (oncomir) or downregulated (tumor-suppressor) and on their mRNA targets, they have different roles in the cancer process.[11] Studies have shown that miRNAs also undergo changes with chemotherapy and could be used as therapeutic and/or diagnostic biomarkers in GC.[12] MiR-106b involves in migration, invasion, and proliferation of several cancers and is suggested as a diagnostic, prognostic, and even responsiveness biomarker to chemotherapy.[13] Another oncogenic miRNA that could be used as a biomarker in GC is miR-20a that is upregulated in GC and has a relation with the proliferation and invasiveness of GC cells.[14] MICA is one of the targets that these two miRNAs have in common.[15]

MHC class I-related chain A (MICA) is one of the NKG2D ligands expressed on the cell surface within cellular stress, malignant transformation, and viral infection. Given that cancer is also a condition of cellular-stress, the expression of MICA on cancer cells increases. In fact, MICA serves as a signal to alert the immune system and activation of natural killer (NK) cells to attack tumor cells. According to previous studies, MICA could be employed as the potential therapeutic target in cancer therapy,[16] and one of the regulatory factors of MICA expression is miRNAs.[17] In this study, we aimed to evaluate the effect of LA along with docetaxel on the expression level of miR-106b and miR-20a, and MICA in MKN-45 metastatic GC cell line.

Methods and Materials

Cell line and reagents

In the present experimental in vitro study, MKN-45 as the metastatic human GC cell line was obtained from the National Cell Bank of Iran (NCBI, Tehran, Iran) and were maintained in complete Roswell Park Memorial Institute-1640 medium (Gibco Inc, El Paso, TX) containing 10% fetal bovine serum (Gibco, USA), and 100 units/ml

penicillin and 100 µg/ml streptomycin (Gibco, USA). The cells were cultured in 5% CO2 incubator (Memmert, Germany) at 37 °C. LA stock solution (Sigma, USA) was prepared in absolute ethanol (EtOH) and 1mM BSA (Bovine Serum Albumin). Docetaxel (Taxotere) was prepared from Sanofi-Aventis (France).

Cells treatment, RNA extraction, and cDNA synthesis

A total of 4.5×10^3 MKN-45 cells were seeded into a six-well culture plate, and 50µM LA and IC50 concentration of docetaxel, which was calculated in our previous study,[18] were used to treat the cells. Total RNA was isolated with RiboExTM (Gene All Biotechnology, South Korea) according to the manufacturer's protocol. Subsequently, the quantity and quality of extracted RNA were evaluated with agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo Scientific, USA).

cDNA for miR-106b and miR-20a was synthesized using miRCURY LNATM Universal cDNA Synthesis Kit II (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. 14µl diethylpyrocarbonate (DEPC) water, 1µl random Hexamer, 2µl dNTP, 2µl reaction buffer, and 250 units reverse transcriptase enzyme (Sina Clone, Iran) were utilized to synthesize cDNA for MICA.

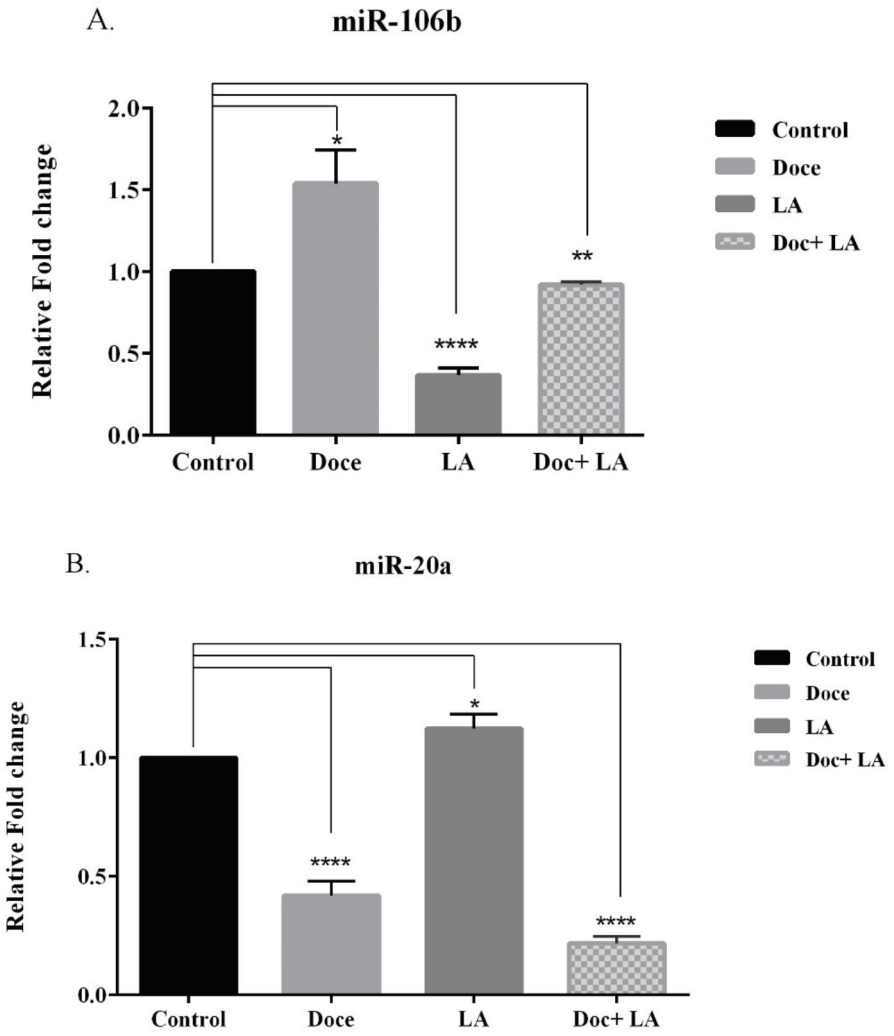

Figure 1. The altered expression level of miR-106b and miR-20a after the treatment of MKN-45 cells within LA and docetaxel. The cells were treated with LA, docetaxel, and their combination for 24 hours. The expression levels of miR-106b (A) and miR-20a (B) in the treated and untreated (control) cells were determined with quantitative real-time PCR. * *P*-value <0.05, ** *P*-value <0.01, **** *P*-value <0.0001.

PCR: Polymerase chain reaction; LA: Linoleic acid

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Using SYBR Green PCR Master Mix (Yecta Tajhiz Azma, Iran), we performed quantitative real-time PCR on a Corbett Rotor-Gene 6000 system (QIAGEN, Germany). The primer of miRNAs was purchased (Exiqon, Vedbaek, Denmark) and the reaction mixture was generated in 10µl volume, according to manufacturer's protocol (5µl master mix, 1µl primer, and 4µl cDNA). The reaction mixture for MICA was prepared with 6.5µl double distilled water (DDW), 7.5µl master mix, 0.3µl primer, and 0.7µl cDNA. U6 snRNA and β-actin were used as endogenous controls for the miRNA and mRNA, respectively. Table 1 depicts the sequences of the primers and table 2 represents the *P*-values and fold changes of these genes.

Ethical approval

This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1396.1028). All the experiments done on cell culture models and clinical samples were not included in this study.

Statistical Analysis

The data were shown as mean ± SD of the fold difference between LA- and docetaxel-treated and untreated cells for triplicate experiments. The obtained data were analyzed with GraphPad Prism software 6.0 (La Jolla, California, USA) employing unpaired student's t-test. *P*-value < 0.05 was considered to be statistically significant.

Results

The expression level of miR-106b and miR-20a in response to docetaxel, LA, and Doce/LA

As shown in figure 1A, miR-106b was upregulated in response to docetaxel ($P = 0.01$ and 0.65 fold), but downregulated with LA and Doc/LA treatment ($P < 0.0001$ and 2.77 fold, $P = 0.002$ and 1.08 fold, respectively). On the other hand, the expression level of miR-20a (Figure 1B) decreased significantly following the treatment with docetaxel and Doc/LA ($P < 0.0001$ and 2.43 fold, $P < 0.0001$ and 4.76 fold, respectively); whereas its expression level increased with LA ($P = 0.02$ and 0.89 fold).

The expression level of MICA in response to LA and Doce/LA

The analysis of our results revealed that the expression level of MICA was downregulated significantly in the MKN-45 GC cell line after the treatment with docetaxel, LA, and a combination of them (Figure 2 and Table. 1). Even though downregulation was seen in the treatment with docetaxel and LA alone ($P < 0.0001$ and 100 fold, $P < 0.0001$ and 7.14 fold), it was lower using the combination of docetaxel and LA ($P = 0.0002$ and 3.03 fold).

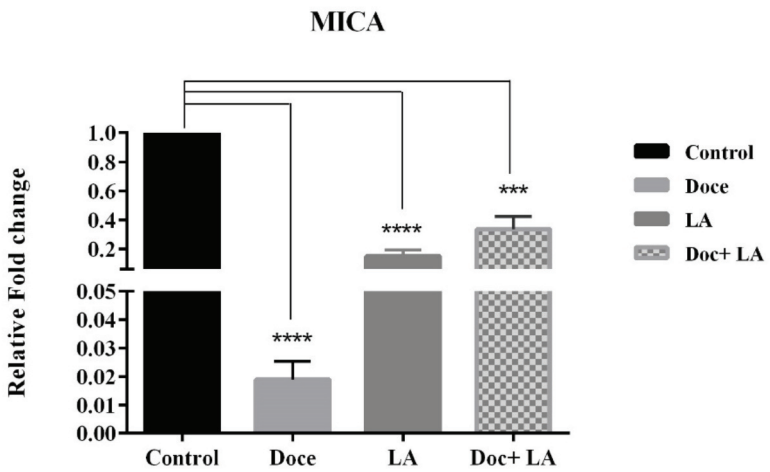

Figure 2. The altered expression level of MICA following the treatment of MKN-45 cells within LA and docetaxel. The cells were treated with LA, docetaxel, and their combination for 24 hours. The expression level of MICA in the treated and untreated (control) cells was determined employing quantitative real-time PCR. *** *P*-value <0.001, **** *P*-value <0.0001.
Doc: Docetaxel, LA: Linoleic acid, MICA: MHC class I-related chain A, PCR: Polymerase chain reaction

Table 1. Primers sequences and annealing temperature in this study

	Primer Sequence(s)	Annealing temperature
miR-106b*	5'- UAAAGUGCUGACAGUACAGUGCAGAU-3'	60 ºC
miR-20a*	5'- UAAAGUGCUUAUAGUGCAGGUAG-3'	60 ºC
U6*	5'-GGG CAG GAA GAG GGC CTA T-3'	60 ºC
MICA	F: 5'-ACACCCAGTTGGGACGAGTGA-3'	62 ºC
	R: 5'- AGTGGAGCCAGTGGACCCAAG-3'	
β-Actin	F: 5'-TCCCTGGAGAAGAGCTACG-3'	59 ºC
	R: 5'-GTAGTTTCGTGGATGCCACA-3'	

*The sequence for the target sequence.
MiR-106b: MicroRNA-106b miR-20a: MicroRNA-20a, U6: U6 small nuclear RNA MICA: MHC class I chain-related protein A, β-actin: beta-Actin

Discussion

According to previous studies, the expression level of miRNAs as biomarkers for responsiveness in GC therapy could be altered by anticancer agents and also PUFAs.[19] Moreover, these changes in miRNAs expression level affect their related targets involved in cellular function. Therefore, investigating the antitumor effect of LA at the molecular level and its effect along with docetaxel in GC patients could improve treatment strategy. In this study, we investigated the positive and synergistic effects of LA along with docetaxel through altering the expression level of miR-106b, miR-20a, and MICA in the GC cell line.

The present results demonstrated that LA significantly downregulated while docetaxel upregulated the miR-106b expression level. In our previous study,[20] LA, similar to DHA, showed the same effect on the expression level of miR-106b. In several cancers, such as brain, ovarian, breast, colon, larynx, prostate, and GC, miR-106b is upregulated and involved in cellular processes, proliferation, migration, and apoptosis for instance.[21] In GC, miR-106b is overexpressed not only in GC tissues, but also in plasma samples. It is suggested as a new tumor marker[22] which suppresses cell migration and invasion and is suggested as a therapeutic agent by affecting the PTEN pathway.[21] The notable point in our study was the proper effect of LA with docetaxel, which in combination form causes a decrease in miR-106b compared to docetaxel alone. This indicates the beneficial effect of the consumption of LA with docetaxel in decreasing miR-106b as an oncomir in MKN-45 cells.

As a result of the therapeutic effect of docetaxel, miR-20a expression level was downregulated, but LA led into the upregulation of miR-20a in MKN-45 cells. MiR-20a as a member of the miR-17-92 cluster functions as oncogenes in several cancers, whose overexpression in GC tissues and cell lines, such as MKN-45, was previously confirmed by Li et al.[23] They found that miR-20a promotes proliferation, invasion, and migration. Based on their results, it is involved in chemotherapy resistance towards cisplatin and docetaxel in SGC7901 and MKN-45 cells. In our previous study, DHA led into the downregulation of miR-20a in the MKN-45 GC cell line and this downregulation was intensified in the combination of DHA and docetaxel.[24] It seems that this might be attributed to the positive effect of LA on tumor progression. Fortunately, this effect was compensated with docetaxel in its combination form whose severity of decrease was intensified in simultaneous utilization of LA and docetaxel.

Considering MICA expression changes, we showed herein that the expression level of MICA was downregulated in all the treated cells. Moreover, the downregulation rate of MICA with LA was less than that with docetaxel alone and in the combination form, the downregulation intensity was less. Several studies have exhibited the regulatory role of miRNAs on MICA expression levels. For example, in a study by Al-Abdallah et al. on thyroid cancer, they found that miR-146b-5p downregulated the expression of MICA and led to immune escape of tumor cells.[16] Among the miRNAs regulating MICA expression, miR-20a, miR-93, miR-15b, miR-16, and miR-106bcan suppress MICA/B expression in several

Table 2. Fold changes and *P*-values of miR-106b, miR-20a, and MICA

		Docetaxel	LA	Doc/ LA
miR-106b	Fold change	+0.65	-2.77	-1.08
	P-value	0.01	<0.0001	0.002
miR-20a	Fold change	-2.43	+0.89	-4.76
	P-value	<0.0001	0.02	<0.0001
MICA	Fold change	-100	-7.14	-3.03
	P-value	<0.0001	<0.0001	0.0002

"+" represents increased and "-" shows decreased expression level MiR-106b: MicroRNA-106b, MiR-20a: MicroRNA-20a, MICA: MHC class I chain-related protein A, LA: Linoleic acid, Doce: Docetaxel

cancers and make immune evasion.[25] Stern-Ginossar et al. showed an inverse relation among MICA, miR-106b, and miR-20a in DU145 and HeLa cell lines related to cancer of the cervix and prostate.[15] A study on GC showed an increased expression level of MICA and proposed it as a poor prognostic factor[26] and a responsive biomarker for in the treatment of GC.[27] Regarding the effect of chemotherapy drugs on the MICA expression levels, various anticancer drugs result in an increase in MICA expression levels in several cancers, such as cervical cancer[28] and pancreatic cancer.[29] The downregulation of MICA after docetaxel treatment was shown previously in the A549 cell line.[30] They proposed that tumor cells begin to reduce NKG2D ligands in order to escape the immune system responses. Furthermore, the changes in this pattern of MICA in response to LA were similar to the effect of DHA on MICA expression in the GC cell line.[24] Therefore, it seems that the reduction of MICA mRNA expression level in the MKN-45 GC cells followed by DHA and LA might be one of the mechanism of tumor cells in order to escape recognition by immune system. Furthermore, more intensity in the downregulation of MICA by docetaxel might be due to the side-effects of docetaxel on tumor cells, which help tumor cells to escape more from immune system responses. Furthermore, as shown in the combination form, LA reduced these side-effects up to an extent and was able to partially eliminate the negative effects of docetaxel and help in gaining good treatment results.

Conclusion

In sum, our findings revealed that LA as a supplementary agent, had the ability to interfere with cellular components, including miRNAs and their related targets in tumor cells and modulate their expression level. On the other hand, the significant side-effects of docetaxel in the therapy of GC patients decreased the therapy outcomes. In line with previous studies, combining LA and docetaxel, these two agents improved each other's therapeutic efficacy and even reduced the unexpected effects. Due to the unwanted effects of docetaxel on the reduction of MICA and promotion of miR-106b in the MKN-45 GC cell line, LA compensated these effects and in the combination form, we obtained proper results. Furthermore, in miR-20a expression changes, docetaxel compensated the LA effects. Ultimately, it seemed that the utilization of LA along with docetaxel treatment had promising results in MKN-45 GC cells via modulation of miR-106b, miR-20a, and MICA expression level. This proposes LA as one of the suitable supplementary fatty acids to be used in GC patient therapy. We also recommend performing in vivo studies and evaluating a wide range of miRNAs in order to validate the obtained results.

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Conflict of Interest

None declared.

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