BMC Immunology

RESEARCH

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Effect of conditioned medium from miRNA-34a transfected gastric cancer-associated fibroblast on peripheral blood mononuclear cells

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Abstract

Background Cancer-associated fibroblast (CAF) cells play an important role in gastric malignancy. MiRNA dysregulation has been detected in CAF cells, which is related to the tumor progression ability of these cells. Therefore, this study aimed to evaluate the function of miRNA34a in CAF cells in gastric carcinoma.

Method We transiently transfected miRNA-34a mimic in CAF cells and examined the effect of the overexpressed miRNA on PD-L1 expression using real-time PCR. Next, we evaluated the role of transfected CAF-conditioned medium (CM) on the immune response and viability of gastric cancer cell lines.

Results We have shown that miRNA-34a significantly reduced PD-L1 expression in CAF cells ($p < 0.05$). However, the conditioned medium of transfected cells had no significant effect on the immune response. We also found that CM of miRNA-34a transfected CAF cells significantly suppressed gastric cancer cell line viability relative to the control group ($P < 0.05$).

Conclusion We indicated that CM of miRNA-34a transfected CAF can reduce gastric cancer cell line proliferation. Additionally, miRNA-34a in these cells may improve immune response via PD-L1 reduction. Thus, miRNA-34a could be a potential therapeutic agent in gastric cancer treatment.

Clinical trial number Not applicable.

Keywords Cancer associated fibroblast, MiRNA-34a, Gastric cancer

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Introduction

Gastric cancer is one of the most common malignancies and the leading cause of cancer-related death worldwide. In 2024, More than 26,000 new cases are diagnosed, and 10,880 patients died of this malignancy in the United States [1, 2]. The Survival rates for patients with advanced-stage gastric cancer remain low despite chemotherapy, surgery, hormone therapy and other treatments [3–5].

The tumor microenvironment (TME) has an important role in cancer progression. TME consists of the extracellular matrix (ECM), blood vasculature, inflammatory cells, immune cells and cancer-associated fibroblast cells [6, 7]. Cancer-associated fibroblast (CAF) cells are the most important and prominent cells in the tumor stroma. These cells can increase tumor invasion, metastasis, and angiogenesis and have an immunosuppressive role in malignancies via the secretion of various mediators and direct interaction with tumor cells and immune cells [8–10].

98% of the genome includes noncoding RNA, such as microRNA (miRNA). MiRNA ——small noncoding RNAs—— contain 19–25 nucleotides that can interact with the 3' untranslated region (3'UTR) of their target gene mRNA and inhibit the protein expression [11, 12]. MiRNAs play a pivotal role in transcriptional and post-transcriptional levels and are related to many biological activities and diseases, such as cell apoptosis, differentiation, metabolism, proliferation, blood pressure, cardiac function, autoimmune disease, viral infection and different types of malignancies. These molecules exhibit an oncogenic or tumor suppressor role in cancers, depending on their target genes [13–16].

MiRNA dysregulation in CAFs, contributes to the tumor progression ability of these cells. MiRNA molecules can influence the reprogramming of normal fibroblasts into CAFs and their function. Also, secreted miRNA from CAF may influence several aspects of a cancer cell's behavior [17–21].

MiRNA-34a is a tumor suppressor miRNA that is down-regulated in numerous types of malignancies, such as gastric cancer, colorectal cancer, and breast cancer. This miRNA involves post- transcriptional regulation of numerous genes such as TGF-βR1, β-catenin, PD-L1, and NOTCH1 that have an effect on tumor cell proliferation, invasion, epithelial-mesenchymal transition (EMT) and immune response [22].

Studies have shown that miRNA can be used as a therapeutic target in malignancies [23]. CAF cells and their secreted exosomes in oral squamous cell carcinoma (OSCC) contain a low level of miRNA-34a, and transfection of this molecule into CAF cells can reduce the tumor genesis of OSCC cells. Therefore, according to previous studies, overexpression of miRNA-34a into CAF can be considered as a possible therapeutic strategy [24].

In this present study, miRNA-34a mimic was transfected into gastric CAF (GC-CAF) cells. Next, we evaluated the effect of miRNA-34a mimic overexpression in these cells on PD-L1 expression as an immune checkpoint molecule. Furthermore, the conditioned media of GC-CAF cells were collected after miRNA transfection. Then, we measured the effect of miRNA-34a transfected GC-CAF conditioned media on the immune response and viability of gastric cancer cell lines. The result of this study may provide new insight into more efficient strategies in cancer therapy.

Material and method

Sample collection

Tumor samples were obtained under sterile conditions from untreated patients with gastric cancer during an endoscopic examination at the Tuba Clinic (Mazandaran, Iran) (the informed consent was obtained from all of the participants). Sterile tubes containing Dulbecco's Modified Eagle Medium F12 (DMEM F12) supplemented with 10% penicillin/streptomycin (Biowest) and 10% amphotericin B (Capricorn, AAS-b, Republic of Germany) were used to transfer the samples. Samples were processed within a maximum time of 24 h after resection.

CAF isolation

Gastric cancer tissue samples were washed in PBS containing 10% penicillin/streptomycin and 10% amphotericin B for 30 min to remove yeast and bacterial contamination. Subsequently, the samples were cut into small pieces. Tissue fragments were digested with collagenase IV (1.5 mg/ml) (Gibco Cat, No: 17104-D19) at 37 ? for 35 min. Next, the suspensions were plated in DMEM F12 with 20% fetal bovine serum (FBS) (Biosera), 1% penicillin/streptomycin, and 1% amphotericin B. After 24 h, the medium was replaced with a fresh medium. After that, the medium was changed every 3 days. Cells typically appeared within one to two weeks after tissue culture.

CAFs are more sensitive than epithelial cells to trypsin; therefore, differential trypsinization was used to separate and purify cancer-associated fibroblast cells from cancer epithelial cells [25]. The cells were characterized by assessing of αSMA (alpha-smooth muscle actin) and EPCAM (Epithelial cell adhesion molecule) expression using real-time PCR. All cells used in this research were from 3 to 7 passages.

Cell lines culture

AGS and Kato3 (gastric cancer cell lines) were cultured in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin under optimal conditions of 37 ? and 5%

CO2. Additionally, HGF, a human gingival normal fibroblast cell line, was cultured in DMEM F12 with 10%FBS and 1% penicillin/streptomycin at 37 ? and 5% CO2.

RNA extraction and reverse transcription (cDNA synthesis)

Total RNA was extracted from cells using Trizol reagent (one-step RNA reagent, Bio Basic, Cat. No: BS410A) according to the manufacturer's instructions. The total RNA concentration was measured using a PicoDrop spectrophotometer.

The extracted RNA was treated with RNase free -DNase I (Thermo Scientific, #EN0521) according to the manufacturer's protocol to remove DNA contamination. CDNA synthesis was performed using a cDNA synthesis kit (Yektatajhiz Azma (YTA), Cat No: YT4500). MiRNA-34a stem-loop primer and U6-specific primer were used for the reverse transcription of miRNA-34a and U6 RNA, respectively. CDNA synthesis from other gene transcripts was performed using Oligo dT and random hexamer primers.

Quantitative real time (qRT) -PCR-

Quantification of miRNA-34a, αSMA, EPCAM, and PD-L1 transcript levels was performed by qRT-PCR analysis using SYBR Green qPCR mastermix 2x (Yektatajhiz Azma (YTA), Cat No: YT2551).

The U6 reference gene was used to normalize miRNA-34a expression, while the GAPDH reference gene was employed to normalize the expression of the other genes. Primers sequences are shown in Table 1.

Transfection of miRNA-34a mimic into CAF cells

CAF cells were seeded in the six-well plates at a density of 2×10^5 cells per well in DMEM F12 + 20% FBS + 1% penicillin/streptomycin medium. After 24 h, the medium was replaced with an antibiotic-free medium (DMEM F12 + 10% FBS). The cells were transfected with the miRNA-34a mimic or miR negative control (NC) at a 20 nM concentration, using Lipofectamin2000 (Invitrogen) according to the manufacturer's instructions. RT qPCR was performed to verify the transfection efficiency 48 h post-transfection.

Table 1 Quantitative real time PCR primers

Primer Name	Primer sequence
miRNA-34a stem-loop (RT)	GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACACAACC
U6 specific (RT)	GAATTTGCGTGTCATCCTTG
GAPDH	F: CATGAGAAGTATGACAACAGCCT R: AGTCCTTCCACGATACCAAAGT
PD-L1	F: GGTGGTGCCGACTACAAGCGA R: TGACTTCGGCCTTGGGGTAGC
αSMA	F: TGGCTATTCCTTCGTTACTACTGCT R: CATCAGGCAACTCGTAACTCTTCTC
EPCAM	F: AATCGTCAATGCCAGTGTACTT R: TCTCATCGCAGTCAGGATCATAA
U6	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTCAT
miRNA-34a	F: GGGATGGCAGTGTCTTAGC Universal R: GTGCAGGGTCCGAGGT

Preparation CAF conditioned medium (CM)

CAF cells were seeded in the six-well plate at a density of 2×10^5 cells per well and were transfected with miRNA34a mimic or miR negative control (NC) at 20nM concentration. Twenty-four hours after transfection, the culture medium was replaced with a new medium containing DMEM F12 without FBS in each well. After 48 h, the supernatant from each group was harvested and centrifuged at 3000 rpm for 10 min to remove cell debris. The conditioned medium was stored at -80 ? for future analysis.

Measurement of protein concentration in CAF's CM

The total protein concentration of CAF's CM in the transfected and NC groups was determined by the Bradford test using the Better Bradford Protein Assay Kit (Bio Basic INC, Cat. No: SK3041) according to the manufacturer's instructions.

Measurement of cytokine concentration using enzyme-linked immunosorbent assay (ELISA)

Peripheral blood mononuclear cells (PBMCs), from healthy donors (n:3) were activated with 1 µg/ml PHA and treated with miRNA-34a-transfected CAF CM or NC group CAF CM at the optimal protein concentration of 2.265 µg/ml for 72 h. Subsequently, the supernatant of the cells was collected, and concentrations of IL-4 and IFN-γ were determined using an ELISA kit (Parsgene) according to the manufacturer's instructions.

Cytotoxic T cells population measurements using flow cytometry method

The percentage of the CD8 + T cell population in PBMC cells from healthy donors was evaluated by flow cytometry after treatment with CAF CM in different groups. For this purpose, 72 h post-treatment, the PBMC cells were collected by centrifugation at 300 g for five minutes. Subsequently, the cells were stained with dual-color antibodies: anti-human CD3-FITC and anti-human CD4 RPE (Dakocytomation, cat. no FR881) in 100µL of phosphate buffered saline (PBS) supplemented with 1% Bovine Serum Albumin (BSA) according to the manufacturer's instructions. Next, the CD8 and CD3 double-positive cells were determined using flow cytometry. The results were analyzed withe FlowJo software 7.6.1 (Tree Star, Inc., San Carlos, CA, USA).

Cells viability assay

The viability of gastric cancer cell lines (AGS and Kato3) was assessed using the methyl thiazolyl tetrazolium test (MTT). Briefly, cancer cells were seeded in complete media (DMEMF12 + 10% FBS + 1% penicillin/streptomycin) at 2.5×10^3 cells/well in 96-well plates. Twenty-four hours later, the cells were treated with miRNA-34a-transfected CAF CM and NC CAF CM at the optimal protein concentration of 2.265 µg/ml for 48 h. Next, MTT solution at a final concentration of 0.5 mg/ml was added to each well, and incubation was performed for four hours at 37 ?. At the end of the incubation time, the medium was removed, and 200 µl of DMSO was added to dissolve the formazan crystal. Next, the plate was shaken for ten minutes. Finally, the absorbance of each well was measured using a microplate reader at 570 nm with a reference of 630 nm.

Additionally, we evaluated PBMC viability 72 h after treatment with CAF cell CM using the MTT test according to the above instructions.

Statistical analysis

An unpaired T-test was used to determine the statistical differences between the two groups. The unpaired ANOVA test was used to determine the statistical differences between the three groups. Normal data were expressed as mean ± SD. P-values < 0.05 were considered significant. Statistical analysis of data and plotting of graphs were performed using GraphPad Prism software version 6.

Result

Characterization of gastric cancer-associated fibroblast

We isolated primary gastric cancer-associated fibroblast cells from the gastric cancer tissue of a patient who had undergone an endoscopic operation in a sterile conditions. The CAF cells exhibited spindle-like morphology (Fig. 1a). The expression of αSMA (fibroblast activation marker) was significantly higher in CAF cells than in the human normal gingival fibroblast (HGF) cell line ($P < 0.05$). Additionally, neither of the cells (CAF and HGF cell line) expressed the EPCAM marker as an epithelial indicator, suggesting the successful isolation of CAF cells (Fig. 1b).

Overexpression of miRNA-34a in CAF cells decreased PD-L1 expression

We transiently transfected the miRNA-34a mimic into CAF cells. Forty eight hours post- transfections, we evaluated the transfection efficacy. The qRT-PCR results showed that miRNA-34a was overexpressed in these cells in comparison with the NC groups (P: 0.0027) (Fig. 2a). Next, we assessed the effect of miRNA-34a overexpression on its target gene, PD-L1, in CAF cells. MiRNA-34a

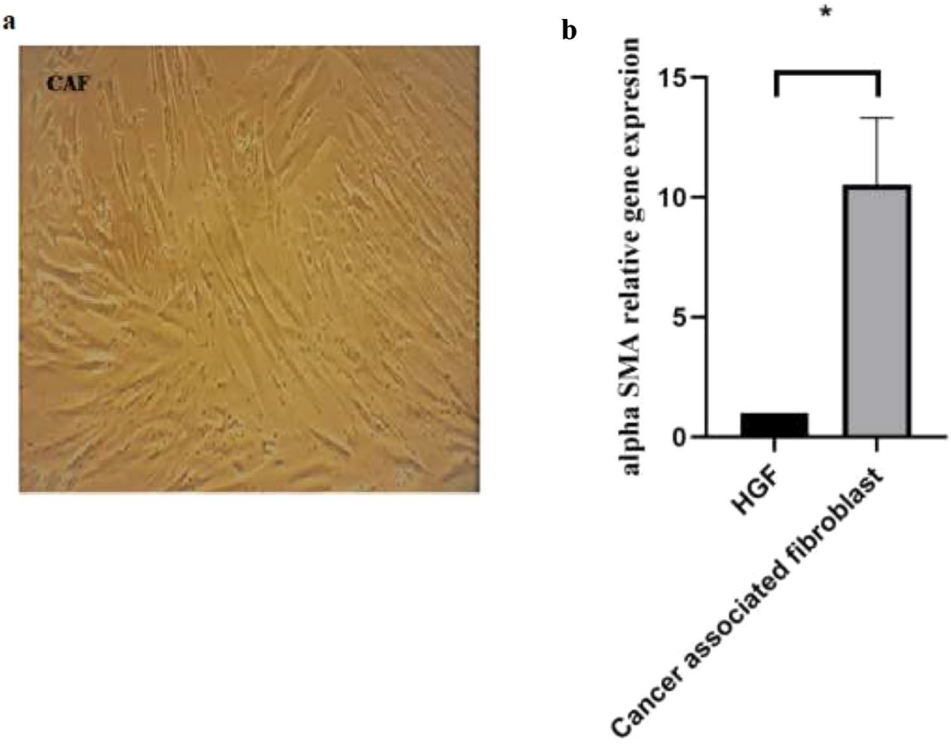

Fig. 1 Characterization of gastric CAF **a**: Gastric CAF showed spindle like morphology. **b**: Analysis of the expression of αSMA marker in CAF and HGF cells using real time PCR: CAF cells had higher level of αSMA than HGF($P < 0.05$)

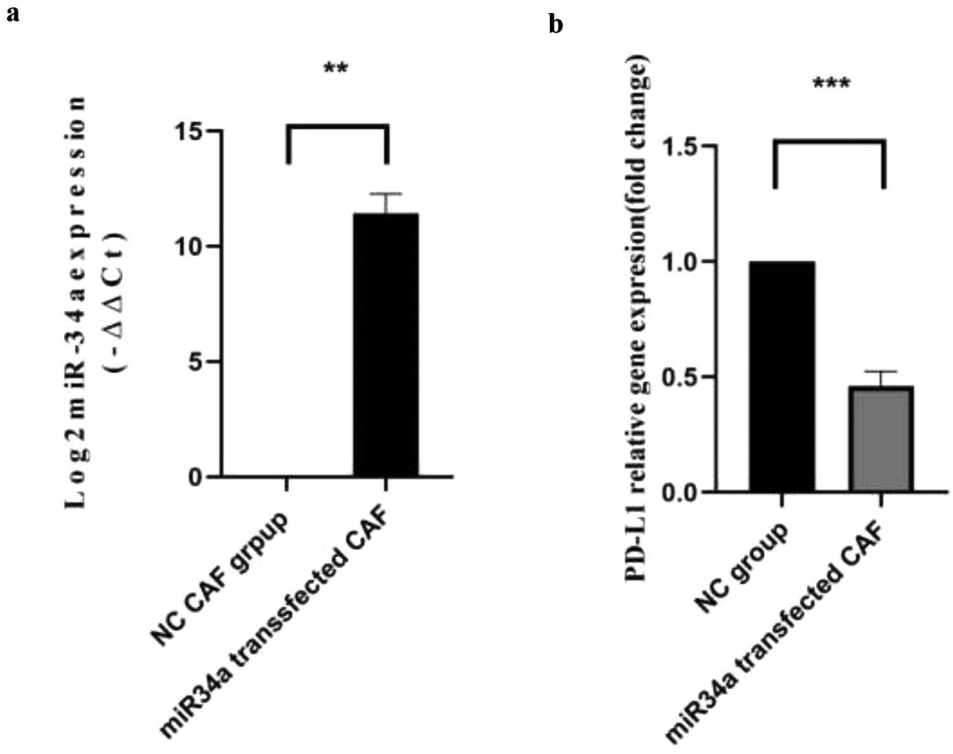

Fig. 2 miRNA-34a transfection in CAF cells miRNA-34a was transfected transiently in CAF cells in 20nM concentration. **a**: The results showed that miRNA-34a was increased in CAF cells relative to NC group (P:0.0027). **b**: MiRNA-34a significantly reduced PD-L1 expression relative to NC group (P:0.0001)

could significantly reduced PD-L1 expression in CAF cells compared to the NC group (P: 0.0001) (Fig. 2b).

miRNA-34a-transfected CAF CM did not affect the immune response

We measured the effect of CM from miRNA-34a-transfected CAF cell on PBMC proliferation, the percentage of CD8-positive T cells, and cytokine (IFN-γ and IL-4) secretion ability 72 h after treatment.

The PBMCs were activated with PHA (1 μg/ml) and treated with miRNA-34a-transfected CAF CM (test group) or miR negative control-transfected CAF CM (NC CAF CM) (control 2) for 72 h and results were compared with data from the group without CM treatment (Control 1).

Our observation showed that cytokine secretion (IL-4 and IFN-γ) and PBMC viability slightly higher in the control 2 group relative to the others (test and control 1), but the differences were not statistically significant($P > 0.05$) (Fig. 3a, b,c). In addition, miRNA-34a-transfected CAF CM did not influence the percentage of CD8-positive T cell population (Test) (24.5 ± 3.928) compared with the two other groups, the non-treated group (control 1) (23.65 ± 5.62) and the treated with NC CAF CM (control 2) (25.63 ± 2.281) (P:0.839) (Fig. 4a, b,c, d).

miRNA-34a-transfected CAF CM reduced gastric cancer cell lines viability

To determine the effect of CM from miRNA-34a-transfected CAF on the viability of gastric cancer cells, AGS and KATO3 cell lines were treated with CAF CM at optimal protein concentrations. Cell viability was measured using the MTT assay. The results revealed that NC CAF CM could significantly increase AGS cell line viability relative to the group without CM treatment (Fig. 5a, P:0.03). It also, improved KATO3 cell line viability, but the effect was not significant (Fig. 5b, P: 0.8). MiRNA-34a-transfected CAF CM significantly suppressed viability in AGS cell line compared to groups treated with NC CAF CM (Fig. 5a, $P < 0.05$, P:0.001) and the control group (Fig. 5a, P: 0.005). It also significantly reduced the viability of KATO3 cell lines relative to the group treated with NC CAF CM (Fig. 5b, P: 0.019).

Discussion

Our study is divided into two parts. In the first part, we measured the effect of miRNA-34a transfection in CAF cells on immune responses by evaluating of PD-L1 expression in these cells and the effect of conditioned media from miRNA-34a-transfected CAF on the CD8 -positive T cell population, as well as the secretion of IFN-γ and IL-4 cytokines in vitro.

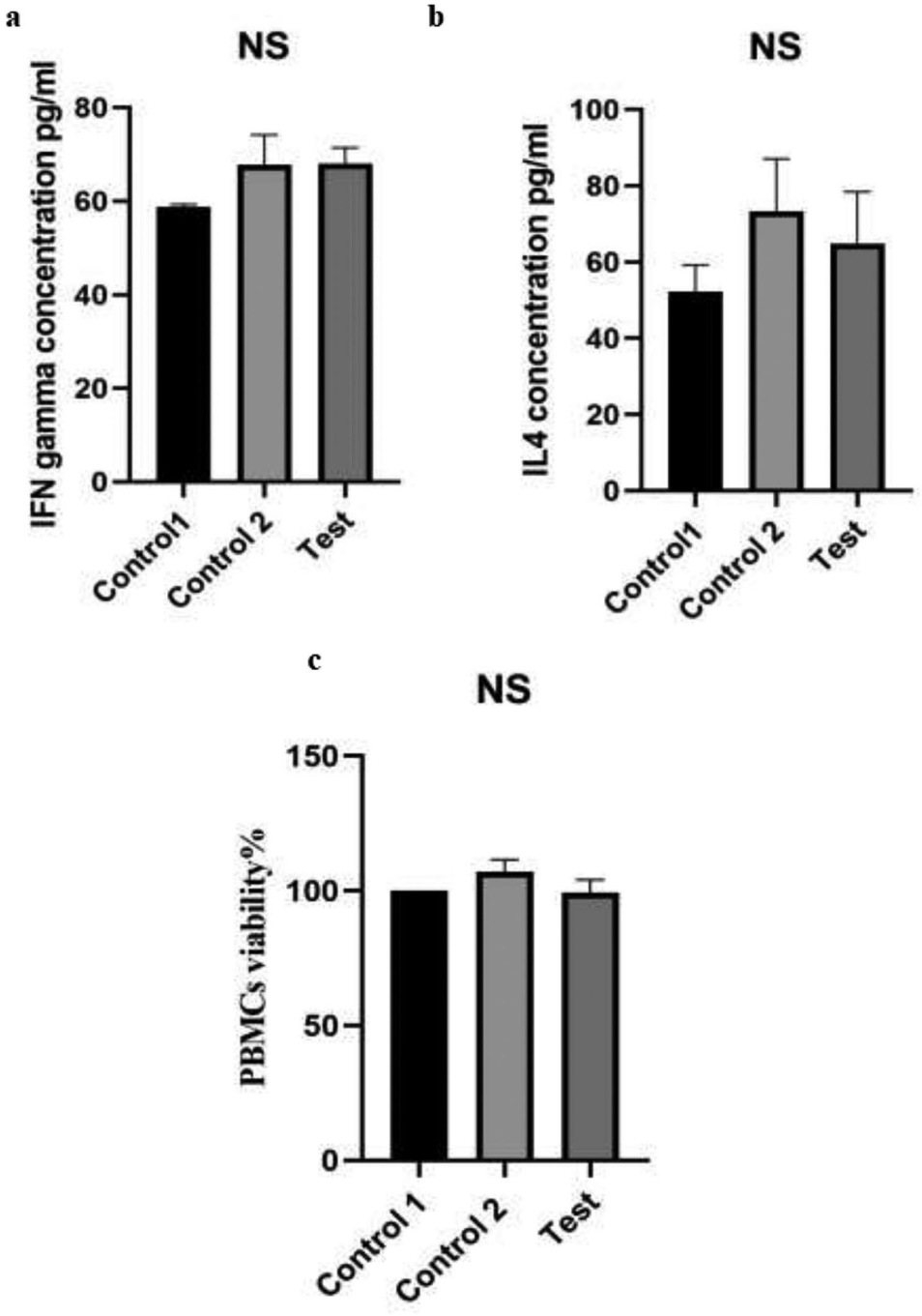

Fig. 3 Effect of CM from miRNA-34a transfected CAF on PBMCs cytokine secretion and viability. **a**, **b**: There wasn't significant differences in IFN gamma and IL-4 concentrations detected in PBMC supernatant after treatment with miRNA-34a transfected CAF and NC CAF CM (a: P:0.0183, b: p:0.339) **c**: There weren't differences in PBMC viability among different groups (P:0.087). Control 1: PBMC+ PHA, Control 2: PBMC+PHA+NC CAF CM, Test: PBMC+PHA+miRNA-34a transfected CAF CM

Our results showed that miRNA-34a transfection in CAF cells decreased PD-L1 expression in these cells ($P < 0.05$) (Fig. 2b).

Tumor cells use different mechanisms to suppress the immune response by inducing T cell exhaustion through the upregulation of immune checkpoint molecules, including, CTLA-4, LAG-3, PD-L1, ITIM and TIGIT [26].

PD-L1 is an immune checkpoint ligand that is often expressed in tumor cells. The binding of PD-L1 to PD-1 on T cells, activates inhibitory signals that can suppress T cell activity. Prevention of PD-1 and PD-L1 binding, leads

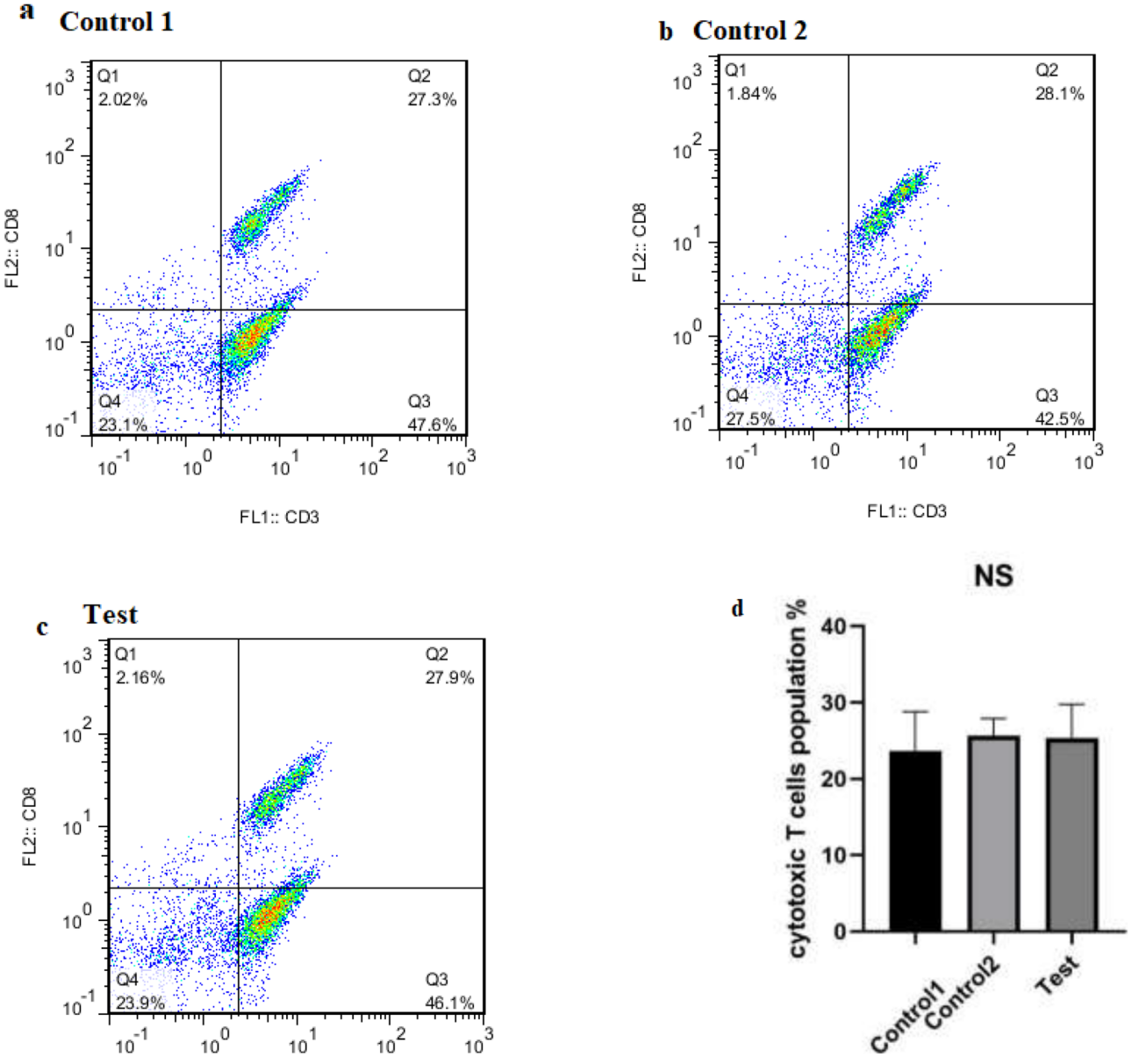

Fig. 4 Effect of miRNA-34a transfected CAF conditioned medium on percentage of cytotoxic T cell population. **a**, **b**, **c**, **d**: Forty-eight hours after treatment PBMCs with CAF conditioned medium we evaluated CD8 positive T cell populations in different groups. CM from miRNA-34a transfected CAF didn't influence on percentage of CD8 positive T cell population (Test) (24.5 ± 3.928) compared with two other groups, control 1 (23.65 ± 5.62) and control 2 (25.63 ± 2.281) (P:0.839). Control 1: PBMC+ PHA, Control 2: PBMC+NC CAF CM, Test: PBMC+miRNA-34a transfected CAF CM

to improved cytotoxic T cell function against tumor cells in several malignancies [27].

$CD8^+$ T cells play an important role in antitumor immunity. These cells recognize tumor cells via HLA class I, secreting cytokines such as TNF- α and IFN-γ, and releasing perforin and granulysin to eradicate tumor cells [28, 29].

MiRNA-34a targets PD-L1 mRNA, resulting in a reduction of PD-L1 [30, 31]. Additionally, the Epstein-Barr virus can increase PD-L1 expression by downregulating miRNA-34a in B-cell lymphoma [32].

PD-L1 expression in CAF cells suppresses the immune response and contributes to the poor prognosis and outcomes in several malignancies, such as esophageal cancer and non-small cell lung carcinoma [33, 34].

IL-1α/β from tumor cells in melanoma, induced PD-L1 and PD-L2 upregulation in CAF cells, contributing to the suppression of the immune response [35].

PD-L1 and PD-L2 molecules overexpression in fibroblast interact with the PD-1 receptors directly, and induce T cells exhaustion that leads $CD8^+$ T cells function abrogating [34–36]. Therefore, miRNA-34a in CAF

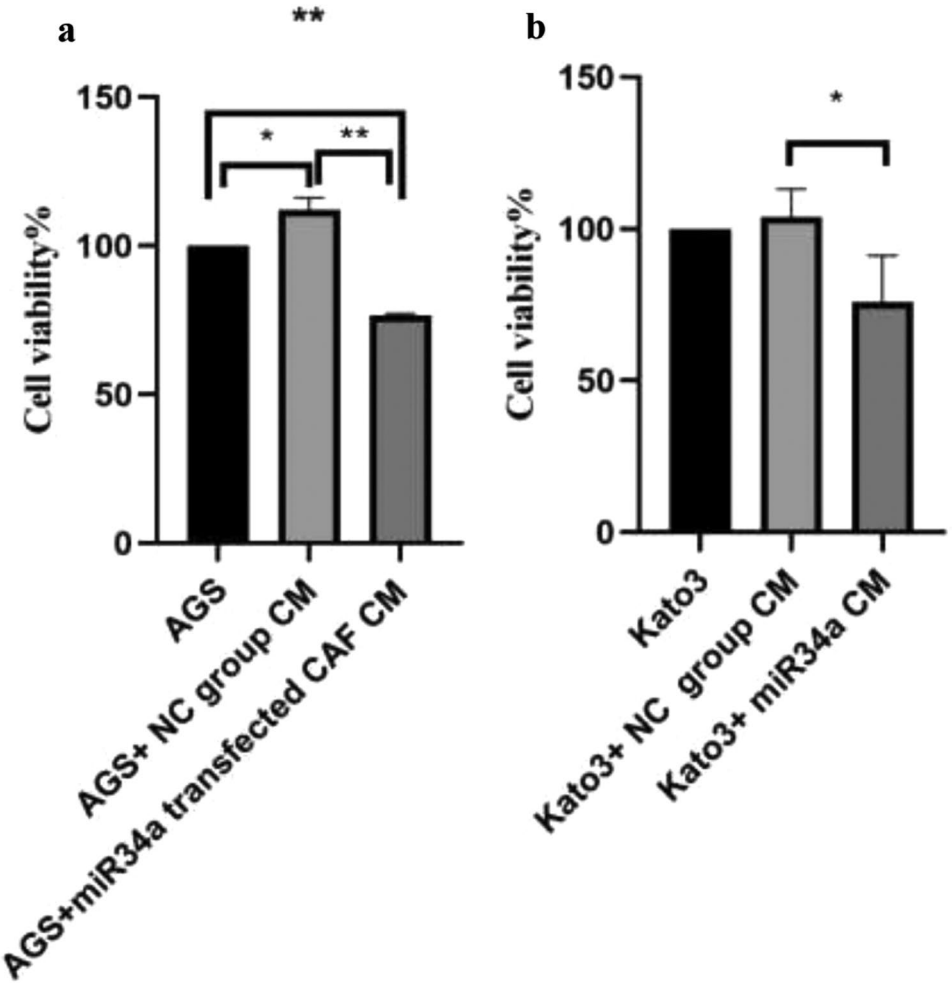

Fig. 5 **a**, **b**: NC CAF CM could significantly increase AGS cell lines viability relative to control group without CM treatment (P:0.03). Also NC CAF CM increased Kato3 cell lines viability but it's not a significant (P: 0.8). MiRNA-34a transfected CAF CM could significantly suppress viability in AGS cell lines compared with groups treated with NC CM (P:0.001) and control group (P: 0.005). Also it could significantly reduce Kato3 cell lines viability compared with group treated with NC CM (P: 0.019)

cells may enhance the immune response by reducing PD-L1 expression.

CAF cells can influence the tumor microenvironment and immune response through the secretion of various factors. Therefore, we also evaluated the effect of conditioned medium from miRNA-34a- transfected CAF cells on the immune response. After treating of PBMCs with CAF CM, the inhibitory effect of CAF CM on the immune response was not observed. Additionally, CM from miRNA-34a-transfected CAFs exhibited a slight inhibitory effect on PBMCs proliferation, cytokine secretion (IFN-γ, IL-4), and the percentage of CD8-positive T cells population. Surprisingly, the immune response was slightly more stimulated in a group treated with NC CAF CM than in, others, but it wasn't significant (Figs. 3a, b and c and 4a and b).

Previous investigations have shown that CAF cells play an important role in T cell polarization, proliferation, cytokine secretion, and possess an immunosuppressive effect through, both direct and indirect pathways [36]. Gorchs and colleagues reported that CAF cells from lung carcinoma maintain their immunosuppressive effect even after high-dose radiation [37]. Conversely, Nazareth and coworkers identified heterogeneous CAF populations in non-small cell lung carcinoma with a different effects on the immune response; one cell subpopulation can suppress the immune response via PD-L2 and PD-L1 expression and TGF-β secretion, while another cell subpopulation can enhance the immune response through direct pathways. However, the supernatant from these cells did not significantly influence the immune response [38].

MRX-34, a liposomal form of miRNA-34a, is the first miRNA to reach a Phase I clinical trial in cancer therapy. Results have shown that MRX-34, in addition to decreasing PD-L1 expression, can also increase $CD8^+$ T cell

infiltration and reduce regulatory T cell and macrophage infiltration in the tumor stroma. Moreover, suppressing the miRNA-34a expression in HBV^+ - hepatocellular carcinoma accelerates the production of CCL22, which enhances regulatory T cell infiltration in the tumor stroma [39, 40].

Conversely, a study showed that the overexpression of miRNA-34a in T cells can inhibit proliferation, activation, and effector function by modulating the NF- κB signaling pathway in these cells [41]. However, in the present study, significant differences were not detected in experiments related to the treatment of PBMCs with CM from miRNA-34a-transfected CAF cells compared to the control group.

In the second part, we evaluated the effect of CM from miRNA-34a transfected CAF cells on the proliferation of gastric cancer (GC) cell lines.

CAF cells contribute to the tumor progression by enhancing cancer cell invasion, metastasis, and angiogenesis through the secretion of various growth factors and ECM (extracellular matrix) degrading proteases [9, 42–45].

In this part, we have revealed that the treatment of gastric cancer cell lines (AGS, Kato3) with CM from miRNA-34a-transfected CAF significantly suppressed the proliferation rate of these cell lines (Fig. 5a and b). In previous reports, the transfection of miRNA-34a in gastric CAF and oral squamous cell carcinoma CAF could increase miRNA-34 in the secreted-exosomes of these cells. Subsequently, miRNA-34a transfected CAF cells can inhibit cancer cell growth and invasion via the transfer of miRNA34a to cancer cells through exosome secretion in-vitro and in-vivo [24, 45]. In contrast to the aforementioned studies, the present study used the supernatant of CAF cells which it contains exosomes and other additional molecules. Therefore, in our research, the effect of CAF CM may not be merely due to the secreted exosomes. In other words, the efficient role of other unknown molecules is inevitable for achieving these results.

The presence of CAF cells in the TME is associated with the poor prognosis of malignancies, Depletion of these cells in pancreatic cancers correlates with immune suppression due to an increased regulatory T cell population in the tumor stroma and reduces patient survival. Additionally, in the early stages of malignancy, normal fibroblast cells can inhibit tumor progression via paracrine signaling through soluble factors, cell– to - cell contact, and ECM-integrity [46, 47]. Therefore, complete removal of these cells is not a good option in cancer treatment. Likely, the best approach is to find a way to reprogram these cells. In this study, we showed that miRNA-34a in CAF cells can reduce tumor cell viability, and probably improve the immune response in malignancies by suppressing PD-L1 expression in CAF cells. Our results indicate that miRNA-34a may have the potential to serve as a novel therapeutic strategy in cancer treatment.

Abbreviations

CAF	Cancer associated fibroblast
miRNA	microRNA
PD-L1	Programmed cell death ligand 1
CM	Conditioned medium
TME	Tumor microenvironment
ECM	Extra-cellular matrix
UTR	Untranslated region
EMT	Epithelial-mesenchymal transition
OSCC	Oral squamous cell carcinoma
GC CAF	Gastric CAF
DMEM F12	Dulbecco's Modified Eagle Medium F12
FBS	Fetal bovine serum
αSMA	αsmooth muscle actin
EPCAM	Epithelial cell adhesion molecule
HGF	Human gingival fibroblast
PBMC	Peripheral blood mononuclear cell
NC	Negative control
PBS	Phosphate buffer saline
BSA	Bovine serum albumin
MTT	Methyl thiazolyl tetrazolium

Acknowledgements
This research was supported by the grant from mazandran-university of medical science, sari, Iran.

Author contributions
M E, performed whole research and wrote the manuscript N J, performed part of experiment and editing manuscript F A, data analysis S M V T, sample resection S A, Corresponding author.

Funding
Not applicable.

Data availability
All data generated or analyzed during this study are available from the corresponding author.

Declarations

Ethics approval and consent to participate
Our study adhered to the declaration of Helsinki. the study protocol was approved by the Ethics Committee at mazandaran University of Medical Sciences (Ethics Code: IR.RUMS.REC.1400.031) and we were obtained informed consent to participate from all of the participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 16 November 2024 / Accepted: 12 February 2025
Published online: 25 February 2025

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