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ORIGINAL ARTICLE

# MicroRNA-23 regulates the growth, metastasis and chemosensitivity of human gastric cancer cells by targeting MAP3K9

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## Summary

***Purpose:*** *The efficient therapeutic targets which could be utilized for gastric cancer are limited. In this context, this study was undertaken to evaluate possible anticancer activity of miR-23 against gastric cancer.*

***Methods:*** *The normal GES-1 and human AGS, SNU-1 and SNU-5 gastric cancer cells were used in this study. qRT-PCR was used for the determination of miR-23 expression. MTT assay was used for cell viability and acridine orange (AO)/ethidium bromide (EB) assay was used for detection of apoptosis. The experiments were performed in triplicate.*

***Results:*** *The microRNA (miR)-23 was downregulated in gastric cancer cells and showed inhibitory effect on cell growth which was manifested as decline in cell survival. Additionally, the chemosensitivity of gastric cancer cells to cisplatin was enhanced with miR-23 overexpression. Furthermore, miR-23 also inhibited the migration and invasion of cancer cells. MAP3K9 was shown to be the target gene of miR-23 and silencing of MAP3K9 was seen to mimic the growth inhibitory effect of miR-23. Overexpression of MAP3K9 reversed the growth inhibition in miR-23 mimics transfected gastric cancer cells.*

***Conclusion:*** *miR-23 exerts growth inhibitory effect against gastric cancer cells and negatively regulates the cell migration and invasion along with enhancement of chemosensitivity of cancer cells.*

***Key words:*** *gastric cancer, metastasis, cell proliferation, migration, invasion, chemosensitivity*

## Introduction

Gastric cancer is one the leading causes of mortality currently ranking as the 5th most frequent type of cancer globally [1]. It has been reported that gastric cancer caused 0.84 million deaths in 2013 and approximately 0.98 million new cases of gastric cancer [2]. The treatment of gastric cancer is based on cytotoxic chemotherapeutic agents. Thus, the development of new potent and efficient chemotherapeutic agents and their identification is the need of the hour [3]. Researchers have proved that there is an active involvement of an important class of RNA molecules, the micro RNAs (miRs), in most of the human cancers. miRs are small non-coding RNAs, usually 20-22 nucleotides in length,

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which act at post-transcriptional level and repress their target genes by binding to mRNA untranslated (3' and 5') regions [4]. Almost 60% of eukaryotic genes are believed to be regulated by miRs [5]. miRs are generally repressed in human cancers but this is not true for all cases [6]. They act either as tumor suppressors or oncogenes to regulate the tumorigenesis and progression of human cancers along with playing a significant role in invading surrounding tissues and develop metastasis [7]. Studies have revealed the dysregulation of miRs in gastric cancer [8-10]. This study was designed to determine the expression of miR-23 in gastric cancer and to explore its role and therapeutic potential.

## Methods

*Cell lines and culture conditions*

The normal GES-1 and human AGS, SNU-1 and SNU-5 gastric cancer cells were procured from Cancer Research Institute of Beijing, China, cells were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) in a incubator at 37°C with 5% $CO_2$ and 95% air.

*RNA isolation and qRT-PCR analysis*

Total RNA was isolated from the cells with RNAiso reagent (Takara, Kyoto, Japan). The extracted RNA was exposed to DNAse I (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA synthesis was performed with the help of Primescript™ reverse transcription reagent (Takara, Kyoto, Japan). Quantitative real time-PCR was performed on QuantStudio 3 Real Time-PCR system (Thermo Fisher Scientific) following the manufacturer's instructions. The relative expression of miR-23 was normalized with U9 snRNA and GADPH. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression of miR-23. RT primers were synthesized through Primer3 v. 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) online software. The AGS cells exhibited the lowest expression of miR-23 and so only this cell line was used for further experiments.

*MTT assay*

The estimation of proliferation of gastric cancer cells was performed through MTT assay. In brief, the AGS cancer cells were stably transfected with miR-NC, miR-23 mimics or miR-23 inhibitor for 48 h. Cells were also transfected with si-MAP3K9 and its negative control, si-NC. Co-transfection of pcDNA-MAP3K9 with miR-23 mimics was also done. Transfected cells were subsequently cultured in 96-well plates for 24, 28, 72, 96h at 37°C and 5% $CO_2$. For chemosensitivity assessments, miR-23 cancer cells and non-transfected cells were administered 5 nM paclitaxel or 2.5 µM doxorubicin. Ten µl of culture medium from each well was replaced with 10µl of 5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Thermo Fisher Scientific) and incubation was continued for another 4 h at 37°C. Then, 150µl of DMSO was added to each well for dissolving the formazan crystals. Subsequently, absorbance at 450nm was recorded with microplate photometer (BioTek, Synergy2, USA).

*AO/EB staining assay*

For estimating the viability of transfected AGS cancer cells, after fixing with paraformaldehyde, the cells were stained with DAPI and examined for nuclear fluorescence under fluorescent microscope.

*Migration and invasion assay*

Transwell assay with or without matrigel coating was used to assess the migration and invasion of transfected cancer cells, respectively. Briefly, 100µl of cell culture containing 6000 cells was added to the upper chamber and lower chamber was filled with 750µl of DMEM supplemented with 10% FBS. After 48 h of incubation at 37°C/5%$CO_2$, cells from the surface of membrane's upper side were removed carefully with cotton swabs while those sticked to lower side of membrane were fixed with 70% ethyl alcohol and stained with 0.1% crystal violet. Light microscope (x100) was used for visualization of cells and photographs were taken. At least 7 random fields were used for counting the migratory or invasive cells.

*Bioinformatics for target identification*

TargetScanHuman7.2 (http://www.targetscan.org/vert_72/) was used to identify the targets of miR-23. Target identification was further validated through assessment of base complementarity and binding energies performed through microRNA.org (http://34.236.212.39/microrna/home.do) and miRDB (http://www.mirdb.org/) online bioinformatics software tools.

*Dual luciferase assay*

Dual luciferase assay was performed for interactional study of miR-23 with 3'-UTR of MAP3K9. Here, the AGS cancer cells were co-transfected either with miR-NC and pGL3-wiltype (WT)/mutated (MUT) 3' UTR stretches or miR-23 mimics and pGL3-wiltype (WT)/mutated (MUT) 3' UTR of MAP3K9, following which, the measurement of luciferase activity was made through Dual Luciferase Reporter system (Promega Corporation) using *Renilla* luciferase for normalization.

*Western blotting*

The transfected AGS cells were lysed in RIPA lysis buffer and the protein extracts were prepared. Centrifugation at 12000 g for 10 min was performed and the protein concentration of each extract was assessed by bicinchoninic acid (BCA) assay. Subsequently, each sample was incubated for 10 min at 99°C. Next, 30 µg of proteins from each sample were loaded on SDS-PAGE and subsequently shifted to PVD membrane which was later subjected to RIPA lysis buffer for 55 min at 22°C. This was followed by incubation with specific primary

antibodies overnight at 4°C and then with secondary antibody. Odyssey Infrared Imaging system was utilised for the detection of protein bands of interest.

*Statistics*

The experiments were performed in triplicate. The values are shown as mean ± SD. Student's t-test was used for comparisons between two samples and $p<0.05$ was taken as statistically significant.

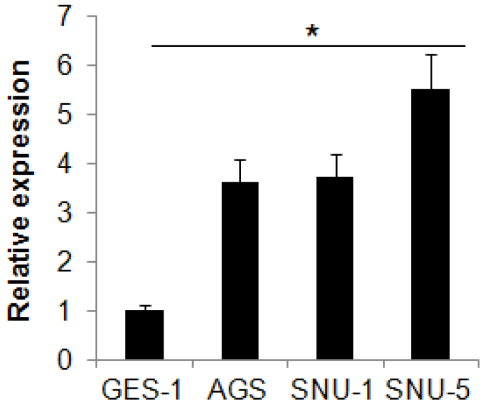


**Figure 1.** Expression of miR-23 in normal GES-1 and human gastric cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

## Results

*The expression of miR-23 is downregulated in gastric cancer cells*

The qRT-PCR analysis was performed for examining the relative expression levels of miR-23 in gastric cancer cell lines (SNU-1, AGS and SNU-5) and normal gastric cell line (GES-1). The expression of miR-23 was up to 5.3 fold lower in gastric cancer cell lines (Figure 1). The miR-23 was seen to have significantly lower expression in all three cancer cell lines, being lowest in AGS cell line (Figure 1). This suggested a most likely regulatory role of miR-23 in gastric cancer.

*miR-23 inhibits the cancer cell growth by inducing apoptosis*

To explore the regulatory role of miR-23 on gastric cancer growth, miR-23 mimics and miR-NC were transfected into AGS cancer cells for 48 h and stable transfection was confirmed by qRT-PCR (Figure 2A). MTT assay was performed following the culturing of transfected cells for 0, 12, 24, 48 and 96 h. At all the time points, the cell growth was significantly lower under miR-23 overexpression (Figure 2B). Again, the assessment of cell viability



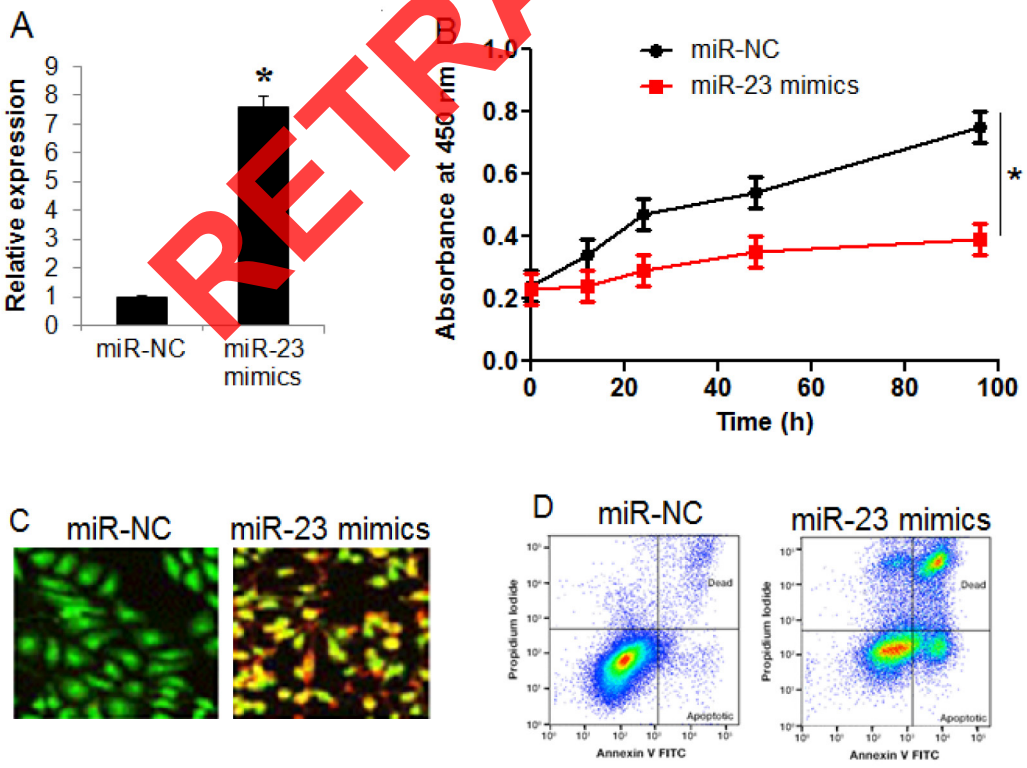


**Figure 2.** miR-23 overexpression inhibits the proliferation of gastric cancer cells. **A:** relative expression of miR-23 in miR-NC and miR-23 mimics transfected AGS gastric cancer cells. **B:** MTT assay showing cell viability of miR-NC and miR-23 mimics transfected AGS cells. **C:** AO/EB staining showing apoptosis of miR-NC and miR-23 mimics transfected AGS cells. **D:** Annexin V/PI staining of miR-NC and miR-23 mimics transfected AGS cells showing the percentage of apoptotic cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

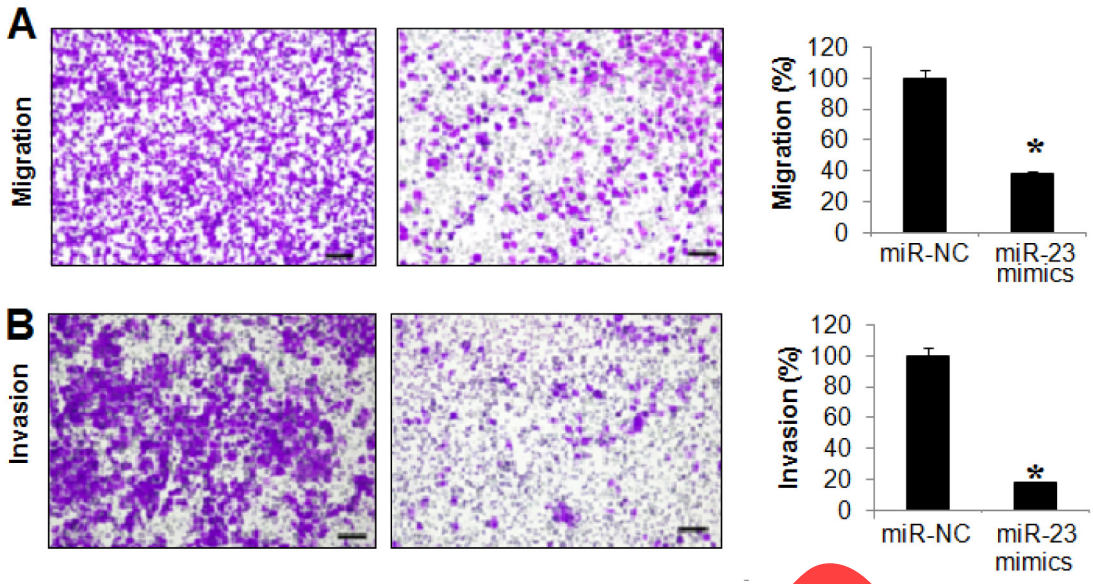


**Figure 3.** Transwell assay showing miR-23 overexpression inhibits migration **(A)** and invasion **(B)** of the AGS gastric cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

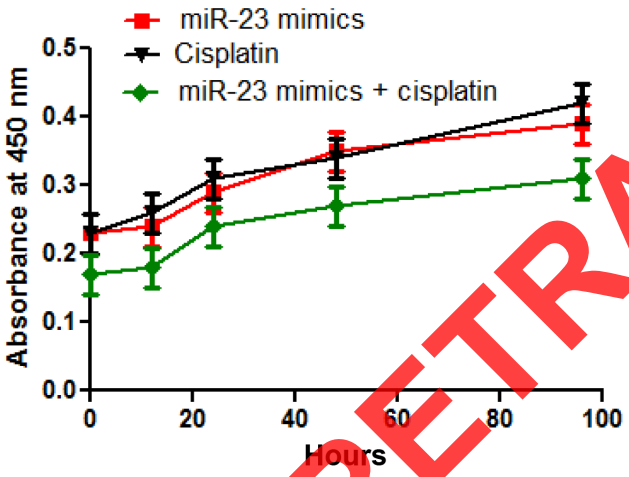


**Figure 4.** miR-23 enhances the chemosensitivity of the human gastric cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (p<0.05).

by DAPI staining revealed that transfection of miR-23 mimics induced apoptosis in AGS cells (Figure 2C). The induction of apoptosis was confirmed by annexin V/PI staining which showed increase in the apoptotic cell percentage (Figure 2D).

*miR-23 inhibits the migration and invasion of the AGS cells*

The migratory and invasive potential of AGS cancer cells under miR-23 overexpression and downregulation was determined through transwell chamber assay using miR-NC transfected cells as negative control. Both the migration and invasion of cancer cells decreased significantly under miR-23 overexpression but the downregulation of miR-23 was seen to enhance the cell migration and invasion (Figure 3A and B). The migration and invasion of cancer cells was reduced by 62 and 78%, respectively under miR-23 overexpression. Thus, it can be concluded that miR-23 negatively regulates the migration and invasion of gastric cancer cells.

*miR-23 overexpression enhances the chemosensitivity of AGS cells*

The sensitivity of AGS cancer cells under miR-23 overexpression was determined for paclitaxel and doxorubicin. Assessment of proliferation rate was made through MTT assay for miR-NC, miR-23 mimics transfected cells along with cisplatin (0.5 μM) administered untransfected and miR-23 mimics transfected cancer cells. Cancer cell proliferation was lowest under miR-23 overexpression plus paclitaxel treatment than cisplatin or miR-23 overexpression alone (Figure 4). Together, the results are indicative that miR-23 has a potential to increase the chemosensitivity of gastric cancer cells to drug treatment and thus it is strongly advocated the application of combinatorial molecular and chemotherapeutics against human gastric cancer.

*MAP3K9 is the target gene of miR-23*

Taking the sequence complementarily and binding energy parameters into consideration, the online bioinformatics software tools predicted MAP3K9 as a target gene of miR-23 (Figure 5A). To validate the target prediction, wild type (WT) and mutated (MUT) 3'-UTR stretches of MAP3K9 were designed and their interaction with miR-23 was

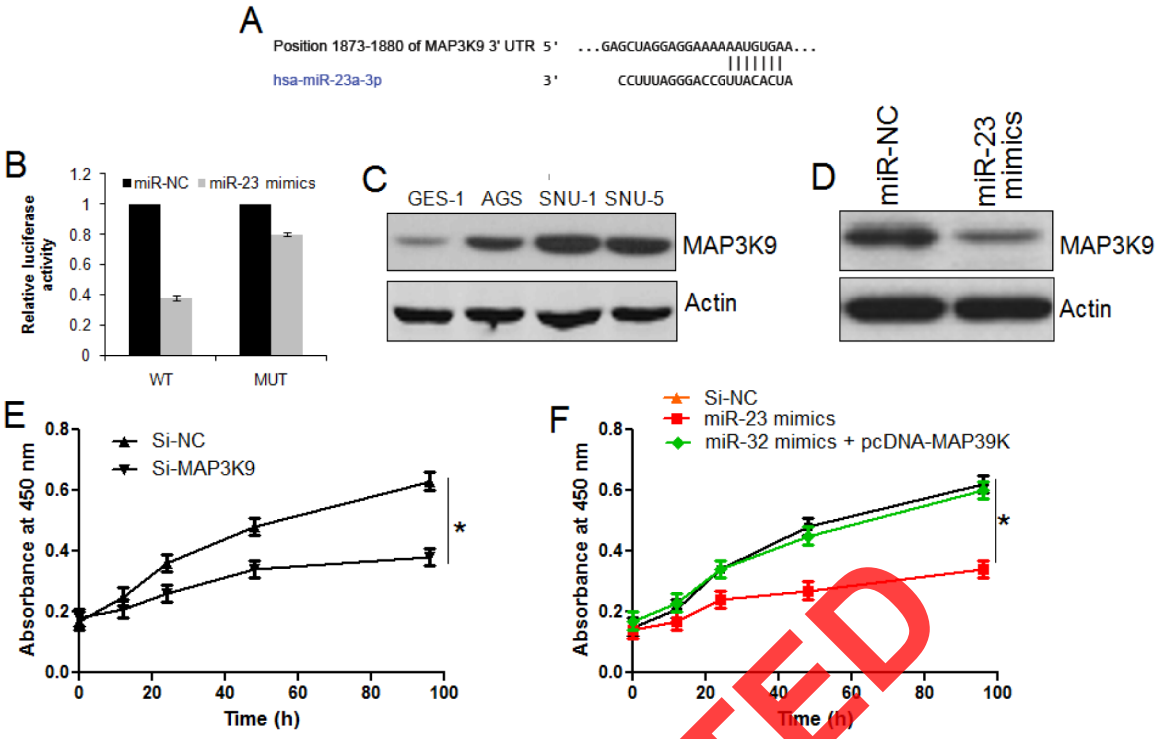


**Figure 5.** miR-23 exerts its effects by targeting MAP3K9. **A:** TargetScan analysis showing miR-23 targets MAP3K9 in AGS cells. **B:** Dual luciferase assay and **C:** Western blot showing the expression of MAP3K9 in normal gastric cells. **D:** Western blot expression of MAP3K9 in miR-NC and miR-23 mimics transfected AGS cells showing the decrease in the MAP3K9 in miR-23 overexpressing AGS cells. **E:** Cell viability of si-NC and si-MAP3K9 cells. **F:** Cell viability of si-NC, miR-23 mimics and miR-23 mimics+pcDNA-MAP39K cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).



determined using dual luciferase reporter assay. Interaction of miR-23 with WT 3'-UTR of MAP3K9 was confirmed by significantly lower luciferase activity in cancer cells transfected with miR-23 mimics and pGL3-WT 3'-UTR (Figure 5B). The cancer cells exhibited very high luciferase activity when co-transfected with miR-mimics and pGL3-MUT 3'-UTR of MAP3K9. Further confirmation was drawn from the protein expression studies of MAP3K9 in three different gastric cancer cell lines and normal gastric cells whereby it was observed that all the cancer cell lines exhibited lower protein levels (Figure 5C). The results were further supported by western blotting of MAP3K9 in miR-23 mimics transfected AGS cancer cells which showed that miR-23 overexpression inhbited the expression of MAP3K9 (Figure 5D). Taken all together, the results reveal that miR-23 represses MAP3K9 post-transcriptionally by interacting its 3'-UTR in a sequence specific manner.

*MAP3K9 repeals the inhibitory effect of miR-23 on cancer cell growth*

The knockdown of MAP3K9 gene in AGS cancer cells was performed through RNA interference by transfecting the cancer cells with si-MAP3K9 RNAi construct. The proliferation rate was also determined for si-MAP3K9 transfected cells using and compared (in percentage terms) with MAP3K9 silencing control cells. The si-MAP3K9 cancer cells exhibited significantly lower proliferation and the effects were the same as under miR-23 overexpression (Figure 5E). Again, when the pcDNA-MAP3K9 overexpression vector construct of MAP3K9 was co-transfected with miR-23 mimics into AGS gastric cancer cells, the anticancer growth effects of miR-23 overexpression were seen to be reversed and cancer cells exhibited the proliferation rates similar to miR-NC transfected control cells (Figure 5F).

## Discussion

For the first time it was found in Caenorhabditis elegans worm that miRs regulate the expression of genes. Since then, extraordinary research has been made in the field of miRs. They have been reported to control not only the overall development of animals but also exhibit vital role in fine designing of cellular fate and differentiation [9,10]. Disease development in animals is one of the prime aspects of animal biology falling under the regulation of

this important group of regulatory RNAs. The dysregulation of miRs has been shown to influence the onset and proliferation of almost all human cancers and researchers are actively involved in elucidating the role of miRs in different human cancers henceforth to understand the molecular mechanics which lead to deviation of controlled cellular plan and initiates the development of cancer growth [10]. Studies on gastric cancer have enlightened the involvement of a number of miRs in growth and development of this malignancy [11]. miR-23 has been implicated as a prognostic factor in cervical cancer and was shown to be repressed in cancer tissues [12]. The miR-23 has also been observed to be involved in epithelial-to-mesenchymal transition (EMT) of endometrial carcinoma [13]. In yet another study, miR-23 targeted the VDR to regulate the EMT in human mesothelial peritoneal cells [14]. The results of our study also suggest a similar type of regulation in gastric cancer and miR-23 was seen to negatively regulate the gastric cancer cell growth and its overexpression decreased the viability of cancer cells. Cisplatin is included in the drugs recommended for the treatment of gastric cancer [15]. When gastric cancer cells were administered cisplatin along with miR-23 overexpression, the cancer cell growth was decreased significantly and it was very low in comparison to the drug treatment only. Hence, our study explored one more candidate miR having potential to increase the chemosensitivity of gastric cancer cells. Cell migration and invasion are the two vital processes required for the metastasis of cancer cells [16] and herein we found that miR-23 overexpression inhibited both the migration and invasion of cancer cells. Furthermore, miRs have been reported to exert their effects by targeting different genes [17]. Herein we reported for the first time that miR-23 exerts its effects by suppressing the effects of MAP3K9 in the AGS cells. To sum up, this study explored the anticancer role of miR-23 against the gastric cancer proliferation together with its regulatory potential to enhance the chemosensitivity of cancer cells and to reduce the cancer metastasis through targeting of MAP3K9.

## Conclusion

The results of this study depicted miR-23 as a potential molecular marker and anticancer regulator of gastric cancer with a key finding of chemosensitivity enhancement of cancer cells by this miR. The study strongly advocates the employment of combinatorial administration of molecular and chemotherapeutic anticancer approaches in gastric cancer management which may act as leading base for conducting more such studies on human cancers in the future.



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## Conflict of interests

The authors declare no conflict of interests.

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