ACTA ABP BIOCHIMICA POLONICA

Vol. 69, No 2/2022
357–362
https://doi.org/10.18388/abp.2020_5798

Regular paper

MicroRNA-145 regulates the proliferation of the human gastric cancer cells by targeting tuftelin 1 (TUFT1)

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MicroRNA-145 (miR-145) has been shown to regulate the development of different human cancer. However, the role of miR-145 *via* modulation of tuftelin 1 (TUFT1) expression has not been studied in gastric cancer. TUFT1The results showed that gastric cancer tissues and cell lines exhibit significant ($P<0.05$) downregulation of miR-145. Overexpression of miR-145 significantly ($P<0.05$) inhibited the viability and colony formation of the MGC-803 gastric cancer cells. Annexin V/PI staining revealed that miR-145 exerts its tumor-suppressive effects *via* induction of apoptosis. The apoptotic cell percentage increased from 5.75% in negative control to 22.95% in miR-145 overexpressing MG-803 cells. This was also accompanied by upregulation of Bax and downregulation of Bcl-2 expression. TargetScan analysis and the dual luciferase assay revealed TUFT1 as the functional target of miR-145. The expression of TUFT1 was significantly ($P<0.05$) upregulated in gastric cancer tissues and cell lines. However, overexpression of miR-145 causes inhibition of the TUFT1 expression. Silencing of TUFT1 mimicked the tumor-suppressive effects of miR-145. However, tuftelin 1 overexpression attenuated the tumor-suppressive effect of miR-145 in MGC-803 gastric cancer cells. Taken together, the results of the present study indicate that miR-145 targets TUFT1 at translational level to exert its tumor suppressive effects in gastric cancer.

Keywords: gastric cancer, micro-RNA, miR-145, apoptosis, tuftelin 1, flow cytometry

Received: 24 July, 2021; revised: 19 October, 2021; accepted: 20 October, 2021; available on-line: 25 May, 2022

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Abbreviations: MTT, Thiazolyl Blue Tetrazolium Bromide; PVDF, polyvinylidene fluoride; TBST, Tris-buffered saline and Tween-20 TUFT1, Tuftelin 1

INTRODUCTION

Gastric cancer is one of the serious and aggressive human cancers and accounts for more than a million cancer-related deaths at the global stage annually (Thrift & El-Serag, 2020). Although, the overall incidence of gastric cancer has decreased recently, nevertheless it is still ranked as the fifth most common and third most lethal human cancer (Arun *et al.*, 2018). Moreover, gastric cancer is considered as the second most-lethal cancer in China (Nie *et al.*, 2018). Despite vast number of research efforts towards improvement in the understanding of the pathogenesis and therapeutic options, the overall 5-year survival rate of gastric cancer is still less than 25% because of the late diagnosis and higher disease invasiveness (Okajima *et al.*, 2016; Van *et al.*, 2016). Therefore, it becomes very crucial to explore the progression of gastric cancer at molecular level and identify the genetic factors involved in its pathogenesis. Micro-RNAs (miRs) have been shown to be linked with the growth and progression of gastric cancer (Zhang *et al.*, 2017). MiRs target various critical genes and regulate multiple pathways in gastric cancer (Haung *et al.*, 2016; Martin *et al.*, 2019). The miRs either act as oncogenes or tumor-suppressors in gastric cancers and can promote or restrict the tumorigenesis of gastric cancer (Chen *et al.*, 2018; Gao *et al.*, 2018; Jia *et al.*, 2019). Kim *et al.*, reported the role of miR-145 in breast cancer (Kim *et al.*, 2011). Zhou and others (Zhou *et al.*, 2017) found that miR-145 regulates the proliferation and metastasis of cervical cancer. Similarly, Ye and others (Ye *et al.*, 2015) found that miR-145 enhances the radiosensitivity of cervical cancer cells. In yet another study, miR-145 has been shown to regulate the development of prostate cancer (Zaman *et al.*, 2010). However, the role of miR-145 *via* modulation of Tuftelin 1 (TUFT1) has not been studied in gastric cancer. TUFT1 has been shown to act as an oncogene in different cancer cells (Liu *et al.*, 2017) but has not been studied in gastric cancer. Therefore, the present study was designed to unveil the role of miR-145/TUFT1 in gastric cancer and to explore the therapeutic implications.

MATERIALS AND METHODS

Human tissues

In total, 69 gastric cancer and normal adjacent tissues were obtained from patients who underwent surgery at Wusong Central Hospital, Baoshan District, Shanghai, China. The tissues were collected prior to any chemo- or radiotherapy. The tissues were transported in liquid nitrogen and stored at –80ºC for further use. The collection of samples was made according to the standard scientific ethical guidelines and only after obtaining proper signed patient consents. All animal experiments were approved by the research ethics committee of the Wusong Central Hospital, Baoshan District, Shanghai, China with number WCH-910-2019.

Cell lines

Four human gastric cancer cell lines (BGC-823, MGC-803, MKN-45, and SGC-7901) and the normal gastric epithelial cell line (GES-1) were procured from the American Type Culture Collection (Manassas, Virginia, United States). The cell lines were cultured using Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific HyClone, Beijing, China) containing 10% fetal bovine serum (FBS, HyClone), 100 U/mL of penicillin,

and 100 U/mL of streptomycin (both from HyClone) at 37ºC. A humidified CO_2 incubator was used for the maintenance of cell lines in an atmosphere of 95% air and 5% CO_2 at 37ºC.

Cell transfection

The MGC-803 gastric cancer cells were transfected with miR-145 mimics or its negative control miR-NC, small interfering oligos against TUFT1 (si-TUFT1) or its negative control si-NC, overexpression plasmid of TUFT1 (pcDNA-TUFT1) or vector alone pcDNA-3.1 with the help of Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, NM, USA) for 48 h as per the manufacturer protocol and using final concentrations of 50 nM for oligos and 2 μg/mL for plasmids. The qRT-PCR was used for evaluating the efficiency of cell transfection.

Quantitative RT-PCR

Trizol reagent (Invitrogen, Carlsbad, NM, USA) was used for homogenization of gastric cancer and matched normal tissues as well as the cell lines to isolate the total RNA according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using GoScript RT System (Promega, USA). The qRT-PCR was then performed using SYBR Green RT-PCR Master Mix (Promega, USA). The thermo-cycling program was as follows: 10 min at 95°C and 40 cycles of 30 sec at 95°C and 1 min at 60°C. Three replicates were used per reaction. Human GADPH and snRNA U6 were used as an internal controls and relative expression was quantified using 2^{-ddCt} method.

Cell viability assays

The proliferation of stably transfected MGC-803 cancer cells was analyzed using Thiazolyl Blue Tetrazolium Bromide (MTT, Amresco, Solon, OH, USA). In brief, 5×10^4 transfected cells were placed into each well of 96-well plates. Each well was added with MTT reagent at 0, 20, 40, 60, 80 or 100 h incubation at 37ºC. After further 4 h incubation at 37ºC, MTT was replaced with dimethyl sulfoxide in each well. Lastly, the OD_{570} was examined for each well using an FLx800 Fluorescence Microplate Reader (BioTek, Winooski, VT, USA).

Colony formation assay

The miR-145 mimics or miR-NC transfected MGC-803 cancer cells were plated into a six-well plate. The cells were then incubated for 15 days. The colonies were ethanol fixed and stained with 0.2% crystal violet. Following, the colonies were photographed and manually counted.

Annexin V/PI staining assay

An apoptosis detection kit (BD Biosciences) was used to analyze the apoptosis of MGC-803 cancer cells stably transfected with miR-145 mimics or miR-NC. After their plating into 12-well plate, the cancer cells at a density of 2×10^6 per well were incubated with 1X binding buffer containing 50 μL/ml Annexin V-FITC and 50 μL/mL propidium iodide (PI) for 30 min at room temperature. Next an additional 250 μL 1X binding buffer was added per well. Finally, the cells were examined by flow cytometry (BD Biosciences).

Bioinformatics analysis

The online software TargetScan (http://www.targetscan.org) was used to predict the potential targets of miR-145.

Dual luciferase reporter assay

A 3'-UTR fragment (100 bp) of TUFT1 was synthesized carrying either wild-type (WT) or mutant (MUT) binding site and subsequently cloned into the psiCHECK-2 reporter vector (Applied Biosystems, USA). The reporter plasmid (WT or MUT) was co-transfected with miR-145 mimics or miR-NC into MGC-803 cancer cells. Cells were collected after 48 h of transfection. The Dual Luciferase Reporter Assay System (Promega, Madison, USA) was used to examine their luciferase activity.

Western blotting

The protein lysates were obtained from the stably transfected MGC-803 cancer cells with the help of RIPA lysis buffer. The lysates were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This was followed by blotting of resolved proteins to the polyvinylidene fluoride (PVDF) membranes. 5% skimmed milk was used to block the membranes. The membranes were then incubated with the primary antibodies at 4°C overnight and rinsed with Tris-buffered saline and Tween-20 (TBST). This was followed by incubation of the membranes with horseradish-labeled secondary antibody (1:10 000) for 2 h at room temperature. The membranes were again washed with TBST. ECL reagent was finally used for detecting the protein bands. Actin was used as reference protein.

Statistical analysis

Statistical analysis was performed with the help of SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Three replicates were used for performing each experiment and results were given as average ± standard experimental error. Statistical significance was analyzed by Student's *t*-test. The $P<0.05$ was considered as a statistically significant difference between two groups.

RESULTS

miR-145 is downregulated in gastric cancer

Analysis of the expression of miR-145 in human gastric cancer tissues and the normal adjacent tissues showed that gastric cancer tissues showed a significantly lower ($P<0.05$) expression of miR-145 relative to the normal adjacent tissues (Fig. 1A). The expression analysis was further extended to four gastric cancer cell lines (BGC-823, MGC-803, MKN-45, and SGC-7901) using normal gastric epithelial cells, GES as control. It was found that miR-145 exhibits significantly lower ($P<0.05$) transcript levels in all the four gastric cancer cell lines relative to the normal epithelial cells (Fig. 1B). The MGC-803 gastric cancer cell line exhibited least expression of miR-145 among all the four cancer cell lines and thus it was used for further characterization of miR-145.

miR-145 inhibits growth of gastric cancer cells

To investigate for the role of miR-145 in regulating proliferation of gastric cancer cells, miR-145 was over-

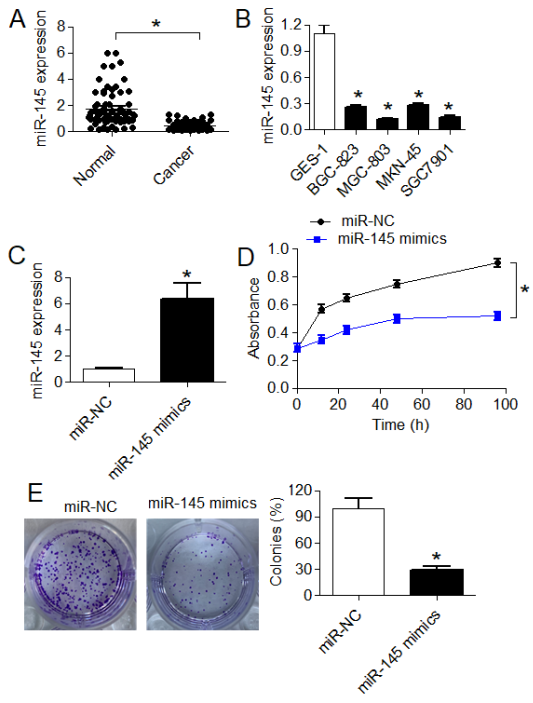

Figure 1. miR-145 overexpression inhibits proliferation of gastric cancer cells.
(**A**) Expression analysis of miR-145 in gastric cancer and normal adjacent tissues. (**B**) expression analysis of miR-145 in gastric cancer cell lines (BGC-823, MGC-803, MKN-45 and SGC7901) relative to GES gastric epithelial cells. (**C**) Expression of miR-145 in MGC-803 cells transfected with miR-145 mimics or miR-NC. (**D**) Cell viability of MGC-803 cells transfected with miR-145 mimics or miR-NC. (**E**) Clonogenic assay of MGC-803 cells transfected with miR-145 mimics or miR-NC. Three replicates were used for performing the experiments and $P<0.05$ was taken as indicative of statistically significant difference.

expressed in MGC-803 cancer cells. Overexpression of miR-145 was confirmed by qRT-PCR which showed 6.2 folds upregulation of miR-145 in MG-803 cells (Fig. 1C). Next, the proliferation rates of gastric cancer cells overexpressing miR-145 was examined by MTT assay. It was found that MGC-803 cancer cells overexpressing miR-145 proliferated at significantly ($P<0.05$) lower rates as compared to the negative control cells (Fig. 1D). Again, miR-145 overexpression in gastric cancer cells impeded their colony forming potential significantly ($P<0.05$) and the number of colonies declined by 72% relative to the control (Fig. 1E). The results thus confirm the tumor-suppressive role of miR-145 in gastric cancer.

miR-145 induces apoptosis in gastric cancer cells

Whether apoptosis is induced in gastric cancer cells overexpressing miR-145 to suppress their proliferation *in vitro*, MGC-803 cells overexpressing miR-145 were evaluated for the level of apoptosis with the help of flow cytometry using negative control transfected cells as reference. Results clearly indicate that the relative proportion of apoptosis was significantly higher for miR-145 cancer cells as compared to the negative control cancer cells. The apoptotic cell percentage increase from 5.75% in control to 22.95% in miR-145 overexpressing cells (Fig. 2A). The MGC-803 cancer cells also expressed significantly ($P<0.05$) higher Bax and significantly ($P<0.05$) lower Bcl-2 transcripts in comparison to the negative control cells (Fig. 2B and 2C). Together, the results indicate that miR-145 induces apoptosis in gastric cancer cells to exert its tumor-suppressive role.

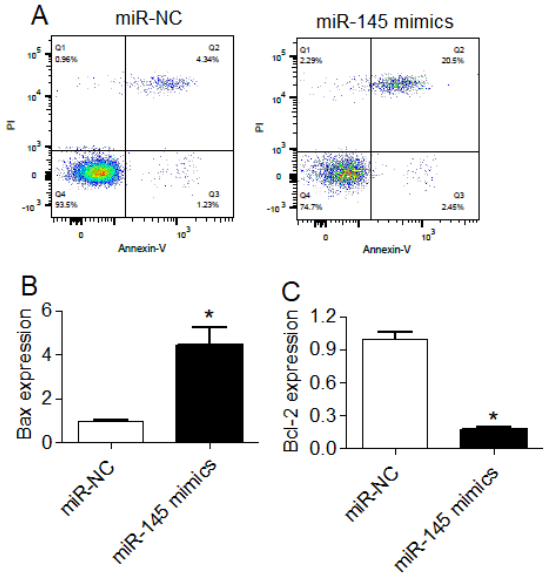

Figure 2. miR-145 induces apoptosis in gastric cancer cells.
(**A**) Analysis of apoptosis of MGC-803 cells transfected with miR-145 mimics or miR-NC by flow cytometry. (**B**) Bax expression in MGC-803 cells transfected with miR-145 mimics or miR-NC. (**C**) Bcl-2 expression in MGC-803 cells transfected with miR-145 mimics or miR-NC. Three replicates were used for performing the experiments and $P<0.05$ was taken as indicative of statistically significant difference.

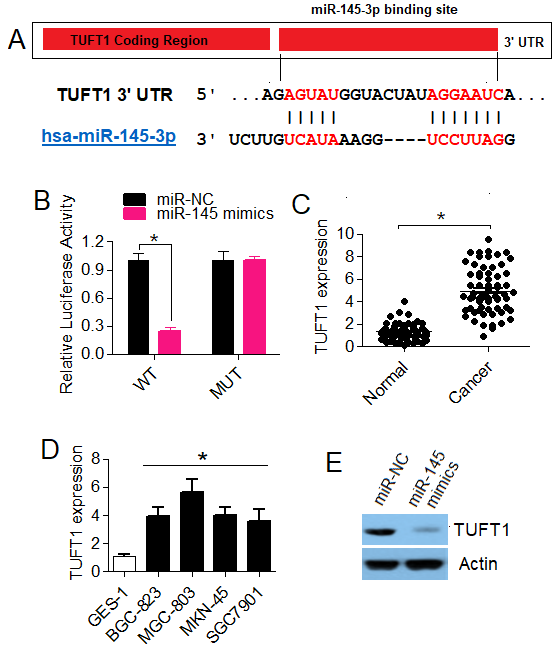

Figure 3. miR-145 targets TUFT1 in gastric cancer at post-transcriptional level.
(**A**) Prediction of miR-145 potential target in gastric cancer by in silico analysis. (**B**) dual luciferase reporter assay. (**C**) Expression of TUFT1 in gastric cancer and adjacent gastric cancer tissues. (**D**) Expression of TUFT1 in normal and gastric cancer cells. (**E**) Assessment of TUFT1 protein levels in MGC-803 cells transfected with miR-145 mimics or miR-NC. Three replicates were used for performing the experiments and $P<0.05$ was taken as indicative of statistically significant difference.

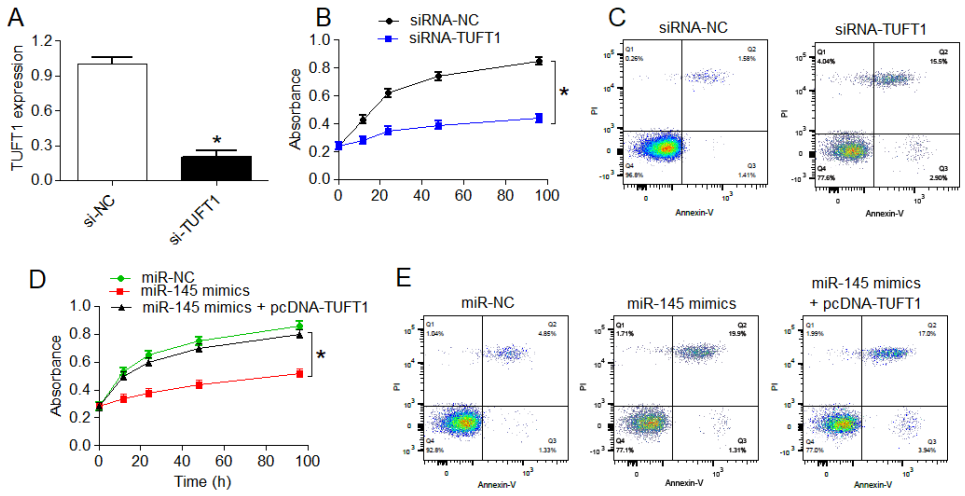

Figure 4. TUFT1 mediates anti-proliferative role of miR-145 in gastric cancer.
(**A**) Expression of TUFT1 in si-NC and si-TUFT1 transfected MGC-803 cells. (**B**) Cell viability of MGC-803 cells transfected with si-TUFT1 or si-NC by MTT assay. (**C**) Percentage of apoptosis in si-TUFT1 or si-NC transfected MGC-803 cells by annexin V/PI assay. (**D**) Cell viability of MGC-803 cells transfected with miR-145 mimics, miR-NC or miR-145 mimics plus pcDNA-TUFT1 by MTT assay. (**D**) Percentage of apoptosis in MGC-803 cells MGC-803 cells transfected with miR-145 mimics, miR-NC or miR-145 mimics plus pcDNA-TUFT1 by annexin V/PI assay. Three replicates were used for performing the experiments and $P<0.05$ was taken as indicative of statistically significant difference.

miR-145 targets TUFT1 in gastric cancer

To point out the specific target of miR-145 in gastric cancer, online bioinformatics was used. The *in silico* analysis predicted that miR-145 targets tuftelin 1 (TUFT1) at post-transcriptional level and identified the specific region in TUFT1 3'-UTR which acts as a binding site for miR-145 (Fig. 3A). The 3'-UTR of TUFT1, carrying miR-145 binding site in native form (WT) or mutated (MUT), was used to generate the luciferase reporter plasmids which were subsequently co-transfected into MGC-803 cancer cells along with miR-145 mimics or negative control miR-NC. The interaction of miR-145 with its binding site was confirmed as the significant ($P<0.05$) decline in the luciferase activity (Fig. 3B). Next, the expression of TUFT was examined in human gastric cancer and normal adjacent tissues. The results showed significant ($P<0.05$) upregulation of TUFT1 in gastric cancer tissues relative to the normal tissues (Fig. 3C). Consistently, the expression of TUFT1 was also found to be significantly ($P<0.05$) upregulated in the gastric cancer cells relative to the normal cells (Fig. 3D). However, the western blot analysis showed that overexpression of miR-145 suppresses the expression of TUFT1 in MGC-803 cancer cells further confirming TUFT1 as the target of miR-145 (Fig. 3C).

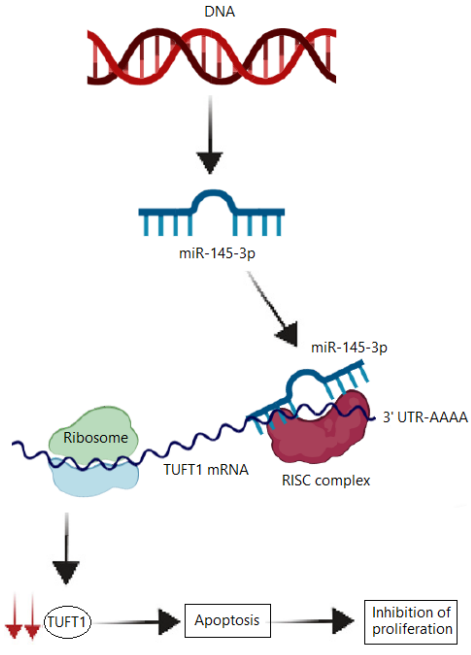

Figure 5. Model for molecular mechanism of miR-145/TUFT1 axis in gastric cancer signaling.
MiR-145 is synthesized and binds to the 3′-UTR of TUFT1 through the RISC complex. This binding inhibits TUFT translation eventually leading to apoptosis mediated inhibition of cell proliferation.

TUFT1 overexpression prevents tumor-suppressive effects of miR-145 in gastric cancer

Whether TUFT1 is responsible for mediating the regulatory control of miR-145 in gastric cancer, TUFT1 was knocked down in MGC-803 cancer cells and the silencing was confirmed by qRT-PCR (Fig. 4A). It was found that silencing of TUFT1 in MG-803 in cancer cells led to a significant ($P<0.05$) decline in the viability of MG-803 cells (Fig. 4B). Annexin V/PI staining showed that silencing of TUFT1 induced apoptosis of MGC-803 cells (Fig. 4C). However, TUFT1 expression prevented the decline in proliferation of gastric cancer cells overexpressing miR-145 (Fig. 4D). These results were also confirmed by annexin V/PI staining assay which showed that overexpression of TUFT1 prevented the induction of apoptosis (Fig. 4E). The results thus proved that miR-145 targets TUFT1 for its post-transcriptional repression to regulate the gastric cancer cell growth (Fig. 5).

DISCUSSION

Gastric cancer is one of the devastating cancers (Van Custem *et al.*, 2016). The unavailability of efficient biomarkers, therapeutic targets, and potent and safe drugs form major hurdles in its management (Singh, 2013). The present study was designed to decipher the role

and therapeutic potential of miR-145/TUFT1 axis for the first time in gastric cancer. In the present study we found miR-145 to be significantly downregulated in gastric cancer. This is confirmation with several other studies wherein miR-145 has been found to be downregulated. For instance, Zhang and Lin (Zhang & Lin, 2015), found miR-145 to be downregulated in lung cancer. Similarly, Feng and others (Feng *et al*., 2014) reported the downregulation of miR-145 in colorectal cancer. To decipher the role of miR-145, it was overexpressed in MCG-803 gastric cancer, which lead to considerable inhibition in their viability indicating the tumor-suppressive role of miR-145. Moreover, the findings of the present study suggest that the downregulation of miR-145 in gastric cancer might be avoiding its tumor-suppressive role, eventually leading to the development of gastric cancer. Apoptosis is one of the critical functions of cells which helps in maintaining homeostasis and signifies regular function to discard dysfunctional and excess cells (Carneiro & El-Deiry, 2020). Consistently, in the present study, the miR-145 mediated apoptosis was found to be responsible for the inhibition of the MCG-803 gastric cancer viability.

It is well established in literature that miRs exerts their effects by causing post-transcriptional silencing of target genes. Additionally, single miR may have multiple molecular targets (Hoshimotot *et al*., 2013). MiR-145 has diverse molecular targets, for example, miR-145 has been shown to target ROCK1 in breast cancer (Zheng *et al*., 2016), FSCN1 in lung cancer and colorectal cancer (Zhang & Lin, 2015; Feng *et al*., 2014) and IRS1 in hepatocellular cancer (Wang *et al*., 2014). Nonetheless, our study for the first time reports TUFT1 as the target of miR-145. Studies have shown that TUFT1 has an oncogenic role and has been shown to be involved the development of different human cancers (Xiao *et al*., 2020; Wu *et al*., 2020). Previous studies have shown that TUFT1 promotes the proliferation of breast cancer (Liu *et al*., 2017), hepatocellular cancer (Wu *et al*., 2021), renal cell carcinoma (Lin *et al*., 2021), and thyroid cancer (Liu *et al*., 2018) to name a few. TUFT1 was found to be upregulated in gastric cancer and miR-145 suppresses its expression post-transcriptionally. This blocks the oncogenic function of TUFT1 and eventually leads to the decline in the proliferation of the gastric cancer cells.

Collectively, the findings of the present study suggest miR-145/TUFT1 molecular axis as a therapeutic target for the management of gastric cancer. Different drugs that can selectively upregulate the expression of miR-45 can be developed. Additionally, given the upregulation of miR-145 in gastric cancer tissues, it can be utilized as one of the biomarkers of gastric cancer. However, the evaluation of miR-145 in tissues of different stages needs to be carried out. Moreover, studies directed at the evaluation of the tumor-suppressive role of miR-145 *in vivo* are urgently required.

CONCLUSION

Taken together, the results of the present study are conclusive that miR-145 exhibits significant down-regulation in gastric cancer. Overexpression of miR-145 induces apoptosis in gastric cancer cells *via* post-transcriptional suppression of TUFT1. These findings suggest the therapeutic potential of miR-145/TUFT1 axis in gastric cancer.

Conflicts of interest

The author(s) declare that there are no conflicts of interest

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