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ORIGINAL ARTICLE

MicroRNA-466 regulates the proliferation, migration and invasion of the human lung cancer cells by targeting transcription factor RUNX2

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Summary

Purpose: *Lung cancer causes significant mortality across the globe. This study aimed at the exploration of the regulatory role of microRNA (miR)-466 in lung cancer.*

Methods: *qRT-PCR analysis was used to infer the expression levels of miR-466 and Runt-related transcription factor 2 (RUNX2). CCK8 kit was used for assessment of cell proliferation. Colony forming assay was employed for examining the viability of cancer cells. The wound healing and Matrigel assays were used for investigating the rates of migration and invasion of cancer cells, respectively. Dual luciferase assay was performed to assess the interaction between miR-466 and RUNX2. Western blotting was performed to determine the protein expression.*

Results: *The results indicated that miR-466 is downregulated in lung cancer cells. Its overexpression led to significant decline of proliferation of cancer cells. The migration and invasion of lung cancer cells transfected with mir-466 mimics also got repressed. At molecular level, the regulatory role of miR-466 was exerted through the RUNX2 transcription factor whose silencing mimicked the effects of miR-466 overexpression.*

Conclusion: *Taken all together, miR-466 suppression is associated with the growth and progression of lung cancer. The miR-466 overexpression declined the proliferation and metastasis of cancer cells and these effects were modulated through miR-466/RUNX2 molecular axis.*

Key words: *lung cancer, micro RNA, migration, invasion, proliferation*

Introduction

Lung cancer is among the most disastrous and fatal malignancies of mankind [1] and is the primary cause of cancer-related mortality worldwide [2]. The 5-year survival rate of lung cancer patients is only about 5% which is indicative of the mortality of this disease [3]. In Taiwan, lung cancer is the most prevalent cancer [4]. The high mortality of lung cancer is due to lack of early detection tests and inefficiency of the anticancer treatments used against this disorder [5]. There is an urgent need to look for effective measures for better manage lung cancer. Researchers in the past few years have remained actively engaged in understanding the molecular patterns associated with the growth and progression of human cancer and have concluded that there is crucial involvement of small non-coding RNA entities in maintaining the proliferation of cancer cells [6]. These non-coding RNAs are about 18-24 nucleotides in length called as microRNAs (miRs) [7]. MiRs are seen to be aberrantly expressed in human cancers [8]. The scientific investigations have suggested them to serve as vital prognostic markers in the detection of particular human cancers [9]. MiRs

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also exert regulatory control on the proliferation and metastasis of lung cancer [10]. The miR-466 has been deduced to be involved in the progression of various human cancers [11,12]. However, little is known about the role of miR-466 in regulating human lung cancer. So, the present study was undertaken to search the molecular regulatory control exerted by miR-466 on human lung cancer.

Methods

Culture of cell lines and cancer cell transfection

All the cell lines used in the study were procured from the American Type Collection Center, ATCC (USA). The lung cell lines used were the normal (MRC-5) and the cancer (A427, A549 HCC827 and SK-LU-1). To culture the cells lines, Roswell Park Memorial Institute medium (RPMI, Thermo Scientific, Waltham, Mass, USA) was used with a supplementation of 10% fetal bovine serum (FBS), 100U/mL ampicillin and 100µg/mL streptomycin. The cell lines were kept in a humidified incubator at 37°C with 5% CO_2.

Transfection

The miR negative control (NC) and miR-466 mimics were designed and realized from RiboBio (Guangzhou, China). The siRNA mediated silencing of RUNX2 was performed to achieve its downregulation and si-NC was used as negative control. Transfection of cancer cells was performed using Lipofectamine 2000 (Thermo Scientific) and the transfected cells were treated with trypsin at 80% transfection confluence to obtain a homogeneous cell mix.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA from the cell lines was extracted by Trizol method. Exactly 2 µg RNA were reverse-transcribed to cDNA with SuperScript First-Strand Synthesis System (Thermo Scientific). The qRT-PCR analysis was performed to determine the expression levels of the genes of interest through SYBR Green method. The expression levels were quantified using 2^{-ddCt} method. To normalize the expression alues, the human GADPH was used to serve as an internal expression control.

Cell proliferation assay

The proliferation of lung cancer cells was analyzed using the Cell counting kit-8 (CCK8, Thermo Scientific)

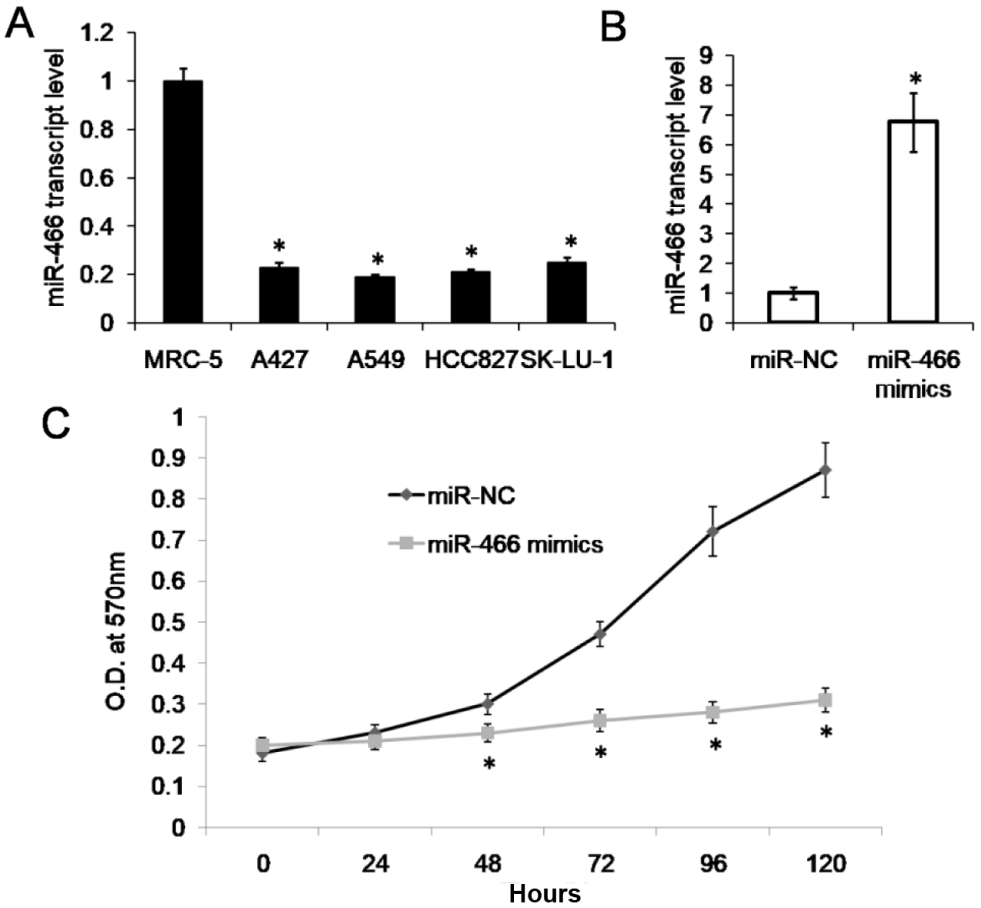

Figure 1. miR-466 is downregulated in human lung cancer and its overexpression declines the cancer cell proliferation. **A:** Expression analysis of miR-466 in normal human lung cell line MRC-5 and cancer cell lines A427, A549 HCC827 and SK-LU-1. **B:** Expression analysis of miR-466 in A549 lung cancer cells transfected with miR-NC or miR-466 mimics. **C:** Assessment of proliferation of A549 cancer cells transfected with miR-NC or miR-466 mimics. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

as per the manufacturer's protocol. Briefly, the cells were placed in 96-well plate at 2x10^5 cells/well. The cells were then cultured for 0, 24, 48, 72, 96h or 120h at 37°C. Their proliferation rates were estimated at the respective time intervals by adding CCK8 reagent and optical density (OD) calculations were performed at 570nm using spectophotometer.

Colony formation assay

The viability of lung cancer cells was determined by culturing them in 6-well plates for 7 days using 100µl initial inoculums. The cells were then collected and fixed with methanol and the colonies were visualized using crystal violet staining solution (0.1%) under light microscope.

Migration and invasion assays

Wound healing and Matrigel Boyden chamber assays were used to assess the migration and invasion of lung cancer cells, respectively. In the wound healing assay, the cells were placed in the 6-well plate and then cultured for 48h. The cell surface was then wounded by making a scratch with a sterile pipette tip. The scratch was photographed. After 24h of incubation at 37°C the scratch was again photographed to estimate the migration of cancer cells. For determining the invasiveness of cancer cells, 10^5 cancer cells were placed in the upper chamber of Matrigel and the lower chamber was inoculated with 10% FBS only. After 24h incubation at 37°C, the cancer cells sticking to upper side of the Matrigel were removed carefully and the cells invading the lower chamber were evaluated to assess the cell invasion.

In silico target identification and dual luciferase assay

The miR database (miRdb, http://www.mirdb.org/index.html) was used to scan the miR-466 for identifying its molecular target. The dual luciferase assay was used to investigate the interaction of miR-466 and its identified target, i.e., RUNX2. The 3' UTR segments of RUNX2 (wildtype, WT or mutated, MUT) were cloned into luciferase vector pCHECK2.0. The luciferase vector construct of WT 3'-UTR or MUT 3'-UTR of RUNX2 was co-transfected with miR-NC or miR-466 mimics and the luciferase activity of the transfected cancer cells was determined using Dual Luciferase Reporter assay system (Promega, Madison, Wiskonsin, USA). The luciferase activity of renilla gene was used for normalizing the luciferase activity values.

Western blotting

The RIPA lysis buffer (Thermo Scientific) was used to lyse the cancer cells. The total proteins concentrations were then determined by the Lowry assay. About 40µg of total proteins were run on 8% SDS-PAGE gels. The SDS-PAGE contents were transferred to the PVDF membranes which were then incubated with primary and secondary antibodies specially designed for the purpose of protein concentration estimation. The bands of particular proteins were finally visualized using an enhanced chemiluminescence reagent. Human β-actin gene was used as a control in western blotting experiments.

Statistics

The statistical data was validated using Graphpad Prism 7.0 software. The values were given as mean±SD. Student's *t*-test and one-way ANOVA were used for determining statistically significant difference (represented as $p< 0.05$).

Results

miR-466 expression is repressed in lung cancer

When the transcript levels of miR-466 were estimated in the normal human lung cell line (MRC-5) and cancer cell lines (A427, A549, HCC827 and SK-LU-1), it was seen that the miR-466 expression level was significantly lower in all the cancer cell lines than the normal lung cells (MRC-5) (Figure 1A), indicating the probable molecular role of miR-466 in human lung cancer. Furthermore, the expression was lowest in the A549 cell line out of all the four cancer cell lines. Hence, for characterizing the role of miR-466 in cancer, the A549 was used in further experimentations.

mir-466 upregulation reduced the proliferation of lung cancer cells

To infer the role of miR-466 in regulating the proliferation of lung cancer cells, the A549 can-

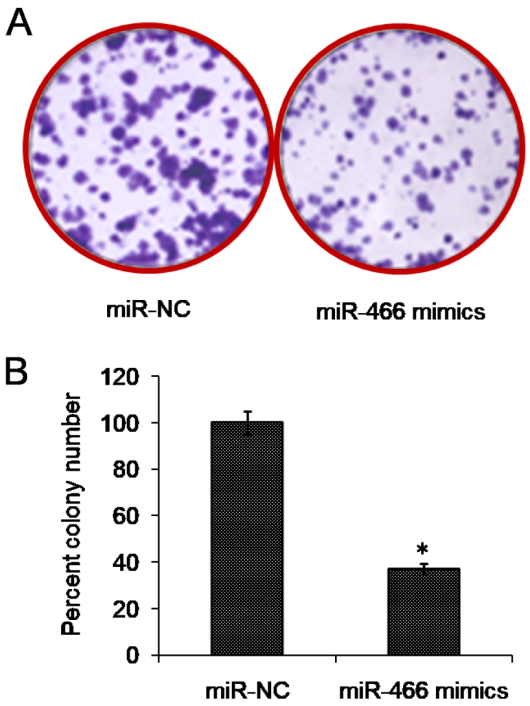

Figure 2. Overexpression of miR-466 declines the viability of A549 lung cancer cells. **A:** Colony forming assay of A549 cancer cells transfected with miR-NC or miR-466 mimics. **B:** Percentage of colonies formed by A549 cancer cells transfected with miR-NC or miR-466 mimics. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

cer cells were transfected with miR-466 mimics to overexpress miR-466 in them. The overexpression was confirmed by the qRT-PCR analysis using miR-NC transfected A549 cells as negative control (Figure 1B). When the proliferation rates of A549 cancer cells overexpressing miR-466 were determined by the CCK-8 kit, they were seen to exhibit remarkably lower proliferation rates (Figure 1C). This finding suggests the therapeutic potential of miR-466 in controlling the lung cancer growth.

Lung cancer cells exhibited lower viability under miR-466 overexpression

The colony forming assay was used to assess the viability of A549 cancer cells transfected with miR-NC or miR-466 mimics. It was found that A549 cells overexpressing miR-466 had significantly lower colony forming potential compared with the control cells (Figure 2A). The relative colony number for A549 cancer cells overexpressing miR-466 was only 37 (Figure 2B), suggesting that miR-466 regulates the viability of lung cancer cells.

Overexpression of miR-466 restricted the metastasis of lung cancer cells

The A549 cancer cells transfected with miR-466 mimics or miR-NC were assessed for their migratory potential by wound healing assay. It was seen that miR-466 mimics-transfected cells exhibited significantly lower migration potential (Figure 3A). The percentage of migration was only 34 for the cancer cells transfected with miR-466 mimics (Figure 3B). Similar findings were obtained regarding the invasion of A549 cancer cells overexpressing miR-466 with invasion cell percentage of only 38 (Figure 4A and 4B). Together, these results revealed the role of miR-466 in regulating the metastasis of human lung cancer cells.

RUNX2 is targeted by miR-466

Online bioinformatics for target identification of miR-466 showed RUNX2 transcription factor is targeted by miR-466 in lung cancer by binding to its 3' UTR region (Figure 5A). The result was then confirmed by the dual lucifearse assay which showed that cancer cells co-transfected miR-466 mimics and pCHECK2.0 vector construct contain-

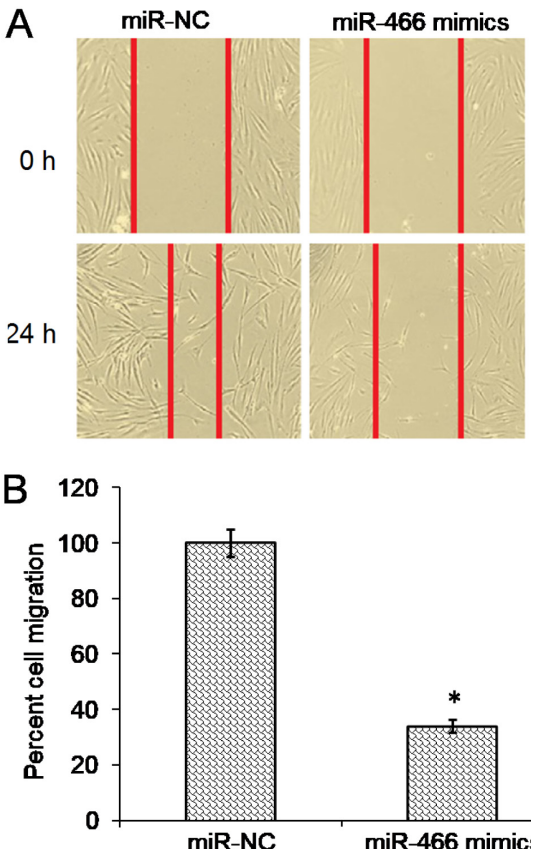

Figure 3. Upregulation of miR-466 represses the migration of A549 lung cancer cells. **A:** Wound healing assay for the assessment of migration of A549 cancer cells transfected with miR-NC or miR-466 mimics. **B:** Percentage of cell migration of A549 cancer cells transfected with miR-NC or miR-466 mimics. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

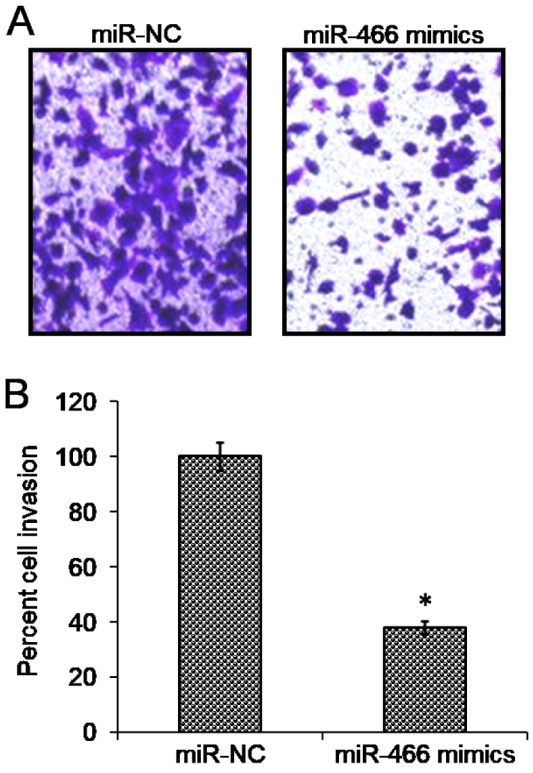

Figure 4. Upregulation of miR-466 represses the invasion of A549 lung cancer cells. **A:** Matrigel chamber assay for the assessment of invasion of A549 cancer cells transfected with miR-NC or miR-466 mimics. **B:** Percentage of cell invasion of A549 cancer cells transfected with miR-NC or miR-466 mimics. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

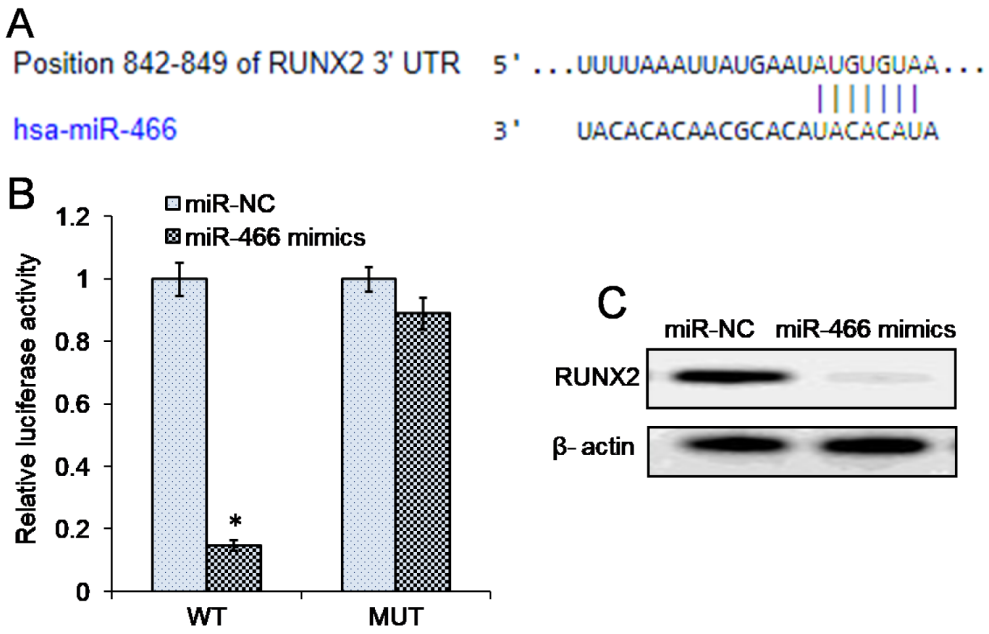

Figure 5. miR-466 targets RUNX2 in human lung cancer. **A:** Target identification of miR-466 by online bioinformatics. **B:** Dual luciferase reporter assay for interaction analysis of miR-466 and RUNX2. **C:** Western blotting of RUNX2 from A549 cancer cells transfected with miR-NC or miR-466 mimics showing miR-466 overexpression inhibits the RUNX2 expression. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

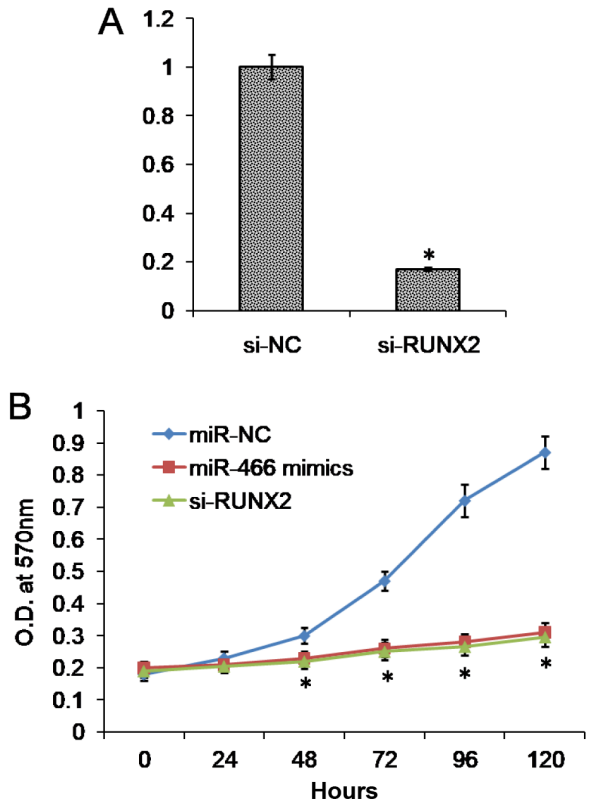

Figure 6. Silencing of RUNX2 mimics the regulatory effects of miR-466 overexpression. **A:** Expression analysis of RUNX2 in A549 lung cancer cells transfected with si-NC or si-RUNX2. **B:** Assessment of proliferation of A549 cancer cells transfected with miR-NC, miR-466 mimics or si-RUNX2 showing RUNX2 silencing inhibits A549 lung cancer proliferation. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

ing WT 3' UTR of RUNX2 against the one with MUT 3' UTR (Figure 5B). The results were also supported by the western blot analysis of RUNX2 transcription factor. The miR-466 overexpressing A549 cancer cells expressed lower concentrations of RUNX2 protein in comparison to control cancer cells (Figure 5C).

RUNX2 mediates the anticancer effects of miR-466 in lung cancer

Whether miR-466 exerts its role by repressing RUNX2 at post-transcriptional level, RNAi based silencing of RUNX2 was performed in A549 cancer cells and the results were confirmed by qRT-PCR (Figure 6A). The A549 cancer cells transfected with silencing construct of RUNX2 exhibited significantly lower proliferation rate (Figure 6B), showing that the regulatory control of miR-466 on lung cancer cell proliferation is exerted through RUNX2 transcription factor.

Discussion

The human lung cancer is ranked among the most deadly malignancies [13]. Despite the current advancements of anticancer approaches, the mortality rates of lung cancer are fairly high. The overall 5-year survival rate of lung cancer is as low as 5% [3]. Such alarming facts reveal that there is an urgent need to better understand the development and growth of lung cancer so as to devise better

anticancer strategies against this deadly disease. At molecular level, the human cancers have been shown to proliferate via dysregulation of molecular regulators of gene expression like miRs [14]. There is growing evidence that miRs play crucial role in the growth and proliferation of human cancers [15,16]. They are also important for regulating the metastasis of various human cancers [17]. miR-466 has been implicated by previous reports to control the proliferation and metastasis of a number of cancers like bone and prostate cancers, etc [18,19]. Here in our current work, we showed that miR-466 expression is repressed in the human lung cancer cells. This is in accordance with previous finding about miR-466 [20]. The overexpression of miR-466 in lung cancer cells markedly declined their proliferation rates as was previously reported by Jia et al for its role in hepatocellular carcinoma [21]. The reduction of lung cancer cell proliferation was also evident as the fall of their colony forming potential. Furthermore, research studies of the past have shown that miR-466 regulates the metastasis of cancer cells [19]. Confirming the same, when the miR-466 transcriptional upregulation was performed in the lung cancer cells, their metastatic potential got decreased significantly. The *in silico* analysis revealed that miR-466 targets the human Runt-related transcription factor 2 (RUNX2) and performs its post-transcriptional silencing. MiR-466 achieves this by interacting with a specific binding site in the 3' upstream region (UTR) of RUNX2. The human RUNX2 is a transcription factor which regulates the development of osteoblast and the bone [21]. RUNX2 has been proved to play a vital role in the metastasis of human cancers [22]. In our present work, we confirmed that RUNX2 is the molecular modulator of effects of miR-466 in lung cancer. The silencing of RUNX2 mimicked the anticancer effects miR-466 upregulation in lung cancer cells. A similar conclusion was reported about the role of miR-466 in prostate cancer [23]. Summing up, the current study is a vital illustration of the molecular potential of miR-466 in regulating the growth and metastasis of human lung cancer cells.

Conclusion

The results of the present study support the molecular potential of miR-466 in regulating the growth and metastasis of human lung cancer cells. The study further highlighted the regulatory control of miR-466/RUNX2 axis in the growth and proliferation of lung cancer cells.

Conflict of interests

The authors declare no conflict of interests.

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