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MiR-181b is a possible non-invasive molecular marker for the aggressiveness of non-small cell Lung cancer

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Lung cancer is considered the foremost predominant cancer all over the world. MIR-181bis involved in multiple critical roles in malignant tumors. Its function in non-small cell lung cancer (NSCLC) remains vague. The current study aims to assess the function of MIR-181b in NSCLC and the possibility of its use as a molecular marker for NSCLC aggressiveness. This study included 76 NSCLC patients with early or locally advanced tumors and underwent surgical resection. MIR-181bexpression in NSCLC tissues, H23, and H522 cells, and their adjacent normal lung parenchyma was quantified by QR-PCR. The H23 and H522 cell lines were transfected with MIR-181b. We analyzed the bioinformatics and utilized western blot and luciferase reporter studies to assess the invasiveness and metastases of MIR-181b transfected cell lines. Results: MIR-181b was significantly downregulated in NSCLC tissues and cell lines. Downregulation of MIR-181bwas found to prevent invasion and metastases in NSCLC cells by triggering the High Mobility Group Box-1 (HMGB1). In NSCLC patients, the downregulation of MIR-181bwas significantly associated with adverse clinical and pathological features in addition to short disease and overall survival. MIR-181b was downregulated in NSCLC. Its downregulation was associated with invasiveness and metastases by targetingHMGB1 in cell lines. In NSCLC patients, MIR-181bdownregulation was related to adverse clinical and pathological features and short survival.

Keywords: lung cancer; NSCLC; MIR-181b

INTRODUCTION

Lung cancer is the most typical malignant tumor and is a widely recognized reason for cancer-related death (Barta et al. 2019). It is the fourth prevalent cancer in Egypt (Ibrahim et al. 2014). Non-small cell lung cancer (NSCLC) is the most prevalent type constituting 80% of lung cancer; it incorporates adenocarcinomas and squamous cell carcinomas (Inamura., 2017). The precise molecular processes utilized by lung cancer for progression and metastases are currently not fully identified. MicroRNAs are involved in critical activities in the malignant cells, such as division, spread, invasion, differentiation, apoptosis, and cell cycle control (Ambros, 2004). Modified expression of several miRNAs in NSCLC permits its initiation, growth, and progression as MIR-205 (Larzabal et al. 2014), MIR-21 (Wang et al. 2011), MIR-574-5p, and MIR-1254 (Foss et al. 2011). MiRNAs carry a wide range of tasks in the 54 NSCLC; however, most of these activities remain to be studied. The MIR -181 group consisted of MIR -181a, MIR -181b, MIR -181c, and MIR -181d (Liu et al. 2014). The activities performed by miR-181b remain to be studied in lung cancer and other malignancies. It remains unclear whether it behaves as a tumor suppressor or promoter (Zhi et al. 2010). MIR -181b has been identified as a tumor suppressor that inhibits glioma cells from proliferation, invasion, migration, and apoptosis (Shiet al. 2013) and has also been shown to reduce their resistance to Temozolomide (Slaby O et al. 2010) via MEK1 signaling mechanisms (Wang et al. 2013). It modulates the response to many chemotherapeutic agents in different malignant tumors such as carcinoma of the stomach (Jiang et al. 2013), colorectal cancer (Nakajima et al. 2006), hepatocellular cancer (Wang et al. 2010), and breast carcinoma (Lu et al. 2011).

The purpose of this research was to assess the function of MIR-181b in the NSCLC and whether it could be used as a possible molecular marker for tumor aggressiveness and thepatient's prognosis.

MATERIALS AND METHODS

This study was performed at the Department of Clinical Oncology, Faculty of Medicine, Zagazig University, between June 2015 and February 2019. It

included 76 NSCLC patients who had early or locally advanced tumors and underwent surgery. Tumor and normal nearby non-tumorous tissues (NATs) situated at least 3 cm away from the primary and not invaded by the tumor have been collected from all patients. The collected tissues were flash-frozen at-80 ° C by nitrogen and preserved until further analysis. No previous chemotherapy or radiation therapy was given to any of the patients before entry in the study. The staging of all patients was based on the American Joint Committee on Cancer (8th edition) (Amin et al. 2017). The Ethical board of our institution accepted the current research. A consent form to participate was obtained from all patients.

RNA extraction and real time-quantitative polymerase chain reaction (RT-qPCR) for MIR-181b expression

RNA was extracted from NSCLC and NATs according to the producer's protocol of the miRNeasy extraction kit. NanoDrop spectrophotometer was used to determine the quantity and efficiency of the RNA. MIR-181b and U6 (as a reference), cDNA was synthesized from the RNA utilizing (iNtRON Biotechnology Kit) according to instructions of the manufacturer.

The primer sequences were forward 5′-AAC ATT CAT TGC TGT CGG TG-3′ and reverse, 5′-GCT GTC AAC GAT ACG CTA CGT-3′;(for miR-181b), and for U6, they were forward 5′-GCT TCG GCA GCA CAT ATA CTA AA-3′ and reverse, 5′-GCT TCA CGAATT TGC GTG TCA T-3′ (Invitrogen). PCR was performed in 96-well plates with a volume of 10 µl (0.67 µl RT ingredients, 1 × TaqMan ® Universal PCR master mixture, and 1µlTaqMan® miRNA assay primer and probe mix).

The amplification program comprised the following steps: denaturation 10 min at 95°C, second 45 denaturation cycles for 15 seconds at 95 °C, annealing lasts 30 seconds at 55°C, elongation 30 seconds at 70 °C and final elongation for 10 minutes at 60 °C (Lawrie et al. 2008). qRT- PCR was done using Stratagene, MX3000P quantitative PCR System, and data were analyzed using MxPro QPCR Software (Agilent technologies). The tests were analyzed in triplicate. The relative proportion of miR-181b to U6 was computed using equation 2–ΔCT, where ΔCT = (CT MIR-181b − CT U6) (Rao et al. 2013).

Cell transfection

The luciferase plasmid reporter, mimic MIR-181b, and the mimic miRNA have been used as a control, obtained from GenePharma, Shanghai, China. The mimic MIR-181b sequence was 5′-AACAUUCAUUGCUGUCGGUGGGU-3′, and the normal control mimic sequence was 5′-UUCUCCGAACGUGUCACGUTT-3′. We utilized Lipofectamine 2000(11668027; Invitrogen; Waltham, MA, US) for transfection with the mimic MIR-181b, negative control, or the plasmid of luciferase reporter based on the company instructions (Yu et al. 2018).

Assay of cell invasiveness and migration

An 8µm pore size Transwell chambers from Costar, Corning, NY, US., has been used to evaluate the invasiveness and migration of NSCLC cells. To study the migration, the transfected cells (about 5×104) were placed in the upper compartment with 200 µl of theRPMI-1640 medium and 0.1% serum. To analyze the invasiveness, the transfected cells (about 5×104) were mounted in the upper container with 200 µl of the RPMI-1640 medium,0.1% serum, and encased by Matrigel obtained from BD Biosciences, CA, US.

In the lower compartment, 0.5 ml of RPMI-1640 medium with 20% FBS was placed for attraction of the cells, followed by incubation of cells for 12h to assess the migration and for 24h to evaluate the invasiveness. A cotton wool wipe was used to gently clean the cells that could not move or infiltrate via the holes of the polycarbonate membrane. Finally, fixation of the cells was performed by methanol 100%, followed by staining with crystal violet 0.5%. The number of stained cells was evaluated with the EVOS XL Core Cell Imaging System by Invitrogen, US (Pijuan et al. 2019).

Western blotting

A radioimmunoprecipitation lysis buffer solution was utilized to isolate the cellular protein after 72 h of cellular transfection, the buffer solution contains [50 mMTris-HCl (pH7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mMNaCl, 1 mM EDTA, 1 mMphenylmethyl-sulfonyl fluoride, aprotinin, leupeptin and pepstatin (1 µg/ml each), 1 mM Na3VO4 and1 mMNaF].

The concentration of protein was calculated utilizing a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, US). Consequently, identical volumes of protein were isolated using 10% SDS-PAGE and electro transferred with Durapore® PVDF Membrane Filters (Merck KGaA, Darmstadt, Germany).

The membrane filters were obstructed by 5% skimmed milk placed in 0.1 % TBST for12 h with the anti-high-mobility group box-1 (HMGB1) rabbit antibodies protein (SA39-03) at 4°C. (1:1,1000; Catalog # MA5-31967; Invitrogen, Waltham, MA US) and the mouse monoclonal anti-β-actin antibody (1:1,000; Anti-beta Actin Monoclonal (AC-15), Catalog 268# AM4302; Invitrogen, Waltham, MA US).

Following the TBST wash, the membranes were incubated with Goat anti-Rabbit IgG(H+L) Secondary Antibody, HRP (Catalog # 65-6120; Invitrogen, Waltham, MA US) and anti-Mouse Goat Cross-Adsorbed Secondary Antibody (1:5,000-1:20,000; Catalog # SA5-10149; Invitrogen, Waltham, MA US) at room temperature for 1h. Protein bands have been established by chemiluminescence substrate (Catalog # 32109; Pierce; Thermo Fisher, US), pictures have been processed using the FluorChem Imaging platform (Alpha Innotech,CA, US) (Tian et al. 2018).

Luciferase assay

In an attempt to identify whether High mobility group box protein 1 (HMGB1) is the trigger for MIR-181b, analyses of the luciferase reporter have been implemented. The plasmid of the luciferase reporter of the wild type pmirGLO-HMGB1–3′UTR (WT) and the mutant pmirGLO-HMGB1–3′UTR mutant (MUT) was obtained from GenePharma,Shanghai, China. We used the Lipofectamine 2000 guided by the instructions to transfect0.5µg of plasmid reporter, 40 nmol of the mimic miR-181b or normal control into the H23and H522 placed in 12 well plates. After 48h of transfection, the Dual-Luciferase Reporter System (E1910; Promega, Germany) was utilized to assess the luciferase activity of thetransfected cells (Tan et al. 2015).

Anaplastic Lymphoma Kinase (ALK) and Epidermal Growth FactorReceptor (EGFR) determination

Evaluation of ALK and EGFR was performed to select a further post-surgical systemic treatment and analyze their relation with MIR-181b. Sections of 3 -4µm thickness were cut from the formalin-fixed paraffin-embedded tumor tissues, deparaffinized, and rehydrated. Immunostaining for ALK and EGFR was carried out on poly L-lysine coated slides. The Streptavidin Biotin immunoperoxidase technique was utilized for immunohistochemistry (Verma et al. 2017). The anaplastic large cell lymphoma and the human placental tissues were used as positive controls for ALK and EGFR, respectively.

Evaluation of Immunostaining:

Table 1: ALK and EGFR expression in the studied NSCLC patients

ALK	No	Positive	%
All patients	76	2	2.6
Adeno.	55	2	3.6
SCC	21	0	0
EGFR	**No**	**Positive**	**%**
All patients	76	54	71.05
Adeno.	55	42	76.4
SCC	21	12	57.1

Statistical evaluation

Data were expressed as the mean ± standard deviation and compared using the student's (t-test) and one-way analysis of variance (ANOVA). Survival was evaluated by the Kaplan-Meier curve. All Statistical estimates were carried out by SPSS version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

MIR-181b levels in NSCLC and NATs

Estimating the MIR-181b levels in the tumor tissues and their adjacent normal tissues revealed that MIR-181b was downregulated in NSCLC significantly relative to their nearby lung tissues. The mean level of miR-181b in NSCLC was 1.47 versus 4.86 in NATs (t=15.32, P <0.0001). The results indicate the suppressive role of miR-181b on the pathogenesis of NSCLC. A receiver operating characteristic curve was plotted to evaluate the ability of MIR-181b to distinguish NSCLC from normal lung tissues; it revealed high specificity of MIR-181b (AUC = 0.96, 95% CI: 0.93 to 0.99, P < 0.0001), Figure 1.

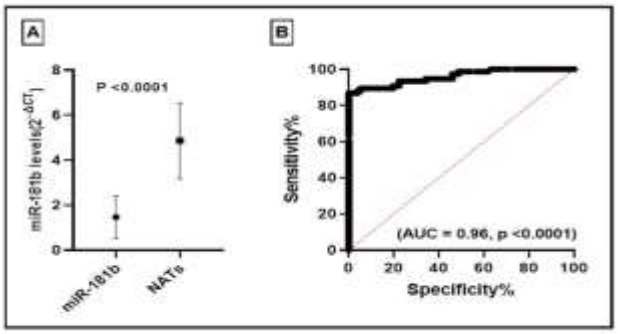

Figure 1: miR-181b in NSCLC and NATs

A) miR-181b was significantly downregulated in NSCLC tissues compared to their adjacent normal tissues (P < 0.0001). B) ROC curve revealed a specificity of MIR-181b for NSCLC (AUC = 0.96, 95% CI: 0.93 to 0.99, P < 0.0001).

Clinical and pathological features of patients

The study included 76 patients with NSCLC, 47 males, and 29 females. The age ranged between 34 years and 73 years, there were 25 patients less than 50 years, and 51 patients were more than fifty years. Adenocarcinomas were the most common histopathological type in 55 patients, SCC was found in 21 patients. TNM staging revealed that 31 patients were stage III, 23 with stage II, and 22 with stage I.

Clinical and pathological characteristics and miR-181b expression

In the present research, we studied the relation between the clinical and pathological features and the MIR-181b levels. We noted that MIR-181b was significantly downregulated in female patients (P = 0.038), in smokers (P =0.04), in poorly differentiated tumors (P < 0.0001), in squamous cell carcinomas (P = 0.0003), in ALK-negative tumors (P = 0.024), in EGFR negative tumors (P <0.0001), and in patients with advanced tumor stage (P <0.0001). The observed results denote that the downregulation of MIR-181b was linked to poor clinical and pathological features (Table 2).

Table 2: Association between MIR-181b levels and clinical and pathological features

		MIR-181b			
	Number	Mean	SD		P
Age					
< 50	25	1.332	0.9638	0.8832*	0.38
≥ 50	51	1.534	0.9269		
Sex					
Male	47	1.642	0.892	2.114*	0.0379
Female	29	1.184	0.9558		
Smoking					
Yes	38	1.007	0.7056	2.087*	0.0404
No	38	1.402	0.9273		
Tumor grade					
I-II	32	2.441	0.364	16.9*	<0.0001
III-IV	44	0.7596	0.4692		
Histopathology					
SCC	21	0.6585	0.4419	3.772*	0.0003
Adeno	55	1.467	0.9409		
ALK					
Positive	2	2.936	0.01344	2.308*	0.0238
Negative	74	1.428	0.9179		
EGFR					
Positive	54	1.908	0.7221	9.498*	<0.0001
Negative	22	0.3859	0.3147		
TNM stage					
I	22	2.632	0.2631	211.1**	<0.0001
II	23	1.589	0.4295		
III	31	0.551	0.3746		

*Student's t-test, **one way analysis of variance (ANOVA), SCC: squamous cell carcinoma, Adeno: adenocarcinoma, ALK: alkaline lymphoma kinase, EGFR: epidermal growth factor receptor.

MIR-181b and cell invasion and migration in H23 and H522 NSCLC cells

We studied the role of MIR-181b on invasion and migration of cells by Transwell analysis. We found a significant impact of MIR-181b on invasion and migration of H23 and H522 NSCLC cells. In the H23 cells, for invasion and migration, the P-value was <0.0001. The same results were observed in the H522 cells regarding the invasion and migration (P <0.0001). Figure 2.

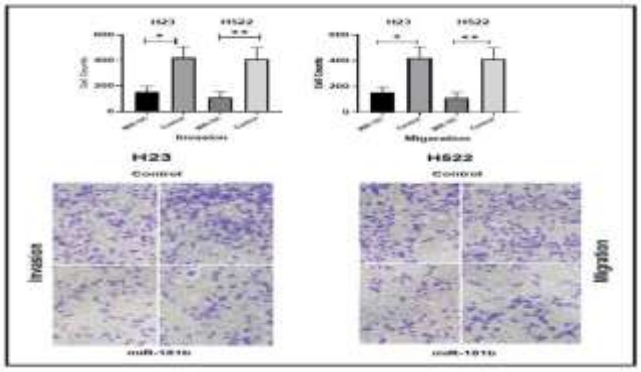

Figure 2: In vitro transwell study of the role of MIR-181b on invasion and migration of NSCLC cells.

When the H23 and H522 NSCLC cells were transfected with miR-181b, their ability to invade and migrate was inhibited.

HMGB1 as a direct target gene for miR-181b in NSCLC

In NSCLC, we used the Target Scan data system (TargetScan: targetscan.org) to recognize the target of MIR-181b. HMGB1 was assumed to be the trigger of MIR-181b. To validate if the MIR-181b mainly targets HMGB1, we conducted western blotting to assess if the transfected H23 andH522 with MIR-181b the HMGB1; we noted that transfection of these cells with MIR-181bsignificantly downregulate HMGB1 in both types of cells (P <0.0001), (Figure 3).

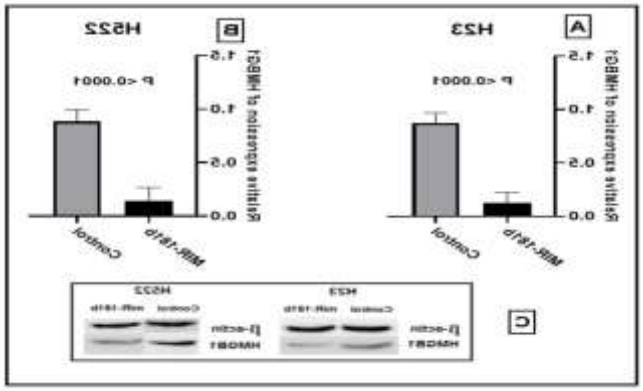

Figure 3: HMGB1 expression in H23 and H522 NSCLC

cells.

A) HMGB1 was significantly downregulated in the H23 cells after transfection with MIR-181b (P <0.0001). B) HMGB1 expression in H522 NSCLS cells (P <0.0001). C) Western blot study to evaluate the levels of HMGB1 in H23 and H522 and normal control NSCLC cells transected with MIR-181b.

Besides, we carried the luciferase reporter analysis; these analyses revealed that the wild type (WT) of HMGB1were suppressed by MIR-181b. The P-value was 0.0009 for WT compared to 0.67 for mutant HMGB1 in H23 cells, The same observation was noted in H522; P-value was 0.0015 for WT compared to 0.06 for mutant HMGB1(Figure 4).

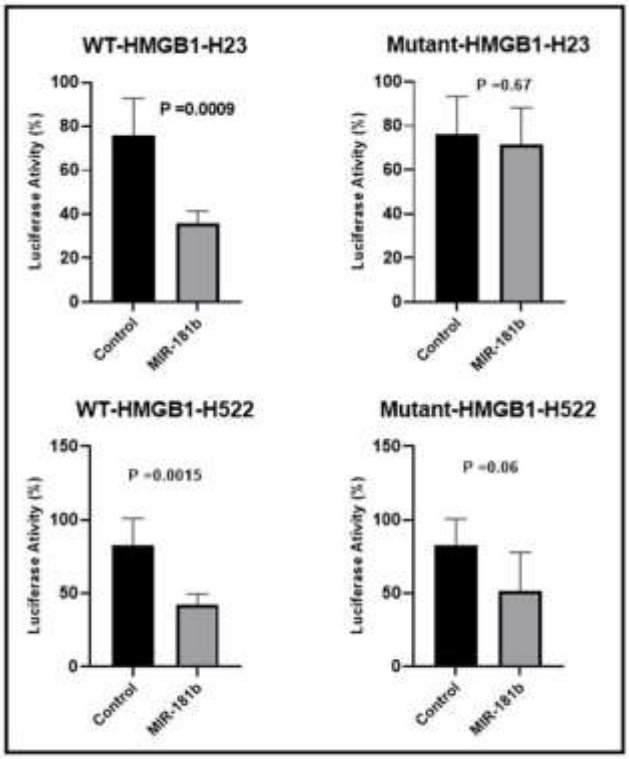

Figure 4: Luciferase analysis in H23 and H522 NSCLC cells transfected with MIR-181b mimics and the luciferase reporter with wild type HMGB1 (WT) and the mutant HMGB1.

The wild type HMGB1 was suppressed significantly in H23 (P=0.0009) and H522 cells (P = 0.0015).

Survival and MIR-181b expression

The relation between MIR-181b and survival in patients with NSCLC has been analyzed. The Kaplan-Meier survival curve was plotted (Figure 5). The median value of MIR-181b expression was utilized to stratify the patients. At 60 months of follow-up, the overall survival was 58% in patients with high MIR-181b levels compared to 20% in patients with low expression (P =0.0031). Moreover, disease-free survival was significantly higher in patients with high MIR-181b expression (P = 0.002).

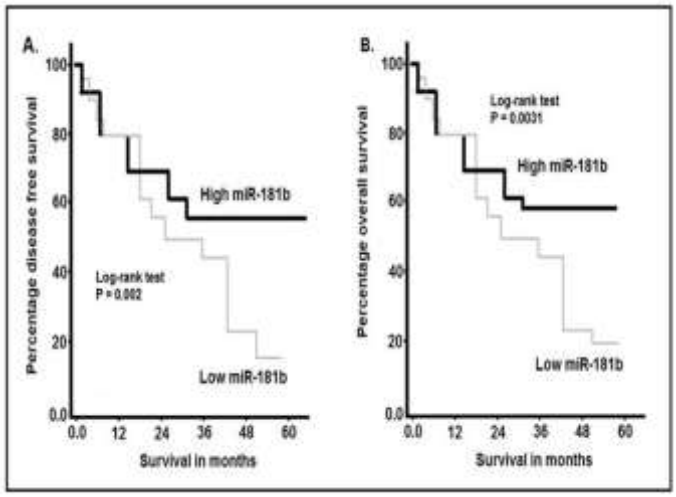

Figure 5: Kaplan-Meier survival curves of NSCLC patients based on the MIR-181b expression.

DISCUSSION

The five-year survival rate for patients with early localized NSCLC is about 56% and decreases to 5% in metastatic disease. This adverse survival provokes investigators to search for molecular markers for early diagnosis (Barta et al. 2019). Much data suggest that miRNAs play a principal role in malignancy. Several reports have been concluded that miRNAs are involved in many cancers initiation and progression (Liu et al. 2014).

In NSCLC, miRNAs play a significant role. MIR-21 has been found to promote growth and invasion (Zhanget al. 2010). Besides MIR-21, it was found that MIR-101, MIR-1254, MIR-143, MIR-494, MIR-143, MIR-574, and MIR-181a involved in NSCLC (Guan et al. 2012; Wang et al. 2012 and Tao et al. 2013). The MIR-181 family comprises MIR-181a, MIR-181b, MIR-181c, and MIR-181d (Guo et al. 2012). The MIR-181b expression has been observed in various human cancers (Pekarsky et al. 2006; Gibert et al. 2014 and Liu et al. 2016).

The miRNAs may function either as a tumor promoter or as a tumor suppressor. In one study, MIR-181b had a regulatory role on the genes of tumor suppression through the inhibition of Tcl1 oncogene which would impact the growth of tumor cells (Gibert et al. 2014). In addition, MIR-181a and b were acted as a tumor suppressor by regulation of the tumor suppressor gene, CDON and acted as a bad prognosis marker for aggressive neuroblastoma stage 4 compared to stage 1, 2, 3 (Gibert et al. 2014). Therefore, MIR-
181b function seems to vary depending on its target.

In the current research, we observed the downregulation of MIR-181b in NSCLC. The mean MIR-181b expression was 1.467 in NSCLC relative to the mean value of 4.862 in NAT (P < 0.00001). Liu et al. also observed the significant downregulation of MIR-181b in

their study comprising 62 NSCLC patients (Liu et al. 2016). Zhou and colleagues also support our results in NSCLC (Zhouet al. 2019). Our findings endorse the concept of the oncogenic suppressor function of MIR-181b in NSCLC. Our results were also confirmed by other studies in other malignant tumors (Lawrie et al. 2008; Gibert et al. 2014 and Marton et al. 2008). In a study on MIR-181b in chronic lymphocytic leukemia (CLL), Visone et al. 2012, found that MIR-181b downregulation was noted in the aggressive types of CLL. MIR-181b was also reported to be downregulated in brain gliomas (Shi et al. 2013). Jiang et al. 2011, observed that MIR-181b was up-regulated in gastric cancer compared with normal gastric tissues; besides, patients with low MIR-181b expression had better overall survival than those with high levels.

The clinical and pathological features have been analyzed based on the levels of MIR-181b expression. There was no significant correlation between miR-181b expression and all clinical and pathological features except the clinical stage. Yang et al. 2013, also observed the down regulation of MIR-181b in NSCLC compared with their normal adjacent tissues, and its downregulation was associated with advanced clinical stage. Our results also confirmed the downregulation of MIR-181b in NSCLC as Yang et al. and Liu et al. However, the expression and functions of MIR-181b need to be more studied to explore underlying mechanisms of regulation.

The current study offered proof that MIR-181b was low expressed in NSCLC that supports its suppressive role in NSCLC. To emphasis this suppressive role, we carried an in vitro study to estimate the levels of MIR-181b in NSCLC cell lines. We noted that MIR-181b over expression was associated with less invasion and metastases of malignant cells invitro.

The recognition of the target genes of MIR-181b is crucial for recognizing the function of miRNA in carcinogenesis and to search for new targeted therapy. The results of the current study identified that MIR-181b suppressed cell invasion and metastasis by influencing HGMB1 directly. HMGB1 is one of the high-mobility protein groups and enhances malignant cell invasion, development, angiogenesis, and metastasis (Wu et al. 2018). In the current study, we noted that at the levels of protein and mRNA, HMGB1was significantly downregulated and linked to malignant growth, invasion, and metastasis in NSCLC. Based on our observed data, we can report that MIR-181b triggers HMGB1 to govern the invasion, aggressiveness, and metastases of NSCLC. More research is required to further explore the MIR-181b functions.

The down regulation impact of MIR-181b on the survival of NSCLC patients has been studied. We found that low expression of MIR-181b was significantly linked with prolonged disease and overall survival. This observation was also reported by Jiang et al.

CONCLUSION

The current research provided data about the role of MIR-181b in the regulation of NSCLC aggressiveness, invasiveness, and metastases by targeting HMGB1 gene. It also showed that the MIR-181b was downregulated in NSCLC. Its downregulation was related to the tumor aggressiveness and adverse clinical and pathological features and survival.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

A F Gharib: designed the research study; performed the research; analyzed the data and wrote the paper.

Howaida M. Hagag: performed the research and analyzed the data.

Amani A. Alrehaili: performed the research and analyzed the data

Maha M. Bakhuraysah: performed the research, analyzed the data and contributed essential reagents.

Afaf Alharthi: performed the research and analyzed the data

Fouzeyyah Ali Alsaeedi: analyzed the data

Hayaa M. Alhuthali::analyzed the data and contributed essential reagents.

W H Elsawy: designed the research study; performed the research; analyzed the data and wrote the paper.

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