RESEARCH PAPER

Exploring the potential of AgNPs in modulating the PI3K/AKT/mTOR pathway via miR-133a regulation in MCF-7 breast cancer cells

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ABSTRACT

Objective(s): Breast cancer is the most common malignancy in women. MiRNAs modulate the PI3K/AKT/mTOR (PAM) pathway, functioning as either tumor suppressors or oncogenes. This research explores the impact of AgNPs on breast cancer cells while emphasizing the interplay between miR-133a and the PAM pathway and uncovering regulatory mechanisms.

Materials and Methods: To assess the impact of AgNPs on cell growth and survival, we performed an MTT assay. Additionally, we employed bioinformatic methodologies to predict potential targets of miR-133a within the PAM pathway. We quantified the expression levels of miR-133a, PI3K, AKT, PTEN, and mTOR in MCF-7 cells after exposure to AgNPs using qRT-PCR. Furthermore, we employed Western blotting to evaluate the protein expression of mTOR.

Results: The MTT assay results demonstrated a significant dose- and time-dependent inhibition of breast cancer cells by AgNPs. The qRT-PCR analysis revealed an upregulation in the mRNA expression levels of PI3K and AKT, accompanied by a downregulation in the mRNA expression levels of PTEN and mTOR upon exposure to AgNPs. However, the efficacy and expression level of miR-133a as a tumor suppressor in breast cancer cells remained unchanged following exposure to AgNPs (IC_{50}).

Conclusion: The study found that AgNPs inhibit breast cancer cell growth, affecting the PAM pathway, but miR-133a remained unchanged, suggesting AgNPs may not primarily act through miR-133a. Further research is needed, but caution is advised when using AgNPs for cancer control and treatment.

Keywords: *Breast Neoplasms, MicroRNAs, Nanoparticles, Phosphatidylinositol 3-kinases*

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INTRODUCTION

Breast cancer is a prevalent form of cancer among adults, with an annual incidence of over 2.3 million cases. Additionally, it significantly impacts the overall mortality rates of women with cancer, serving as the primary or secondary leading cause in 95% of countries [1]. The fight against cancer remains one of the greatest challenges confronting science, despite significant research achievements. Drug resistance, adverse side effects, and limited selectivity represent just a few of the challenges associated with current cancer treatments. Therefore, the challenge lies in identifying sensitive compounds that are efficient, cost-effective, and targeted to specific cells, while also enhancing their sensitivity [2].

A groundbreaking research field known as cancer nanomedicine is emerging, with the goal of developing advanced tools for oncological applications. The primary advantage of cancer nanomedicine in advancing healthcare and management lies in the utilization of nanoparticles (NPs) that operate at the molecular level [3]. Silver nanoparticles (AgNPs) are attracting growing attention in cancer research due to their unique physicochemical properties and inherent antiproliferative effects [4]. Studies have provided evidence suggesting that NPs can have detrimental effects on human health. However, paradoxically,

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there are instances where NPs may appear innocuous or even beneficial to the human body. NPs commonly interact with receptors on the cell surface or undergo endocytosis, potentially leading to the activation or inhibition of specific signaling pathways [5]. Cell signal transduction, which plays a pivotal role in the development and progression of cancer, involves alterations in multiple pathways that facilitate the development and progression of tumor cells [6, 7]. As a result, research focused on the PI3K/AKT/mTOR (PAM) pathway has also been directed toward investigating its role in cancer. Alterations in the PAM pathway have been identified in nearly all human tumors, including breast cancer, where up to 60% of tumors exhibit diverse variations that lead to the overactivation of this pathway [8]. The PAM pathway is essential in breast cancer because it regulates various cellular processes, including cell growth, cell metabolism and survival. Dysregulation of this pathway can lead to uncontrolled cell growth and contribute to the development of resistance to antineoplastic treatments in cancer cells. This emphasizes the critical importance of investigating PAM signaling to understand the progression and severity of this disease [9] and suggests that this pathway holds promise as both a prognostic and diagnostic tool for patients with breast cancer, as well as a potential therapeutic target [10]**.** Some studies have indicated that miRNAs play a crucial role in modulating the growth, migration, and metastasis of breast cancer cells through their interaction with the PAM oncogenic signaling pathway [11].

MicroRNAs (miRNAs) serve as an epigenetic mechanism that significantly contributes to our understanding of the biological and therapeutic properties of AgNPs, as well as their implications for future research endeavors [12]. MiRNAs, which are non-coding RNAs consisting of 18–25 nucleotides, play a critical role in post-transcriptional gene expression by specifically targeting RNA and inhibiting mRNA translation [13]. Substantial evidence suggests that various types of NPs have the capacity to modulate the expression of miRNAs. To date, investigations into the impact of AgNPs on miRNA expression have primarily been conducted *in vitro*, revealing significant alterations in miRNA expression in various cell types, including human dermal fibroblasts, Jurkat cells, and brain cells [2]. Considering the pivotal role which miRNA plays in cancer development, a substantial body of research has demonstrated the efficacy of miRNA in combating the disease [14, 15]. Among the evaluated miRNAs, miR-133a has emerged as a promising tumor suppressor and prognostic biomarker for various cancers, including osteosarcoma, esophageal cancer, non-small cell lung cancer, colorectal cancer, and breast cancer [16]. MiR-133a functions as a tumor suppressor in breast cancer by effectively targeting and modulating genes, such as EGFR, LASP1, MAMC1, and UCP2, as well as pathways like the PAM and MAPK/ERK signaling cascades. In doing so, it helps to prevent uncontrolled cancer cell growth and mitigate disease progression. Dysregulation of miR-133a during early tumorigenesis, coupled with reduced expression, is associated with an unfavorable prognosis, aligning with its role as a tumor suppressor [16]. Despite significant efforts, our current understanding of the intricate involvement of miRNAs in cancer development and their role in cancer chemotherapy remains limited [17]. Therefore, the objective of this study is to examine the efficacy of miR-133a in MCF-7 human breast cancer cells when exposed to AgNPs, and subsequently elucidate the role of miR-133a in modulating the PAM signaling pathway.

MATERIALS AND METHODS

Preparation and characterization of AgNPs

Spherical AgNPs, measuring 5-8 nm in size and coated with Poly Vinyl Pyrrolidone (PVP), were obtained from NANOSANY, a pioneering Iranian nanomaterials company (Mashhad, Iran). Table 1 displays the Certificate of Analysis for AgNPs, reflecting a NP purity of 99.99%. Additionally, the X-Ray Diffraction (XRD) patterns and Transmission Electron Microscopy (TEM) images of these NPs are depicted in Fig. 1A and Fig. 1B, respectively. XRD is a reliable method for identifying crystalline phases and providing detailed chemical composition information.

Cell culture

The MCF-7 cell line, derived from human breast adenocarcinoma, was obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran). The cells were cultured in accordance with standard laboratory protocols. Specifically, they were maintained at a temperature of 37°C with 5% CO_2 and a humidity level of 90-95%. The cell culture medium used was Dulbecco's modified Eagle medium (DMEM; Bio Idea, Iran, Tehran), supplemented with 10% fetal bovine

Table 1. Certificate of Analysis for AgNPs - ppm.

Silver Nanoparticles Certificate of Analysis -ppm (AgNPs: 99.99%)				
Cu^{2+}	Bi^{3+}	Fe^{2+}	Pb^{2+}	Sb^{2+}
10	2	3	2	2

The certificate has been derived from Nanosany company. The proportional concentration of certain materials within the AgNPs is indicated in parts per million (ppm), and the purity of the AgNPs stands at 99.99%

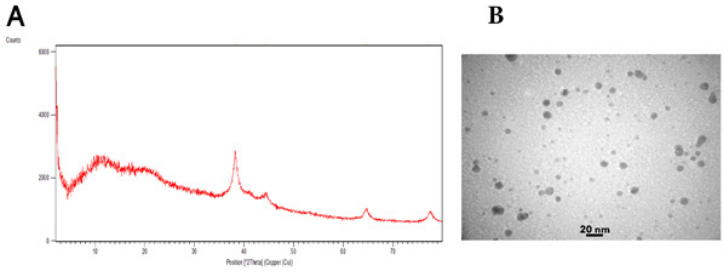

Fig. 1. (A) X-ray diffraction (XRD) patterns and (B) transmission electron microscopy (TEM) analyses were performed on AgNPs coated with Poly Vinyl Pyrrolidone (PVPAgNPs)

serum (FBS; Bio Idea, Iran, Tehran) and 1% penicillin/streptomycin (Bio Idea, Iran, Tehran). All experiments were conducted using logarithmically growing cells. To minimize potential genomic drift caused by instability, the MCF-7 cell line utilized in the study was limited to the initial 10 passages from the original flask. The cell culture medium, DMEM, was replenished every three days, and the cells were subcultured when they reached a confluency range of 65% to 80%. The NPs were appropriately diluted in DMEM medium to achieve the desired concentrations. Afterwards, the MCF-7 cells were exposed to various concentrations of AgNPs.

Cytotoxicity assay

To evaluate the impact of AgNPs on the viability and proliferation of MCF-7 cells, an MTT assay was conducted. This assay employs a colorimetric system based on the reduction of MTT by mitochondrial dehydrogenase in viable cells, which leads to the formation of a purple formazan product. In summary, MCF-7 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated for a period of 24 hr. After confirming the adhesion of cells to the plate surface, various concentrations of AgNPs ranging from 0 to 250 µg/mL were added to each well in triplicate. Subsequently, the cells were incubated for 24, 48, and 72 hr (Fig. 2). Next, the MTT reagent (5 mg/ml in PBS) was added to each well, followed by incubating the cells at 37 °C for 4 hr. The supernatant was carefully aspirated, and 100 µl of dimethyl sulfoxide (DMSO; Kiazist, Iran, Tehran) was added to dissolve the insoluble

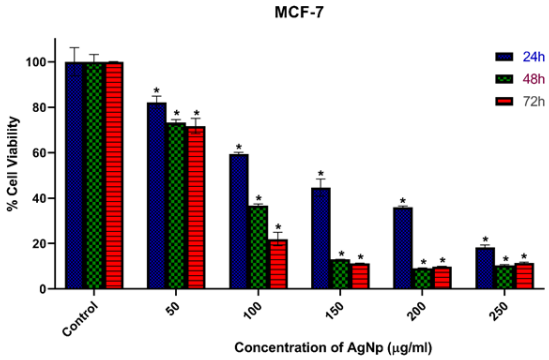

Fig. 2. The survival capabilities of MCF-7 cells were suppressed by the presence of AgNPs. The MCF-7 cells were subjected to different concentrations of AgNPs (ranging from 0 to 250 µg/mL) for durations of 24, 48, and 72 hr, respectively. Cellular viability was assessed by performing the MTT assay. The results are reported as the mean ± SD (n=3). * P<0.05

formazan crystals generated by mitochondrial dehydrogenases in viable cells, and incubated for 30 min. Finally, the optical density (OD) of both the control and the samples from each well was measured at a wavelength of 570 nm using an ELISA plate reader (RT-2100C Microplate Reader, China). The survival rate of MCF-7 cells was determined using the following formula:

Percentage of viability = Absorption (treated well) / Absorption (control well) × 100.

GraphPad Prism 8 software was utilized to calculate the 50% inhibitory concentration (IC_{50}).

Prediction of miRNA target genes

Potential targets of miR-133a were identified through the utilization of bioinformatics tools.

This process involved the use of several online algorithms, including TargetScan, miRDB, miRWalk, miRmap, and miRTarbase. The analysis primarily focused on identifying targets within the PAM pathway. These algorithms are designed to screen potential miRNA targets by evaluating the thermodynamic advantages of interactions between miRNAs and their targets. This assessment relies on identifying complementary binding sites in the 3'-untranslated region (3'-UTR) and calculating minimal binding energies. Target genes predicted by all four software tools and listed in at least one database were carefully selected, taking into account the intersection of results from multiple databases to avoid overprediction. To unravel the role of miR-133a in AgNPs-treated cells, in silico predictions were used to identify potential targets of miR-133a within the PAM pathway (Table 2).

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from both treated and untreated MCF-7 cells using the RNX-plusTM kit, following the manufacturer's protocol (Sinaclon; Iran, Tehran). The quantity and purity of the isolated RNA were evaluated by measuring the optical density ratios (260/280 and 260/230 nm) using a NanoDrop One UV-Vis spectrometer (Thermo Fisher Scientific; Waltham, MA). Furthermore, the quality of the RNA was assessed by conducting 1% agarose gel electrophoresis. The mRNAs were reverse transcribed into cDNA using the Pars Tous Company Kit (Iran, Mashhad), following the manufacturer's instructions.

To generate specific cDNA for miR-133a, a stem-loop primer was designed, and the synthesis of miRNA cDNA was performed using an Anacell kit (Anacell; Iran, Tehran) following the manufacturer's instructions. The primer pairs were designed by utilizing Integrated DNA Technologies (IDT) and Clustal Omega tools, and their specificity was assessed using the NCBI Primer-BLAST platform (Table 3). The specific sequence primers for miR-133a (accession ID MIMAT0000427) were obtained from miRBase (http://www.mirbase.org)

Table 2. The target gene of the microRNA was determined by evaluating the target prediction scores obtained from five web applications. Several web applications, such as miRDB, miRWalk, TargetScan, miRmap, and miRTarBase, were utilized for prediction purposes

			hsa-miR-133a-3p		
Targets	miRDB (target Score) †	miRWalk (Score) ††	TargetScan (total context + Score) †††	miRmap (Score) ††††	miRTarBase
					✔
PIK3C2A	79	1.00	-0.21	91.28	---

† A greater value of this score indicates an increased probability of target interaction
†† The closer the value approaches one, the greater the likelihood that this interaction is successful
††† A higher negative value of this score corresponds to a greater degree of effective suppression
†††† The predicted strengths of miRNA repression are presented as "Percentile" values, ranging from 0% to 100%, where 100% represents the highest level of repression
✔ Validation methods with strong evidence (qPCR, Western blot and Reporter assay)

Table 3. The primer sequences used for qRT-PCR analysis

Gene		Primer sequence (5′ → 3′)	Length of production (bp)
PIK3C2A	Forward	TACCAATCACCGCACAAACC	150
	Reverse	ACAGTAGAACTCACATCACACG	
PTEN	Forward	AGTCCAGAGCCATTTCCATC	183
	Reverse	GATAAATATAGGTCAAGTCTAAGTCG	
AKT-1	Forward	ACACCACCTGACCAAGATGA	164
	Reverse	TACAGATCATGGCACGAGG	
mTOR	Forward	CAATAGGAGAATTGGCACAGG	206
	Reverse	CAGTAGCACCTCAAGCAAAGT	
β-Actin	Forward	AATGTGGCCGAGGACTTTG	261
	Reverse	GGCACGAAGGCTCATCATT	

Standard curves were generated for each gene prior to data analysis. Subsequently, PCR products were visualized by electrophoretic separation on a 3% agarose gel and analyzed for resolution

and designed by Anacell. The qRT-PCR analysis was performed using the Real Q Plus 2x Master Mix Green kit (Amplicon, Denmark) and the LightCycler® 96 system (Roche Life Science, Deutschland GmbH Sandhofer, Mannheim, Germany). The expression levels of mRNAs and miRNAs were normalized by utilizing β-actin and U6 genes as internal controls. Furthermore, the specificity of the primers was verified through a melting curve analysis. The relative expression levels of mRNAs and miRNA were quantified using the $2^{-\Delta\Delta CT}$ method.

Western blotting analyses

MCF-7 cells (8 × 10^5 cells) were exposed to different concentrations of AgNPs (40, 70, and 100 µg/mL) for 48 hr. After the incubation period, the cells were lysed using RIPA buffer supplemented with cocktails of phosphatase and proteinase inhibitors (cOmplete Mini Tablets, PhosSTOP, and ProSTOP, Roche, Basel, Switzerland). Protein extraction for western blotting analysis was performed according to the following protocol. In brief, the protein concentration was quantified using the BCA Protein Assay Kit (DNA biotech; Iran, Tehran). Twenty micrograms of total protein samples were transferred from a 4-10% precast polyacrylamide gel onto nitrocellulose membranes using a Bio-Rad device, following the manufacturer's instructions. The membranes were incubated overnight at 4 °C in Tris-buffered saline with primary antibodies against mTOR (ab134903, diluted at 1:1000) and p-mTOR (ab109268, diluted at 1:1000). After washing, the membranes were incubated with HRP-conjugated secondary antibodies (ab97051, diluted at 1:200-500). The β-actin protein was used for protein quantification and as an internal loading control. The protein bands were detected using an ECL Kit (Chemiluminescence system). Unless otherwise stated, all chemicals were procured from Sigma Chemicals. Finally, the intensity of the bands was evaluated using Image J software, with normalization to the loading control.

Statistical analysis

The differences among groups were analyzed using GraphPad Prism version 8.0, employing a one-way analysis of variance (ANOVA), followed by a post hoc Tukey test. The results were presented as the mean ± standard deviation (SD), and statistical significance was determined for values with a $P<0.05$.

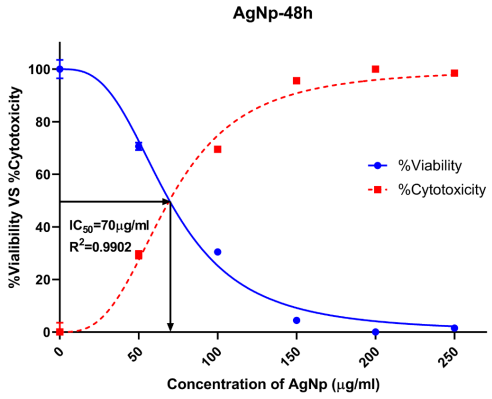

Fig. 3. To evaluate the cytotoxic impact of various concentrations of AgNPs and analyze cell viability after 48 hr, the MTT assay was employed

RESULTS

Effects of AgNPs on MCF-7 cell viability

As depicted in Fig. 2, the viability of MCF-7 cells was reduced by AgNPs in a dose- and time-dependent manner, with an estimated half-maximal IC_{50} value of 70 µg/mL after 48 hr of incubation (Fig. 3). The viability of MCF-7 cells significantly decreased ($P<0.05$) when exposed to AgNPs at doses ranging from 50-250 µg/mL, compared to the control untreated cells (100% cell viability). Based on the IC_{50} concentration, we selected doses of 40, 70, and 100 µg/mL of AgNPs for subsequent experiments.

AgNPs induce changes in the PAM signaling pathway

As demonstrated in Fig. 4, AgNPs significantly altered the expression of PI3K, PTEN, AKT, and mTOR genes in MCF-7 cells. The qRT-PCR results revealed a significant upregulation of PI3K and AKT (oncogenic genes) in cells exposed to 40, 70, and 100 µg/mL of AgNPs (Fig. 4A, 4C; $P<0.01$). Meanwhile, the expression levels of PTEN (a tumor suppressor gene) and mTOR (a crucial growth-related molecule) were significantly downregulated in the treated cells compared to the control (Fig. 4B, 4D; $P<0.001$). Indeed, the expression of oncogenic genes PI3K and AKT was upregulated by AgNPs, which was accompanied by the downregulation of PTEN expression.

Effects of AgNPs on miR-133a gene expression

To gain a better understanding of the anticancer effects of AgNPs at 48 hr, we conducted a qRT-PCR analysis to evaluate the expression of miR-133a in both AgNPs-treated and untreated MCF-7 cells

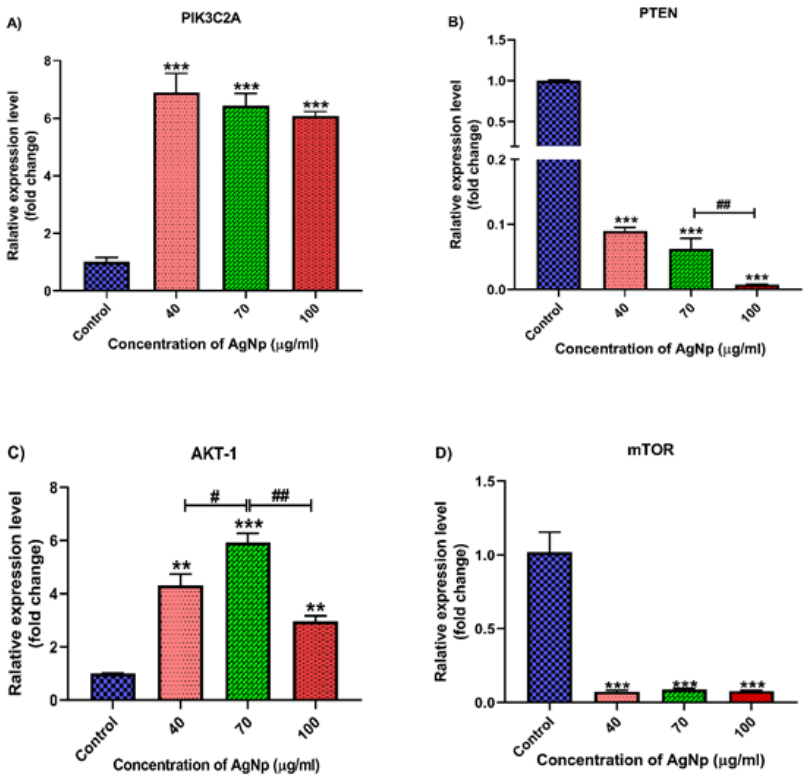

Fig. 4. The relative expression levels of PIK3C2A, PTEN, AKT-1, and mTOR were quantitatively compared between AgNPs-treated cells and untreated cells. The expression levels of four genes were normalized to β-actin as a reference. The results are reported as the mean ± SD (n=3). ** *P*<0.01, *** *P*<0.001, # Compare with groups *P*<0.05, ## *P*<0.01

The expression level of miR-133a did not show significant changes after treatment with 70 µg/mL of AgNPs. However, at 40 and 100 µg/mL, the expression level of miR-133a increased compared to the control (Fig. 5B; *P*< 0.01).

The bioinformatic analysis indicated that PIK3C2A mRNA has the potential to function as a molecular target of miR-133a in the PAM signaling pathway (Fig. 5A). Moreover, our analysis revealed that the PTEN, AKT-1, and mTOR mRNAs did not possess any binding sites for miR-133a. Throughout the course of this inquiry, our initial expectation was based on the potential to observe a direct relationship between the expression of miR-133a and the PIK3C2A gene. Nonetheless, the intricate impact of AgNPs on the expression of miR-133a yielded results that diverged from our initial projections, consequently obstructing the establishment of the envisaged correlation. Based on the results, it can be concluded that miR-133a did not effectively influence its target gene in the PAM signaling pathway at a concentration of 70 µg/mL of AgNPs (IC_{50}).

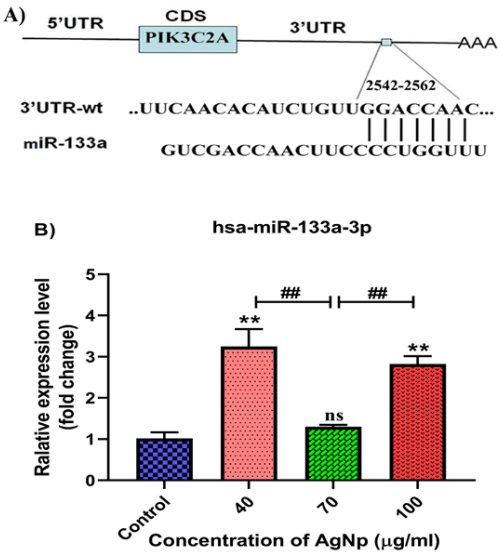

Fig. 5. (A) A schematic diagram illustrating the predicted binding sites of miR-133a in the 3'UTR of PIK3C2A. (B) The relative expression of miR-133a was compared between cells treated with AgNPs and untreated cells. The expression levels of miR-133a were normalized using U6 small nuclear RNA as an internal control. The results are reported as the mean ± SD (n=3). ** *P*<0.01, ## Compare with groups *P*<0.001.

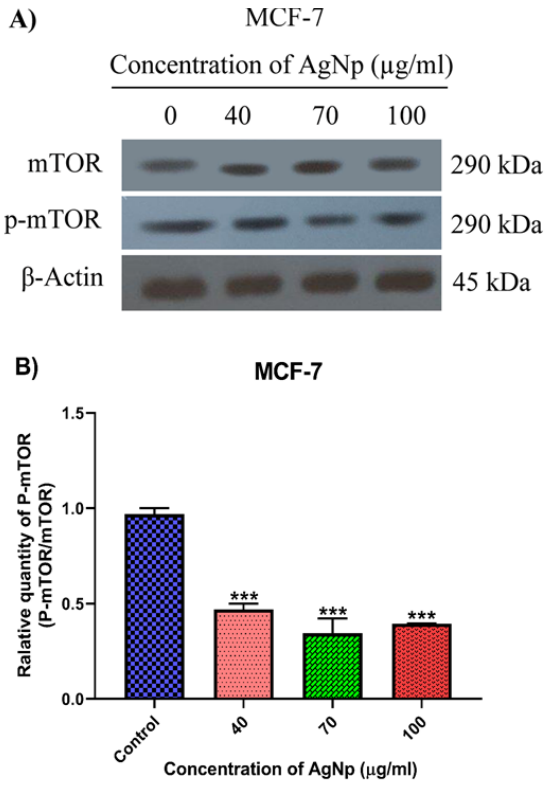

Fig. 6. The expression of mTOR protein in MCF-7 cells was examined. (A) The impact of various concentrations of AgNPs (40, 70, 100 µg/mL) on the relative protein expression of mTOR and p-mTOR in MCF-7 cells after 48 hr. (B) Immunoblotting analysis was performed to assess the ratio of p-mTOR / mTOR. The results are reported as the mean ± SD (n=3). *** *P*< 0.001.

Influence of AgNPs on the protein levels of p-mTOR in treated MCF-7 cells

As depicted in Fig. 6, the protein levels of p-mTOR were evaluated to investigate the mechanisms underlying the effects of AgNPs on the treated cells. In this study, we examined the expression level of mTOR, a crucial element essential for cell survival in the PAM signaling pathway. The protein levels of p-mTOR, a downstream component of the PAM signaling pathway, were lower in cells exposed to AgNPs (Fig. 6B; *P*<0.001). This suggests that AgNPs may have induced cancer cell death by suppressing the expression of p-mTOR.

DISCUSSION

Breast cancer is the leading type of cancer among women, affecting over one million individuals worldwide annually. However, significant variation exists in terms of prognosis and observed outcomes among patients who share similar clinical and pathological characteristics [11]. Luminal breast cancers account for approximately 70% of all cases of breast cancer. Unfortunately, despite the availability of treatment options, breast cancer remains a considerable threat to the lives of women, resulting in substantial mortality rates. Hence, it is crucial to understand the molecular mechanisms underlying breast cancer and identify new therapeutic targets in order to improve patient care [18]. AgNPs are commonly used in pharmacology and medicine because of their distinctive physicochemical properties [19]. Despite extensive research on the cytotoxicity of these NPs, the underlying biological mechanisms of toxicity remain largely unknown. Therefore, it is crucial to have a comprehensive understanding of their adverse effects [12]. In this study, our findings provide supporting evidence that NPs can elicit adverse effects due to their extensive surface areas, heightened chemical reactivity, increased internal volumes, and enhanced cell penetrability in breast cancer cells via the PAM signaling pathway. Furthermore, these effects may be mediated by miRNAs. The aforementioned findings can contribute to a better understanding of the biology and medicine of AgNPs, while also providing valuable insights for future research endeavors.

Tumor cells exhibit specific features such as uncontrolled proliferation, genomic instability, and apoptosis evasion. These traits involve changes in cellular signaling networks that support tumor development and survival. Cell transduction plays a vital role in the development and progression of cancer [7]. The PAM signaling pathway, commonly altered in different human cancers, including breast cancer, plays a significant role in drug resistance of cancer cells [10, 20]. As a result, it is not surprising that when the PAM signaling pathway undergoes mutations, modifications, and imbalanced expression, it contributes to the development of serious illnesses, including diabetes, dementia, cardiovascular problems, and cancer [21]. Although “PI3K” may appear as a single entity, it is, in fact, divided into three distinct classes of related molecules based on their regulation, performance, and structure. Among these classes, class I PI3Ks exhibit the strongest associations with oncogenic processes [22]. PI3Ks are a crucial family of lipid enzymes that associate with the plasma membrane and become activated by receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). The

structure of a class I PI3K enzyme is comprised of catalytic (p110) and regulatory (p85) subunits [23, 24]. Following dimerization and activation of the extracellular receptor, PI3K is activated, leading to the phosphorylation of AKT, which is also known as protein kinase B (PKB) and is a serine-threonine kinase with three isoforms encoded by separate genes: AKT-1 (expressed in most tissues), AKT-2 (found in insulin-sensitive tissues), and AKT-3 (predominantly in the brain and testes). Among these isoforms, AKT-1 has been strongly associated with cancer. The tumor suppressor PTEN (phosphatase and tensin homolog) negatively regulates this pathway by inhibiting PI3K, thus maintaining cellular homeostasis [24–26]. The PTEN gene has undergone mutations in malignant tumors, resulting in abnormal PTEN that cannot function as an inhibitor of the PAM pathway [27]. Therefore, as plasma PIP3 levels rise, AKT activity is continuously stimulated [28]. AKT interacts with numerous substrates, including transcription factors, cell cycle progression inhibitors, protein kinases, compounds that induce apoptosis, and mTOR (mammalian target of rapamycin) [9]. mTOR is a crucial element essential for the survival, growth, and proliferation of tumor cells [29]. The PI3K-AKT and mTOR, two critical intracellular signaling pathways, are closely related and essential for cell cycle control. They interact with various other pathways, including Notch, Mitogen-Activated Protein Kinase (MAPK), and Hypoxia Inducible Factors (HIFs) [30]. Studies have revealed that the PIK3CA gene, which encodes the p110α catalytic subunit of the PI3K pathway, is the most commonly mutated oncogene in luminal breast cancers (found in 35% of clinical cases), whereas AKT-1 and PTEN mutations occur in only 4% of patients [9]. Therefore, the MCF-7 cell line was utilized as a representation of luminal breast cancer in the present study. The PAM signaling pathway is an altered pathway that, when affected by PI3K catalytic subunit mutations, AKT activation, and loss of PTEN function, increases resistance to treatment [31].

The present study uncovered that, although chemically synthesized AgNPs exhibit a cytotoxic effect on breast cancer cells, the PAM signaling pathway remains resistant to AgNPs treatment. Our study primarily focused on PAM signaling, as it has previously been shown to be crucial in breast cancer. Furthermore, we utilized a mTOR-specific primary antibody in western blot analysis to provide a more precise characterization of the interactions between the end product of the pathway and other compounds in this signaling pathway.

Grzelak et al. found that commercial AgNPs with a diameter of approximately 20 nm were not acutely cytotoxic to MCF-7 cells. In contrast, green-synthesized AgNPs around 48 nm inhibited cell migration, while AgNPs of approximately 20 nm in diameter promoted migration [19]. This study demonstrated that AgNPs with a diameter ranging from approximately 5-8 nm were effective in inhibiting cancer cell growth and proliferation. This effect is supported by decreased mTOR expression, a critical molecule for tumor cell survival and growth. According to multiple research studies, it has been found that AgNPs have a negative impact on various signaling pathways, including the HIF pathway, the Src kinase pathway, and the PAM signaling system [2]. For example, Qi et al. observed a reduction in AKT and Erk1/2 levels in human bladder cancer cells (T24) following treatment with Au-Ag [32]. Other studies have demonstrated that the cytotoxicity of AgNPs in HT22 and endothelial cells is mediated by the activation of the PAM signaling pathway [33, 34]. Additionally, it was observed that treatment of MCF-7 and Vero cells with AgNPs resulted in increased levels of PTEN expression compared to the control [35]. However, we discovered that AgNPs suppressed PTEN expression in cancer cells. It is plausible to assume that these effects could be influenced by the size of NPs and their synthesis method [2].

MiRNAs are small noncoding RNAs of about 20-25 nucleotides that regulate gene expression by binding to the 3'-UTR of target mRNAs [16]. MiRNAs are involved in various cellular processes, and their dysregulated expression is linked to human carcinogenesis and cancer progression, indicating their potential as both tumor suppressor genes and oncogenes [36]. Therefore, miRNAs have the potential as therapeutic targets in medicine due to their role as crucial epigenetic regulators of gene expression in various physiological pathways [12]. Among miRNAs, miR-133a has emerged as a tumor suppressor and prognostic biomarker for various cancers, including osteosarcoma, esophageal cancer, colorectal cancer, bladder cancer, gastric cancer, and breast cancer. MiR-133a was initially experimentally identified in mice and subsequently found to be highly conserved

across mice, flies, and humans. Overall, miR-133a is frequently dysregulated in early tumorigenesis, and its low expression is associated with a poor prognosis. This is consistent with its role in drug resistance and tumor suppression in cancer progression [16]. Extensive research has focused on miRNA interference as a cancer treatment strategy. However, challenges with miRNA delivery and instability have impeded the development of RNAi-based therapies. Thus, there is a pressing need for targeted cancer treatments that spare healthy cells. Genetic anomalies are a major contributor to the complex nature of cancer [37].

In a study investigating the effects of five different-sized AgNPs on human dermal fibroblasts (HDFs) - namely AgNP-5, AgNP-20, AgNP-50, AgNP-100, and AgNP-200 - it was observed that AgNPs inhibited cell growth, induced apoptosis, and ultimately triggered cytotoxicity through MAPK signaling. Based on the miRNA sequencing results, HDFs treated with five AgNPs exhibited differential expression of 457, 76, 177, 461, and 341 miRNAs. Notably, a strong correlation was observed between the expression of miR-424-5P and miR-340-5P and the cytotoxicity of AgNPs with varying sizes [38]. Kamil et al. examined DNA methylation and miRNA expression in HepG2 cells in response to AgNPs, gold nanoparticles (AuNPs), and superparamagnetic iron oxide nanoparticles (SPIONs) at low cytotoxic doses. They found significant effects on nine miRNAs, with AgNPs affecting six, AuNPs affecting four, and SPIONs affecting two miRNAs. However, they cautioned against drawing conclusions about the NPs' pro- or anti-tumor properties solely based on miRNA expression changes [39]. In this study, the presence of AgNPs did not affect the efficiency of miR-133a in cancer cells. Given the commercial availability of AgNPs in solution form, it is reasonable to speculate that other non-coding RNAs, such as lncRNAs or circRNAs, could potentially impact the functionality of miR-133a through their interaction with the miRNA (Fig. 7) [16].

Discussions on AgNPs are challenging due to conflicting literature reports and the controversial nature of the IC_{50} parameter. AgNPs are widely recognized as cytotoxic, with their biological effects known to be size-dependent. A fundamental principle underlies this observation: smaller NPs are more toxic due to their enhanced cell-penetrating ability. The majority of published studies are based on AgNPs synthesized using green methods, which often contain a sufficient quantity of silver ions [19]. However, in this study, we employed a commercially available solution of chemically synthesized AgNPs. Therefore, it can be inferred that the utilization of AgNPs is influenced by factors such as their origin or size. However, to develop a safe and effective anticancer drug, further investigations into the *in vitro* and *in vivo* mechanisms of the anticancer effects of AgNPs are necessary.

CONCLUSION

In this study, the impact of AgNPs on breast cancer cells was investigated, with a focus on the

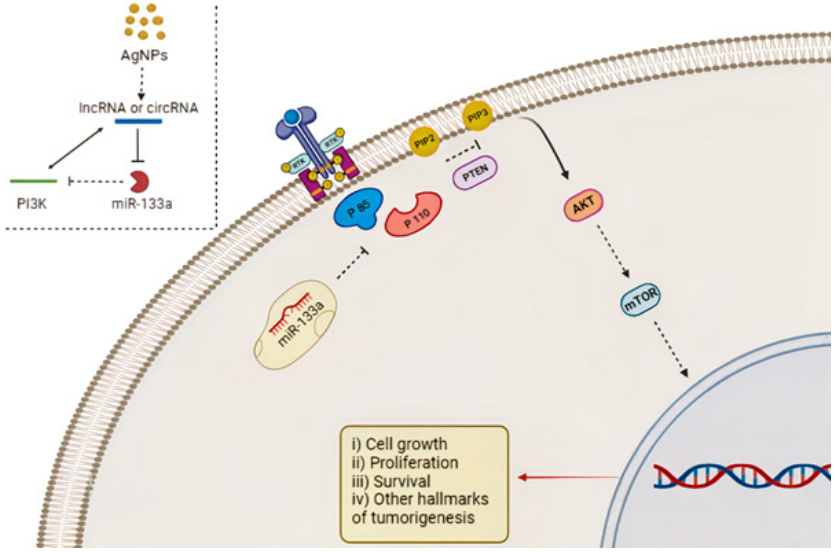

Fig. 7. An overview of the AgNPs/miR-133a/PAM signaling pathway. PTEN acts as a tumor suppressor, inhibiting the PAM signaling pathway. Dysregulation in the expression of miR-133a and the PAM pathway may contribute to tumorigenesis

interplay between miR-133a and the PAM pathway. The results indicate that AgNPs significantly inhibit breast cancer cell growth in a dose- and time-dependent manner. While the PAM pathway's key components were affected, the tumor suppressor miR-133a remained unchanged following exposure to AgNPs. This finding suggests that the modulation of miR-133a expression may not be a primary mechanism through which AgNPs exert their anti-cancer effects in breast cancer cells. Further research is needed, but caution should be exercised when considering AgNPs for cancer control and treatment.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

IR.UMSHA.REC.1400.685.

AVAILABILITY OF DATA AND MATERIAL

Not applicable.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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