The Effect of Micro RNA-34a and carboplatin combination on the inducing apoptosis of Breast Cancer Cell line

Sajjad Eslamkhah
Tabriz University of Medical Sciences
Nazila Alizadeh
Tabriz University of Medical Sciences
Sahar Safaei
Tabriz University of Medical Sciences
Mohammad Amini
Tabriz University of Medical Sciences
ahad Mokhtarzadeh
Tabriz University of Medical Sciences
Amir Baghbanzadeh
Tabriz University of Medical Sciences
Behzad baradaran (behzad_im@yahoo.com)
Tabriz University of Medical Sciences https://orcid.org/0000-0001-6701-7446

Research Article

Keywords: Breast cancer, miR-34a, carboplatin, Apoptosis, Chemosensitivity

DOI: https://doi.org/10.21203/rs.3.rs-289066/v1

Abstract

Aim: Breast cancer (BC) has been classified among the main causes of death owing to females' cancer. Carboplatin is a platinum-based chemotherapeutic drug that is an important treatment option for BC. But high and frequent doses of carboplatin usually reducing the reaction of cancer cells to medication. There is an immediate need to establish methods for increasing the carboplatin susceptibility to BC cells. For instance, micro RNAs (miRNAs) such as MiR34a demonstrate significant potential. Considering that, this research was planned to explore the better clinical effect and underlying mechanism of miR-34a as a possible tumor inhibitor and drug resistance regulator in compound with carboplatin chemotherapy drug in the cell lines of BC in humans.

Methods: MCF-7 cell line was transfected with miR-34a to perform functional analyses. Subsequently, the MTT assay was applied to assess cell viability. Cell viability and cell death associated gene expression amounts including Bax, Bcl-2, caspase-3, MDR1, P53, and mir34-a, were examined through real-time quantitative PCR.

Results: Findings showed that miR-34a upregulation significantly decreased MCF7 cell viability in comparison with control group. Furthermore, separate treatment of cells with miR-34a mimics and carboplatin could significantly increase Bax, Caspase-3, P53, and decrease in Bcl-2 mRNA expression levels evaluated to the non-treated group. Moreover, by reduction in expression levels of the MDR1 gene, BC cells' reaction to carboplatin has increased via miR-34a.

Conclusion: In line with the findings, it could be inferred that miR-34a may improve the responsiveness of breast cancer cells to carboplatin chemotherapy with downregulation of MDR1.

Introduction

One of most common malignancies in females is breast cancer, responsible for about one in three cancers reported by women in the North America, and the second leading factor of fatality due to cancer in females [1]. Medical methods such as chemotherapy, operations, radiation therapy, and immunotherapy can be used in breast cancer treatments. However, these therapies have some drawbacks, such as recurrence, toxic effect, high expense, and other possible complications. Therefore, the discovery of new treatment methods was an important necessity to solve these patients' problems. MicroRNA therapy could be one of the most successful methods for treating breast cancer [2]. Over the past years, small RNA regulators were discovered for the development of cancer research. MicroRNAs (miRNAs) are tiny single-stranded RNAs with 20 to 24 nucleotides that do not specifying the genetic codes [3] and are stabilized or located in post-transcription gene expression regulation in multicellular organisms [4]. Recent research has demonstrated that MicroRNAs are aberrantly represented in different forms of tumors [5]. They also have an important role in human tumorigenesis and/or metastatic process by targeting oncogenes or tumor suppressors [6]. Altering the MicroRNA profile expression has been established as a modulator of proliferation and apoptosis in breast cancer therapy [7], and Dysregulation

of certain miRNAs is related to breast cancer [8]. Particular MicroRNA expression pattern and the expression of the associated genes give the scientists insight into choosing the best-targeted therapy [9]. MicroRNA 34a (miR-34a) is one of the human microRNAs coding by the gene MIR34A and considered as a tumor suppressor [10]. The production of this MIR in healthy cells reduces the formation of cancer cells such as breast cancer. [11]. Carboplatin is platinum derived anti-tumor drug of the second generation. It acts as both an alkylating agent and a safer chemical agent for malignancies that are newly diagnosed, like breast tumors [12]. The intention of this research was to exploration of the impacts of the MIR-34a and carboplatin combination on apoptosis of BC Cells.

Materials And Methods

Cell Culture

The breast cancer cell line MCF-7, still maintaining mammary epithelium properties, including estrogen in the cell cytoplasm. MCF-7 cells are less aggressive and non-invasive, resulting in a versatile xenograft therapy response model for early and advanced ER + breast tumor development [13–15]. In the current investigation, the MCF-7 cell line has been provided from the Iran National Cell Bank (Pasteur institution, Tehran, Iran). This cell line was cultivated at 37 ° C in the 10% fetal bovine serum RPMI-1640 medium (Gibco), including streptomycin, and penicillin antibiotics (100 µg / mL and 100 IU / mL respectively), providing 5% CO2 and 95% humidity. The cells were isolated with Trypsin-EDTA (0.25 percent) and cultivated each 24–48 h with a 70–80 percent confluence of cell monolayers.

MicroRNA transfection

MicroRNA 34a was transfected into MCF-7 cells at different doses (40, 60, 80 and 100 pmol) using (Bio-Rad) Gene Pulser electroporation system, according to supplied protocols. Transfection was performed with a total volume of 1×10^6 MCF-7 cells per 500 mL electroporation buffer in voltage of 150 v for 12 ms. Then, 6×10^5 transfected MCF-7 cells were cultured into each well of 6-well plate, then followed up for 24, 48, and 72 hours. After harvesting the cells for evaluating optimized time and dose of drug and miRNA, RNA extraction was done, and the qRT-PCR was assessed the expression amount.

Extraction of RNA and quantitative real-time PCR

The Trizol RNA isolation package (GeneAll, Korea) was applied to separate the whole RNA following the producer's instructions. To measure the concentration and quantity of the extracted RNA, nano Drop spectrophotometer (Thermo Fisher Scientific Life Sciences) was used at A260/A280 nm. For cDNA synthesis, purified RNA (1µg) was used with RT Master Mix in the Bio-Rad T100 thermal cycler. To demonstrate the amounts of mRNA expression of miR-34a-5p, P-53, Bax, Caspase-3, MDR1, and Bcl-2 quantitative real-time PCR was utilized, and as a reference gene, GAPDH was used (Table 1). With the StepOnePlus Real-Time PCR Device and the BioFACT 2X RealTime PCR Master Mix, the reactions were performed triplicated. To assess the relevant quantitation presentation of genes levels, the $2^{-\Delta\Delta Ct}$ procedure was used.

Table 1
Primer sequences

Genes Name	Primers	Sequence of primers
miR-34a-5p	Target sequence	5´ UGGCAGUGUCUUAGCUGGUUGU 3´
GAPDH	F	5’- CAAGATCATCACCAATGCCT – 3’
	R	5’- CCCATCACGCCACAGTTTCC-3’
P53	F	5´ ACTTGTCATGGCGACTGTCC 3´
	R	5´ CACCCCTCAGACACACAGGT 3´
Bax	F	5'-GACTCCCCCCGAGAGGTCTT-3'
	R	5'-ACAGGGCCTTGAGCACCAGTT-3'
Bcl-2	F	5´ CCTGTGGATGACTGAGTACC 3'
	R	5´ GAGACAGCCAGGAGAAATCA 3´
Caspase-3	F	5´ TGTCATCTCGCTCTGGTACG 3´
	R	5´ AAATGACCCCTTCATCACCA 3´
MDR1	F	5'CCATCATTGCAATAGCAGG3'
	R	5' GAGCATACATATGTTCAAACTTC 3'

Cell Viability Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium decrement assay has been used to detect cell survival with (Sigma-Aldrich, St. Louis, MO) kit [16]. First, the MTT assay was used to test the carboplatin IC50 value. After obtaining the IC50 amount for carboplatin, the cell transfection was performed subsequently with miR-34a and treated with varying amounts of carboplatin. Thus, various doses of carboplatin including 0.25 to 100 μM (Mylan, Saint-Priest, France) were applied to every electroporated well after 24 hours of transfection. Tests were carried out in triplicate.

Statistical analysis

All quantitative analyses have been carried out by Graph Pad Prism edition 6.00 (GraphPad, San Diego, CA), except for annexin V / PI assay. All concepts were stated as mean ± SD, and triple trials (n = 3) were conducted. Student's t test and variance analyzis were applied to assess the statistical significance of the discrepancies between groups. A $p < .05$ was seen as demonstrating a statically important discrepancy.

Results:

miR-34a overexpression sensitized MCF-7 cells to carboplatin treatment

As mentioned, MTT testing was carried out to investigate miR-34a toxic impact on BC cells combined with carboplatin. The obtained results implied that miR-34a exogenous overexpression could significantly ($p < 0.001$) decrease MCF-7 cell viability compared to the controls. Meanwhile, not meaningful differences were observed in cell survival rates among control and miR-control groups (Fig. 1a). Furthermore, as depicted in Fig. 1b, MTT results illustrated medication of MCF-7 cells by carboplatin decreased survival of cells based on dosage, determining the carboplatin IC50 at 222.3 µg/mL. However, pretreatment of MCF-7 cells with miR-34a mimics significantly ($p < 0.001$) increased carboplatin cytotoxic effect and remarkably reduced the IC50 value to 159 µg/mL. Hence, it was presented that miR-34a is a crucial component in the sensitivity of BC cells to carboplatin treatment.

miR-34a modulated the expression of genes involved in apoptosis

To further explore underlying molecular processes through which miR-34a could increase carboplatin cytotoxic effect on MCF-7 cells, the function of genes were quantified using qRT-PCR. As represented in Fig. 2, the outcome illustrated that separate treatment of cells with miR-34a mimics and carboplatin could considerablly ($p < 0.0001$) increase Bax mRNA expression levels evaluated with control group. However, combination therapy led to more upregulation of the pro-apoptotic Bax gene compared to separate treatments. Furthermore, the evaluation of Bcl-2 expression level in treatment groups established that miR-34a and carboplatin could cooperatively downregulate this pro-survival gene expression compared to the control. Besides, miR-34a overexpression was able to increase carboplatin-induced upregulation of caspase-3 expression, as an important executer caspase in apoptosis pathways. Therefore, miR-34a was suggested to increase BC cell chemosensitivity to carboplatin through modulating the activity of genes associated with cell apoptosis. Furthermore, the P53 tumor suppressor expression as an important molecular agent participating in BC tumorigenesis and therapeutic responses was investigated in treatment groups. The qRT-PCR results showed that miR-34a overexpression alone or in combination with carboplatin could significantly ($P < 0.0001$) regulate up P53 mRNA expressions evaluated with control cells, indicating that miR-34a may also be involved in sensitization of BC cell to carboplatin through regulating P53-mediated cell death.

Transfection Of Mir-34a Downregulated Mdr1 Expression

The amount of expression of MDR1 in the current investigation, as an important modulator of chemoresistance of tumor cells, also was investigated. This gene encodes P-glycoprotein 1 that functions as an efflux pump removing chemotherapy drugs from the cell. The obtained results from qRT-PCR illustrated that MCF-7 transfected cells with miR-34a mimics significantly ($p < 0.05$) reduced MDR1 expression compared to control cells. Controversy, carboplatin treatment led to remarkable upregulation

of this gene in MCF-7 cells. However, pretreatment of these cells with miR-34a mimics impaired carboplatin induced upregulation of MDR1 and decreased its expression to normal levels (Fig. 3).

Discussion

The most frequent cancer detected in females is breast cancer which is the prominent reason for cancer fatality across the globe. The critcal care strategy for this illness is chemotherapy [17]. Presently, resistance to tumor medications is challenging for acquiring effective anti-cancer therapeutic breast cancer outcomes [18]. MicroRNAs (miRs) are small non-coding RNAs whose ability to bind to the three non-Translated parts of their selected mRNAs negatively regulates gene expression [19]. Over 1,000 human miRNAs have also been recognized until now, and several of them are found to have expression disorderliness in cancer [20–22]. As a well-researched miRNA, various cancer forms have been relevant to miR-34a, for instance neuroglioma, breast cancer, prostate cancer, and lung cancer; miR-34a is known to be a potent tumor suppressor [23, 24]. In this research, the synchronous effects of miR-34a and carboplatin on the MCF-7 breast cancer cell line were investigated. The effectiveness of miR-34a on cell formation was examined for showing miR-34a's function on breast cancer cells, the findings of which revealed that miR-34a was capable of breast cancer cells apoptosis. The MTT assay results in the present study demonstrated that after curement of MCF-7 cell line with miR-34a and carboplatin combination, the survival rate of this cell line was significantly reduced in evaluation with carboplatin treatment and miR-34a transfection alone and also the control group. Afterwards, the expression levels of genes involved in apoptosis and cell survival were assessed in treatment groups using qRT-PCR. It was observed that mir-34a and carboplatin combined therapy lead to further upregulation of pro-apoptotic genes such as Bax, caspase-3, and P53 and downregulation of the expression level of Bcl-2, leading to the diffusion of cytochrome c and initiation of apoptosis [25]. Furthermore, qRT-PCR results also showed that, miR-34a overexpression results in downregulation of MDR1 expression level in the transfected cells. Yet incredibly, treatment with carboplatin has led to upregulation of this gene in MCF-7 cells. Nonetheless, therapy of these cells by miR-34a mimics inhibited the upregulation of MDR1 by carboplatin and limited its expression to usual amounts. Therefore, it could be assumed that miR-34a also would boost the responsiveness of the BC cells for carboplatin chemotherapy via downregulation of MDR1 gene.

Conclusion

This study illustrated that miR-34a would prevent breast cancer cell proliferation via decrement of Bcl-2 expression level. Moreover, with targeting MDR1, the sensibility of breast cancer cells would enhance to carboplatin through miR-34a. This report allows researchers to discuss new combination therapy methods with miR-34a and carboplatin in further studies.

Declarations

Acknowledgments

We acknowledge the Immunology Research Center (IRC), Tabriz University of Medical Sciences for providing excellent technical support and also research environment. Current investigation was facilitated by funds of the Ministry of Health and Medical Education (No, 700/95, 1394.12.24).

References

1. DeSantis CE, et al. Breast cancer statistics, 2019. Cancer J Clin. 2019;69(6):438–51.
2. Tang G, Tang M, Xie Y. The role of miRNAs in gastric cancer. J Gastroint Dig Syst. 2013;3(129):2161–069.
3. Wong GL, Abu Jalboush S, Lo H-W. *Exosomal MicroRNAs Organotropism in Breast Cancer Metastasis* Cancers. 2020;12(7):1827.
4. Soheilifar MH, et al. Angioregulatory microRNAs in colorectal cancer. Cancers. 2020;12(1):71.
5. Mohammadi A, Mansoori B, Baradaran B. The role of microRNAs in colorectal cancer. Biomed Pharmacother. 2016;84:705–13.
6. Babaei K, et al. An insight of microRNAs performance in carcinogenesis and tumorigenesis; an overview of cancer therapy. Life Sci. 2020;240:117077.
7. Tian F, et al. LncRNA SNHG7/miR-34a-5p/SYVN1 axis plays a vital role in proliferation, apoptosis and autophagy in osteoarthritis. Biol Res. 2020;53(1):1–11.
8. Tanman Ü, Yangın S, Cansaran-Duman D. *Determination of Dysregulated miRNA Expression Levels by qRT-PCR after the Application of Usnic Acid to Breast Cancer.* Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 2020. 20(5): p. 548–558.
9. Martinez-Gutierrez AD, et al. Identification of miRNA Master Regulators in Breast Cancer. Cells. 2020;9(7):1610.
10. Behzad M, et al. Micro RNA 34a and Let-7a expression in human breast cancers is associated with apoptotic expression genes. Asian Pac J Cancer Prev. 2016;17(4):1887–90.
11. Irani S, et al., *Evaluation of miR-34a Effect on CCND1 mRNA Level and Sensitization of Breast Cancer Cell Lines to Paclitaxel*. Iranian Biomedical Journal, 2020: p. 0–0.
12. Karanam V, et al. Poly (□-caprolactone) nanoparticles of carboplatin: Preparation, characterization and in vitro cytotoxicity evaluation in U-87 MG cell lines. Colloids Surf B. 2015;130:48–52.
13. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. Breast cancer research. 2011;13(4):1–7.
14. Studebaker AW, et al. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6–dependent manner. Cancer research. 2008;68(21):9087–95.
15. Valiyari S, et al. Cytotoxic and apoptotic activity of Scrophularia oxysepala in MCF-7 human breast cancer cells. Toxicological Environmental Chemistry. 2013;95(7):1208–20.
16. Ghazanchaei A, et al. Restoration of miR-152 expression suppresses cell proliferation, survival, and migration through inhibition of AKT–ERK pathway in colorectal cancer. Journal of cellular

physiology. 2019;234(1):769–76.
17. Khordadmehr M, et al. Key microRNAs in the biology of breast cancer; emerging evidence in the last decade. Journal of cellular physiology. 2019;234(6):8316–26.
18. Wu H, et al. Anticancer agent shikonin is an incompetent inducer of cancer drug resistance. PloS one. 2013;8(1):e52706.
19. Li X, Ren Z, Tang J. MicroRNA-34a: a potential therapeutic target in human cancer. Cell death disease. 2014;5(7):e1327–7.
20. Kasinski AL, Slack FJ. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. Nat Rev Cancer. 2011;11(12):849–64.
21. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic acids research. 2010;39(suppl_1):D152–7.
22. Mansoori B, et al. MicroRNAs in the diagnosis and treatment of cancer. Immunol Investig. 2017;46(8):880–97.
23. Li Y, et al. MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. Cancer research. 2009;69(19):7569–76.
24. Yang S, et al. MicroRNA-34 suppresses breast cancer invasion and metastasis by directly targeting Fra-1. Oncogene. 2013;32(36):4294–303.
25. Frenzel A, et al. Bcl2 family proteins in carcinogenesis and the treatment of cancer. Apoptosis. 2009;14(4):584–96.