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ORIGINAL ARTICLE

MicroRNA-138 targets SOX4 to regulate the proliferation and metastasis of human lung cancer cells

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Summary

Purpose: *The current study was set with a purpose to assess the regulatory role of micro RNA (miR)-138 in human lung cancer cells with emphasis on the underlying mechanism of action.*

Methods: *RT-PCR based analysis was employed for gene expression studies. MTT assay was used to determine the proliferation rates of lung cancer cells. Colony forming assay was performed for the analysis of colony forming potential. DAPI and Annexin V-FITC/propidium iodide (PI) double staining methods were performed for the analysis of apoptosis. Migration and invasion of cancer cells were assessed using wound healing and transwell assays, respectively. Dual luciferase reporter assay was performed for interactional study. Western blotting was used to determine the protein concentrations.*

Results: *Cancer cells had lower levels of miR-138 transcripts. The overexpression of miR-138 reduced the proliferation of cancer cells and cells were seen to form lower number of viable colonies. This was due to the induction of cancer cell apoptosis under miR-138 overexpression. miR-138 also inhibited the metastasis of lung cancer cells. miR-138 was found to interact with SOX4 intracellularly and SOX4 protein levels decreased under miR-138. The anticancer effects of miR-138 were shown to be modulated through SOX4.*

Conclusion: *MiRs have a potential to act as molecular markers in cancer prognosis. There is a need to screen for miRs specific to particular types of cancer and to look for their potential to function as anticancer entities at molecular level.*

Key words: *Micro RNA, lung cancer, cell proliferation, metastasis, anticancer*

Introduction

MicroRNAs (MiRs) are a heterogeneous group of small non-coding RNA molecules which are about 22 nucleotides in length [1]. MiRs are regulatory in function and are synthesized by the action of RNA polymerases II in multicellular organisms [2]. MiRs play a role in post-transcriptional gene regulation by either repressing the mRNAs or by targeting them for degradation [3] and are involved in a number of metabolic pathways and their involvement in human cancer development is no longer hidden [4-6]. They are seen to regulate the proliferation and metastasis of human cancer cells by modulating different signaling pathways. The human cancers proceed with change in the expression levels of miRs and as such they are seen with a scope of being used as important molecular markers in cancer diagnosis [7,8]. There is a good number of reports about the role of miRs in human lung cancer [9,10]. The miR levels are even reported to predict the survival of lung cancer patients [11]. Recent studies have envisaged the role of specific miR molecules like miR 148, 320, 105,

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181 etc in lung cancer [12-15]. Taking a lead from such reports, the role of miR-138 in lung cancer was elucidated in the present study. Human lung cancer is one of the deadliest cancers. The current chemotherapy-based approaches used against lung cancer are having marginal success rates and overall survival rates are still very low [16]. Therefore, it is necessary to look for alternative anticancer approaches including the molecular ones against lung cancer. This study was undertaken to evaluate the expression profile and to explore the role and therapeutic potential of miR-138 in lung cancer cells

Methods

Cell culture and transfection

The normal lung cell line MRC-5 and lung cancer HCC827, A549, SK-LU-1 and A427 cell lines were obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cell lines were maintained in an incubator at 37°C with 5% CO_2. RPMI-1640 medium containing 100U/mL ampicillin and 100µg/mL streptomycin with 10% fetal bovine serum (FBS) was used for cell culture.

The miR-negative control (NC) and miR-138 mimics were designed using RiboBio (Guangzhou, China). Overexpression of SOX4 was achieved by using pCDNA3.1. RNA interference-based method was used for SOX4 gene silencing. Cell transfection was done using Lipofectamine (Invitrogen, Carlsbad, California, USA). Posttransfection, when the confluence reached about 80%, trypsinization was used after phosphate buffered saline (PBS) washing to obtain a homogeneous cell resuspension.

Gene expression studies

The RNA was isolated from cancer cells using TriZOL (Invitrogen) as per the manufacturer's guidelines. RevertAid First strand cDNA kit (Invitrogen) was used to synthesize the complementary DNA. Real Time PCR (Applied Biosystems) was used for expression studies using human GAPDH gene as internal control employing SYBR Green method. The primers used were miR-138-1 5'-GCCGCAGCTGGTGTTGTGAAT-3', and miR-138-2 5'-GCGAGCACAGAATTAATACGAC-3', SOX4-1 5'-TCTGCACCCCCAGCAAGA-3' and SOX4-2 5'-CACCCCGGAGCCTTCTGT-3', GAPDH-1: 5'-AGGTCGGTGTGAACGGATTTG-3' and GAPDH-2: 5'-GGGGTCGTTGATGGCAACA-3'. The PCR amplification conditions were 95°C for 10 min for initial denaturation, 40 cycles (95°C for 30s, 55°C for 30s and at 72°C for 15s). Finally, $2^{(-\Delta\Delta Ct)}$ method was used for comparing the expression levels.

Determination of cell proliferation rates

Cell proliferation was determined using MTT assay. Briefly, about 3×10^3 cells were plated per well of 96-well plates. MTT 5% (20µl/100µl culture) was added to each well and the plates were incubated at 37°C with 5% CO_2 for 4h. Then, 150µl DMSO were added to solubilize the product formed, i.e, formazan. Optical density $(OD)_{570}$

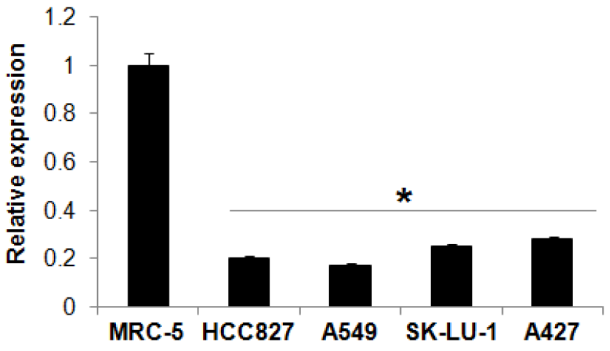

Figure 1. Expression of miR-138 in normal and lung cancer cell lines as determined by the qRT-PCR. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

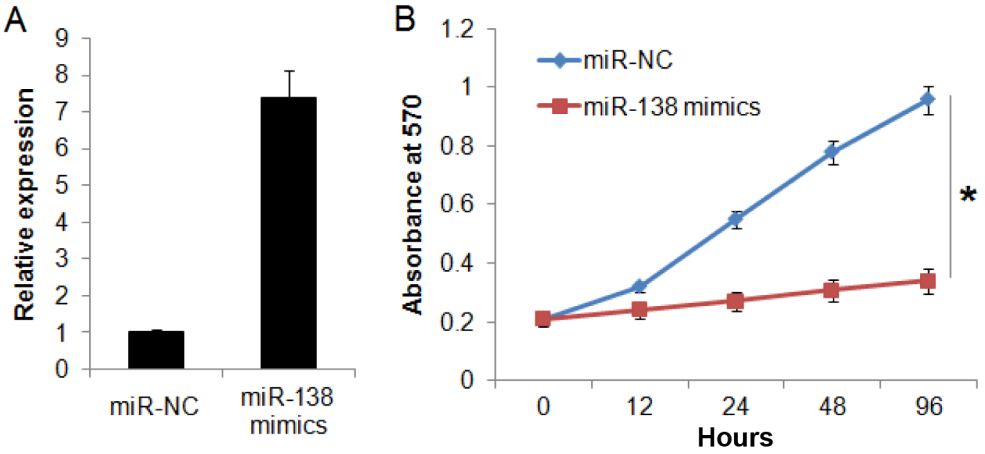

Figure 2. A: Expression of miR-138 in miR-NC and miR-138 mimics transfected lung cancer cell lines as determined by the qRT-PCR. **B:** Viability of the miR-NC and miR-138 mimics transfected lung cancer cells as determined by MTT assay. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

was recorded for each sample using spectrophotometer to determine the proliferation rate of cancer cells.

Proliferation of cancer cells was further assessed using the colonogenic assay. In brief, the cell counting was done using hemocytometer and about 200 cells were seeded per well in 6-well plates. The cells were allowed to grow for 6 days before being harvested. The cells collected were fixed using methanol and stained with crystal violet. Finally, the colonies were counted and pictures were taken.

Examination of cell apoptosis by DAPI staining and flow cytometry

To assess the cellular apoptosis, cancer cells were seeded at a density of 2×10^5 cells/well in 6-well plates and kept at 37°C for 24h. The cells were then collected by centrifugation, washed with PBS and fixed using 70% ethanol. This was followed by DAPI staining and fluorescent microscopic examination of cancer cells. The cells were also examined with flow cytometer using Annexin V-FITC/PI double staining for further analysis of apoptotic cell death.

Migration and invasion assays

The migration of cancer cells was analyzed through wound healing assay. The cells were plated in 6-well plates at a density of 2×10^5 cell/well. The plates were incubated for 24h at 37°C with 5% CO_2. Perpendicular to cell surface, a scratch line was made and its width was recorded. After 48h of incubation at 37°C, the width of scratch was again examined to determine the cell migration. The cell invasion was determined using transwell assay. In brief, the cells were placed in the upper chamber of transwell and in the lower chamber only culture medium was kept. This was followed by incubation at 37°C for 24h. The cells which had invaded the lower chamber were fixed using 4% isoformaldehye followed by subsequent crystal violet staining. Then, the invaded cells were visualized and photographs taken using high magnification inverted microscope.

MiR-138 target analysis and luciferase assay

To identify the intracellular target of miR-138, online bioinformatics using TargetScan (http://www.targetscan.org) software was performed. The dual luciferase report-

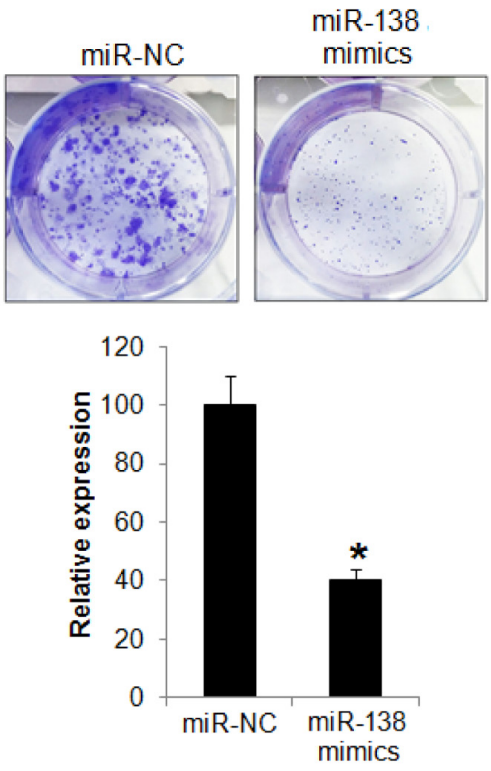

Figure 3. Colony formation assay of miR-138 in miR-NC and miR-138 mimics transfected lung cancer cell lines as determined by the colony formation assay. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

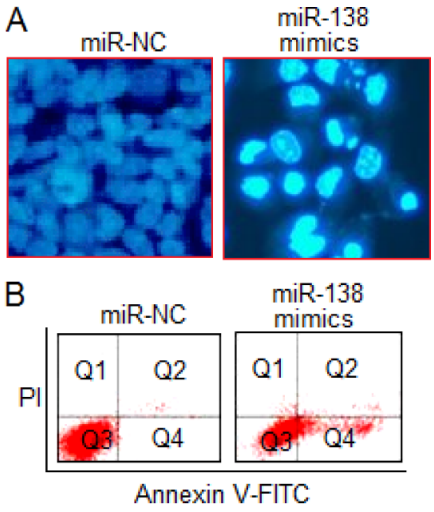

Figure 4. A: DAPI staining and **B:** annexin V/PI staining of miR-138 in miR-NC and miR-138 mimics transfected lung cancer cell lines showing induction of apoptosis. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

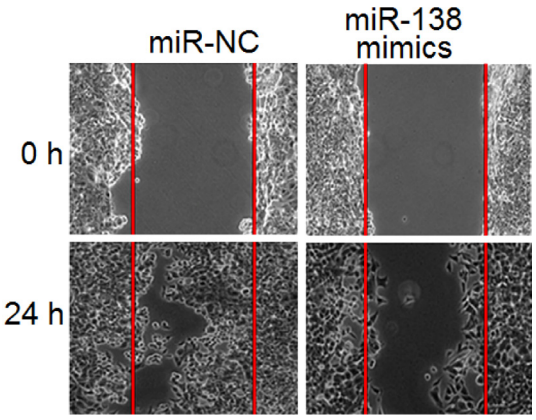

Figure 5. Wound healing assay of miR-138 in miR-NC and miR-138 mimics transfected lung cancer cell lines showing induction of apoptosis. The experiments were performed in triplicate and expressed as mean ± SD (p<0.05).

er assay was performed using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) following miR-138 mimics/miR-NC co-transfection of cancer cells with pGL3-SOX4-3´UTR-WT or pGL3-SOX4-3´UTR-MUT.

Western blotting

For protein expression studies, western blot technique was performed. Here, cancer cells were subjected to cell lysis with RIPA buffer (Invitrogen). The cell lysates were examined for total protein concentration using Bradford's method. Then, 45µg of proteins from each lysate was run on 10% SDS-PAGE gel. The gel was blotted to PVDF membrane which was exposed to primary antibodies. Final detection was made through chemiluminescence method after exposing membrane to secondary antibodies. Human actin was used as internal control in protein expression studies.

Statistics

All the experimental procedures were carried out using three replicates. The final values were represented as mean±SD. P value was calculated using Student's *t*-test performed through Graphpad Prism7. The inter value difference was considered statistically significant if calculated p value less than 0.05 was obtained in *t*- test.

Results

MiR-138 expression is declined in lung cancer

Real Time PCR-based expression analysis of miR-138 showed that the lung cancer cell lines (HCC827, A549, SK-LU-1 and A427) have relatively very lower transcript abundance of miR-138 transcripts than normal (MRC-5) cells (Figure 1). This suggests that miR-138 expression is inhibited in lung cancer indicating its regulatory role in lung cancer.

Inhibition of cancer cell proliferation and induction of cell apoptosis by miR-138

The overexpression of miR-138 was performed by transfecting the A549 cancer cells with miR-138 mimics and overexpression was confirmed by RT-PCR analysis (Figure 2A). When the proliferation of A549 cancer cells transfected with miR-138 mimics along with those transfected with miR-NC was determined at different time intervals after transfection, it was observed that proliferation rates were significantly lower for cancer cells transfected with miR-138 mimics but the miR-NC transfected cells had higher proliferation rates (Figure 2B). Also, the colony forming potential of miR-138 overexpressing cells was remarkably low in comparison to miR-NC cancer cells (Figure 3). Also, when the transfected cancer cells were assessed for morphological examination using DAPI staining, clear signs of nuclear deformation, indicative of cellular apoptosis, were observed (Figure 4A). The results were further confirmed by flow cytometric analysis (Figure 4B). This confirms that the inhibition of lung cancer cell proliferation by miR-138 was because of its potential to induce apoptosis in cancer cells.

miR-138 reduces cancer cell metastasis

The analysis of metastatic parameters (migration and invasion) revealed that the overexpression of miR-138 in A549 cancer cells led to decline in the migration of cancer cells (Figure 5). The miR-138 overexpression also caused sufficient decline in cancer cell invasion and the number of invading cells declined to more than half under miR-138 overexpression (Figure 6).

miR-138 interacts with and targets

SOX4 SOX4 was identified as the intracellular target of miR-138 and miR-138 was predicted to bind the SOX4 in 3'-UTR region through nucleotide complementarity (Figure 7A). SOX4 as target of miR-138 was further confirmed through luciferase assay. It was observed that when wild type (WT) and mutated (MUT) 3'- UTR region of SOX4 was co-transfected with miR-138, the cells bearing WT- UTR were having higher luciferase activity

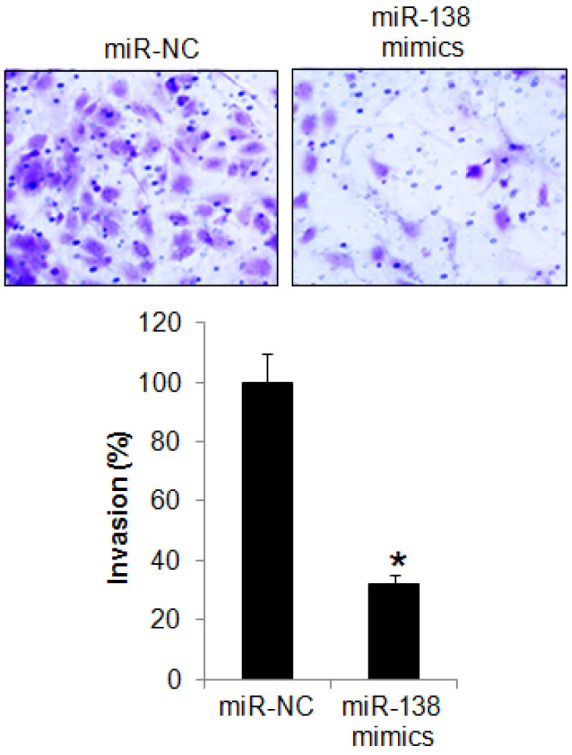

Figure 6. Transwell assay of miR-138 in miR-NC and miR-138 mimics transfected lung cancer cell lines showing inhibition of cell migration. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

in comparison to those bearing mutated 3'- UTR (Figure 7B). This indicates that miR-138 represses SOX4 by binding to 3'-UTR and the statement was further supported by western blotting of SOX4 where SOX4 was having very low protein count under miR-138 overexpression (Figure 7C).

SOX4 modulates the effects of miR-138

To see whether the antiproliferative effects of miR-138 are modulated through SOX4 protein, RNAi based silencing of SOX4 was carried out in A549 cancer cells (Figure 8A). It was seen that silencing of SOX4 reduced considerably the prolif-

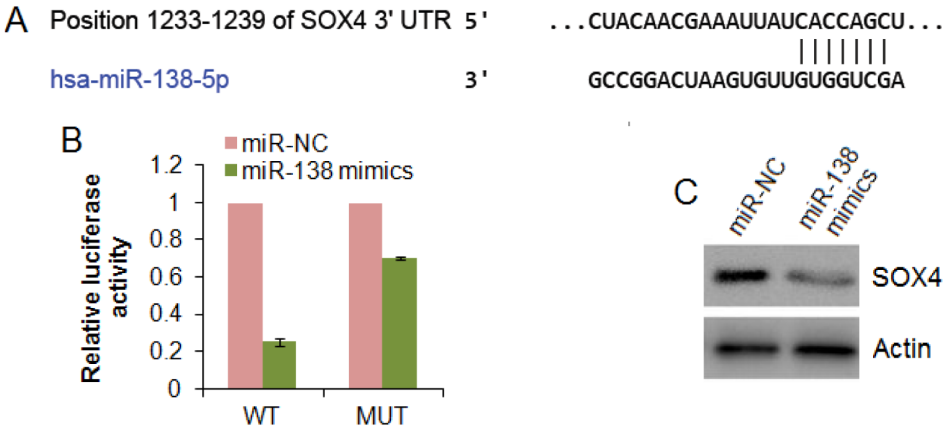

Figure 7. A: TargetScan analysis of miR-138. **B:** Dual luciferase assay and **C:** western blot analysis showing the expression of SOX4 in miR-NC and miR-138 mimics transfected lung cancer cell lines showing inhibition of cell migration. The experiments were performed in triplicate and expressed as mean ± SD ($p<0.05$).

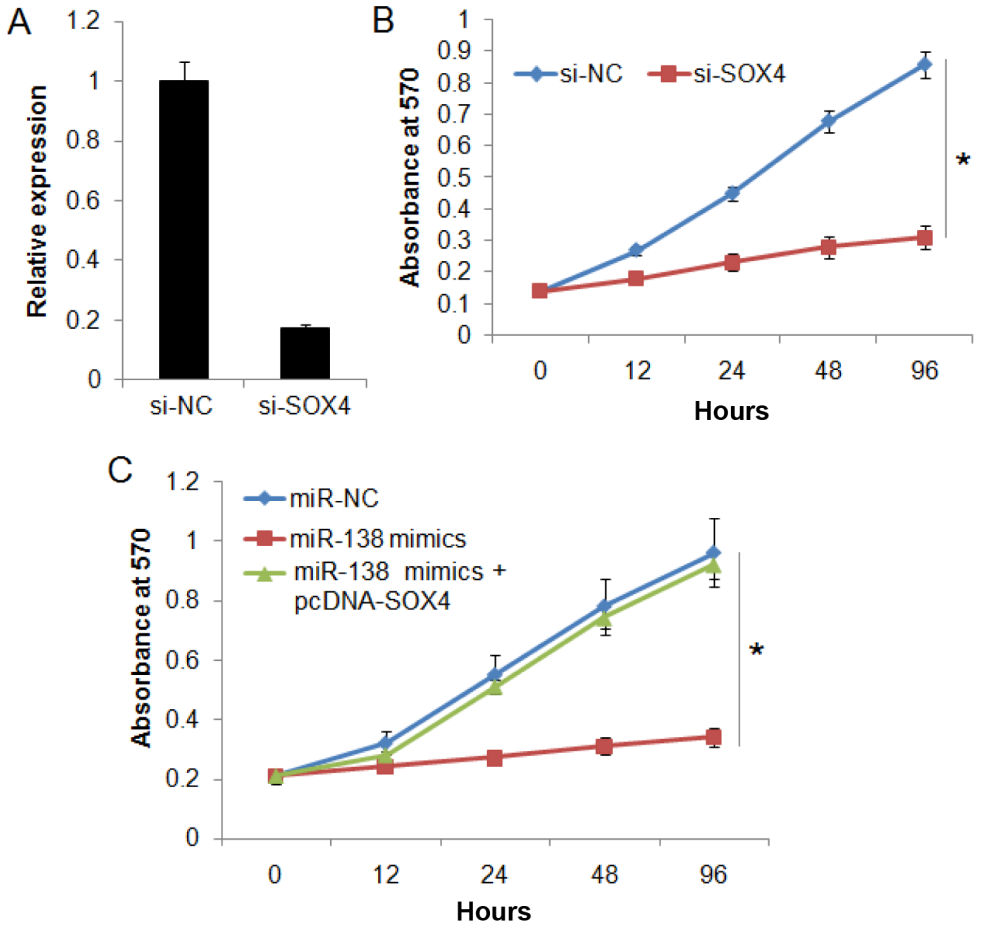

Figure 8. A: Expression of SOX4 in si-NC and si-SOX4 transfected lung cancer cell lines. **B:** Cell viability of the si-NC and si-SOX4 transfected lung cancer cells. **C:** Cell viability of the miR-NC, miR-138 mimics and miR-1388 mimics + pcDNA-SOX4 transfected lung cancer cell. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

eration rates of cancer cells (Figure 8B). Further, the antiproliferative effects of miR-138 were reverted when SOX4 expression level was enhanced in miR-138 overexpressing cancer cells (Figure 8C). These results suggest that the anticancer effects of miR-138 are modulated through SOX4, intracellularly.

Discussion

Lung cancer is amongst the most deadly human cancers. The higher mortality rate of lung cancer is because of the very less effective diagnosis at initial stages. The current anticancer treatments employed against lung cancer patients are based on chemotherapeutic approaches which are having very low success and as such, the survival rates for lung cancer patients are still very low. Recent advances in the molecular biology have enabled researchers to better understand the basic molecular mechanics behind cancer development. The role of miRs in cancer progression has been well recognized and they are reported to help not only in diagnosis but also in the management of such malignancies. The current study focused on the regulatory role of miR-138 in human lung cancer. It was observed that miR-138 is downregulated in lung cancer cells but the expression levels were quite higher in normal lung cells. Lung cancer progression with downregulated miR-138 levels has also been previously reported [17]. To understand the regulatory role experienced by miR-138 in lung cancer, overexpression of miR-138 was performed in A549 cancer cells. It was seen that the proliferative ability of cancer cells decreased significantly revealing the antiproliferative role of miR-138, which is in concordance with the results obtained for miR-138 against colon cancer cells [18]. The assessment of the miR-138 role to reduce cell proliferation revealed that it triggered apoptotic cell death in overexpressing cells. The miR-138 mediated induction of apoptosis in cancer cells has already been reported by previous workers and our results also confirm the same [19,20]. Further, the cancer cells showed lower migration and invasion, which govern the process of metastasis, under miR-138 overexpression. The potential of miR-138 to inhibit the metastasis of cancer cells has been elucidated in a number of previous reports [21,22]. Hence, our results are in conformity with such studies. MiRs are well known to target mRNA post-transcriptionally and repress the protein levels of their target proteins. So, to identify the intracellular target of miR-138, online bioinformatics was used and the target was identified as SOX4 which was further supported by luciferase assay and western blotting results. SOX4 is an oncogene and plays a definit role in cancer development, as reported earlier [23,24]. Moreover, SOX4 is targeted by miR-138 in ovarian cancer cells as it has been confirmed previously [25]. To infer whether effects of miR-138 are modulated through its intracellular target, SOX4 was silenced in A549 cancer cells and the effects observed were the same as miR-138 overexpression, confirming that miR-138 exerts its regulatory action in inhibiting lung cancer by targeting the SOX4 intracellularly. This was also evidenced by overexpression of SOX4 in miR-138 overexpressing cancer cells where SOX4 reverted the antiproliferative effects of miR-138. Overall, the study highlights the anticancer potential of miR-138 against human lung cancer and its possibility of using miR-138 in prognosis.

Conclusion

Human cancers proceed with enhancement/repression of specific molecular entities. Fine molecular administration to restore the deviated metabolic state in cancer cells along with usage of current drug based anticancer approaches can be employed for better results of cancer therapies and the current study is a step towards understanding the importance of this combinatorial strategy.

Conflict of interests

The authors declare no conflict of interests.

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