The Role of miRNA-377 as a Tumor Suppressor in Lung Cancer by Negative Regulation of Genes Belonging to ErbB Signaling Pathway

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Abstract

The ErbB signaling pathway plays important role in the pathogenesis of lung cancer. We explored the role of miRNA-377 as a tumor suppressor in NSCLC through silencing of some genes in the ErbB pathway. Targeting the effect of miRNA-377 on EGFR, MAPK1, ABL2, and PAK2 was evaluated. The expression levels of these genes and miRNA-377 were surveyed in NSCLC and normal human tissues, Calu-6, and A549 cells. Real-time PCR was used to figure out whether miRNA-377 could decrease the target genes mRNAs in transfected lung cancer cell lines. The effects of miRNA-377 on apoptosis cell and proliferation were analyzed. We showed that miRNA-377 targets EGFR, MAPK1, and PAK2 mRNAs in in-silico and luciferase reporter assay. The expression of miRNA-377 was significantly downregulated in human NSCLC tissues, Calu-6 and A549 cells compared to their controls. We observed a negative correlation between EGFR, MAPK1, PAK2, and miRNA-377 expression in human NSCLC tissues. A significant reduction in EGFR, MAPK1, and PAK2 mRNA levels was detected, following miRNA-377 transfection in Calu-6 and A549 cells. The higher levels of miRNA-377 in Calu-6, and A549 cells induced apoptosis and reduced proliferation, significantly. All these data reveal that miRNA-377 functions as a tumor suppressor in NSCLC and may serve as a potential therapeutic target for the treatment of NSCLC.

Introduction

Lung cancer is the first leading cause of cancer death (18.4% of the total cancer deaths), [1] which is classified into two major groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for approximately 85% of all lung cancers and the treatment efficacy of currently anticancer agents is usually undesirable and patients with advanced diseases scarcely have long life [2–4]. Therefore, it is essential to develop an efficient strategy for the treatment of NSCLC. The ErbB signaling pathway and its receptors with tyrosine kinase activity performs main roles in the molecular pathogenesis of many types of human cancer [5, 6], including lung cancer [7]. The ErbB receptors family has four members, including EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Homo- or heterodimerization of these receptors occurs after ligand binding, leads to activation of downstream signaling cascades like PLC γ , PI3K/AKT, ERK and more with the cell. EGFR mutations are found in some types of lung cancers and this mutation is associated with either HER2 or HER3 activation and triggers downstream signaling pathways like anti-apoptotic signaling [8]. Because overexpression of the EGFR occurs in 40–80% of patients with NSCLC, it has been an attractive target for the development of therapeutic agents [9–11].

miRNAs (microRNAs) are short single-stranded non-coding RNAs, containing approximately 18–24 nucleotides that regulate post-transcriptional gene expression by targeting 3' untranslated regions (3'-UTRs) of mRNAs with their seed sequences (2–7 nucleotides in the 5' end) [12]. Deregulation of several miRNAs, which are involved in various biological and pathological processes, has been found in cancers, [13–15] making them attractive targets for therapeutic intervention. miRNAs such as miRNA-125a, miRNA-125b, miRNA-199a, miRNA-21, miRNA-1 miRNA-125a-5p and miRNA-145 that mediated regulation of the ErbB signaling [16–18] are of special attention due to the important contribution of this pathway to

cancer development. These examples of miRNA ErbB signaling network illustrate the role of miRNA regulation and their potential for therapeutic purposes. In the present study, we targeted EGFR, ABL2, MAPK1 and PAK2 to silencing ErbB pathway in NSCLC. We intended to use just one miRNA to silence the ErbB pathway genes and according to the bioinformatics analysis results, miRNA-377 with a high score targeted all four main genes in this pathway.

The aim of this study was to further prospect the participation of miRNA-377 in NSCLC progression, through the analysis of the connection between miRNA-377 expression and four ErbB pathway elements which their dysregulation have key role in lung cancer development. In this study, we firstly verified the targeting of miRNA-377 on EGFR, MAPK1, and PAK2, by using bioinformatics analyses. Then we confirmed that miRNA-377 directly targeted EGFR, MAPK1, and PAK2 but not ABL2 by luciferase reporter assay. In next step our results showed that miRNA-377 expression significantly decreased in NSCLC tissue and lung cancer cell lines compared with normal tissue. Furthermore, we found that the mRNAs of EGFR, MAPK1 and PAK2 significantly increased in NSCLC tissue. We showed a significant reduction in EGFR, MAPK1 and PAK2 mRNA levels following miRNA-377 transfection in Calu-6 and A549 cells. Moreover, transfection of miRNA-377 set in motion cell proliferation and apoptosis of lung cancer cells. In conclusion, our findings suggest that miRNA-377, assisting as a tumor suppressor, played an inhibit role in the development of NSCLC by negative regulation of EGFR, MAPK1 and PAK2 expression, thereby play a part in the progress of NSCLC.

Materials And Methods

miRNA selection

Bioinformatics studies were conducted to select the appropriate miRNA that could simultaneously contain the four genes required to control the ErbB pathway. miRBase, TargetScan, MiRanda, PicTar, **miRWalk**, RNA22, PITA and MicroCosm Targets were used to find a suitable miRNA and reducing experimental error and trials. The results were checked out with **miRWalk**, TargetScan, RNA22 and DIANA mirPath software programs and the one, which was most effective on ABL2, PAK2, EGFR and MAPK1, was selected.

Cell culture

The human lung cancer cell lines (A549 and Calu-6) were obtained from National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, IRAN) and grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (Thermo Fisher Scientific) and antibiotics (100 units/mL of penicillin and 100 µg/mL of streptomycin). The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Clinical specimens

All thirty-five lung cancer tissue samples and nine non-tumor tissue samples, as control were obtained from the Imam Khomeini Hospital Complex (Tehran, Iran). The present study was conducted under the

instructions accepted by the Ethics Committee of Pasteur Institute of Iran, and written informed consent was obtained from all participants involved in the present study. The tissue samples were stored in liquid nitrogen until analysis. All experiments and analysis procedures were performed in accordance with the relevant guidelines and regulations.

Transfection

Pre-miR precursors of miRNA-377 and control pre-miR precursor (scrambled) were purchased from Ambion (Thermo Fisher Scientific). A549 and Calu-6 cells were grown to 80% confluence in 6-well plate at the density of 5×10^5 per well one day before transfection, cells were transfected with miRNA-377 or control scrambled oligonucleotide (100 nM of the final concentration of mixture) with Lipofectamine 2000 (Thermo Fisher Scientific) according to the procedures described in the manufacturer's protocol. Cells were harvested after 24, 48 and 72 h for subsequent analysis.

Luciferase assay

To test the direct binding of miRNA-377 to the target genes ABL2, PAK2, EGFR and MAPK1, a luciferase reporter assay was done. For luciferase reporter experiments, 3'-UTR of target genes ABL2, PAK2, EGFR, MAPK1 and a special scrambled sequence (AAGCTTCATAAGGCGCATAGC) as a negative control were cloned into psiCHECK™-2 Vector (Promega, C8021) closely downstream to the stop codon of Renilla luciferase (The PCR primers are listed in Table 3). Luciferase assays were performed with Dual-Glo Luciferase Assay System (Promega, E2940). In order to perform this test, $4-5 \times 10^4$ of A549 and Calu-6 cells were cultured into each well of a 48-well plate 1 day before transfection. The cells were transfected with 400 ng psiCHECK™-2 Vector harboring, ABL2 3'-UTR, PAK2 3'-UTR, EGFR 3'-UTR MAPK1 3'-UTR or scrambled sequence and 100 nM of each precursor miRNAs (377 or scrambled miRNAs) using Lipofectamine 2000 reagent (Life Technology) according to the manufacturer protocol. The multi-well plate luminometer Renilla luciferase activity was normalized to that of firefly luciferase. Luciferase activities were determined 24 h after transfection using dual luciferase reporter assay system (Promega) [19].

Designing the primers and stem-loop:

The sequence of the miRNA-377 was retrieved from the miRBase at <http://www.mirbase.org/>. In order to increase the flexibility of stem-loop structure as well as achieving necessary sensitivity, the published sequence of Chen et al. was modified [20]. The modifications included addition of fourteen nucleotides to the original sequence in order to lengthen the loop and enabling the design of a universal reverse primer inside it and substitutions for decreasing the melting temperature of the stem part. This new designed structure was able to specifically detect each miRNA because few nucleotides complimentary to the 3'UTR of miRNA were added to each stem-loop [21]. Almost complete sequence of miRNA-377 was used as the forward primer for real-time PCR assay. miRNA forward primer and the mRNA real-time PCR primers were designed with Allel ID, Gene Runner software programs and Primer blast (Table 4) were

designed by Allele ID6 software (PREMIER Biosoft, USA). Secondary structure analysis of the amplicon was performed with “mfold” software (<http://mfold.rna.albany.edu/?q=mfold/>). All Oligos were purchased from Metabion international AG (Lena- Christ- Strasse, Germany).

RNA isolations and quantitative real-time PCR

miRNeasy Mini kit (Qiagen) was used to extract total RNA from both the cells and tissues. The concentration and purity of total RNA were quantified using UV spectrophotometric analysis at 260 nm. The cDNA synthesis kit (Thermo Fisher Scientific) was used to synthesize cDNA of mRNA. Real-time PCR was carried out using real-time PCR Master Mix (2X Maxima SYBR Green real-time PCR Master Mix, Thermo Fisher Scientific) and the GAPDH was chosen as the endogenous control. The PCR mixtures were made in 25 µL volumes consisted of 12.5 µL of SYBR Green real-time PCR Master Mix, 2 µL of cDNA, 0.4 µL of Forward Primer (10 pmol), 0.4 µL of Reverse Primer (10 pmol) and 7.2 µL of RNase-free H₂O. The thermal cycling profile of real-time PCR was as follows: 10 min initial denaturation at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 40 s. Finally, the melting curve analysis was performed at a range of 65°C up to 95°C. For miRNA following RNA extraction, the samples were reverse transcribed using M-MuLV reverse transcriptase enzyme. Five microliters of extracted miRNA, was added to 1.5 µL of stem-loop RT primers (50 nM) and 4 µL distilled water. The 10.5 µL reactions were incubated in a peQlab thermocycler (peQlab, Germany) at 65°C for 10 minutes. The tubes were hold on ice and 4 µL of 5×RT-Buffer (Roche, Germany), 2 µL of DTT (10 mM) (Merck Germany), 2 µL of dNTP (10 mM), 20unit of RNase inhibitor (Thermo scientific, USA) and 1 µL of by M-MuLV reverse transcriptase enzyme were added to the preheated mixture. The cDNA synthesis was carried out as following: 25°C for 15 min, 37°C for 15 min, 42°C for 40 minutes and finally, 2 minutes’ incubation at 95°C in order to inactivate the enzyme. The cDNA was stored at -20°C until use.

Real-time PCR for miRNA-377 was performed in 12.5 µL PCR mixture volume composed of 6.5 µL 2× Maxima qPCR Master Mix (Takara, Japan), 0.3 µM of each oligonucleotide primers and 2 µL of cDNA. Amplification was performed in the following conditions: Enzyme activation step at 95°C for 10 min and 45 cycles of two thermal amplification steps: 94°C for 10 seconds and 60°C for 40 seconds. Single fluorescence detection was performed in each cycle at the end of extension phase. Reaction set ups were done manually. Amplification, data acquisition and analysis were performed on Rotor-Gene 6000 (Corbett Research, Australia) using Rotor-Gene 6.1 and the Relative expression software tool (REST). All samples were run in triplicate along with pertaining controls such as positive, negative controls. U6 was used as the reference gene. The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Permissible Ct scattering in triplicates of each sample was ≤ 5 %. Statistical analysis for relative miRNA and mRNA expression were performed by REST proposed by Pfaffl. Fold change and P value (≤ 0.05) were estimated by REST, where all requirements of this software were considered.

Apoptosis

Cells were seeded into 12-well plates at a concentration of 3×10^4 cells/well and allowed to adhere to the surface in 24 h. After 48 h of transfection, cells were trypsinized, placed onto 1.5 ml tube. After washing and centrifugation, the harvested cells were incubated in PI and Annexin V FITC according to the manufacturer's protocol (Roche Applied Sciences). Stained cells were analyzed using a flow cytometer (Partec, Germany). In each experiment, 20,000 cells per 200 secs were recorded and each sample was run in triplicate.

Cell proliferation

MTT (Sigma) was used to perform cell proliferation assay. Cells were seeded into 96-well plates at a concentration of 1×10^4 cells/well. After 72 h of transfection, cells were washed with PBS and MTT (5 mg/ml) added. The cells were incubated for 3 h at 37°C in the CO₂ incubator. The crystals were then dissolved by adding 100 µl of DMSO. Spectrophotometrical absorbance of the purple-blue formazan dye was measured in a microplate reader at 540 nm (reference wavelength: 630 nm). Each experiment was repeated at least three times.

Statistical analysis

Real-time PCR experiments, cell proliferation and apoptosis assays were carried out at least three times. The data shown are the mean $\pm$ standard error of the mean. Statistical analysis of data was carried out using the Prism 8 (GraphPad) software using a Student's t-test for the comparison of the difference between the two groups and analysis of variance (ANOVA) followed by post hoc Tukey's Multiple Comparison Test, for comparison among multiple groups. All real-time PCR results were analyzed using $\Delta\Delta$ CT method with REST 2009 software (Qiagen, Hilden, Germany) and expression was normalized against GAPDH for mRNA and U6 for miRNA. Correlation analysis was performed using the Spearman test. The differences were considered statistically significant at P values less than 0.05.

Results

Demographic data of patients

While twenty-two of samples were obtained from men, thirteen samples were from women. The age ranges of patients were 39–80. They all suffered from adenocarcinoma of the lung, TNM stages in patients were T1N1Mx in seven patients, T2N2M1 in thirteen patients, T3N0M0 in ten patients and T4N2M0 in five patients and EGFR mutation had been detected in three patients.

miRNA-377 can target the 3'-UTR of EGFR, PAK2, MAPK1

Eight bioinformatics prediction software programs (miRBase, TargetScan, MiRanda, PicTar, miRWalk, RNA22, PITA and MicroCosm Targets) were applied to predict the best miRNA for silencing ABL2, PAK2, EGFR and MAPK1 genes in ErbB pathway. Four first ranked miRNAs for each gene are shown in Table 1.

hsa-miRNA-377-3p was among the first four miRNAs that could recognize and attach to all four mRNAs. In order to confirm bioinformatics prediction of previous software programs and predict the miRNA-377-target positions interaction and seed matching with ABL2, PAK2, EGFR and MAPK1, prophecy programs including **miRWalk**, TargetScan, RNA22, DIANA, mirPath, miRmap and miRDIP software programs were used (Table 2). Inhibitory effect of miRNA- 377 on all four genes expression was predicted at least in four prediction software programs with moderate to high score.

In order to validate whether ABL2, PAK2, EGFR and MAPK1 are direct target of miRNA-377, we cloned the 3'-UTR of the ABL2, PAK2, EGFR or MAPK1 and a special scrambled sequence into the Renilla luciferase gene in psiCHECK™-2. This plasmid was co-transfected with miRNA-377 and scrambled in A549 and Calu-6 cells. As shown in Fig. 1a, miRNA-377 significantly reduced the luciferase activity in cells which contain vector harboring, PAK2 3'-UTR, EGFR 3'-UTR or MAPK1 3'-UTR compared with the scrambled oligonucleotide. Transfecting miRNA-377 into cells which contain vector harboring, EGFR 3'-UTR, MAPK1 3'-UTR or PAK2 3'-UTR decreased the luciferase activity to $51\% \pm 4.623\%$, $57\% \pm 3.4631\%$ and $52\% \pm 4.3272\%$ respectively in comparison with the control ($P < 0.001$), while in cells which contain vector harboring, ABL2 3'-UTR, the luciferase activity decreased to $90\% \pm 4.376\%$. These results suggested that PAK2, EGFR and MAPK1 could be considered as a candidate target gene for miRNA-377 and also their 3'-UTR is a functional target site for this miRNA in A549 and Calu-6 cells.

Expression level of miRNA-377 in lung cancer tissues and lung cancer cell lines was lower than normal tissues

To investigate the level of miRNA-377 in lung cancer samples, A549 and Calu-6 cell lines and normal tissues, total RNA were extracted from thirty-five NSCLC patients and nine normal samples used for real-time PCR. The results are shown that the average miRNA-377 expression in human NSCLC tissues and cell lines was significantly ($P < 0.05$) lower than that found in non-tumor tissue samples. We compare them due to the fact that we do not have normal cell line (Fig. 1b and c).

There was a negative correlation between the expression of miRNA-377 and some ErbB signaling pathway genes mRNA level in lung cancer tissues

In order to confirm any correlation between the level of miRNA-377 and EGFR, MAPK1, and PAK2 mRNA expression in lung cancer samples and normal tissues, total RNA was extracted from thirty-five NSCLC patients and nine normal samples used for real-time PCR.

The EGFR, MAPK1 and PAK2 mRNAs were significantly increased in the lung tumor tissues compared to normal tissues. ($P < 0.05$; Fig. 2a).

Statistical analysis further revealed that the expression level of miRNA-377 negatively correlated with EGFR and MAPK1 and PAK2 mRNA expression. These values for EGFR and MAPK1 and PAK2 were

$r=-0.7699$ ($P < 0.05$), $r=-0.7863$ ($P < 0.05$) and $r=-0.8990$ ($P < 0.05$) respectively (Fig. 2b).

miRNA-377 introduction to A549 and Calu-6 cells downregulates the expression level of some genes belonging to ErbB signaling pathway.

To determine whether miRNA-377 could target the EGFR, ABL2, MAPK1 and PAK2 mRNAs, we examined the relative expression levels of the target RNAs in transfected A549 and Calu-6 cell lines with miRNA-377 or scrambled oligonucleotide and evaluated with control cells by real-time PCR. In this experiment non-transfected cells and cells transfected with scrambled oligonucleotide were used as controls, respectively. The real-time PCR results demonstrated that miRNA-377 induced a remarkable decrease in levels of EGFR, MAPK1 and PAK2 mRNAs in the two cell lines (Fig. 3). The suppression effect of miRNA-377 on ABL2 levels in A549 and Calu-6 cells was not significant.

Overexpression of miRNA-377 induced apoptosis in Lung cancer cell lines

The apoptotic effect of miRNA-377 in A549 and Calu-6 cells was evaluated by flow cytometry using annexin V and PI. The results obtained demonstrated an increase in cells undergoing early apoptosis when compared controls (Fig. 4a and 4b). Non-transfected cells and cells transfected with scrambled oligonucleotide used as controls for this experiment. The apoptosis ratio significantly increased in cells transfected with miRNA-377 in comparison with controls ($P < 0.05$). The percentage of early apoptotic cells increased from 0.16–30.41% in A549 and 0.2 to 24% in Calu-6 transfected with miRNA-377.

Overexpression of miRNA-377 decreased cell proliferation in lung cancer cell lines

In order to understand the role of miRNA-377 on lung cancer cell lines cancer cell proliferation, MTT assay was used. A549 and Calu-6 cells were seeded in 96-well plates and transfected with miRNA-377 or scrambled oligonucleotide. 48 h after transfecting, the proliferation of these cells in comparison with non-transfected and scrambled cells was evaluated. We demonstrated that the miRNA-377 could significantly ($P < 0.05$) reduce cell growth compared with the scrambled and control groups in the A549 and Calu-6 cell lines (Fig. 4c). Experiments were performed three times.

Discussion

Lung cancer is the leading cause of cancer-related deaths in many countries [22]. The ErbB pathway plays an important role in the pathogenesis of many types of human cancer, including those of the lung [7]. Accumulating evidence suggested that some miRNAs are abnormally expressed in several types of cancer [23, 24]. In this article, we studied the role of miRNA-377 in ErbB pathway in lung cancer and found

the correlation of the downregulation of miRNA-377 and overexpression of three genes in the ErbB pathway in NSCLC tissue. In addition, we demonstrated that miRNA-377 significantly increased the apoptosis and decreased cell proliferation of lung cancer cell lines in vitro.

The interaction between miRNA-377 and 3'-UTR of the mRNA of EGFR, MAPK1, ABL2 and PAK2 was investigated through in-silico analysis. Among all of the miRNAs that targeted the 3'-UTR of mentioned mRNAs, miRNA-377 had a relatively high score for assessment in the present. Also, luciferase reporter data showed that 3'-UTR of PAK2, EGFR or MAPK1 are functional target site for miRNA-377 in A549 and Calu-6 cells. miRNA-377 is one of the miRNAs which belongs to the large miRNA cluster encoded on chromosome 14 (14q32) and plays critical roles in types of human malignancies [24, 25]. The role of miRNA-377 downregulation was identified to be associated with various human malignancies, including prostate tumors, hepatocellular carcinoma, osteosarcoma MG-63, clear cell renal cell carcinoma, glioblastoma, malignant melanoma, pancreatic cancer and ovarian cancer [26–32]. Also, miRNA-377 plays an important role in chemotherapeutic resistance in B-cell lymphoid malignancies [33]. One study has been shown that miRNA-377 functions as an oncogene in human colorectal cancer [34]. Previously, we have identified the role of miRNA-377 in the regulation of DNA methylation in pancreatic cancer [35]. Decreasing of miRNA-377 has been found in lung cancer [36] and inhibition of tumorous behaviors of miRNA-377 in this cancer through targeting AEG-1 and CDK6, has been reported [36, 37].

In this study, we found significant diminution of miRNA-377 and overexpression of EGFR, MAPK1 and PAK2 genes in NSCLC tissue samples in comparison to their control. Real-time PCR was used to evaluate the level of miRNA-377 in both lung tumor tissue and lung cancer cell lines compared to their normal samples. The data showed that miRNA-377 was significantly downregulated in lung cancer samples compared to normal tissues and cells (Fig. 1). To evaluate the levels of EGFR, MAPK1, and PAK2 expression, we analyzed their mRNA levels in all NSCLC and normal samples (Fig. 2). Our results showed that mRNAs of EGFR, MAPK1 and PAK2 were significantly upregulated in tumors compared to normal tissues.

The data is confirming the inverse correlation between miRNA-377 and EGFR, MAPK1 and PAK2 mRNAs. Expression of miRNA-377 was decreased while mRNA of EGFR, MAPK1 and PAK2, the ErbB signaling pathway-related genes were increased in NSCLC tissue when compared with the control group.

To confirm whether miRNA-377 could target EGFR, MAPK1, ABL2 and PAK2 mRNAs in A549 and Calu-6 cells, we analyzed expression levels of them in these cells, following miRNA-377 transfection. The miRNA-377 at the same time reduced the expression level of mRNA of EGFR, MAPK1 and PAK2 genes. While no significant effect was detected with the expression levels of ABL2 mRNA (Fig. 3).

An MTT assay and flow cytometry were used to determine the roles of miRNA-377 in cell proliferation and apoptosis, respectively. The results indicated that, compared with the control and scrambled miRNA, miRNA-377 obviously inhibited the cell proliferation and promoted the apoptosis of A549 and Calu-6 cells, which suggested that miRNA-377 has a role in suppressing lung cancer. These results were in line with previous studies, which reported that of miRNA-377 suppresses, prostate cancer, hepatocellular

carcinoma, human osteosarcoma, clear cell renal cell carcinoma, glioblastoma, pancreatic cancer, ovarian cancer cells proliferation, and promotes prostate cancer and pancreatic cancer cells apoptosis.^{24,27–32} All data indicated that miRNA-377 has a key role in cell proliferation and apoptosis of lung and mentioned cancer cells. The corresponding molecules involved in the mentioned processes and more miRNA-377 targets remain to be clarified.

Conclusions

Taken together, we found the role of miRNA-377 in the regulation of ErbB pathway in lung tumor via targeting the 3'-UTR of the mRNA of EGFR, MAPK1, and PAK2. Transfection of miRNA-377 causes preventing cell proliferation and promoting cell apoptosis in A549 and Calu-6 cells. Future studies will maybe find further miRNA-377 targets to clarify a complete picture of the miRNA-377 regulation network that drives the progression of NSCLC. These data also indicate that miRNA-377 may be a potential therapeutic target for the treatment of NSCLC.

Declarations

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Ethical approval statement

The present study was conducted under the instructions accepted by the Ethics Committee of Pasteur Institute of Iran, **written informed consent to participate**, and **consent to publish forms** was obtained from all participants involved in the present study. This material is the authors' own original work, which has not been previously published elsewhere. The paper is not currently being considered for publication elsewhere. The paper reflects the authors' own research and analysis in a truthful and complete manner.

Data availability statement

The authors confirm that the data supporting the findings of this study are available.

Authors' contributions

MA and SZ designed the study. NY, SH, FRJ, MK and MHR carried out the experiments. MA, FRJ, SH, NY and RM collected and analyzed the data. SH and MA wrote the manuscript. All authors read and

approved the final manuscript.

Disclosure of interest

All authors declare that they have no Conflict of interest.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68(6):394–424
2. Sekine I, Yamamoto N, Nishio K, Saijo N (2008) Emerging ethnic differences in lung cancer therapy. *British journal of cancer* 99(11):1757–1762
3. Götz R (2008) Inter-cellular adhesion disruption and the RAS/RAF and beta-catenin signalling in lung cancer progression. *Cancer cell international* 8(1):1–4
4. Su VY, Yang KY, Huang TY, Hsu CC, Chen YM, Yen JC, Chou YC, Chang YL, He CH (2020) The efficacy of first-line tyrosine kinase inhibitors combined with co-medications in Asian patients with EGFR mutation non-small cell lung cancer. *Scientific reports* 10(1):1–4
5. Ramaiah MJ, Kumar KR (2021) mTOR-Rictor-EGFR axis in oncogenesis and diagnosis of glioblastoma multiforme. *Molecular Biology Reports*, 1–23
6. Obradovic J, Todosijevic J, Jurisic V (2021) Application of the conventional and novel methods in testing EGFR variants for NSCLC patients in the last 10 years through different regions: a systematic review. *Molecular Biology Reports*, 1–12
7. Hynes NE, MacDonald G (2009) ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol* 21(2):177–184
8. Engelman JA, Jänne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, Cichowski K, Johnson BE, Cantley LC (2005) ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proceedings of the National Academy of Sciences* 102(10):3788–93
9. Torigoe H, Yamamoto H, Sakaguchi M, Youyi C, Namba K, Sato H, Shien K, Soh J, Suzawa K, Tomida S, Tsukuda K (2018) Tumor-suppressive effect of LRIG1, a negative regulator of ErbB, in non-small cell lung cancer harboring mutant EGFR. *Carcinogenesis* 39(5):719–727
10. Deng Q, Fang Q, Sun H, Singh AP, Alexander M, Li S, Cheng H, Zhou S (2020) Detection of plasma EGFR mutations for personalized treatment of lung cancer patients without pathologic diagnosis. *Cancer medicine* 9(6):2085–2095
11. Hirsch FR, Varella-Garcia M, Cappuzzo F (2009) Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. *Oncogene* 28(1):S32–S37
12. Zhang B, Chen J, Ren Z, Chen Y, Li J, Miao X, Song Y, Zhao T, Li Y, Shi Y, Ren D (2013) A specific miRNA signature promotes radioresistance of human cervical cancer cells. *Cancer cell international* 13(1):1–8

13. Shi ZM, Wang L, Shen H, Jiang CF, Ge X, Li DM, Wen YY, Sun HR, Pan MH, Li W, Shu YQ (2017) Downregulation of miR-218 contributes to epithelial–mesenchymal transition and tumor metastasis in lung cancer by targeting Slug/ZEB2 signaling. *Oncogene* 36(18):2577–2588
14. Cao S, Jiang L, Shen L, Xiong Z (2019) Role of microRNA-92a in metastasis of osteosarcoma cells in vivo and in vitro by inhibiting expression of TCF21 with the transmission of bone marrow derived mesenchymal stem cells. *Cancer cell international* 19(1):1–7
15. Bischoff A, Bayerlová M, Strotbek M, Schmid S, Beissbarth T, Olayioye MA (2015) A global microRNA screen identifies regulators of the ErbB receptor signaling network. *Cell Communication Signaling* 13(1):1–5
16. Soliman SES, Abdelaleem AH, Alhanafy AM, Ibrahim RAL, Elhaded ASA, Assar MF (2021) Circulating miR-21-5p and miR-126-3p: diagnostic, prognostic value, and multivariate analysis in non-small-cell lung cancer. *Mol Biol Rep* 48(3):2543–2552
17. Kawana S, Saito R, Miki Y, Kimura Y, Abe J, Sato I, Endo M, Sugawara S, Sasano H (2021) Suppression of tumor immune microenvironment via microRNA-1 after epidermal growth factor receptor-tyrosine kinase inhibitor resistance acquirement in lung adenocarcinoma. *Cancer medicine* 10(2):718–727
18. Amri J, Molaee N, Karami H, Baazm M (2021) Combination of two miRNAs has a stronger effect on stimulating apoptosis, inhibiting cell growth and increasing erlotinib sensitivity relative to single miRNA in A549 lung cancer cells. *Biotechnology and Applied Biochemistry*
19. Sherf BA, Navarro SL, Hannah RR, Wood KV (1996) Dual-luciferase reporter assay: an advanced co-reporter technology integrating firefly and Renilla luciferase assays. *Promega Notes* 57(2):2–8
20. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ (2005) Real-time quantification of microRNAs by stem–loop RT–PCR. *Nucleic acids research* 33(20):e179–
21. Mohammadi-Yeganeh S, Paryan M, Samiee SM, Soleimani M, Arefian E, Azadmanesh K, Mostafavi E, Mahdian R, Karimipoor M (2013) Development of a robust, low cost stem-loop real-time quantification PCR technique for miRNA expression analysis. *Molecular biology reports* 40(5):3665–3674
22. Barta JA, Powell CA, Wisnivesky JP (2019) Global epidemiology of lung cancer. *Annals of global health* 85(1)
23. Zou X, Wei J, Huang Z, Zhou X, Lu Z, Zhu W, Miao Y (2019) Identification of a six-miRNA panel in serum benefiting pancreatic cancer diagnosis. *Cancer Med* 8:2810–2822
24. Wu J, Shen Z (2020) Exosomal miRNAs as biomarkers for diagnostic and prognostic in lung cancer. *Cancer Med* 9(19):6909–6922
25. Chang W, Liu M, Xu J, Fu H, Zhou B, Yuan T, Chen P (2016) MiR-377 inhibits the proliferation of pancreatic cancer by targeting Pim-3. *Tumor Biology* 37(11):14813–14824
26. Zehavi L, Schayek H, Jacob-Hirsch J, Sidi Y, Leibowitz-Amit R, Avni D (2015) MiR-377 targets E2F3 and alters the NF-kB signaling pathway through MAP3K7 in malignant melanoma. *Mol Cancer*

27. Formosa A, Markert EK, Lena AM, Italiano D, Finazzi-Agro E, Levine AJ, Bernardini S, Garabadgiu AV, Melino G, Candi E (2014) MicroRNAs, miR-154, miR-299-5p, miR-376a, miR-376c, miR-377, miR-381, miR-487b, miR-485-3p, miR-495 and miR-654-3p, mapped to the 14q32. 31 locus, regulate proliferation, apoptosis, migration and invasion in metastatic prostate cancer cells. *Oncogene* 33(44):5173–82
28. Chen G, Lu L, Liu C, Shan L, Yuan D (2015) MicroRNA-377 suppresses cell proliferation and invasion by inhibiting TIAM1 expression in hepatocellular carcinoma. *PloS one* 10(3):e0117714
29. Wang L, Shao J, Zhang X, Xu M, Zhao J (2015) microRNA-377 suppresses the proliferation of human osteosarcoma MG-63 cells by targeting CDK6. *Tumor Biology* 36(5):3911–3917
30. Wang R, Ma Y, Yu D, Zhao J, Ma P (2015) miR-377 functions as a tumor suppressor in human clear cell renal cell carcinoma by targeting ETS1. *Biomed Pharmacother* 70:64–71
31. Zhang R et al (2014) MicroRNA-377 inhibited proliferation and invasion of human glioblastoma cells by directly targeting specificity protein 1. *Neurooncology* 16:1510–1522
32. Yu R, Cai L, Chi Y, Ding X, Wu X (2018) miR377 targets CUL4A and regulates metastatic capability in ovarian cancer. *Int J Mol Med* 41(6):3147–3156
33. Al-Harbi S, Choudhary GS, Ebron JS, Hill BT, Vivekanathan N, Ting AH, Radivoyevitch T, Smith MR, Shukla GC, Almasan A (2015) miR-377-dependent BCL-xL regulation drives chemotherapeutic resistance in B-cell lymphoid malignancies. *Mol Cancer* 14(1):1–7
34. Liu WY, Yang Z, Sun Q, Yang X, Hu Y, Xie H, Gao HJ, Guo LM, Yi JY, Liu M, Tang H (2018) miR-377-3p drives malignancy characteristics via upregulating GSK-3 β expression and activating NF- κ B pathway in hCRC cells. *Journal of cellular biochemistry* 119(2):2124–2134
35. Azizi M, Fard-Esfahani P, Mahmoodzadeh H, Fazeli MS, Azadmanesh K, Zeinali S, Teimoori-Toolabi L (2017) MiR-377 reverses cancerous phenotypes of pancreatic cells via suppressing DNMT1 and demethylating tumor suppressor genes. *Epigenomics* 9(8):1059–1075
36. Meng F, Zhang L, Shao Y, Ma Q, Lv H (2015) MicroRNA-377 inhibits non-small-cell lung cancer through targeting AEG-1. *Int J Clin Exp Pathol* 8(11):13853
37. Zhang J, Zhao M, Xue ZQ, Liu Y, Wang YX (2016) miR-377 inhibited tumorous behaviors of non-small cell lung cancer through directly targeting CDK6. *Eur Rev Med Pharmacol Sci* 20(21):4494–4499

Tables

Table 1. Sixteen first ranked predicted miRNAs for silencing ErbB pathway

Genes	Predicted miRs			
MAPK1	hsa-miRNA-130a-5p	hsa-miRNA-137	hsa-miRNA-377-3p	hsa-miRNA-148b-3p
EGFR	hsa-miRNA-432-5p	hsa-miRNA-186-3p	hsa-miRNA-148a-3p	hsa-miRNA-377-3p
PAK2	hsa-miRNA-455-3p	hsa-miRNA-377-3p	hsa-miRNA-222-3p	hsa-miRNA-137
ABL2	hsa-miRNA-200a	hsa-miRNA-377-3p	hsa-miRNA-141-3p	hsa-miRNA-107

Table 2. 3'-UTRs contain predicted miRNA-377-binding site

Position 136-142 of EGFR 3'-UTR	CUGCCACAUGCCAGCUGUGUGAC
Hsa-miRNA-377	UGUUUUCAACGGAA-ACACACUA
Position 3558-3563 of PAK2 3'-UTR	AAUCAGUUUUUUGAUAGUGUGAA
Hsa-miRNA-377	UGUUUUCAACGGAAACACACUA
Position 2202-2209 of MAPK1 3'-UTR	AUGAAAUUAUAUUUGCUGUGUGAC
Hsa-miRNA377	UGUUUUCAACGGAA—ACACACUA
Position 3397-3402 of ABL2 3'-UTR	ACCGACAAUCAGCCUGUGUGGU
Hsa-miRNA-377	UGUUUUCAACGGAAACACACUA

Table 3. Relative primers used for Luciferase Reporter Assay. Bold sequences indicate the NOT1 and Xcho1 endonuclease restriction sites.

Gene Name	Primer Sequence
ABL2	CTGGCACACAATGAGCACAC CTCGAG Forward:
	AGGGAATGTAGGACTAACTAGGT GCGGCCG Reverse:
EGFR	CTCGAG AGCTCCTATGGGGGACAGCForward:
	AAACAAACAGGCATCCACTGC GCGGCCG Reverse:
PAK2	CTCGAG TGAGTATTTCTTTTAGGGCTCACForward:
	GCGGCCG TGAACAAAGTCAAAATACAGCAGTTReverse:
MAPK1	CTCGAG TTCCCTCAGCTTGCTGGTAForward:
	GCGGCCG AGACAAACACCAAAATAGGTACARReverse:

Table 4. Relative primers used for real-time PCR and stem-loop sequences

Gene Name	Primer Sequence	Product Size
ABL2	GACACTTCACTTTGCTGCCTGForward:	138bp
	ACGATGCAAAGCTTCTGGACTARReverse:	
EGFR	TCGGCACGGTGTATAAGGForward:	127bp
	ACGTAGGCTTCATCGAGGReverse:	
PAK2	ATTAACGAGATTCTGGTGATGForward:	195bp
	ATTAACGAGATTCTGGTGATGReverse:	
MAPK1	Forward: AGGGCTCCAGAAATTATGTTG	157bp
	Reverse: ATCCAAGAATACCCAAAATGTG	
GAPDH	Forward: GTCTCCTCTGACTTCAACAGCG	131bp
	Reverse: ACCACCCTGTTGCTGTAGCCAA	
miRNA-377	Forward: GAATCACACAAAGGCAACTTTTGT	60bp
	Reverse : CAGTGCAGGGTCCGAGGTATTC	
U6	Forward: CGCAAGGATGACACGCAAATTC	58bp
	Reverse: CAGTGCAGGGTCCGAGGTATTC	
miRNA-377 stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAAAA	
U6 stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATGG	

Figures

Figure 1

a. MiRNA-377 directly targets PAK2, EGFR and MAPK1. A549 and Calu-6 cells were transfected with the Renilla and firefly luciferase expression construct psiCHECK K™-2 harboring ABL2 3'-UTR, PAK2 3'-UTR, EGFR 3'-UTR or MAPK1 3'-UTR and miR-377 or scrambled oligonucleotides. After 48 h, Dual-Luciferase assays were performed and the decrease in relative Renilla luciferase activity was observed, while no effect was detected with the scrambled miR and scrambled sequence in pSicheck™-2 vector. The diminution of luciferase activity in cells harboring ABL2 3'-UTR was not significant. Bars indicate Firefly luciferase activity normalized to Renilla luciferase activity $\pm$ SD. Each reporter plasmid was transfected three times, and each sample was assayed in triplicate (**P < 0.001). b. The chart shows the comparison of miRNA-377 expression in lung cancer cells A549, Calu-6 and lung normal tissues. miRNA-377

expression significantly decreases in cell lines. c. Comparing differences in the expression levels of miRNA-377 between lung tumor tissues and the normal lung tissues.

Figure 2

The correlation between miRNA-377 expression and EGFR, MAPK1, PAK2 and ABL2 level in lung cancer. The correlation between miRNA-377 expression and the mRNA level of EGFR, MAPK1, PAK2 and ABL2 in human lung cancer specimens. a. Diagram illustrates EGFR, MAPK1, PAK2 and ABL2 mRNA differential expression in lung tumor and normal tissues. The expression of EGFR and MAPK1 are significantly increasing in lung tumor tissue compared with normal lung tissues. n=9; *P < 0.05, NL: non lung cancer, LC: Lung cancer. b-d. Correlation analysis between relative expression level EGFR, MAPK1, and PAK2 mRNA and miRNA-377 in lung tumor tissue was measured by Pearson correlation.

Figure 3

The level of EGFR, MAPK1, PAK2 and ABL2 mRNAs following miRNA-377 or scrambled transfection. a and b. A549 and Calu-6 cells were transfected with miRNA-377 or scrambled oligonucleotide. The miRNA-377 simultaneously reduced the expression level of mRNA of EGFR, MAPK1 and PAK2 genes, while no significant effect was detected with the expression levels of ABL2 mRNA. Results are shown as mean $\pm$ SE from three independent experiments, which performed in triplicates. *P < 0.05 compared with control.

Figure 4

The effects of miRNA-377 on apoptosis and cell proliferation in A549 and calu6 cells. a representative histogram, non-transfected cells used as a negative control. It is known that Q1, Q2, Q3 and Q4 quadrant represent the necrosis (annexin negative and PI positive), late apoptosis (double annexin/PI positive), live (double annexin/PI negative) and early apoptosis (annexin positive), respectively. The experiments were carried out three times. b. Percentage of three independently apoptosis after transfection of miRNA-377 in A549 and Calu-6 cell lines. This results representative of three independently performed and demonstrated that miRNA-377 significantly increases the apoptosis ratio in A549 and Calu6 cells (* $P < 0.05$). c. The effects of miRNA-377 on cell proliferation in A549 and calu-6 cells. The proliferation of A549 and Calu-6 cells transfected with miRNA-377, scrambled oligonucleotide and control cells were determined using the MTT assay. Experiments were performed for three times and data are shown as mean $\pm$ SD. The results showed that miRNA-377 could markedly inhibit cancer cell proliferation.