International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2024.552.560

Research Article
Long Non-Coding RNA MAFG-AS1 Regulates the Proliferation and Epithelial to Mesenchymal Transition of Human Esophageal Carcinoma Cells

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Abstract

Background and Objective: Esophageal carcinoma (EC) is considered a major type of neoplastic disorder of the human gastrointestinal tract and causes significant mortality and morbidity across the globe. This research was done to find out if the Long Non-coding RNA (lncRNA) MAFG-AS1 affects the growth of human esophageal carcinoma (HEC) cells and their change from an epithelial to a mesenchymal state. **Materials and Methods:** The 44 pairs of tissue specimens were taken from carcinoma patients between the ages of 38 and 73. The HEC cell lines (EC109, TE-10 and KYSE-410) together with Het-1A and human esophageal epithelial cells, were utilized in this study. Transient transfection, RNA isolation, cDNA synthesis, RT-PCR analysis, MTT proliferation and clonogenic assays, EdU incorporation assays and the transwell assay were performed by standard methods. **Results:** The outcomes of this investigation showed that EC samples and cell lines exhibit considerable ($p<0.05$) expression of MAFG-AS1. The MAFG-AS1 knockdown substantially ($p<0.05$) decreased the growth of EC109 and KYSE-410 esophageal cancer cells via apoptosis, a rise in Bax and a reduction in Bcl-2 in EC109 and KYSE-410 esophageal cancer cells. Furthermore, in EC109 and KYSE-410, ES knockdown enhanced E-cadherin and α-catenin expression while reducing fibronectin and vimentin expression. **Conclusion:** The MAFG-AS1 can be used as a therapeutic target because it may control how HEC cells grow and change from being epithelial to mesenchymal transition (EMT) cells.

Key words: Esophageal, lncRNAs, MAFG-AS1, oncogene, apoptosis, cancer

Citation: Kang, S., H. Guo, K. Lan, N. Guo, S. Lu, F. Lv and Y. Ni, 2023. Long non-coding RNA MAFG-AS1 regulates the proliferation and epithelial to mesenchymal transition of human esophageal carcinoma cells. Int. J. Pharmacol., 20: 552-560.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Esophageal carcinoma (EC) is a more frequent neoplastic tumor of the human gastrointestinal system, affecting the upper section of the alimentary canal[1,2]. Currently, EC has the sixth highest mortality rate in the world and is the most prevalent and severe form of EC[3,4]. Furthermore, the overall five-year survival percentage for esophageal squamous cell carcinoma becomes less than 20%[5]. The higher mortality has been suggested to be a consequence of the low prognosis, metastasis and recurrence of esophageal carcinoma[6]. In these situations, it is important to find better ways to diagnose and treat HEC to improve clinical success.

The utilisation of non-coding RNAs as molecules of prognosis and therapy has shifted from concept to reality[7]. Among such approaches, lncRNAs, which control the post-transcriptional expression of specifically targeted genes, are emerging as the therapeutic molecules of choice[8,9]. Several studies have previously shown that lncRNAs are regulated in the formation and pathophysiology of a wide variety of patient malignancies[10,11]. The HECC have been shown to develop and advance as a result of abnormal lncRNA expression[12]. The lncRNAs regulate the formation and development of HEC by functioning as oncogenes or tumour regulators[13,14]. Several studies revealed that MAFG-AS1 is significantly overexpressed in numerous human malignancies, indicating that it has an oncogenic function in cancer growth, proliferation and migration[15,16]. Several researchers previously reported that MAFG-AS1 regulates breast and bladder cancer growth[17,18]. However, the significance of the lncRNA MAF-BZIP transcription factor G antisense RNA 1 (MAFG-AS1) in esophageal cancer is currently poorly understood. So, this investigation intended to identify the impacts of the lncRNAs MAFG-AS1 on HEC development and EMT.

MATERIALS AND METHODS

Study area: This investigation was carried out at the Changzhou Tumor Hospital, Soochow University, from March, 2017 to January, 2020.

Tissue samples and cell lines: In this study, 44 tumor and surrounding non-tumor samples were obtained from patients aged 38 to 73 years (26 males and 18 females) diagnosed with EC and admitted to the Institute Hospital. After surgery, the specimens were snap-frozen in cold liquid nitrogen and maintained at -80°C for a long time. The human EC cell lines (EC109, TE-10 and KYSE-410), together with Het-1A, normal human esophageal epithelial cells, were acquired from the American Type Culture Collection, USA. The cell cultures were cultivated at 37°C in RPMI-1640 medium with fetal bovine serum (10%), penicillin sodium (100 IU/mL) and streptomycin sulphate (100 g/mL). The cell cultures were grown at a temperature of 37°C in a (5%) CO_2 incubator.

Transient transfection: To knock down MAFG-AS1, small interference RNA (si-MAFG-AS1) was introduced into the cancer cells KYSE-410 and EC109, while si-NC-introduced cells served as the -ve control. The cell transfections were carried out using synthetic si-MAFG-AS1 and si-NC oligos (GenePharma, Shanghai, China), as well as Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Finally, 48 hrs after transfection, cells were harvested.

RNA isolation, cDNA synthesis and RT-PCR study: The TRIzol reagent (Invitrogen, USA) was utilized to recover total RNA from tumor and nontumor cells (Invitrogen, USA). The recovered RNA was processed by DNAse I before being reverse-transcribed into complementary DNA (cDNA) by utilising the RevertAid First-Strand cDNA Synthesis Kit. The Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) was utilised for quantitative Real-Time PCR (qRT-PCR). The QuantStudio 3.0 Real-Time PCR System was used for the qRT-PCR experiments (Applied Biosystems). Human GADPH and the U6 gene were used as endogenous controls. Table 1 expresses the primers used in the current investigation.

Table 1: Sequences of primers used in the present study

Primer	Direction	Sequence
MAFG-AS1	Forward	5'-ATGACGACCCCCAATAAAGGA-3'
	Reverse	5'-CACCGACATGGTTACCAGC-3'
Bax	Forward	5'- CTACAGGGTTTCATCCAG-3'
	Reverse	5'- CCAGTTCATCTCCAATTCG-3'
Bcl-2	Forward	5'- GTGGATGACTGAGTACCT-3'
	Reverse	5'- CCAGGAGAAATCAAACAGAG-3'
E-cadherin	Forward	5'-GACCAGGACTATGACTACTT-3'
	Reverse	5'-ATCTGCAAGGTGCTGGGTG-3'
α-catenin	Forward	5'-CAACCCTTGTAAACACCAAT-3'
	Reverse	5'-ACTGAACCTGACCGTACAC-3'
Fibronectin	Forward	5'-CCACCCCCATAAGGCATAGG-3'
	Reverse	5'-GTAGGGGTCAAAGCACGAGTCA-3'
Vimentin	Forward	5'-TGTCCAAATCGATGTGGATGTTTC-3'
	Reverse	5'-TTGTACCATTCTTCTGCCTCCTG-3'
GAPDH	Forward	5'-CAGCTAGCCGCATCTTCTTTT-3'
	Reverse	5'-GTGACCAGGCGCCCAATAC-3'
U6	Forward	5'- GTCCGGTTTCAGCATGTTT-3'
	Reverse	5'-CTCGCTTCGGCAGCACA-3'

MTT proliferation and clonogenic assay: The EC109 or KYSE-401 cancer cells stably transfected with si-MAFG-AS1 (5'-GGGCAAUUCCAACCAAGAAAC-3') or si-NC (5'-AAUUCUCCGAACGUGUCUCGU GU-3') were collected and plated into a 96-well plate with an initial inoculation of 2×10^5 cells per well. At 0, 12, 24, 48 and 96 hrs of maintenance at 37°C. Newly prepared RPMI-1640 medium supplemented with 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-Yl)-2, 5-Diphenyl tetrazolium Bromide (MTT) was used as the culture medium. After 2 hrs of maintenance, the medium was supplemented with 150 L of Dimethyl Sulfoxide (DMSO). The microplate reader was utilised to assess the absorption at 570 nm. Stable-transfected EC109 or KYSE-401 cells were grown in 6-well plates for 3 weeks until colonies formed. Grown colonies were studied via the colony formation assay. The colonies were preserved with 70% ethanol and stained for 15 min with 0.1% crystal violet solution (Sigma-Aldrich). An inverted microscope was utilised to assess the colonies (>50 cells).

EdU incorporation assay: The Si-MAFG-AS1 or si-NC were introduced for 48 hrs into EC109 or KYSE-401 cells; after that, 10 M EdU (5-ethynyl-2-deoxyuridine) was applied to the cells for an hour. The cells were then analysed by the Click-iTTM EdU Cell Proliferation Kit for Imaging and the Alexa FluorTM 488 dye kit (Thermo Fisher Scientific) and the proportion of EdU-+ve cells was analyzed. The nuclei were labelled with 4', 6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich).

Flow cytometry: To detect apoptosis in transfected EC109 or KYSE-401 cells, the Annexin V-FITC Apoptosis Staining and Detection Kit (Abcam) was utilized. Cells were harvested and dark stained for 20 min at 25°C with 7.5 µL Annexin V-FITC and 15 µL propidium iodide (PI) and analyzed by flow cytometry. The analysis of flow-cytometric data was made using Cyflogic software (Perttu Terho, Mika Korkeamaki, CyFlo Ltd.).

Transwell migration and invasion study: Transwell chambers (8.0 m pore size; Millipore, Billerica, Massachusetts, USA) were utilised to examine the migration and invasion of EC109 or KYSE-401 cells with si-MAFG-AS1 or si-NC, respectively. In summary, the upper chamber received 105 transfected cells added to 150 uL serum-free RPMI-1640, whereas the bottom chamber received 450 µL serum-free RPMI-1640 with 10% FBS. In the top chamber, the cells were removed by using cotton buds and those migrating or invading the bottom chamber were treated with methanol for 20 min and identified with 0.1% crystal violet for 25 min at ambient temperature for 24 hrs at 37°C. An upright microscope (Nikon, Japan, 200×magnification) was used for manual cell counting.

Ethical consideration: This investigation was done with the permission of the institute's ethical committee (Reg. No. 36445/2021/CPC/EsoCan/12.01.2017). The sample collection form and informed permission were received from the involved patients.

Statistical analysis: The GraphPad Prism 7.0 software was utilised for data assessment. The final data were revealed as Mean±Standard Deviation (SD) and statistical variations were assessed using a student's t-test. The statistically relevant variation was determined at $p<0.05$.

RESULTS

MAFG-AS1 knockdown suppresses the proliferation of EC cells: The MAFG-AS1 expression in EC and surrounding usual tissues was determined using qRT-PCR. According to the findings (Fig. 1a), MAFG-AS1 expression was considerably ($p<0.05$) elevated in the EC compared to normal adjacent tissues. Additionally, the expression of MAFG-AS1 in EC cell lines (EC109, TE-10 and KYSE-410) in comparison to normal epithelial cells was investigated (Het1A). The cancer cell lines expressed substantially ($p<0.05$) greater transcript values of MAFG-AS1 relative to normal Het1A cells, with the expression of MAFG-AS1 being highest in the EC109 cell line (Fig. 1b). The MAFG-AS1 was silenced in EC109 and KYSE-401 cancer cells to see if it influences EC cell proliferation and the MAFG-AS1 reduction was confirmed by qRT-PCR (Fig. 1c). Following that, an MTT experiment expressed that MAFG-AS1 knockdown inhibits the development of EC109 and KYSE-401 EC cells (Fig. 1d). Following that, an EdU test was performed to validate the decrease in cell growth caused by MAFG-AS1 knockdown. It was found that MAFG-AS1 knockdown caused significantly lower incorporation in EC109 and KYSE-401 cancer cells, suggesting inhibition of cell proliferation relative to control cells (Fig. 2a). The MAFG-AS1 inhibition was also tested on EC109 and KYSE-401 cell colony formation. The MAFG-AS1 inhibited colony formation in both EC109 and KYSE-401 esophageal cancer cells ($p<0.05$). These results thus indicate that MAFG-AS1 controls the development of EC cells (Fig. 2b).

Silencing MAFG-AS1 promotes apoptosis in EC cells: To gain insights into the possible growth-inhibitory mechanism of MAFG-AS1 knockdown, flow cytometry was

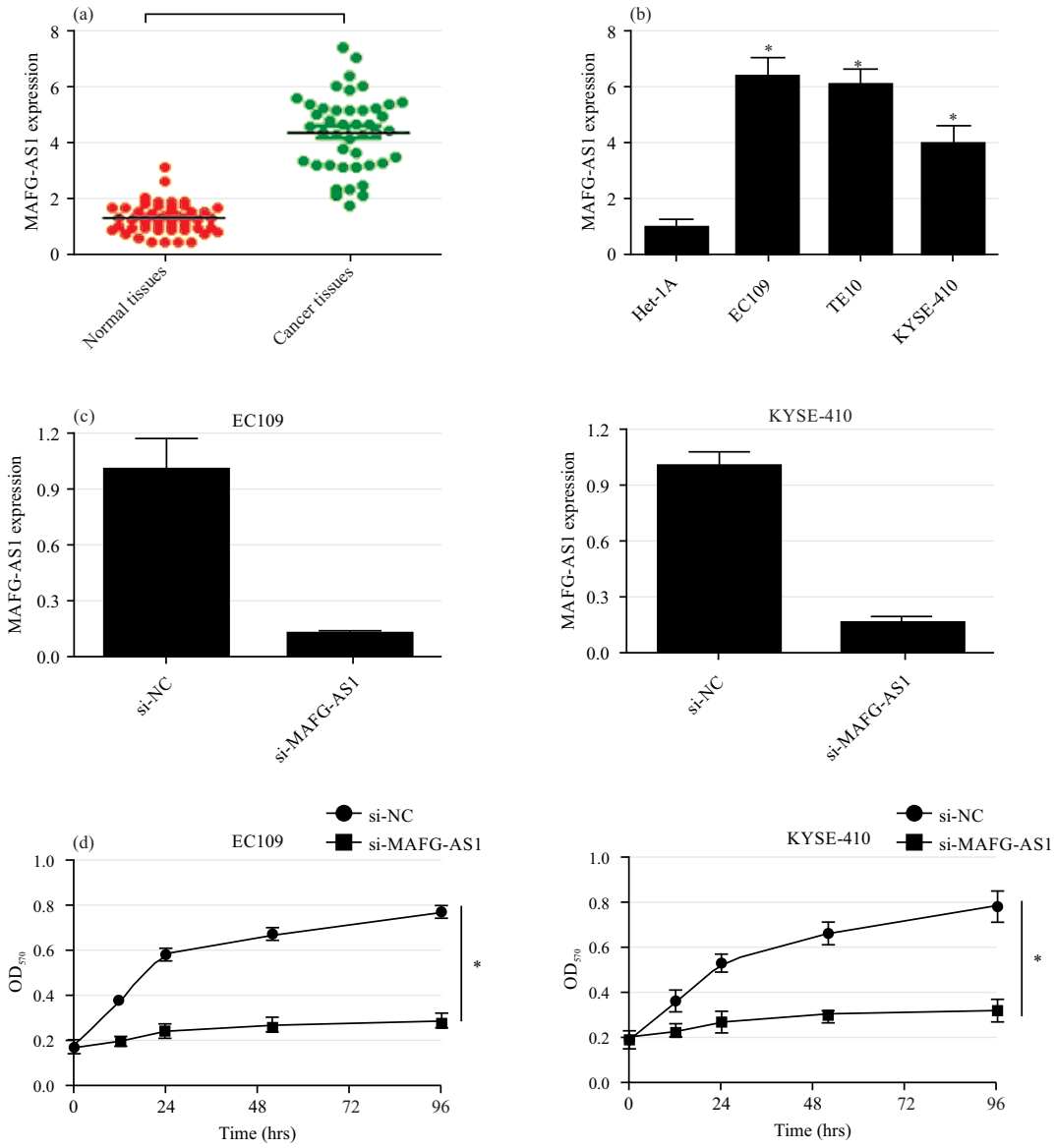

Fig. 1(a-d): Knockdown of MAFG-AS1 inhibits the esophageal cancer cell proliferation, (a) Expression of MAFG-AS1 in esophageal carcinoma and adjacent normal tissues as determined by qRT-PCR, (b) Expression of MAFG-AS1 in esophageal carcinoma cell lines (EC109, TE-10 and KYSE-410) relative to normal esophageal epithelial (Het1A) cells as determined by qRT-PCR, (c) Expression of MAFG-AS1 in si-NC or si-MAFG1-AS1 transfected EC109 and KYSE-410 cells as determined by qRT-PCR and (d) Viability of EC109 and KYSE-410 cells transfected with si-NC or si-MAFG-AS1

Experiments were performed independently in triplicates and expressed as Mean±SD (*p<0.05)

used. The results reveal that when MAFG-AS1 is silenced, EC109 and KYSE-401 cancer cells exhibit much greater levels of apoptosis than the corresponding negative control cells (Fig. 2c). The proportions of early and late apoptosis in si-MAFG-AS1 transfected cells were 6.29 and 14.7%, respectively, compared to 2.88 and 2.55% in si-NC transfected EC109 cells. Similarly, in si-MAFG-AS1 transfected cells, the proportions of early and late apoptosis were 4.20 and 14.7%, respectively, compared to 2.16 and 3.28% in si-NC transfected KYSE-401 cells (Fig. 2c). Additionally, the qRT-PCR study revealed that Bax expression was considerably upregulated and Bcl-2 was downregulated in EC109 and KYSE-401 EC upon MAFG-AS1 knockdown (Fig. 2d). The results thus confirmed that MAFG-AS1 knockdown induces apoptosis in EC cells to inhibit their proliferation.

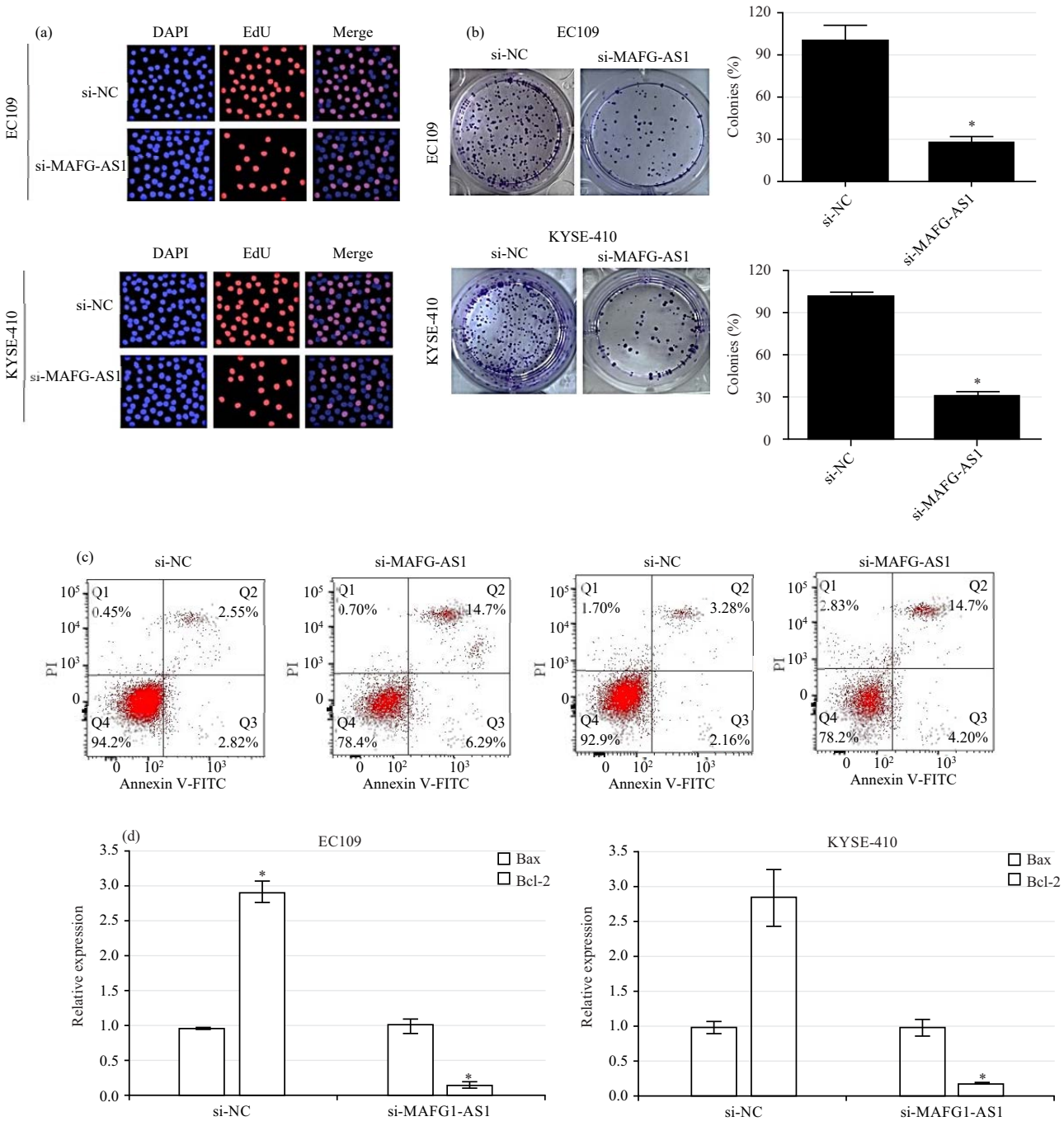

Fig. 2(a-d): Knockdown of MAFG-AS1 induces apoptosis in esophageal carcinoma cells, (a) EdU staining of EC109 and KYSE-410 cells transfected with si-NC or si-MAFG-AS1 showing cell proliferation, (b) Colony formation assay of EC109 and KYSE-410 cells transfected with si-NC or si-MAFG-AS1, (c) Annexin V/PI assay showing apoptosis in of EC109 and KYSE-410 cells transfected with si-NC or si-MAFG-AS1 and (d) Expression of Bax and Bcl-2 in EC109 and KYSE-410 cells transfected with si-NC or si-MAFG-AS1

Experiments were performed independently in triplicates and data is presented as Mean±SD (*p<0.05)

MAFG-AS1 knockdown prevents EMT of EC cells: The transwell studies performed to investigate the silencing of MAFG-AS1 did not influence the movement and infiltration of EC cells. Based on the findings, MAFG-AS1 substantially ($p<0.05$) prevented the migration of EC109 and KYSE-401 cancer cells. The movement and infiltration of EC109 and

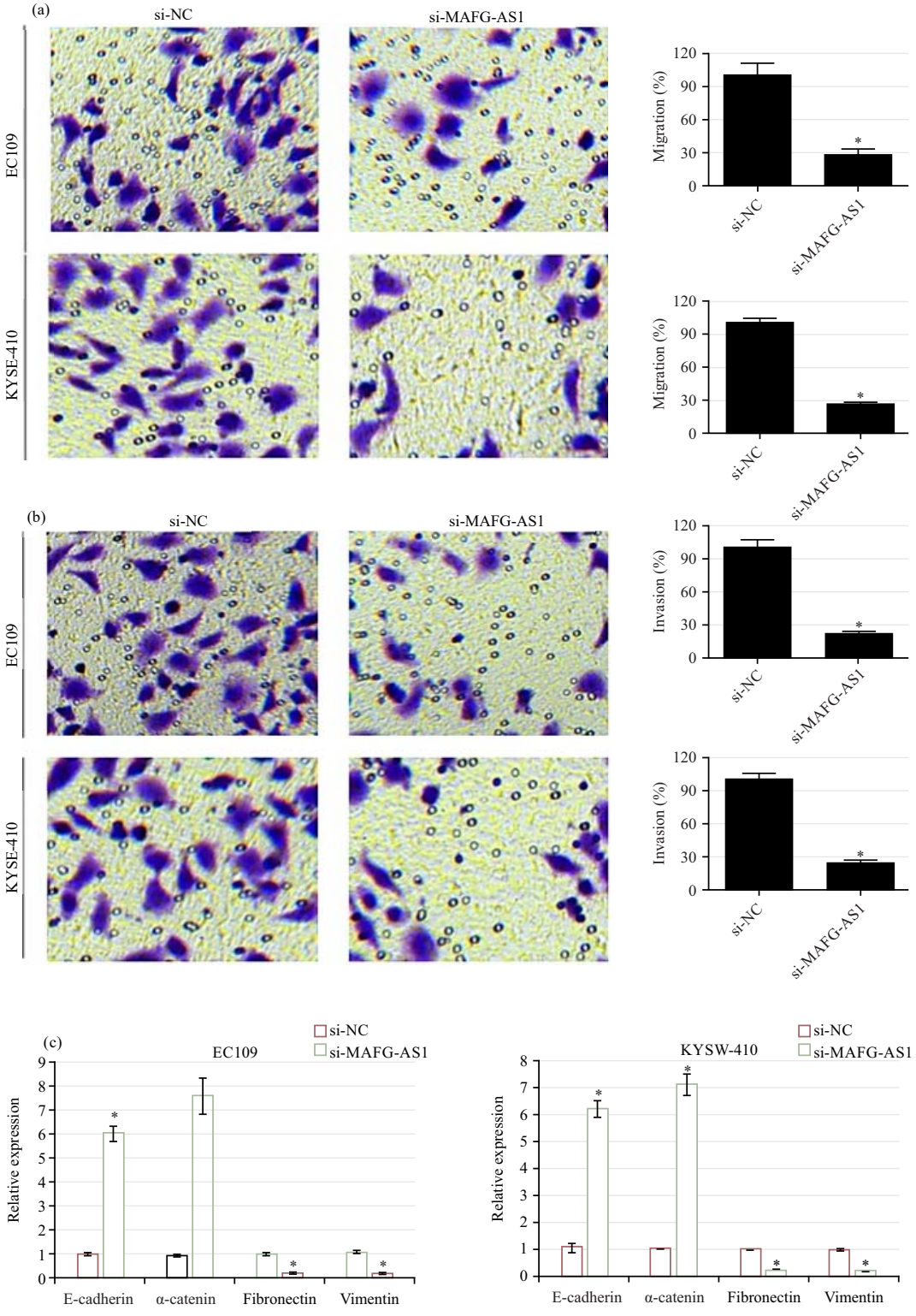

Fig. 3(a-c): MAFG-AS1 knock down inhibits epithelial to mesenchymal transition, (a) Transwell assay showing migration of si-NC or si-MAFG-AS1 transfected EC109 or KYSE-410 cancer cells, (b) Transwell assay showing invasion of si-NC or si-MAFG-AS1 transfected EC109 or KYSE-410 cancer cells and (c) Expression of epithelial marker (E-cadherin and α-catenin) and mesenchymal marker (Fibronectin and Vimentin) in si-NC or si-MAFG-AS1 transfected EC109 or KYSE410 cells
Experiments were performed independently in triplicates and data is presented as Mean±SD (*p<0.05)

KYSE-401 cells were inhibited by 72 and 74% (relative to control), respectively (Fig. 3a). Similarly, MAFG-AS1 knockdown significantly ($p<0.05$) hampered EC cell invasion. The percentage of invasion of EC109 and KYSE-401 cells was inhibited by 83 and 74%, respectively, relative to the control (Fig. 3b). Furthermore, when compared to negative control cells, MAFG-AS1 knock-down dramatical improved ($p<0.05$) in the expression of epithelial proteins such as E-cadherin and α-catenin while significantly suppressing ($p<0.05$) the expression of mesenchymal proteins (fibronectin and vimentin) in EC109 and KYSE-401 cells (Fig. 3c). The findings suggest that MAFG-AS1 inhibits the EMT of HEC cells.

DISCUSSION

The lncRNAs are non-protein-coding RNA transcripts that are four times longer in length than the average protein-coding RNA[19]. However, the generally accepted classification recognizes lncRNAs with sizes greater than 200 nucleotides[20]. The lncRNAs control eukaryotic genes, mostly at the post-transcriptional level, where they have been shown to affect many cellular, physiological and disease-related processes[21,22]. Recent research has shown that lncRNAs have abnormal expression patterns in several kinds of human malignancies and influence the carcinogenesis process[23,24]. Based on this knowledge, researchers are attempting to characterise the functional properties of lncRNAs in various human cancers to investigate their potential applications in cancer diagnosis and therapy.

The HEC is responsible for a considerable number of cancer-related fatalities worldwide[25]. Because of poor prognosis and disease recurrence, the current therapeutic procedures used to treat this cancer have a very low clinical success rate[26]. Considering this, the appraisal of molecular irregularities becomes crucial and the same may lead to the formulation of better and more effective diagnostic and curative strategies against EC in the future. This study set is intended to investigate just that to clarify the function of lncRNA MAFG-AS1 in controlling the development and metastasis of esophageal cancer. The upregulation of MAFG-AS1 in various human malignancies has previously been identified and its oncogenic function in controlling cancer growth has been hypothesized[27,28].

The current study's findings led to similar conclusions. The EC tissues and cell lines expressed significantly higher transcript levels of MAFG-AS1. Experimental knock-down of MAFG-AS1 in EC cells inhibited their growth significantly by inducing apoptosis, as shown by flow cytometry. The rise in the Bax/Bcl-2 protein ratio indicated apoptotic initiation. It has been suggested that the latter causes caspase-driven cell death. The latter has been proposed to activate caspase-driven cell death[29]. These findings were corroborated by prior research that demonstrated MAFG-AS1 to trigger apoptosis in cancer cells. Dai *et al.*[30] revealed that MAF-AGS1 controls carcinogenesis in breast cancer cells via apoptosis. Also, Ruan *et al.*[31] discovered that MAFG-AS1 knockdown causes apoptosis in colorectal cancer cells.

Furthermore, the esophageal cancer cells showed a significant decline in migration and invasion rates once transfected with silencing oligos against MAFG-AS1. As previously described, evidence suggests that MAFG-AS1 has a positive regulatory function in controlling cancer cell migration and invasion[32]. Additionally, it has been discovered that MAFG-AS1 knockdowns can suppress EMT in esophageal cancer cells. The EMT promotes metastasis by hastening cancer cell invasion[33,34]. Thus, the findings suggested that MAFG-AS1 might be used as a vital prognostic and therapeutic molecular target against HEC; however, the findings need to be verified *in vivo*.

CONCLUSION

The current study's findings demonstrated a considerable elevation of MAFG-AS1 in EC. By increasing apoptotic cell death, MAFG-AS1 silencing reduced the development and viability of esophageal cancer cells. Furthermore, MAFG-AS1 knockdown inhibited EC cell motility, invasion and EMT, demonstrating its therapeutic potential. Nonetheless, further studies, especially *in vivo*, are required to further confirm the findings.

SIGNIFICANCE STATEMENT

Esophageal carcinoma (EC) is a prominent neoplastic condition of the human gastrointestinal system and it's the 6th largest cause of cancer death rates globally. This investigation intended to study the long non-coding RNA (lncRNA) MAFG-AS1 and its influences on the proliferation and transition of human esophageal cancer (HEC) cells from an epithelial to a mesenchymal state. The present investigation's outcomes revealed a significant increase of MAFG-AS1 in EC. The MAFG-AS1 knockdown slowed the proliferation and viability of esophageal cancer cells by promoting apoptotic cell death. The MAFG-AS1 knockdown also decreased EC cell motility, invasion and EMT, indicating its therapeutic benefits.

ACKNOWLEDGMENT

The authors thank the facilities offered by the higher officials.

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