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Evaluation of the Cytotoxic Effects of MicroRNA-34a: An Experimental Interference in Human Cancer Cell Lines

Mariam G. Saadeldin[a], Nashwa El-Khazragy[b*], Ahmed Abdo Elsherif[a], Azza M. El Amir[a]

[a] *Department of Biotechnology, Faculty of Science, Cairo University, Giza 12613, Egypt*

[b] *Department of Clinical Pathology-Hematology and AinShams Medical Research Institute (MASRI), Faculty of Medicine, Ain Shams University, Cairo 11566, Egypt*

Abstract

MicroRNA-34a (miR-34a) is recognized for its role as a tumor suppressor, influencing critical cellular processes such as cell cycle arrest, apoptosis, and senescence. Its dysregulation is linked to various cancers, making it a potential target for therapeutic intervention. This study investigates the cytotoxic effects of miR-34a in three human cancer cell lines: colorectal adenocarcinoma (Caco2), non-small cell lung cancer (NSLC), and hepatocellular carcinoma (HepG2). The research involved transfecting these cell lines with miR-34a mimic and inhibitor, followed by a 48-hour incubation and assessment of cytotoxicity through cell proliferation assays. Additionally, Notch1 gene expression was evaluated using Syber-green-based Real-time quantitative PCR (qPCR). Results showed that miR-34a mimic significantly reduced cell viability in all three cancer cell lines, with a 30% decrease in Caco2 cells. Conversely, the miR-34a inhibitor increased cell viability, with Caco2, A549, and HepG2 cells showing 16%, 11%, and 14% increases, respectively. The Notch1 gene expression was notably decreased with miR-34a mimic—an 8-fold reduction in Caco2, a 4-fold reduction in A549, and a 6-fold reduction in HepG2 cells. In contrast, miR-34a inhibitor led to a significant increase in Notch1 expression. These findings highlight miR-34a's potent cytotoxic and anti-proliferative effects through the downregulation of the Notch1 signaling pathway, suggesting its potential as a therapeutic target in cancer treatment.

Keywords: miR-34a; Hepatocellular carcinoma; Non-small cell lung cancer; Colorectal cancer; Cytotoxicity

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play a crucial role in the regulation of gene expression by targeting messenger [1, 2]. RNAs (mRNAs) for degradation or translational repression. Among the myriad of miRNAs, microRNA-34a (miR-34a) has garnered significant attention for its tumor-suppressive functions, acting as a critical regulator in various cellular processes[3, 4], including cell cycle arrest, apoptosis, and senescence[5]. Its dysregulation is often implicated in the pathogenesis and progression of several malignancies, making it a promising target for cancer therapeutics[6, 7].

The dysregulation of the microRNA-34 family has been demonstrated in various types of cancers associated with tumorigenesis[8], growth, and progression. For instance, microRNA-34a has been identified as a tumor suppressor in solid tumors, including lung cancer [9, 10], multiple myeloma[11], neuroblastoma, cervical carcinoma, breast cancer, and colon cancer [12, 13]. microRNA-34a promotes epithelial-to-mesenchymal transition (EMT)-mediated invasion and metastasis via feedback and feedforward loops with other microRNAs and proteins [14]. In addition, it has been suggested that microRNA-34a mimics were a novel potential therapeutic agent targeting multiple myeloma and pancreatic cancer [15].

The miR-34a/Notch1 signaling pathway plays a crucial role in cancer biology, where miR-34a acts as a tumor suppressor by targeting the Notch1 gene. Notch1 is a key regulator of cell fate decisions, influencing processes like cell proliferation, differentiation, and apoptosis.[16]. In many cancers, the Notch1 pathway is aberrantly activated, leading to uncontrolled cell growth and tumor progression. miR-34a suppresses this pathway by directly binding to the 3' untranslated region (UTR) of Notch1 mRNA, leading to its degradation or translational repression[17]. This inhibition of Notch1 reduces its oncogenic effects, thereby promoting cancer cell apoptosis and reducing proliferation. The miR-34a/Notch1 axis is, therefore, a critical mechanism through which miR-34a exerts its anti-cancer effects, and its modulation offers a potential therapeutic approach for targeting Notch1-driven malignancies. Understanding this pathway is vital for developing novel cancer treatments that leverage miR-34a's regulatory functions[17].

*Corresponding author e-mail: nashwaelkhazragy@med.asu.edu.eg (Nashwa El-Khazragy)

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Hepatocellular carcinoma (HCC)[18, 19], non-small lung carcinoma (NSLC) [20], and colorectal cancer (CRC) [21] are among the most prevalent and lethal forms of cancer worldwide, characterized by their aggressive nature, resistance to conventional therapies, and poor prognosis [22]. The aberrant expression of miR-34a in these cancers suggests its potential as a therapeutic agent, either alone or in combination with other treatments, to enhance cytotoxicity towards cancer cells while sparing normal cells [22-24].

The current study aims to evaluate the cytotoxic effects of miR-34a in human cancer cell lines, specifically HCC, NSLC, and CRC, by employing experimental interference techniques. Through the restoration of miR-34a levels in these cancer cell lines, this study seeks to elucidate the mechanistic pathways through which miR-34a exerts its tumor-suppressive effects, including the induction of apoptosis, inhibition of cell proliferation and modulation of key signaling pathways involved in cancer progression.

Additionally, the study explores the potential of miR-34a as a biomarker for early detection and prognosis of these cancers, given its pivotal role in tumor suppression. Understanding the differential expression and function of miR-34a across various cancer types will provide valuable insights into its application in precision oncology. By comprehensively analyzing the cytotoxic effects of miR-34a, this research could pave the way for novel therapeutic strategies aimed at improving clinical outcomes in patients with HCC, NSLC, and CRC, potentially offering a new avenue for targeted cancer therapy.

2. Methods

2.1. Passaging & Propagation of cancer cells:

Human colorectal adenocarcinoma (Caco-2), adenocarcinoma alveolar basal epithelial (A549), and Caco-2 cell lines, all obtained from ***Nawah Scientific*** (Cairo, Egypt), were cultured and passaged at least three times to reach the log phase. The procedure began by passaging pre-cultured cancer cells in an Airflow Cabinet (Biosafety level II), ***Smart Fast, Italy***. Media containing dead cells was removed from the culture flask, and the remaining cells were washed with 5 ml of Phosphate buffer saline (PBS), ***Gibco, Thermosientific, USA***, ensuring thorough coverage of the flask. After aspirating the PBS, 1 ml of Trypsin-EDTA, ***Gibco, Thermosientific, USA*** was added to detach the cells, and the T- 75 culture flask was incubated in a CO2 incubator, CB210 CO2 Mammal Cells incubator, ***Binder, Germany*** for 5 minutes. Next, 10 ml of serum-containing media *Dulbecco's Modified Eagle's Medium* (DMEM), ***Gibco, Thermosientific, USA,*** was added to a T-75 culture flask. The detached cells were collected in a 15 ml falcon tube and centrifuged at 800 rpm for 5 minutes. After centrifugation, the supernatant was aspirated, and the cell pellet was resuspended in 15 ml of fresh culture media. The cells were sub-cultured in a new flask containing DMEM media, 1% penicillin-streptomycin, and 1% Fetal Bovine serum (FBS), ***Gibco, Thermosientific, USA***. When the cells reached 70-75% confluence, they were harvested using 0.25% trypsin EDTA, incubated for 5-10 minutes at 37°C in 5% CO2, and then observed under a microscope. The cells were centrifuged at 800xg for 10 minutes, the supernatant was discarded, and the pellet was washed twice with PBS. Finally, the pellet was resuspended in 1 ml of media, and the cell count was adjusted to 1 x 10^6 cells [25].

2.2. Transfection of Adherent Cells with miRNA 34a mimic in a 96-well plate

The protocol outlines the transfection of adherent cancer cells with miRNA-34a mimic and inhibitor in a 96-well plate to evaluate the impact of miRNA-34a on cancer cell elimination. The assay aims to mimic or inhibit miRNA-34a action across three cancer cell lines to determine its efficacy in inducing cell death. Before transfection, 0.4-1.6 × 10^3 cells were seeded per well in a 96-well plate, with 150 µl of Roswell Park Memorial Institute media (RPMI-1640), ***Sigma-Aldrich, USA*** culture medium 1% Penicillin /Streptomycin antibiotics. The cells were incubated under standard conditions (37°C, 5% CO2). For each well, 0.15 µl of miR-34a mimic (***Syn_hsa_miR -34a-5p, cat no: MSY0000255)*** or 0.5 µl of miR-34a inhibitor (***Anti_hsa_miR-34a-5p, cat no: MIN0000255), Qiagen, Hilden, Germany*** was mixed with 3 µl of RNase-free water, forming a complex. This mixture was added to the wells to achieve final concentrations of 5 nM for the mimic and 50 nM for the inhibitor. Subsequently, 0.75 µl of HI Perfect Transfection Reagent, cat no: 301704, ***Qiagen, Hilden, Germany,*** was combined with 24.25 µl of RPMI media (without serum) and incubated at 15-20°C for 10 minutes to form transfection complexes. These were then added to the cells, followed by 175 µl of DMEM media. The cells were incubated for 5-10 minutes at room temperature to allow the formation of the transfection complex. The mixture was gently added to the cells, ensuring even distribution. The cells were then incubated under normal growth conditions. For validation, AllStars siRNA negative control, ***Qiagen, Hilden, Germany,*** was used, and un-transfected cells served as a baseline for untreated negative control cells [26].

2.3. Assessment of cell viability by cell proliferation assay (MTT)

The cell cytotoxicity assay was performed using the ***Vybrant® MTT Cell Proliferation Assay Kit***, cat no: M6494 (***Thermo Fisher, Germany***). The cell viability was tested in the cancer cells, transfected with miR-34a mimic or inhibitor, using the cell proliferation assay. At the end of incubation, 100µL of media was removed and replaced by new media. Twenty µL of *4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT) solution (1mg/mL) (***Invitrogen, ThermoScientific, Germany***) was added to each well and the plates were incubated at 37 °C and 5% CO2 for four hours. Finally, the MTT solution was removed, and 100 µL of sodium dodecyl sulfate with hydrochloric acid (SDS-HCL) was added to the wells [27]. Cell viability was determined by measuring the optical density at 570 nm on a spectrophotometer (***ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA***).

2.4. Gene expression analysis by Real-time PCR

2.4.1. Total and miRNAs extraction and purification

Total mRNA and miRNAs were extracted using miRNeasy Serum/Plasma Advanced Kit, cat no: 217204, ***Qiagen, Hilden, Germany***. Total mRNA and miRNAs were extracted using ***miRNeasy Serum/Plasma Advanced Kit***, cat no: 217204 (**Qiagen, Hilden, Germany**) [28, 29]. The miRNeasy Serum/Plasma Kit was used to isolate total RNA from samples using a combination of phenol/guanidine-based lysis and silica-membrane purification. The kit's QIAzol Lysis Reagent, a mixture of phenol and guanidine thiocyanate, effectively lyses cells, denatures proteins, and removes DNA and proteins from the lysate through organic extraction. The protocol begins with thawing frozen cell lines, followed by the addition of five volumes of QIAzol Lysis Reagent to the samples, which are mixed thoroughly. The lysate is incubated at room temperature for 5 minutes before adding 3.5 µl of RNeasy Serum/Plasma Spike-In Control, which is thoroughly mixed. An equal volume of chloroform is added to the lysate, shaken vigorously, and incubated for 2-3 minutes. The samples are then centrifuged at 12,000 x g for 15 minutes at 4°C in Cooling centrifuge 1-8 (D-37520), ***Sigma-Aldrich, USA***, resulting in three phases: an upper aqueous phase containing RNA, a white interphase, and a lower red organic phase. The aqueous phase is carefully transferred to a new tube, avoiding the interphase, and mixed with 1.5 volumes of 100% ethanol. The mixture is then passed through a RNeasy MinElute spin column and centrifuged. The column is washed sequentially with Buffer RWT, Buffer RPE, and 80% ethanol, followed by a final spin to dry the membrane. RNA is eluted by adding 14 µl of RNase-free water to the spin column, and the eluate is collected after centrifugation. For higher RNA concentration, a smaller volume of elution water can be used, although the yield decreases slightly.

2.4.2. cDNA synthesis by Reverse Transcription

Total RNA was reversibly transcribed using a miScript II RT kit, cat. No. 218161, Qiagen, Hilden, Germany [30]. First, template RNA is thawed on ice, and the necessary components, including 10x miScript Nucleics Mix, RNase-free water, and 5x miScript HiSpec Buffer, are thawed at room temperature (15–25ºC). All solutions are mixed by flicking the tubes and briefly centrifuged to collect any residual liquid, then kept on ice. A reverse transcription master mix is prepared on ice, containing all the components required for first-strand cDNA synthesis except for the template RNA. The template RNA is then added to each tube containing the master mix, mixed gently, centrifuged briefly, and stored on ice. The mixture is incubated at 37ºC for 60 minutes to allow the synthesis of cDNA. Following this, the reaction is incubated at 95ºC for 5 minutes to inactivate the miScript Reverse Transcriptase. The resulting undiluted cDNA is then transferred to a -20ºC freezer for storage. This protocol ensures the efficient synthesis of cDNA, suitable for subsequent analyses.

2.4.3. Measurement of Notch1 gene expression in transfected cancer cells

Relative expression of the Notch1 gene was amplified by using Quantitect SYBR Green PCR Kit, cat. No. 218300, ***Qiagen, Hilden, Germany***, and Quantitect Primer Assay, cat no: 249900 for *Notch1" Hs_NOTCH1,* assay ID: QT00231056", and ***Hs_ACTB_1_SG*** QuantiTect Primer Assay ***(β-actin)*** cat no: 249900, as housekeeper gene. All samples were analyzed using the 5-plex Rotor-Gene Real-Time PCR Analyzer (Qiagen, Germany)[31].

The components for the PCR reaction, including 2x QuantiTect SYBR Green PCR Master Mix, 10X primer assay, template cDNA, and RNase-free water, were thawed at room temperature (15–25ºC). A reaction mixture was prepared with a final volume of 20 µl per well. Each well contained 10 µl of 2x QuantiTect SYBR Green PCR master mix, 2 µl each of 10x primer assay, 4 µl of RNase-free water, and 2 µl of template cDNA. The PCR plate was then sealed with an adhesive sheet. The PCR cycling protocol involved an initial activation step at 95ºC for 15 minutes, followed by 40 cycles of denaturation at 94ºC for 15 seconds, annealing at 55ºC for 30 seconds, and extension at 70ºC for 30 seconds. The cycle threshold (CT) is determined during the first cycle, where there is a significant increase in fluorescence above the background level, which is inversely proportional to the initial quantity of the target sequence of the PCR product[32].

2.5. Statistical Analysis

The collected data was reviewed, coded, organized, and entered into a computer using **GraphPad Prism version 8.0.0 for Windows**, developed by GraphPad Software in ***San Diego, California, USA*** (www.graphpad.com). Data was presented and analyzed based on the type of data collected for each parameter. To ensure the reproducibility and reliability of the experimental results, each biological sample was processed in triplicates. For all assays, three independent replicates were performed under identical conditions to account for potential variability and to strengthen the statistical significance of the findings. Data from these triplicates were analyzed, and the mean values along with standard deviations were calculated to reflect the consistency of the results. Descriptive statistics included the mean, standard deviation (± SD), and range for parametric numerical data, while the median and interquartile range (IQR) were used for non-parametric numerical data. Analytical statistics involved the use of the unpaired Student's t-test to compare differences between two independent groups. A one-way ANOVA was conducted to identify statistical differences between multiple groups (e.g., control and treated groups), followed by Dunnett's post-test for specific comparisons. The level of significance was determined with p-values, where ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ indicated increasing levels of significance, while $p > 0.05$ was considered non-significant (NS).

3. Results

3.1. Comparative analysis for the cytotoxic effect of miR-34a mimic and inhibitor in Caco-2, A549, and HepG2 cells

This study evaluated the in vitro cytotoxic effects of miR-34a on three cancer cell lines: Caco-2 (colorectal), A549 (lung), and HepG2 (liver). The cells were transfected with either a miR-34a mimic to elevate miR-34a levels or a miR-34a inhibitor to reduce them, and cell viability was assessed using the MTT assay. The results demonstrated that the miR-34a mimic significantly decreased the viability of all three cancer cell lines compared to untreated controls, with a 30% reduction

observed in Caco-2 cells (**Table 1, Fig. 1**). In contrast, the miR-34a inhibitor significantly increased cell viability (**Table 2, Fig.2**), with Caco-2 showing a 16% increase, A549 an 11% increase, and HepG2 a 14% increase, compared to untreated cells (**Table 3**, **Fig. 3**). These findings indicate that miR-34a exerts a strong cytotoxic effect on these cancer cell lines, suggesting that enhancing miR-34a levels could be a promising therapeutic approach, while inhibiting miR-34a may enhance cancer cell survival.

Table 1: Descriptive analysis for the Coco-2 cell viability after transfected with miR-34a mimic or inhibitor for 48 hours

	mean±SD	Range	Statistics
Caco-2 cells	100±3.79	95.8 – 103	F: 80.88
Mock cells	95.9±4.36	91.5 - 100	P-value: <0.0001 [HS]
Anti_hsa_miR-34a-5p	116±1.09	115 - 117	
Syn_hsa_miR -34a-5p	70.6±4.29	66.6 – 75.1	

F: ANOVA test value, ANOVA: Analysis of variances, HS: High significant difference

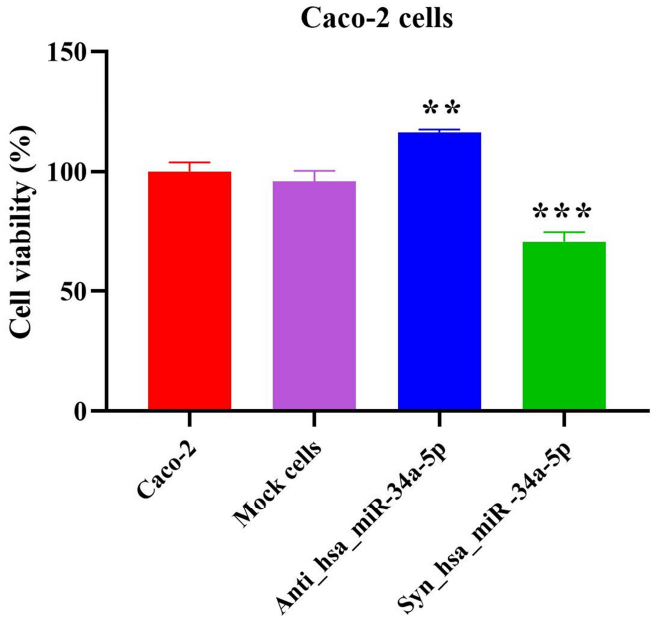

Figure 1: Bar chart illustrating a significant reduction in the percentage of cell viability of Caco-2 cells after treatment with miR-34a inhibitor, while a significant increase in % of cell viability in Caco-2 cells following transfection with miR-34a mimic ($p<0.01$), compared to untreated cells

Table 2: Descriptive analysis for the A549 cell viability after transfected with miR-34a mimic or inhibitor for 48 hours

	mean±SD	Range	Statistics
A549 cells	100±4.58	96.6 – 105	F: 95.83
Mock cells	94.6±3.89	90.1 – 96.9	P-value: <0.0001 [HS]
Anti_hsa_miR-34a-5p	111±3.64	107 - 114	
Syn_hsa_miR -34a-5p	54.9±5.01	49.2 – 58.5	

F: ANOVA test value, ANOVA: Analysis of variances, HS: High significant difference

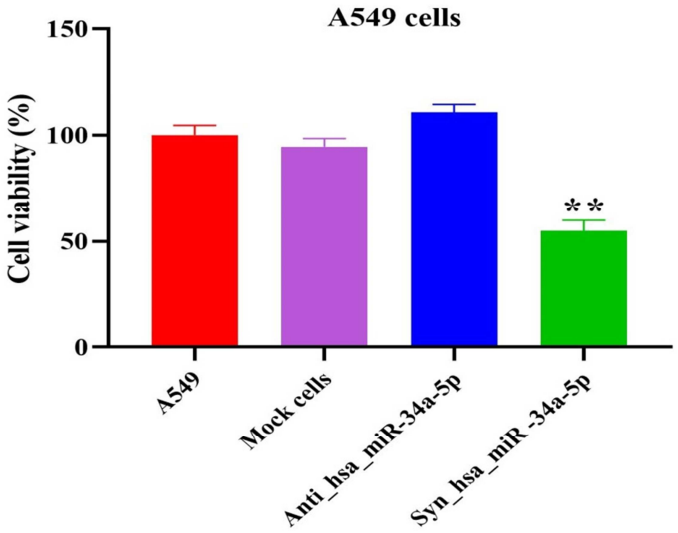

Figure 2: Bar chart illustrating a significant reduction in the percentage of cell viability of A549 cells after treatment with miR-34a inhibitor, while no significant change was observed in % of cell viability in A549 cells following transfection with miR-34a mimic ($p<0.001$), compared to untreated cells

Table 3: Descriptive analysis for the HepG2 cell viability after transfected with miR-34a mimic or inhibitor for 48 hours

	mean±SD	Range	Statistics
HepG2 cells	100±2.46	97.8 – 103	F: 208.4
Mock cells	98.1±1.80	96.5 – 100	P-value: <0.0001 [HS]
Anti_hsa_miR-34a-5p	114±3.67	111 - 118	
Syn_hsa_miR -34a-5p	65.9±1.13	64.8 – 67.1	

F: ANOVA test value, ANOVA: Analysis of variances, HS: High significant difference

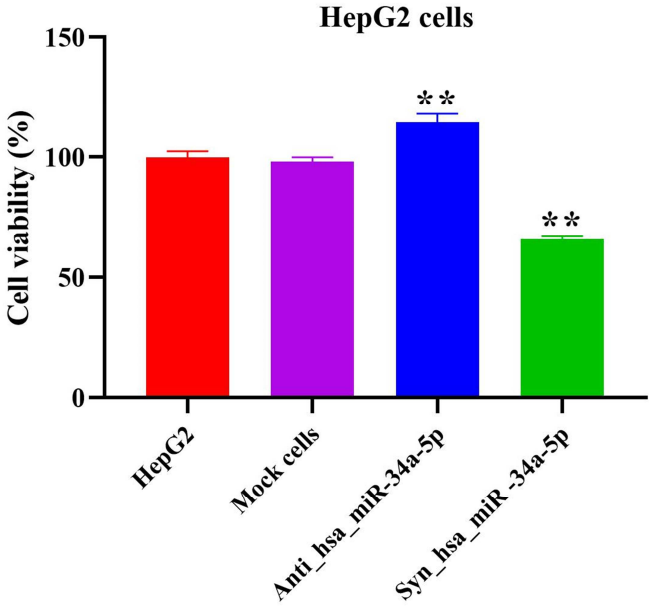

Figure 3: Bar chart illustrating a significant reduction in the percentage of cell viability of HepG2 cells after treatment with miR-34a inhibitor, while no significant change was observed in % of cell viability in HepG2 cells following transfection with miR-34a mimic ($p<0.001$), compared to untreated cells

3.2. Comparative analysis for the expression level of Notch1 gene "miR-34 target" in Cancer cells after transfection with miR-34a mimic and inhibitor

To investigate the genotoxic effect of miR-34a on three cancer cell lines "Caco2, A549, and HepG2", the Notch1 gene "cell proliferative gene," known as a direct target for miR-34a was measured in cancer cells after being transfected with miR-34a mimic or inhibitor for 48 hours using Quantitative Real-time PCR. The gene expression was determined as fold change after normalizing it to the expression level in untreated "unstimulated" cells. **Tables 4, 5, 6 Fig. 4, 5, and 6 present the Notch1 gene expression (fold change) in Caco2, A549, and HepG2 cells,** respectively.

The results show that the miR-34a mimic significantly reduced the expression of Notch1 in all three cancer cell lines, with an 8-fold decrease in Caco-2 cells **(Table 4 and Fig. 4)**, a 4-fold decrease in A549 cells **(Table 5 and Fig. 5)**, and a 6-fold decrease in HepG2 cells **(Table 6 and Fig. 6)**, compared to untreated controls. In contrast, the miR-34a inhibitor led to a significant increase in Notch1 expression, with a 7-8-fold increase in Caco-2 cells, a 3-4-fold increase in A549 cells, and a 5-6-fold increase in HepG2 cells. These findings suggest that miR-34a exerts its cytotoxic effects, at least in part, by downregulating the expression of the Notch1 gene, a key regulator of cell proliferation. Inhibiting miR-34a, on the other hand, appears to increase Notch1 expression, which may contribute to the observed increase in cancer cell viability.

Table 4: Descriptive analysis for the Notch1 gene expression in Caco-2 cells after transfected with miR-34a mimic or inhibitor for 48 hours

	mean±SD	Range	Statistics
Caco-2cells	1.03±0.32	0.73 – 1.38	F: 145.7
Anti_hsa_miR-34a-5p	8.01±1.02	6.92 – 8.94	P-value: <0.0001 [HS]
Syn_hsa_miR -34a-5p	0.13±0.03	0.104 – 0.160	

F: ANOVA test value, ANOVA: Analysis of variances, HS: High significant difference

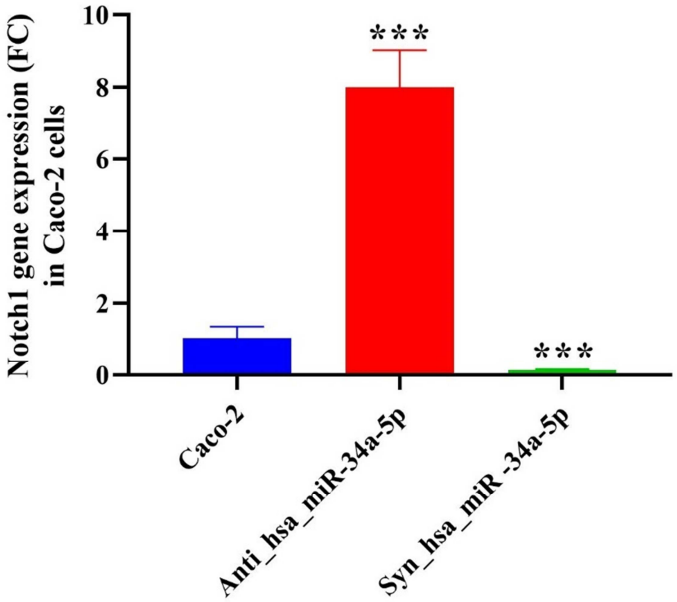

Figure 4: Bar chart illustrating a significant reduction in Noch1 gene expression (Fold change) in Caco-2 cells after treatment with miR-34a mimic ($p<0.001$), while a significant increase was observed in the expression of Noch1 gene in Caco-2 cells following transfection with miR-34a inhibitor ($p>0.05$), compared to untreated cells

Table 5: Descriptive analysis for the Notch1 gene expression in A549 cells after transfected with miR-34a mimic or inhibitor for 48 hours

	mean±SD	Range	Statistics
A549 cells	1.02±0.26	0.84 – 1.32	F: 39.0
Anti_hsa_miR-34a-5p	4.0±0.92	3.03 – 4.86	P-value: <0.0004 [HS]
Syn_hsa_miR -34a-5p	0.23±0.07	0.14 – 0.29	

F: ANOVA test value, ANOVA: Analysis of variances, HS: High significant difference

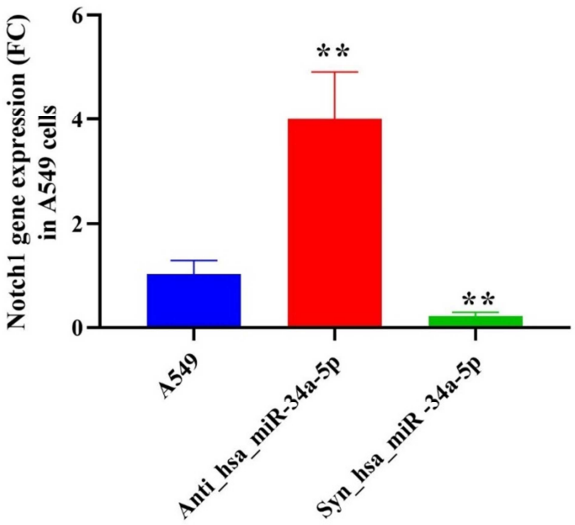

Figure 5: Bar chart illustrating a significant reduction in Noch1 gene expression (Fold change) in A5492 cells after treatment with miR-34a mimic ($p<0.001$), while a significant increase was observed in the expression of Noch1 gene in A549 cells following transfection with miR-34a inhibitor ($p>0.05$), compared to untreated cells.

Table 6: Descriptive analysis for the Notch1 gene expression in HepG2 cells after transfected with miR-34a mimic or inhibitor for 48 hours

	mean±SD	Range	Statistics
HepG2 cells	1.07±0.48	0.65– 1.59	F: 180
Anti_hsa_miR-34a-5p	6.1±0.494	5.66 – 6.64	P-value: <0.0001 [HS]
Syn_hsa_miR -34a-5p	0.49±0.03	0.46 – 0.511	

F: ANOVA test value, ANOVA: Analysis of variances, HS: High significant difference

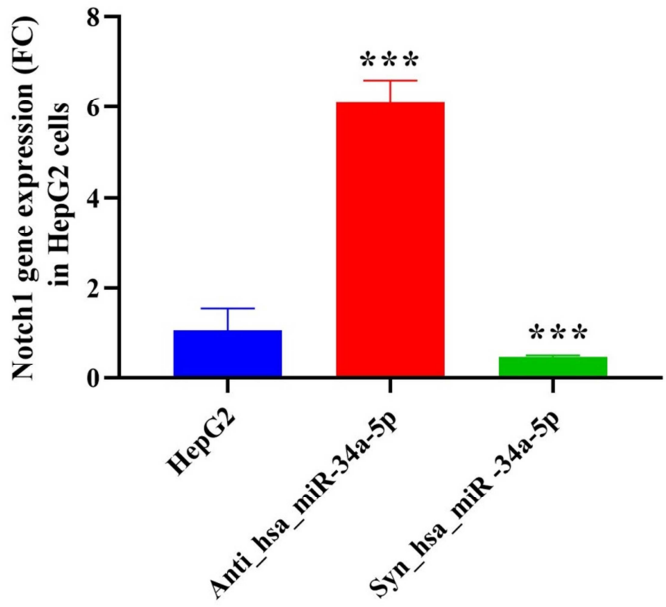

Figure 6: Bar chart illustrating a significant reduction in Noch1 gene expression (Fold change) in HepG2 cells after treatment with miR-34a mimic ($p<0.001$), while a significant increase was observed in the expression of Noch1 gene in HepG2 cells following transfection with miR-34a inhibitor ($p>0.05$), compared to untreated cells

4. Discussion

The present study aimed to investigate the cytotoxic and anti-proliferative effects of miR-34a in three different human cancer cell lines - Caco-2 (colorectal carcinoma), A549 (non-small cell lung carcinoma), and HepG2 (hepatocellular carcinoma) - and to elucidate the underlying molecular mechanisms, with a focus on the regulation of the Notch1 signaling pathway. The results demonstrate that modulation of miR-34a levels significantly impacts the viability, proliferation, and colony-forming capacity of these cancer cells, suggesting miR-34a could be a promising therapeutic target.

The MTT assay results revealed that the miR-34a mimic potently reduced the viability of all three cancer cell lines compared to untreated controls. In Caco-2 cells, the miR-34a mimic decreased viability by around 30%, in A549 cells by 45%, and in HepG2 cells by 34%. Conversely, the miR-34a inhibitor led to a significant increase in cancer cell viability, with Caco-2 showing a 16% increase, A549 an 11% increase, and HepG2 a 14% increase compared to untreated cells.

These findings are consistent with a growing body of evidence demonstrating the tumor-suppressive role of miR-34a in various cancer types. miR-34a has been well-characterized as a key regulator of cell cycle progression, apoptosis, epithelial-mesenchymal transition, and other critical cellular processes dysregulated in cancer **[**12**,** 33**].** The observed cytotoxic effects of the miR-34a mimic are likely due to its ability to target and downregulate the expression of numerous oncogenes and pro-survival factors [34].

For instance, miR-34a has been shown to directly target and repress the expression of the anti-apoptotic protein Bcl-2, leading to increased apoptosis in cancer cells **[**35**,** 36**]**. Additionally, miR-34a can inhibit the expression of c-MYC, CDK4/6, and other cell cycle regulators [37], thereby inducing cell cycle arrest and suppressing proliferation [9]. The miR-34a-mediated downregulation of EMT-inducing transcription factors such as Snail, Slug, and Zeb1 has also been linked to its anti-metastatic effects [38-40].

The contrasting effects observed with the miR-34a inhibitor further highlight the crucial tumor-suppressive role of this miRNA. By blocking the endogenous miR-34a activity, the inhibitor likely allows for the increased expression of miR-34a target genes, thereby promoting cancer cell survival and proliferation. These results are in line with previous studies demonstrating that downregulation or loss of miR-34a is frequently observed in various cancer types and is associated with poor prognosis [41]

To elucidate the molecular mechanisms underlying the cytotoxic effects of miR-34a, the researchers investigated the expression of the Notch1 gene, a known target of miR-34a that plays a crucial role in regulating cell proliferation, survival, and differentiation[42].

The qRT-PCR analysis revealed that the miR-34a mimic significantly reduced the expression of Notch1 mRNA in all three cancer cell lines. In Caco-2 cells, the miR-34a mimic decreased Notch1 expression by around 8-fold, in A549 cells by 4-fold, and in HepG2 cells by 6-fold, compared to untreated controls. Conversely, the miR-34a inhibitor led to a significant increase in Notch1 mRNA levels, with a 7-8-fold increase in Caco-2 cells, a 3-4-fold increase in A549 cells, and a 5-6-fold increase in HepG2 cells.

The Notch signaling pathway is known to play a pivotal role in regulating various cellular processes, including cell fate determination, proliferation, survival, and differentiation [11, 43]. Aberrant activation of the Notch pathway has been implicated in the pathogenesis of numerous cancers, including those investigated in this study [44]. The Notch1 receptor, in particular, has been shown to promote tumor growth, angiogenesis, and metastasis in various cancers [45].

The present study's findings demonstrate that miR-34a can effectively downregulate Notch1 expression at both the mRNA and protein levels in the investigated cancer cell lines. This miR-34a-mediated repression of Notch1 is likely a key mechanism by which miR-34a exerts its anti-cancer effects. By targeting and inhibiting the Notch1 signaling pathway, miR-34a can disrupt critical cellular processes that drive tumor initiation, progression, and metastasis.

miR-34a directly targets Notch1, a key player in the Notch signaling pathway, which is crucial for cell differentiation, proliferation, and apoptosis. miR-34a binds to the 3' untranslated region (3'UTR) of Notch1 mRNA, leading to its degradation or translational repression, thereby reducing Notch1 protein levels. The suppression of Notch1 diminishes its downstream signaling, particularly affecting the transcription of genes involved in cell survival and proliferation. According to Kai et al., 2021, NOTCH-1 was the target gene of miR-34a in Chronic heart disease, as confirmed by dual luciferase reporter assay[46]. In addition, it was demonstrated that miR-34a loaded on nanoparticles can downstream Notch1 in Triple-negative breast cancer [47]

The inverse relationship observed between miR-34a levels and Notch1 expression is consistent with previous studies reporting the direct targeting of Notch1 by miR-34a [48]. Furthermore, the upregulation of Notch1 in response to miR-34a inhibition suggests that endogenous miR-34a plays an important role in maintaining Notch1 homeostasis in these cancer cells [49]

The comprehensive results of this study suggest that the regulation of the Notch1 signaling pathway is a key mechanism by which miR-34a exerts its cytotoxic and anti-proliferative effects in the investigated cancer cell lines.

The observed downregulation of Notch1 gene expression in response to the miR-34a mimic indicates that miR-34a can directly target and suppress Notch1 in these cancer cells. Numerous studies have previously reported the direct targeting of the Notch1 3'-UTR by miR-34a, leading to the inhibition of Notch1 translation and/or the induction of Notch1 mRNA degradation [50-52].

The aberrant activation of the Notch1 signaling pathway has been closely linked to the pathogenesis of various cancers, including colorectal, lung, and liver cancers [44]. Notch1 can promote tumor growth, angiogenesis, invasion, and metastasis through the transcriptional regulation of genes involved in cell proliferation, survival, epithelial-mesenchymal transition, and other critical processes [53]

By targeting and inhibiting the Notch1 pathway, miR-34a can disrupt these pro-tumorigenic signaling cascades, leading to the observed cytotoxic and anti-proliferative effects in the cancer cell lines. The increased Notch1 expression in response to the miR-34a inhibitor further supports the notion that endogenous miR-34a plays a crucial role in maintaining Notch1 homeostasis and limiting its oncogenic activity in these cancer cells.

In addition to the direct targeting of Notch1, miR-34a may also indirectly regulate the Notch1 pathway through the modulation of other signaling molecules and transcription factors. For instance, miR-34a has been shown to target the Notch ligand Jagged1, as well as the Notch transcriptional regulators Hes1 and Hey1, further contributing to the inhibition of Notch1 signaling [54, 55]

Moreover, miR-34a can crosstalk with other pathways that converge on the Notch1 axis, such as the p53 [56], Wnt/β-catenin [57, 58], and TGF-β signaling cascades [59]. These intertwined regulatory networks involving miR-34a likely play a crucial role in fine-tuning Notch1 activity and its downstream cellular responses in the context of cancer.

The findings of this study highlight the potential of miR-34a-based therapeutic strategies for the treatment of colorectal, lung, and liver cancers. The potent cytotoxic and anti-proliferative effects of miR-34a, mediated in part through the downregulation of the Notch1 signaling pathway, suggest that targeting this tumor-suppressive miRNA could be a promising approach for cancer management.

Several studies have already explored the therapeutic potential of miR-34a mimics in preclinical cancer models. For instance, miR-34a mimics have been shown to inhibit tumor growth, metastasis, and angiogenesis in various solid tumors [60], including lung, prostate, and pancreatic cancers [9, 61]. Moreover, the systemic delivery of miR-34a mimics has demonstrated favorable safety profiles and anti-tumor efficacy in early-phase clinical trials [24].

Furthermore, the observed upregulation of Notch1 in response to the miR-34a inhibitor highlights the potential of miR-34a inhibitors as a means to activate the Notch1 pathway in certain contexts, such as in the treatment of neurodegenerative diseases or tissue regeneration applications [62, 63].

Future studies should further investigate the therapeutic potential of miR-34a-based interventions in relevant in vivo cancer models, evaluating their efficacy, safety, and potential synergistic effects when combined with standard-of-care treatments. Additionally, a deeper understanding of the complex regulatory networks and signaling crosstalk involving miR-34a and the Notch1 pathway in the context of cancer progression and metastasis could inform the development of more targeted and personalized therapeutic strategies.

According to the obtained results, miR-34a holds significant potential as a therapeutic target in cancer due to its role as a tumor suppressor that regulates key oncogenic pathways, including Notch1, p53, and Bcl-2. By inhibiting these pathways, miR-34a can reduce tumor cell proliferation, induce apoptosis, and prevent metastasis, making it a promising candidate for cancer therapies. Clinical studies have explored miR-34a-based therapeutics, such as MRX34, a synthetic miR-34a mimic, showing initial efficacy in some cancer types [64]. However, there are several challenges and limitations. One major challenge is the efficient and targeted delivery of miR-34a to tumor cells without affecting healthy tissues, as systemic delivery can lead to off-target effects and toxicity. Additionally, cancer cells may develop resistance mechanisms, such as miRNA degradation or compensation by other pathways. The complexity of miRNA regulation, along with potential immune responses, presents further limitations that need to be addressed for miR-34a to become a reliable therapeutic option in clinical settings[65].

5. Conclusion

In conclusion, the present study provides compelling evidence that miR-34a exerts potent cytotoxic and anti-proliferative effects in colorectal, lung, and liver cancer cell lines, at least in part through the downregulation of the Notch1 signaling pathway. The ability of miR-34a to suppress cancer cell viability, colony formation capacity, and Notch1 expression suggests that this tumor-suppressive miRNA could be a promising therapeutic target for the management of these malignancies.

6. Limitations and Future Directions

The study offers valuable insights into miR-34a's role in regulating the Notch1 signaling pathway and its impact on cancer cell viability and proliferation, but it has limitations. Conducted solely in vitro, the findings may not fully represent the complexity of human cancers, necessitating further validation in animal models and clinical studies. The study focused on the

Notch1 pathway, although miR-34a targets various other signaling cascades involved in cancer. Exploring these interactions could provide a more comprehensive understanding of miR-34a's anti-cancer effects. Additionally, the study did not examine miR-34a's potential synergy with standard therapies, which could reveal novel treatment strategies. Expanding research to include more cancer types and patient-derived samples would enhance the generalizability of the findings. Future studies should address these limitations, using in vivo models and broader analyses to explore miR-34a's therapeutic potential and its relevance as a biomarker in cancer treatment.

7. Conflict of interest

The authors declare that there is no conflict of interest.

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9. References

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