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RESEARCH ARTICLE

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Effects of HMGA2 gene downregulation by siRNA on lung carcinoma cell migration in A549 cell lines

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Abstract

Background: Although there are multiple treatments for lung cancer, the death rate of this cancer remains high because of metastasis in earlier stages. So a novel treatment for overcoming metastasis is urgently needed. Overexpression of high-mobility group AT-hook 2 (HMGA2), a nonhistone chromosomal protein has been observed in metastatic cancers. So, we suggested that HMGA2 upregulation may play a critical role in treating lung cancer.

Methods: The A549 cells were transfected with specific HMGA2 small interfering RNA (siRNA) using transfection reagent. Relative HMGA2 and matrix metallopeptidase 1 (MMP1), C-X-C chemokine receptor type 4 (CXCR4), vimentin, and E-cadherin messenger RNA expression levels were measured by quantitative real-time polymerase chain reaction. To diagnose cytotoxic effect of HMGA2 siRNA and other components of transfection process, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was applied. The migration capacity after transfection with HMGA2 siRNA was detected by wound-healing assay.

Results: HMGA2 siRNA significantly reduced HMGA2 expression in a dose-dependent manner 48 hours after transfection. Expression levels of MMP1, vimentin, and CXCR4 were reduced, but E-cadherin level was not changed meaningfully. HMGA2 knockdown significantly reduced cell survival rate and also led to the inhibition of cell migration.

Conclusions: Our results indicated that RNA interference by downregulation of HMGA2 gene expression and affecting downstream genes led to the inhibition of cell migration and proliferation. Therefore, HMGA2 siRNA might be an alternative treatment option for metastatic lung cancer.

KEYWORDS

A549, epithelial-mesenchymal transition, high-mobility group AT-hook 2, small interfering RNA

1 | INTRODUCTION

Lung cancer, which originates from genetically and epigenetically altered normal epithelial cells remains the deadliest cancer among both genders.[1-4] Despite the advances in early detection, diagnosis, and variety of remedies that exist including surgery, chemotherapy, immunotherapy, targeted therapy, and radiation therapy,

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as well as the therapeutic progresses that had occurred in last decades, less than 20% of all patients were alive for 5 years after the lung cancer was diagnosed.[5-9] This low rate of survival was due to the fact that most patients at the time of diagnosis had reached an inoperable and metastatic stage.[4,7] Therefore, understanding the molecular mechanism of metastasis and pin pointing the genes involved, with new treatment modalities for effectively preventing this process is urgently required and may contribute to lower morbidity and mortality rate.[8-10]

Among gene therapy methods, RNA interference (RNAi) is known because of several factors, such as reducing toxicity and side effects, higher specificity, potency and efficiency, carrying out numerous rounds of messenger RNA (mRNA) cleavage, low cost in comparison to other cancer therapy methods, and simultaneous targeting various other genes that can be considered as a proper candidate to attain this purpose.[11-14]

RNAi is a mechanism of post-transcriptional gene regulation. This is mediated by short interfering RNAs (siRNAs). The siRNAs are 21 to 23 bp long double-stranded RNA molecules with two nucleotides overhanging at 3′ ends.[12,15] These siRNA duplexes may originate from endogenous sources like LINE1 transposons or exogenous double-stranded RNA (dsRNAs). In this case, long exogenous dsRNAs after entering cytoplasm are cleaved by DICER, a dsRNA-specific RNAase III family ribonuclease, into 21 to 23 nucleotide fragments.[16] After separation of duplex fragments by helicase and degradation of sense strand by the endonuclease, the antisense strand is loaded onto RNA-induced silencing complex (RISC). This complex is guided by siRNA to the target mRNA, which is highly complementary to the sequence of siRNA. The mRNA will be cleaved by a member of a RISC called Argonaute 2. So, RNAi by suppression of gene expression ceases its role-playing in the cell.[17-20]

High-mobility group AT-hook 2 (HMGA2) previously called HMGI-C, a member of high-mobility group A-T hook genes, encodes a nonhistone chromosomal protein. Previous research has demonstrated a role of this multifunctional protein in several biological processes including chromatin organization; cell growth; differentiation; embryogenesis; gene expression; participates in malignant transformation, epithelial-mesenchymal transition (EMT) process; apoptosis; cell cycle control invasion; and metastasis.[21-26] HMGA2 regulates these processes by binding to A-T rich regions at minor groove of DNA and changing its structure to call transcription factors and/or by interaction with them. HMGA2 is expressed during embryogenesis and after that tissue differentiation is not detectable.[22,27,28] But overexpression of this gene has been observed in several cancers, such as pancreas, gastric, lung, ovarian, breast, and other cancers.[11,29] Also HMGA2 by regulating EMT-related proteins, such as snail, twist, C-X-C chemokine receptor type 4 (CXCR4), vimentin, and E-cadherin is correlated with invasion and metastasis.[26,29-31]

During EMT process epithelial cells acquire mesenchymal properties and show morphological changes, for example, losing ECM interaction and increased invasive behavior. This process lets cancerous cells to migrate and metastasize from primary site to distant sites and make secondary tumors.[32-35]

The aim of this study was to find out the effect of HMGA2 gene knockdown by siRNA on cell migration of A549 lung cancer cell line and expression changes of CXCR4, vimentin, matrix metallopeptidase 1 (MMP1), and E-cadherin genes that are involved in EMT and cell migration processes.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human lung cancer-derived cell line, A549 (Pasteur Institute, Tehran, Iran) was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) (Sigma-Aldrich), 1% sodium pyruvate, and 2 mM glutamine; and was incubated at 37°C, 5% CO_2, 95% humidified atmosphere. The cells were subcultured for 24 and 48 hours later with an initial concentration of 4×10^4 cells/mL and used in the logarithmic phase in all the experiments.

2.2 | SiRNA transfection

A549 cells with a concentration of 5×10^5 cells per well were seeded onto six-well plates and were incubated at 37°C, 5% CO_2, 95% humidified atmosphere till they reached 60% to 70% confluency. Just before transfection, the culture medium was removed and cells were cultivated in RPMI-1640 medium free of FBS and antibiotics. Transfection of siRNA that was purchased from Santacruz Biotechnology (Santa Cruz, CA) was performed using transfection reagent (Santacruz Biotechnology) according to the manufacturer's recommendations.

To sum up, 80 pmol of siRNA and 80 pmol of transfection reagent were separately diluted in siRNA transfection medium (Santacruz Biotechnology). After 10 minutes incubation of diluted solutions at room temperature, they were mixed and incubated for

20 minutes more at room temperature. Eventually, this mixture was added to wells, containing cells and transfection medium. (The cells incubated only with transfection reagent were considered as controls.) Cell culture plate was incubated for 6 hours at 37°C in a CO_2 incubator. Afterward, RPMI-1640 medium containing 20% FBS was added to the transfected cells. The plate was incubated under above-mentioned conditions.

Suppression of HMGA2, CXCR4, MMP1, vimentin, and E-cadherin genes expression was assessed 48 hours later by quantitative real-time polymerase chain reaction (qRT-PCR).

2.3 | Total RNA extraction, reverse transcription, and qRT-PCR

To measure quantitative mRNA expression, at different time points after transfections and also the expression of downstream genes, total cellular RNA was extracted by the RiboEx reagent (GeneAll, Seoul, Korea) according to the manufacturer's instructions. To synthesize Complementary DNA (cDNA) 5 μg of total RNA plus 1 μg random hexamer primer, MMLV reverse transcriptase (Promega, Madison, WI) was used according to the manufacturer's instructions. qRT-PCR reactions to determine transcript amounts of target genes were run in the light cycler 96 Roche system (Roche, Germany) using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan).

The reaction system of PCR was 5 μL of master mix 2×, 0 to 25 μL of primers, 1 μL of cDNA template, and 3.75 μL of nuclease-free distilled water. The primer sequences are shown in Table 1. The mRNA levels of genes were normalized to endogenous β-actin. PCR conditions consisted of one predenaturation cycle of 10 minutes at 95°C, which was followed by 45 cycles at 95°C for 10 seconds and 60°C for 35 seconds and 72°C for 20 seconds. Relative HMGA2 and other genes mRNA expression (Table 1) was measured with the $2^{-\Delta\Delta C_t}$ method. Reactions were carried out in triplicate.

2.4 | Cytotoxicity assay

The cytotoxicity effects of different doses of HMGA2 siRNA, transfection reagent, and taxol on the A549 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich). The experiment was subdivided into eight groups: different concentration of siRNA (20-80 pmol), transfection reagent, scrambled siRNA, taxol, and control. In brief, to assess cell viability, 15 000 cells were seeded onto each well of 96-well cell culture plates and incubated. After reaching 60% to 70% confluency, cells were exposed to the different concentration of siRNA plus transfection reagent, 48 hours later medium of wells was removed and substituted with fresh medium and 50 μL of MTT solution (2 mg/mL in PBS) and incubated for 4 hours. Afterward, 200 μL of DMSO plus Sorensen phosphate were added and incubated for 30 minutes. The amount of formazan dye was determined by quantifying its absorbance (A) at 570 nm using a microplate reader (Awareness Technology, Palm City, FL). The survival rate (SR) was measured from the following equation: $SR\ (\%) = (A_{\text{Treatment}} / A_{\text{Control}}) \times 100\%$.

2.5 | Wound-healing assay

To investigate cell migration capacity after transfection, wound-healing assay was carried out in this assay. Nearly, 50 000 cell were seeded onto each well of 24-well plate and incubated. When the A549 cells in the monolayers were more than 90% confluent, the "wound" was made by scratching each well with a sterile yellow micropipette tip and cell debris were washed with serum-free medium. Images of cell migration at 0, 6, 12, 24, and 48 hours after transfection were captured by the light microscope. The migration capacity was attained by measuring the distance between the wound edges. Wound-healing assay was repeated in triplicate.

TABLE 1 Sequences of forward and reverse primers used in qRT-PCR reactions

Gene	Forward primer	Reverse primer
HMGA2	5′-TGGGAGGAGCGAAATCTAAA-3′	5′-TCCCTGGAGAAGAGCTACG-3′
MMP1	5′-GCGCACAAATCCCTTCTACC-3′	5′-ATCCGTGTAGCACATTCTGTCC-3′
CXCR4	5′-AACTTCAGTTTGTTGGCTGC-3′	5′-TTTAACATGTACTTTTATTA-3′
Vimentin	5′-CAGGCAAAGCAGGAGTCCA-3′	5′-AAGTTCTCTTCCATTTCACGCA-3′
E-cadherin	5′-TGCCCAGAAAATGAAAAAGG-3′	5′-GTGTATGTGGCAATGCGTTC-3′
B-actin	5′-TCCCTGGAGAAGAGCTACG-3′	5′-GTAGTTTCGTGGATGCCACA-3′

Abbreviations: CXCR4, C-X-C chemokine receptor type 4; HMGA2, high-mobility group AT-hook 2; MMP1, matrix metallopeptidase 1; qRT-PCR, quantitative real-time polymerase chain reaction.

2.6 | Statistical analysis

Data were presented as mean ± SD, and statistical differences between control and transfected groups were analyzed using the Student *t* test and analysis of variance followed by Dunnet test. And $P < 0.05$ was defined as statistically significant.

3 | RESULTS

3.1 | siRNA decreased HMGA2 expression level in A549 cells

qRT-PCR was used to measure HMGA2 mRNA expression level, mRNA expression in transfected A549 cells in 24, 48, and 72 hours was measured and relative expression was calculated in relationship to control cells, which were considered as 100%. As shown in Figure 1, HMGA2 siRNA lead to a significant mRNA expression reduction in 48 hours after transfection (50%), but in 24 and 72 hours, expression levels were 75% and 78%, respectively. Also mRNA expression levels were 95%, 90%, 75%, and 45% for 20, 40, 60, and 80 pmol of siRNA doses, respectively. So, it is obvious that inhibition rate of HMGA2 is not time dependant but it is dose dependent. All the data compared with control (negative control siRNA) group and the $P < 0.05$ was considered as significant between the groups.

3.2 | Reduction in expression levels of CXCR4, MMP1, and vimentin

To confirm the relationship between HMGA2 and 4 EMT-related genes (CXCR4, MMP1, vimentin, and E-cadherin), relative mRNA expression levels of these genes after HMGA2 downregulation were measured by qRT-PCR and calculated in relation to the control group, as shown in Figure 2; expression of MMP1 was reduced remarkably (7%), CXCR4 (89%) and vimentin (55%) levels were reduced too, but E-cadherin level (104%) did not show significant changes.

3.3 | Reduction of cell viability by HMGA2 siRNA

To assess HMGA2 downregulation effect, at different doses in combination with transfection reagent on cell survival of A549 cells, MTT assay was done. As shown in Figure 3, taxol decreased the cell survival and HMGA2 siRNA at the dose of 80 pmol significantly reduced the cell survival rate to 40% in comparison with the control group. Also, other transfection elements did not have any effect on cell viability.

3.4 | Suppression of HMGA2 led to inhibition of migration

HMGA2 gene downregulation by siRNA blocked A549 cells migration in vitro, this result was obtained from wound-healing assay. HMGA2 gene knockdown with specific siRNA led to reduced migration of cells to scratched area; the number of A549 cells 48 hours after transfection in this area was 320 ± 20 in comparison with control cells 720 ± 15 (Figure 4).

4 | DISCUSSION

Surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy are the existing treatment modalities for lung cancer, which because of its metastatic

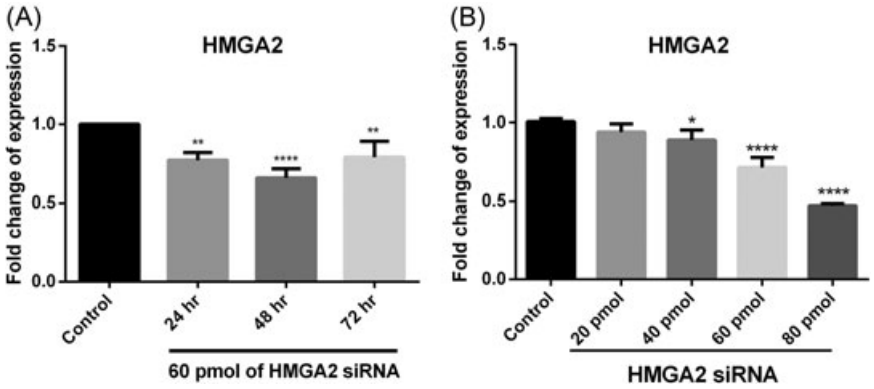

FIGURE 1 Suppression of HMGA2 mRNA expression by siRNA in A549 lung cancer cell line. A, A549 cells were transfected with HMGA2 siRNA for 24, 48, and 72 hours. B, A549 cells were transfected with HMGA2 siRNA with the doses of 20, 40, 60, 80 pmol. Relative HMGA2 mRNA expression was measured by qRT-PCR using $2^{-\Delta\Delta C_t}$ method. The results are expressed as mean ± SD; n = 3; $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p = 0.0001$ and $^{****}p < 0.0001$ vs control. HMGA2, high-mobility group AT-hook 2; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA

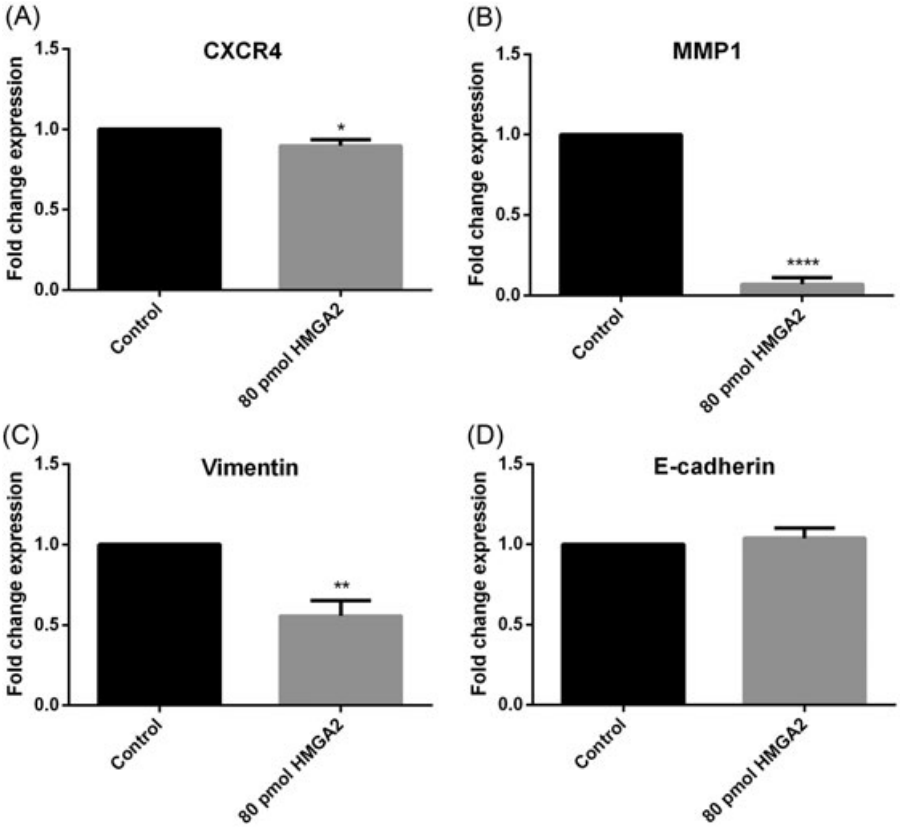

FIGURE 2 Relative CXCR4, MMP1, vimentin, and E-cadherin after HMGA2 silencing in A549 cell line. CXCR4, MMP1, vimentin, and E-cadherin mRNA expression were evaluated by qRT-PCR. Data are presented as means ± SD; n = 3; *$p < 0.05$, **$p < 0.001$, ***$p = 0.0001$, and ****$p < 0.0001$ versus control. CXCR4, C-X-C chemokine receptor type 4; HMGA2, high-mobility group AT-hook 2; MMP1, matrix metallopeptidase 1; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction

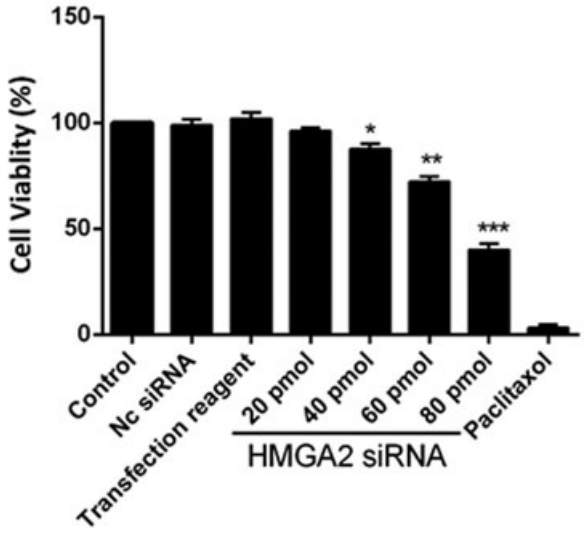

FIGURE 3 Effect of HMGA2 siRNA on cell survival of A549 cell line. After 48 hours of HMGA2 siRNA transfection (20, 40, 60, and 80 pmol), as mentioned in Section 2, survival treatments was determined by MTT assay. The data is represented as the mean ± SD; n = 3; *$p < 0.05$, **$p < 0.$ 0.01, and **$p < 0.001$ versus control group. HMGA2, high-mobility group AT-hook 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; siRNA, small interfering RNA

behavior, at the time of diagnosis cannot increase the survival rate.[3,7] Moreover, RNAi, from its first description in 1998 by Fire and Mello, has been used in multiple experiments because of its high efficacy, potency, specificity, and low cost is an upgrowing field as cancer treatment.[11,12] Since the first observations of HMGA2 involvement in malignancies was about 30 years ago, a lot of studies have identified its misexpression in numerous cancers.[29,36] These include colorectal, breast, lung, gastric, ovarian, pancreas, oral, colorectal, thyroid, bladder retinoblastoma, melanoma, nasopharyngeal and hepatocellular carcinomas, and mesenchymal tumors.[11,26,29,37-39]

HMGA2 is a nonhistone nuclear protein that contains three A-T hook DNA binding motifs that interact with several transcription factors, such as NF-kB, ATF-2/c-Jun, Elf-1, Oct-2, Oct-6, SRF, NF-Y, PU-1, RAR, SNAIL, and SLUG.[23,40] Also, it directly or indirectly alters EMT-related proteins expression.[41]

To silence HMGA2 gene in an ideal circumstance we need to get optimum dose siRNA and transfection reagent at most appropriate time. To attain this purpose different doses of transfection reagent (2, 4, 6, and 8 pmol) with a mean dose of siRNA (6 pmol) were used.

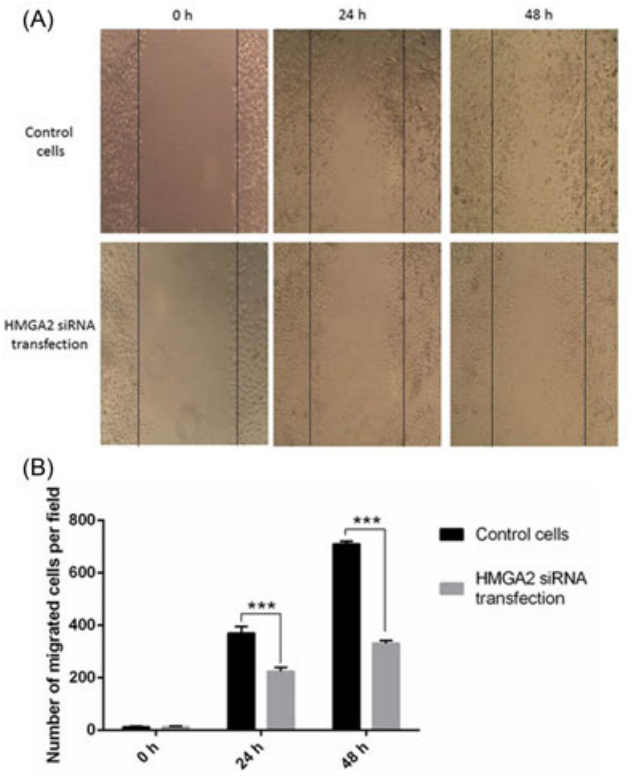

FIGURE 4 The effect of HMGA2 siRNA on A549 lung cancer cells migration. A, Images at 0, 24, and 48 hours after transfection of HMGA2 siRNA and negative control siRNA (control). B, The number of cells that migrated into scratched area quantified in 0, 24, and 48 hours of HMGA2 silenced group and compared with negative control siRNA group

After 24 hours, relative expression of HMGA2 was measured by qRT-PCR and showed that HMGA2 knockdown occurred in a dose-dependent manner. So, 8 pmol of transfection reagent was chosen as an optimum dose. In a similar way, siRNA optimum dose was obtained. Cells were transfected with 8 pmol of transfection reagent and different doses of siRNA (2, 4, 6, and 8 pmol) and again HMGA2 expression levels were measured 24 hours later. This time, inhibition rate of HMGA2 was dose dependent too, and most reduced level was 8 pmol of siRNA. After getting optimum doses, cells were transfected with 8 pmol of siRNA and 8 pmol of transfection reagent. RNA extraction, DNA synthesizes, and qRT-PCR were done 24, 48, and 72 hours later. Significant HMGA2 mRNA reduction was observed in 48 hours after transfection.

MTT assay with the objective of checking cytotoxic effects of siRNA, transfection reagent and other elements of transfection experiment was done. Results indicate that observed cell death was due to HMGA2 gene silencing by siRNA and its effect on A549 cells. Also the results showed the reagents and media did not induce cytotoxic effects. Cationic lipids were among nonviral

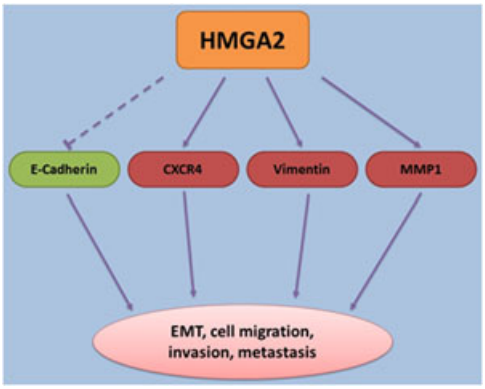

FIGURE 5 Schematic model of the HMGA2 regulatory actions in lung cancer. CXCR4, C-X-C chemokine receptor type 4; EMT, epithelial-mesenchymal transition; HMGA2, high-mobility group AT-hook 2; MMP1, matrix metallopeptidase 1

vectors that because of low cost, easy production, reduced pathogenicity, safety, and low toxicity were used in numerous nucleic acid delivery experiments. Nonetheless, this low rate toxicity of cationic lipid nucleic acid complex is known to be due to cationic lipid components. So, if transfection reagent did not induce cytotoxic effect in this study, it could be due to usage of santacruz transfection reagent.[42,43]

To assess migration rate, wound-healing assay was operated. After transfection at 0, 24, and 48 hours, photos from scratched area were captured. Number of cells migrated to wound side in control group was much more than the transfected group overtime. So HMGA2 downregulation by siRNA could reduce the migration capacity of A549 cells. With respect to this we examined the effect of HMGA2 downregulation with specific siRNA on A549 cell line migration, cell cytotoxicity, and alteration of EMT-related genes expression.

In this study, qRT-PCR results showed that mRNA level of HMGA2 was reduced 48 hours after transfection in a dose-dependent manner. Also, expression levels of MMP1 and vimentin were reduced significantly, and the CXCR4 level was reduced too, but E-cadherin level was not changed. Wound healing and MTT assay results showed that HMGA2 knockdown with siRNA significantly reduced cell migration and cell viability in comparison with control group.

These results are in concordance with the hypothesis of study that stated, HMGA2 knockdown by decreasing vimentin, MMP1, and CXCR4 expression contributed to a reduction of migratory ability and cell survival rate of A549 cells. Nevertheless, it does not approve E-cadherin enhancement after the HMGA2 knockdown.

Previous studies have shown an association between HMGA2 overexpression and progression, and poor prognosis and early metastasis of various cancers.[44] HMGA2

knockdown in colon and breast cancer contributed to growth, invasion, and metastasis reduction.[45] These results are comparable with our results and other studies, such as Mansoori et al[11] results showed that HMGA2 siRNA inhibited MDA-MB 468 cell migration and reduced cell viability. Also, other studies using LET7 micro RNA families had stated same results, for example, LET7g micRNA usage on A549 cell line by targeting HMGA2 inhibited cell migration.[46] LET7a micRNA showed the same effect by reducing HMGA2 translation, but on 95D cells.[10] In a study managed by Wu et al[25] on clinical specimens of NPC, miRNA LET7a by downregulation of HMGA2 protein expression suppressed EMT process, migration, and invasion. Downregulation of HMGA2 by RNAi in SW480 cells decreased the invasive ability of cells.[26] In addition, this study results were in agreement with three similar studies that used HMGA2 siRNA and DOX in combination or alone in HT29, A549, MDA-MB-231 cell lines. In all of them, like our study, HMGA2 knockdown led to vimentin downregulation. Although they had reported that E-cadherin level was increased significantly after the knockdown, in this study E-cadherin level did not change meaningfully.[30,33,45] An explanation that could be considered for slightly increase in E-cadherin expression thereafter HMGA2 knockdown is that E-cadherin, which is a member of classical cadherins and is found in adherence junctions to help to sustain cell shape and polarity, is negatively regulated by snail and twist during EMT process. These two genes in turn are regulated by HMGA2. So HMGA2 knockdown by effecting downstream EMT-related genes could lead to E-cadherin increase.[29,40,47-49]

With respect to previous studies, relationships between HMGA2 and mentioned genes with EMT process could be considered. HMGA2 by affecting EMT-related genes expression has a proven role in cell migration process.[29,41] Among several genes that are regulated by HMGA2, snail and twist are conspicuous because of their involvement in EMT process by downregulation of E-cadherin and upregulation of VIM and MMPs. CXCL12/CXCR4 by increasing transcription factors of genes involved cell survival results in cell growth and metastasis.[50]

According to the studies, we can consider to connect these genes and their pathways to each other. HMGA2 activation increases CXCR4 expression. The role of CXCR4 in migration, metastasis, proliferation, and angiogenesis in multitude cancers has been reported. Also CXCR4 overexpression has been observed in lung cancer and its elevated levels is correlated with invasion and metastasis of NSCLC.[51] In contrast, ECM components degradation, such as collagens or basement membrane is a crucial step in tumor invasion mediated by MMPs.[52] And also, vimentin overexpression has been associated with EMT process and metastasis,[53] so reduced migration of cells is because of their decreased expression following HMGA2 knockdown. However, several studies have reported E-cadherin enhancement after HMGA2 knockdown. In our study E-cadherin did not show significant changes (Figure 5).

To conclude, results of this study indicate that HMGA2 knockdown and downregulation of EMT-related genes, MMP1, CXCR4, and VIM directly or indirectly reduces migration capacity of A549 cell. So these results increase hope for treatment of inoperable and metastatic lung cancer by gene therapy. Investigating other signaling pathways involved in lung cancer, such as EGFR pathway, and also other existing and not well-studied genes would be our future experiment.

We propose future works to be done on other EMT-related genes that are regulated by HMGA2, also evaluating gene expression by Western blot analysis at the protein level. We also recommend assessing apoptosis by TUNEL test to investigate HMGA2 role on cell cycle regulation; besides acting out all experiments in vitro on other lung cancer-derived cell lines.

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