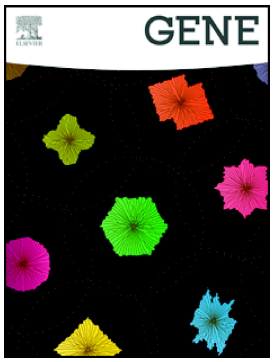

miR-199a-5p and miR-495 target GRP78 within UPR pathway of lung cancer

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PII: S0378-1119(17)30211-1
DOI: doi: 10.1016/j.gene.2017.03.032
Reference: GENE 41838

To appear in: *Gene*

Received date: 9 December 2016
Revised date: 22 March 2017
Accepted date: 24 March 2017

Please cite this article as: Azam Ahmadi, Behzad Khansarinejad, Saman Hosseinkhani, Mostafa Ghanei, Seyed Javad Mowla , miR-199a-5p and miR-495 target GRP78 within UPR pathway of lung cancer. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Gene(2017), doi: 10.1016/j.gene.2017.03.032

miR-199a-5p and miR-495 target GRP78 within UPR pathway of lung cancer

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Running head: Hsa-miR-199a-5p and hsa-miR-495 target GRP78 in UPR pathway

Grant sponsor: This study supported by a PhD grant to the first author from Tarbiat Modares University.

Competing interests: The authors declare no conflicts of interest.

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High lights:

- miRNAs targeting GRP78 within UPR pathway is proposed.
- GRP78 is up-regulated in tissue lung tumors, compared to non-tumor samples. miR-199a-5p and miR-495 are down-regulated in lung tumor samples compared to non-tumor samples.
- Down-regulation of these miRNAs, might be the reason for up-regulation of GRP78 in NSCLC patients.
- miR-199a-5p and miR-495 have interaction with 3'-UTR of GRP78.
- These miRNAs might have effect on ER stress in lung cancer, and can be used as diagnostic biomarkers for activated UPR in NSCLC.

Abstract

Introduction: Non-small cell lung cancer (NSCLC) is the most common subtype of lung cancer. One of the signal transduction pathways related to NSCLC is Unfolded Protein Response (UPR), which is mainly regulated by *GRP78* (*HSPA5*, Gene ID: 3309). The aim of this study was to employ bioinformatics tools to predict microRNAs (miRNAs) affecting *GRP78* expression, experimentally validate interaction of these miRNAs with GRP78 and also evaluating the expression correlation of *GRP78* and its predicted miRNAs in clinical samples.

Materials and Methods: Various software were used to predict miRNAs that simultaneously target all upstream and downstream components of GRP78 in the UPR, as well as the main components of PI3K/AKT, MAPK, ErbB and calcium pathways. For experimental analysis, 36 pairs of Formalin-Fixed Paraffin-Embedded (FFPE) lung tumor and non-tumor tissue samples were obtained. Additionally, A549 and QU-DB lung cancer cell lines were used for expression determination of *GRP78* and its predicted targeting miRNAs. We also employed a luciferase assay to evaluate interactions between candidate miRNAs with the 3'-UTR of GRP78.

Results: hsa-miR-495 and hsa-miR-199-5p were chosen based on several criteria including thermodynamic binding features of miRNAs to the target transcripts, number of recognition sites, and conservation of binding sites within the 3'-UTR of GRP78. RT-qPCR data revealed a significant up-regulation of *GRP78* (3.87 times, P=0.002) and down-regulation of *miR-199a-5p* (0.13 times, P=0.0001) and *miR-495* (0.085 times, P=0.0001) in tumor samples. Luciferase assay confirmed an interaction of hsa-miR-199a-5p and hsa-miR-495 with the 3'-UTR of GRP78 transcript. In addition, over-expression and competitive inhibition of the aforementioned miRNAs, significantly altered the expression of *GRP78* and spliced *XBP1* level.

Conclusion: Our data revealed a significant up-regulation of *GRP78* and a concomitant down-regulation of *miR-495* and *miR-199a-5p* in NSCLC. Accordingly, our data suggest a causative role for miR-199-5p and miR-495 in tumorgenesis of lung and probably other cancer types.

Key words: Non-small cell lung cancer, UPR, GRP78, Luciferase assay

1. Introduction

Lung cancer is the leading cause of cancer death (Ferlay et al., 2010). ~ 80% of lung cancers are non-small cell lung cancer (NSCLC) subtype. The tumorgenic process of lung tissue depends on several factors including inhalation of smoke and air pollution. In general, these factors induce endoplasmic reticulum (ER) stress and unfolded protein response (UPR) pathway in lung cells (Incoronato et al., 2011; Perdomo et al., 2013; Jorgensen et al., 2008). ER stress represents a natural response in lung after exposure to environmental toxins, hypoxia, infectious agents, or accumulation of misfolded proteins (Wu et al., 2014; Vandewynckel et al., 2013). It has now been demonstrated that ER stress and the subsequent UPR play a central role in mediating homeostasis within different cell types of lung (Nana-Sinkam et al., 2014; Choi et al., 2014). GRP78 (Gene ID: 3309), also known as HSPA5, BIP, HEL-S-89n and MIF2, is the main regulator of UPR pathway (Guo et al., 2010). *GRP78* is over-expressed in cancer cells and is associated with proliferation, carcinogenesis, metastasis, and drug resistance (Guo et al., 2010). Upon induction of ER stress, three main stress sensors, activating transcription factor 6 (ATF6), PKR-like ER kinase (PERK) and inositol-requiring enzyme 1 (IRE1), are dissociated from GRP78. Then, these sensors activate down-stream processes related to UPR pathway (Wu et al., 2014). One of this down-stream processes is splicing of X-box binding protein 1 (XBP1) by IRE1, thereby increasing XBP1s (XBP1-spliced) transcript during UPR (Mimura et al., 2012; Back et al., 2005; Romero-Ramirez et al., 2004). XBP1 is essential for survival and growth of tumor cells under hypoxia conditions (Romero-Ramirez et al., 2004).

GRP78 also facilitates apoptosis inhibition and leads to tumor progression and resistance to chemotherapy (Firczuk et al., 2013). Serum levels of GRP78 antibodies and expression levels of GRP78 in patients' tissue biopsies could be considered as a new biomarker for treatment

resistance. In addition of UPR pathway regulation, this chaperon is responsible for quality control of newly made proteins within the ER (Xuemei et al., 2011).

Different signal transduction networks such as UPR, PI3K/AKT, MAPK, ErbB and calcium pathways are involved in NSCLC. Frequently, these pathways interact with each other, for example, PI3K/AKT signaling pathway is related to UPR and GRP78 (Chang et al., 2012) and GRP78 is capable of binding to AKT (Xu et al., 2012).

MicroRNAs (miRNAs) are endogenously made small non-coding RNAs of 20-24 nucleotides that post-transcriptionally regulate gene expression. miRNAs mostly act through imperfect binding to the 3'-untranslated region (3'-UTR) of their target genes. Identification of cancer specific miRNAs and their target genes have important clinical applications (Fabbri et al., 2007). Here, we aimed to find miRNAs that target GRP78, its relationship with other signaling pathways, and its association with tumorigenesis of NSCLC. Softwares such as miRwalk, DIANA microT, Targetscan, mirBase and miRecord tools were used to predict miRNAs that can bind and hence regulate GRP78 transcript in lung cancer (Dweep et al., 2015; Wong and Wang et al., 2015; Agarwal et al., 2015; Vlachos et al., 2015; Betel et al., 2010; Jacobsen et al., 2013). Furthermore, a potential interaction between the predicted miRNAs and GRP78 was experimentally validated.

2. Materials and Methods

2.1. Bioinformatics analysis

Bioinformatics software including DIANA-microT, Targetscan, miRDB, miRwalk, Tarbase and microRNA.org tools, were employed to predict miRNAs that have binding sites on 3`-UTR

region of GRP78 transcript (Supplementary Table 3). miRNAs that simultaneously target several upstream and downstream components of UPR were selected. Additionally, the main components of other KEGG signaling pathways, PI3K/AKT, MAPK, ErbB and calcium pathways, which are involved in NSCLC, were determined using miRwalk software. Considering all these criteria, we finally analyzed miRNAs that target 22 key components genes in NSCLC KEGG pathway (Supplementary Figure 1).

2.2. Functional analysis of miRNAs

2.2.1. Plasmid construction

Genomic DNA was extracted from blood cells using QiaAmp DNA mini kit (Qiagen, Germany). Specific primers were designed (Supplementary Table 1) for cloning pri-miR-199 and pri-miR-495 in pTracer-SV40 vector. PCR amplification was carried out using the mentioned primers and PCR master-mix (Fermentas, USA) in an Eppendorf thermocyler (Germany). The fragments were cloned in the vector using *Eco*RI and *Not*I restriction enzymes (Cinnagen, Iran). Recombinant vectors were extracted by a plasmid extraction kit (Takara, Japan). The vectors were then used to transfect QU-DB and A549 cell lines. Validations of cloning were done by digestion of recombinant plasmids with *Eco*RI and *Not*I enzymes and subsequent sequencing with an ABI Applied Biosystems 3730xl instrument (Macrogen, South Korea). In addition, a scramble fragment was sub-cloned into pTracer-SV40-vector, to be used as a control.

We also designed several sponges for competitive suppression of candidate miRNAs. The designed sponges contained 3 repeated sequences of miRNA binding sites that are complementary to their seed region, as decoy targets of miRNAs.

2.2.2. Cell culture

Human epithelial-like adenocarcinoma lung cancer, A459 (NCBI code: C137), human fibroblasts lung cancer adenocarcinoma, QU-DB (NCBI code: C565), and human embryonic kidney HEK293T (NCBI code: C497) cell lines were cultured in Dulbecco's Modified Eagle Media (DMEM, Bioidea, Iran), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate (Bioidea, Iran), under a humidified condition at 37°C with 5% CO2.

2.2.3. Transfection

Calcium phosphate method was used for transfection. Immediately prior to transfection, cell culture medium was replaced with fresh medium without FBS. Then 2.6 µl of CaCl2 (Merck, USA), 2.5 M, and appropriate amount of plasmid DNA was diluted in double distilled water up to 50 µl. The aforementioned solution was added to 50 µl 2X HBS (8.0 g NaCl, 0.37 g KCl, 106.5 mg Na2HPO4, 1.0 g dextrose and 5.0 g HEPES). After 30 minutes of incubation in room temprature, the solution was added directly to the cells. After 48 hours the expression levels of selected miRNAs, GRP78 and spliced XBP1 were evaluated.

2.3. MTT test

A549 and QU-DB cell lines were cultured in 96-well multiplates. After incubation at 37ºC and 5% Co2, groups of wells were transfected with a series of different concentrations of recombinant plasmids, miR-199 Tracer and miR-495 Tracer. After 24 hours, a volume of 10ul MTT (Sigma, USA), 50mg /ml PBS, added to each well. Following incubation for 3 hours at 37ºC, reduced MTT was observed. The supernatant of plates carefully discarded and 150 ul of

dimethyl sulphoxide (DMSO) (Cinagen, Iran) were added to each well and mixed thoroughly to dissolve the crystals. Optical density was read using a microplate ELISA reader (BioTEK, USA) at a wavelength of 570 nm.

2.4. Dual Luciferase Assay

Different constructs were produced for luciferase assay: 1. Recombinant psiCHECK-2 containing 1744 bp of wild-type-3'UTR-GRP78, 2. Recombinant psiCHECK-2 containing 1744 bp of mutant-3'UTR-GRP78, 3. Recombinant pTracer-SV40 containing 261 bp hsa-miR-199a precursor, 4. Recombinant pTracer-SV40 containing 311 bp of hsa-miR-495 precursor sequence. The designed primers for construction in psiCHECK-2 are summarized in supplementary table 1. Creation of point mutations in 3'-UTR-GRP78 is performed by SOE-PCR method. This technique is described completely in legend of Supplementary figure 2.

5×10^4 HEK293T cells were transfected with 200 ng of the recombinant psiCHECK-2 construct and mock vector as control. Transfection was carried out in 24-well plates and lysates were used for evaluation of luciferase activity, 48 hours after transfection. Dual luciferase assay was performed in two steps. At first, lysates were assessed for firefly luciferase (EC 1.13.12.7) activity by Firefly luciferin substrate complex (Luciferin 2mM, ATP 4mM, Tris 20mM and MgSo4 10mM). In the next step, lysates were assayed for Renilla reniformis luciferase (Rluc) activity using Renilla substrate, Coelenterazine. The light-emitting of these molecules were read by Berthold detection systems sirius illuminator.

2.5. Sample collection

2.5.1. Ethics statement

The clinical sample collection procedure was approved by the ethics committee of TMU.

2.5.2. Tissue sample preparation

A total number of 36 NSCLC archival FFPE tissue samples were obtained from pathology departments of Arak Vali-Asr hospital and Sina laboratory (Supplementary Table 2). All samples had been collected during last 5 years and had an ICD-O (International classification of diseases for oncology) code of 34, which is the universal code for classification of lung cancers.

2.6. RNA extraction, cDNA synthesis and RT-qPCR

FFPE samples were trimmed and deparaffinized in xylol and digested with Proteinase K (Cinnagen, Iran) as explained before (Nouraee et al., 2013). Total RNA was extracted using RNX-Plus reagent (Cinnagen, Iran) according to the manufacturer instructions. RNAs were treated with DNase I (Sinaclon, Iran) and 50-100 ng of total RNA were used for reverse transcription step. A two-step protocol of synthesis was performed with stem-loop and random-hexamer primers and M-MuLV enzyme (Cinnagen, Iran). Finally, real-time polymerase chain reaction (q-PCR) was performed using SYBR green master-mix kit (YTA, Iran) on a lightCycler real-time PCR machine (Roche, Germany). To rule out a potential non-specific amplification related to possible contamination of samples with genomics DNA, for each sample a no-reverse transcription (no-RT) control was used. Expression levels of *GAPDH* and *SNORD47* (U47) were used for normalizing the expression levels of mRNAs and miRNAs, respectively. The expression analyses were determined in three technical replicates and the relative changes were calculated by the comparative Cq method using the relative expression software tool (REST).

2.7. Statistics analysis

Relative RT-qPCR data were calculated by REST 2009. The difference between groups was compared by Student's t-test as well as unpaired t test GraphPad prism 7 software. The

efficiencies of gene expression were calculated with the LightCycler96 Roche software. P values of less than 0.05 were considered as statistically significant.

3. Results

3.1. Bioinformatics prediction of miRNAs targeting GRP78 transcript

miRNA target prediction tools predicted a list of ~ 575 miRNAs capable of targeting the 3'-UTR of GRP78 (Supplementary Table 3). In order to narrow-down the list of predicted miRNAs, we then selected miRNAs that also target some upstream and downstream components of UPR as well as master components of main pathways involved in NSCLC (Supplementary Figure 1). Accordingly, exported results from miRwalk algorithm narrowed-down the original predicted miRNAs to a list of 52 miRNAs (Supplementary Table 4). Finally, according to the scoring system of the software, binding power, conservation and the number of miRNA recognition element (MRE), five miRNAs were selected including miR-495, miR-199a-5p, miR-942, miR-373, and miR-302. These 5 miRNAs had overlaps between UPR, PI3K/AKT, MAPK, ErbB and calcium signaling pathways (Figure 1). Hsa-miR-495 and hsa-miR-199-5p had the highest scores in miRNAs target prediction tools. Moreover, because these two miRNAs are located at conserved sequence of the 3' UTR site of GRP78, as it is evident in UCSC genome browser, we selected them for experimental validation (Figure 2A).

3.2. Down-regulation of *GRP78* and *XBP1s* expression level following hsa-miR-199a-5p and hsa-miR-495 overexpression

Two genomics segments with the length of 261 bp and 311 pb, associated with hsa-mir-199a-5p and hsa-mir-495 were cloned and introduced into A549 and QU-DB cell lines. Transfected cells with recombinant vectors showed ~20 folds over-expression of hsa-miR-199a-5p and hsa-miR-495. Additionally, transfected cells demonstrated a significant down-regulation of *GRP78* (126 folds and *XBP1s* (2 folds) in QU-DB (Figure 3A), and a significant down-regulation of *GRP78* (35.13 folds) and *XBP1s* (0.6 folds) in A549 cell line (Figure 3B).

3.3. Elevated *GRP78* and *XBP1s* expression levels following introducing constructs containing sponge sequences complementary to hsa-miR-199a-5p and hsa-miR-495

Transfecting lung cancer cell lines with competitive sponge vectors containing the binding sites for hsa-miR-199a-5p and hsa-miR-495 caused a 1.92 fold and 1.4 fold up-regulation of *GRP78* in transfected cells, compared to the cells transfected with mock vectors. Moreover, transfection of miRNA sponges, up-regulated *XBP1s* expression level 1.8 fold and 0.72 fold in QU-DB and 3.5 fold and 1.5 fold in A549 cell line (Figure 4).

3.4. hsa-miR-199a-5p and hsa-miR-495 decreased cell viability in A549 and QU-DB cells

The MTT assay was used to evaluate the viability of A549 and QU-DB cells following over-expression of miR-199 and miR-495. Results demonstrated a logical relationship between the formazan crystals produced by cells and the level of over-expressed miRNAs, the more over-expression of miR-199 and miR-495, the less absorbance and cell viability (figure 5).

3.5. hsa-miR-495 and hsa-miR-199a directly interact with 3'-UTR of GRP78

To perform the reporter assay, the 3'UTR region of GRP78 (1744bp) as well as the mutant form of 3'UTR (generated by SOE-PCR) was cloned upstream of the luciferase gene in the psicheck-2 vector. Then, the enzymatic activity of Renilla per enzymatic activity of Firefly (R/F) was calculated, where firefly signal was used to normalize the renilla luciferase signal. Our data demonstrated that R/F ratio of Sicheck-wt-3'-UTR was lower (meaning a stronger interaction between 3'UTR and miRNAs) than mock and Sicheck-mut-3'-UTR groups (respectively, 0.2 and 0.48 folds for miR-199 (Figure 6A), and 0.007 fold and 0.4 fold for miR-495 (Figure 6B).

3.6. Up-regulation of *GRP78* and down-regulation of *hsa-miR-199a-5p* and *hsa-miR-495* in tumor samples of NSCLC patients

The expression levels of *GRP78* and its targeting miRNAs were normalized with internal controls *GAPDH* and *U47*, respectively. RT-qPCR data revealed a significant up-regulation (3.87 times, P=0.002) of *GRP78* (Figure 7A) and a significant down-regulation of *miR-199a-5p* (0.13 times, P=0.0001) and *miR-495* (0.085 times, P= 0.0001) in tumor samples of NSCLC patients, in comparison to the non-tumor tissues obtained from the same patients (Figure 7B and 7C).

4. Discussion

Lung cancer is the most common invasive cancer and cause of cancer death worldwide (Ferlay et al., 2010). The primary etiological factor for NSCLC is cigarette smoking which induces ER stress and UPR in lung cells (Jorgensen et al., 2008). Sustained induction or repression of UPR pharmacologically may have therapeutic effects against cancer (Ma et al., 2014). ER stress leads to up-regulation of *GRP78* within tumor microenvironment (TME) (Lee et al., 2007; Wang et al.,

2009). GRP78, an ER chaperon, is the main regulator of UPR pathway (Lee et al., 2007; Wang et al., 2009). Consequently, IRE1 dissociates from GRP78 and becomes activated, then it induces splicing and hence activation of XBP1 (Mimura et al., 2012). In the present study, we aimed to find miRNAs that involve in the regulation of GRP78 expression.

Different algorithms and software have been developed for miRNAs and miRNA targets prediction. Interaction between miRNA and its mRNA targets takes place based on conservation and thermodynamic stability of the target sequence. A miRNA binds via its seed sequence to its complementary sequences, mostly at 3'-UTR, of its target transcripts. Various studies have described the role of these regulatory molecules, especially their interactions with cancer-related genes (Ruepp et al., 2010; Jacobsen et al., 2013). In this study, various target prediction programs including miRwalk, Targetscan, Diana, miRDB and MirTarBase tools were employed to predict miRNAs capable of targeting GRP78 transcript.

Furthermore, using miRwalk, the long list of predicted miRNAs was narrowed-down to 5 common miRNAs (including miR-495, miR-199a-5p, miR-373, miR-302 and miR-942) which simultaneously target both UPR components including GRP78 and XBP1, as well as the key components of the main signaling pathways of NSCLC. miR-373 is associated with invasion and metastasis in patients with lung and other cancer types (Harel et al., 2015; Wu et al., 2014). miR-302 is mainly expressed in human embryonic stem cells (hESCs) and is involved in stemness process (Cai et al., 2015). A tumor suppressor role of miR-302a, miR-302b and miR-30c has been reported in prostate cancer, Hepatocellular Carcinoma (HCC), glioma cells and ovarian cancer (Zhang et al., 2015; Wang et al., 2015; Guo et al., 2015, Oksuz et al., 2015). In addition, miR-302b has a fundamental role in maintaining pluripotency of hESCs. A role of miR-942 in cervical cancer is already reported (Lui et al., 2007). In addition, altered expression levels

of the aforementioned miRNAs in various cancers can be observed in databases such as CancerMiner and CBioPortal.

Diana-microT web server uses a scoring system named miRNA target gene (miTG) which checks miRNA and mRNA interactions thermodynamically (Maragkakis et al., 2009; Mondanizadeh et al., 2014, Manolis et al., 2009). A higher miTG value indicates that this prediction is closer to reality. miTG score of hsa-miR-495 is the larger and closer to reality. On the other hand, Targetscan database predictions was based on context scores rate of binding sites. This database has a scoring system that named probability of conserved targeting (Pct) (Lewis et al., 2005). This software indicated conserved sites for two selected miRNAs. Pct of the hsa-miR-199a-5p and hsa-miR-495 is shown in Figure 2B. Conservation of the selected miRNAs was also confirmed in mirBase (Griffiths-Jones et al., 2008; Su et al., 2012). The aforementioned software demonstrated that hsa-miR-495 and hsa-miR-199a-5p have interactions with GRP78 transcript. In addition, binding sites of these miRNAs existed at conserved region of the 3'UTR, as it is evident in UCSC genome browser (Figure 2). The conserved region, located at first quarter of GRP78 3'-UTR, has binding sites for hsa-miR-199a-5p, hsa-miR-495, hsa-miR-379, hsa-miR-181, hsa-miR-384-5p and hsa-miR-1192. In our experimental phase of the research, we focused on hsa-miR-199a-5p and hsa-miR-495 based on the novelty issue.

In order to over-express selected miRNAs, their precursors were cloned and expressed in two lung cancer cell lines. Elevated expression level of the mature forms of the mentioned miRNAs indicated a proper processing of the cloned precursors. Additionally, down-regulation of *GRP78* was determined in transfected cells. Similarly, sponges were constructed with repeated binding sites complementary to the seed sequences of the mentioned miRNAs into the 3'-UTR of a

reporter gene encoding GFP (Ebert et al., 2007; Ning et al., 2014). Transfecting the construct into A549 and QU-DB cell lines caused a noticeable up-regulation of *GRP78*, probably via releasing GRP78 transcript from miR-199 and miR-495 regulation. In addition, because one of the indicators of activated UPR is splicing of XBP1, we evaluated XBP1s expression level and splicing as well. We observed a positive correlation between GRP78 and XBP1s in functional analysis of candidate miRNAs.

To perform the dual-luciferase reporter assay, the full length 3'-UTR of GRP78 was cloned to the upstream of luciferase gene in psi-check-2 vector. This vector was introduced into cells along with recombinant miR199-tracer and miR495-tracer vectors. Since 3'-UTR-GRP78 is fused to the luciferase reporter gene; the luciferase activity can be directly correlated to the activity of 3'-UTR-GRP78. This technique further confirmed a direct binding, and hence regulating, of miR-199a-5p and miR-495 with the conserved region of 3'UTR of GRP78.

In accordance with our hypothesis, a significant down-regulation of miR-199a-5p and miR-495 was demonstrated in tumor samples, compared to the marginal tissues of the same patients. Therefore, based on our findings, down-regulation of these two miRNAs might be the reason for an up-regulation of GRP78 in NSCLC samples. The low endogenous expression of these miRNAs, is probably due to a dose-dependent manner of miRNAs for binding to their targets and exerting their biological activity.

We also performed MTT assay to analyse cell viability rate in hsa-miR-199a-5p and hsa-miR-495 over-expressing cells. MTT assay was similar to that originally described by Mosmann (Mosmann., 1983). Our data demonstrated that over-expressed hsa-miR-199a-5p and hsa-miR-495 decrease cell viability in lung tumor cells lines. Many studies have shown that GRP78 is

essential for cell viability. The data of MTT test showing a decrease in cell viability suggests an outcome of inhibition of GRP78 by these two miRNA.

Many studies until 2012 determined the role of 26 miRNAs in UPR pathway in different types of cancers. Only hsa-miR-346 has been investigated in the Calu-3 lung carcinoma cell line and hsa-miR-199a-5p in chronic obstructive pulmonary disease (COPD) lung disease (Su et al., 2012). Expression of hsa-miR-199 increased in the physiological response to tissue damage and mediated lung fibroblasts that are induced with TGF-b by targeting Caveolin-1 (Lino et al., 2013). Hsa-miR-199a-5p targets HIF-1 and VEF that are involved in processes such as response to stress by inducing hypoxia and angiogenesis (Singh et al., 2012). In contrast to our results, this miRNA is up-regulated in gastric cancer (He et al., 2014). However, a tumor suppressor role for this miRNA has already been reported in thyroid carcinoma (Minna et al., 2014). Expression of hsa-miR-495 was increased with tumor progression and resistance to hypoxia in breast cancer cells. In agreement with our findings, this miRNA has shown to have an inhibitory role in gastric cancer (Li et al., 2012) and glioblastoma (Chen et al., 2013). There is a paradoxical role for UPR in inhibition of tumor growth or protection of tumor cells within the TME (Vandewynckel et al., 2013). GRP78 is the main regulator of UPR pathway (Schröder et al., 2005), therefore, a better understanding of the miRNAs targeting it within UPR pathway of lung diseases would have potentially important diagnostic and therapeutic purposes. However, further experiments are needed on evaluating altered expression of *GRP78* and the rest of UPR components at mRNA and protein levels.

5. Conclusion

There are limited data on miRNAs targeting UPR pathway in lung cancer. GRP78 has an important role in drug resistance, mostly via regulation of UPR. Bioinformatics is a cheap

approach to limit number of targets for experimental validation. The aforementioned miRNAs are valuable because targeting main components of KEGG pathways of NSCLC. Therefore, we hypothesized that miRNAs involve in regulating GRP78 expression are probably down-regulated in lung cancer. In this study, we confirmed a direct interaction of hsa-miR-495 and hsa-miR-199a with GRP78 transcript. Based on our data, these miRNAs might have an effect on ER stress, as well as a causative role in lung tumorigenesis. The aforementioned miRNAs have also a potential to be used as diagnostic biomarkers for activated UPR in NSCLC. Furthermore, manipulating the expression of these miRNAs could have a potential therapeutic application in lung cancer.

Acknowledgements

We are thankful to Dr. Hassanpour and Dr. Daneshmand for their assistance in sample collections, and Dr. Baazm for her technical assistance.

Competing interests

None declared.

Author contributions: AA: designed study, performed the experiments, analyzed data, interpreted data and manuscript preparation; MGh: designed study and interpretation of data; BKh: interpretation of data, clinical sample preparation and manuscript preparation; SH: performed the reporter assay experiments; SJM: designed study, performed the experiments, analyzed data, interpreted data, manuscript preparation and approved final manuscript.

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Figures and Legends:

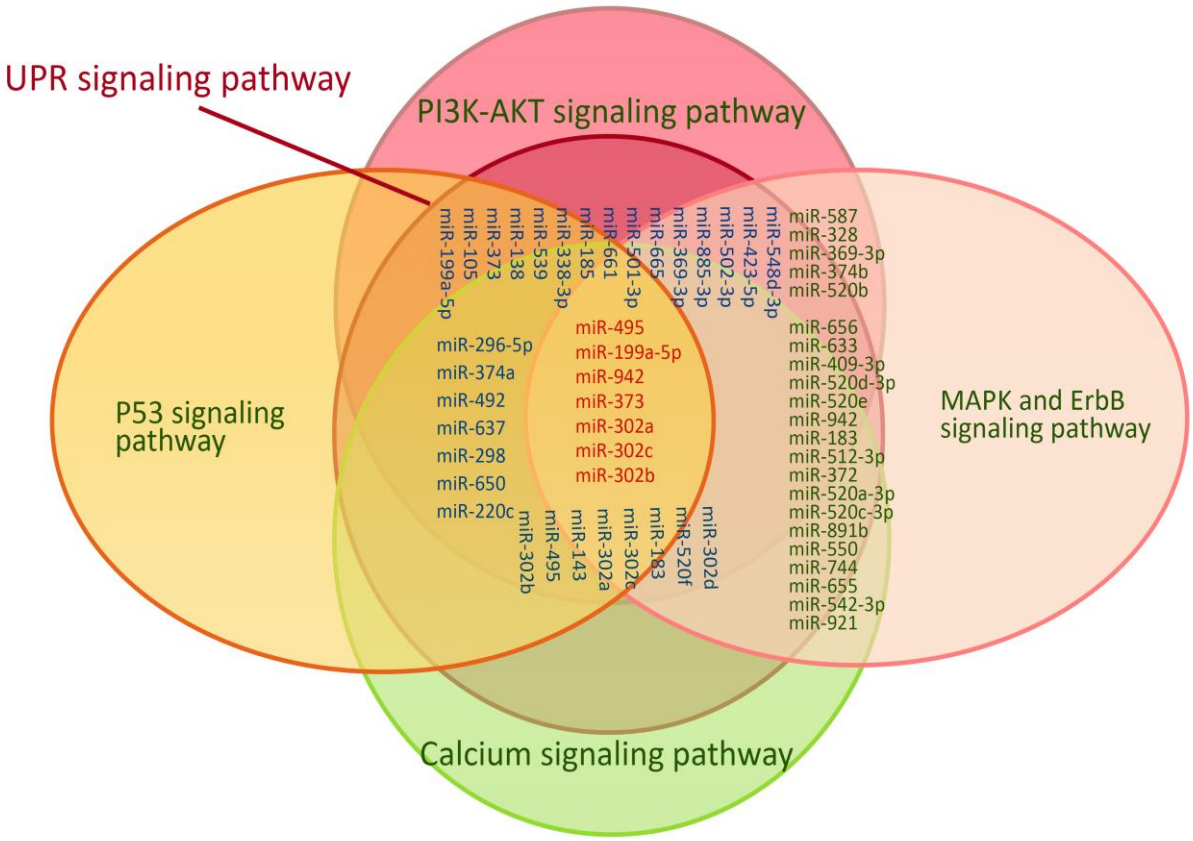
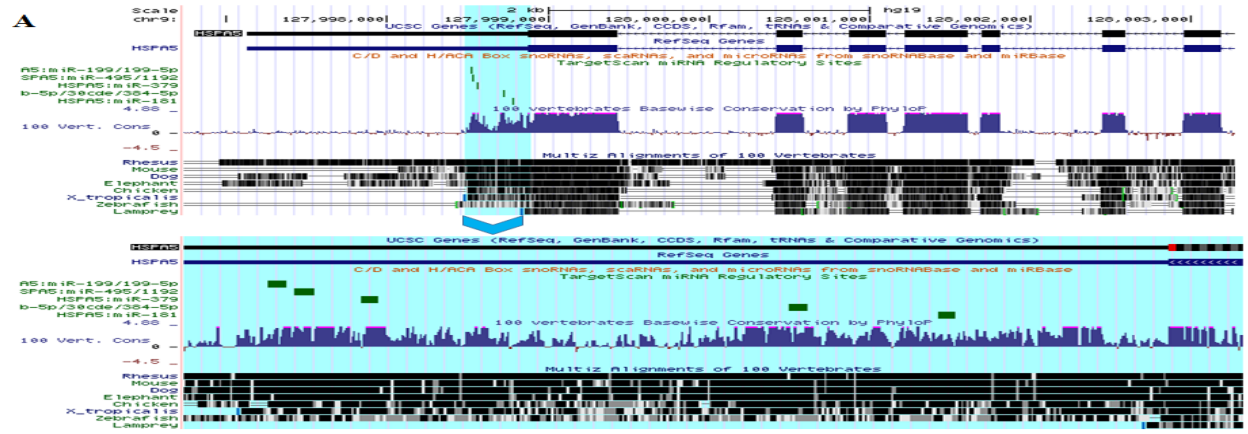


Figure 1: The Venn diagram shows 52 predicted miRNAs (obtained from our bioinformatics analysis, Supplementary table 2) with potential involvement in UPR, PI3K/AKT, P53, MAPK and Calcium signaling pathways in NSCLC. MiRNAs indicated with red color fonts (in the middle of the diagram) are common in all mentioned pathways.



B

miRNA	Conservation (miRbase and DIANA, Targetscan)	Diana	TargetScan	CancerMiner	miRDB	microRNA.org	miRanda-mirSVR
Hsa-miR-495 (14q32.31)	✓	miTG score: 5.09 precision:0.64 SNR:4.39	Context score precentile: 98 & 49 Target scan context score: -0.47	REC score: 0.81 FDR: 0.69	Target score: 96	mirSVR score:-1.2592 PHsatCons score:0.7212	miRanda-mirSVR: -1.31
Hsa-miR-199a-5p (19p13.2)	✓	miTG score: 2.00 precision:0.1 SNR:1	Context score precentile: 90 Target scan context score: -0.26	REC score: 0.44 FDR: 0.88	Target score: 93	mirSVR score:-1.1437 PHsatCons score:0.7212	miRanda-mirSVR: -1.15

Figure 2: A: a schematic diagram of UCSC genome browser showing the conserved site, at the initial region, of GRP78 3'-UTR (highlighted in blue). MRE sites for hsa-miR-199a-5p and hsa-miR-495 are shown within highlighted region. B: different features of selected miRNAs, as have been predicted by various miR-prediction bioinformatics tools.

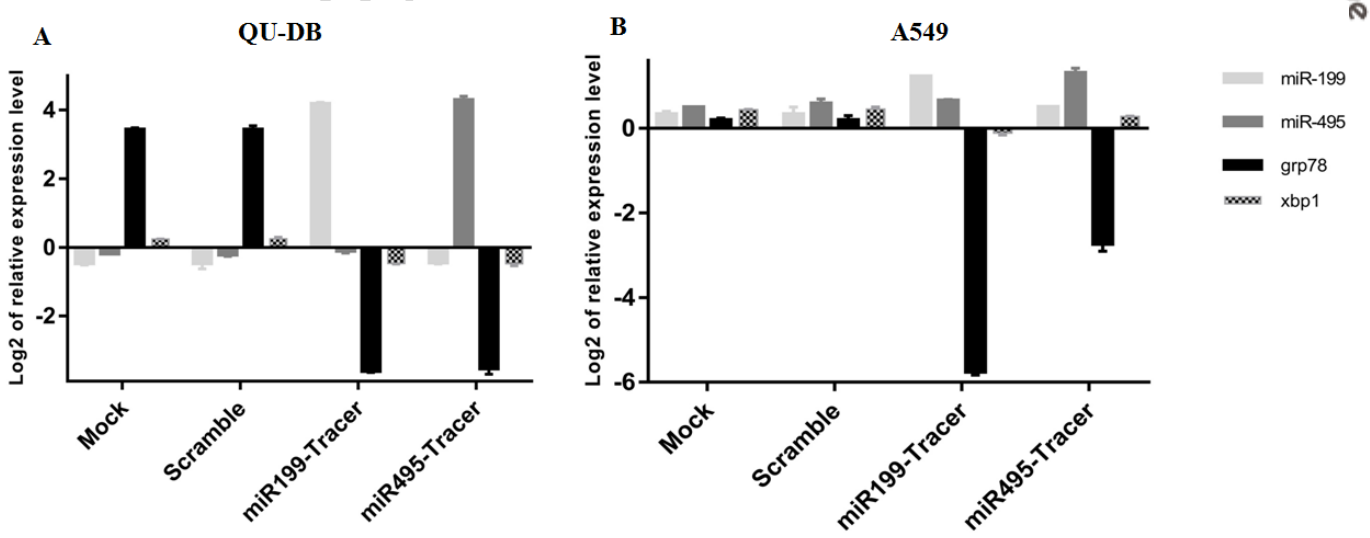


Figure 3: Down-regulation of GRP78 and XBP1s expression level following hsa-miR-199a-5p and hsa-miR-495 overexpression in A549 and QU-DB cell lines (Triplicate repeats, P value: 0.04*).

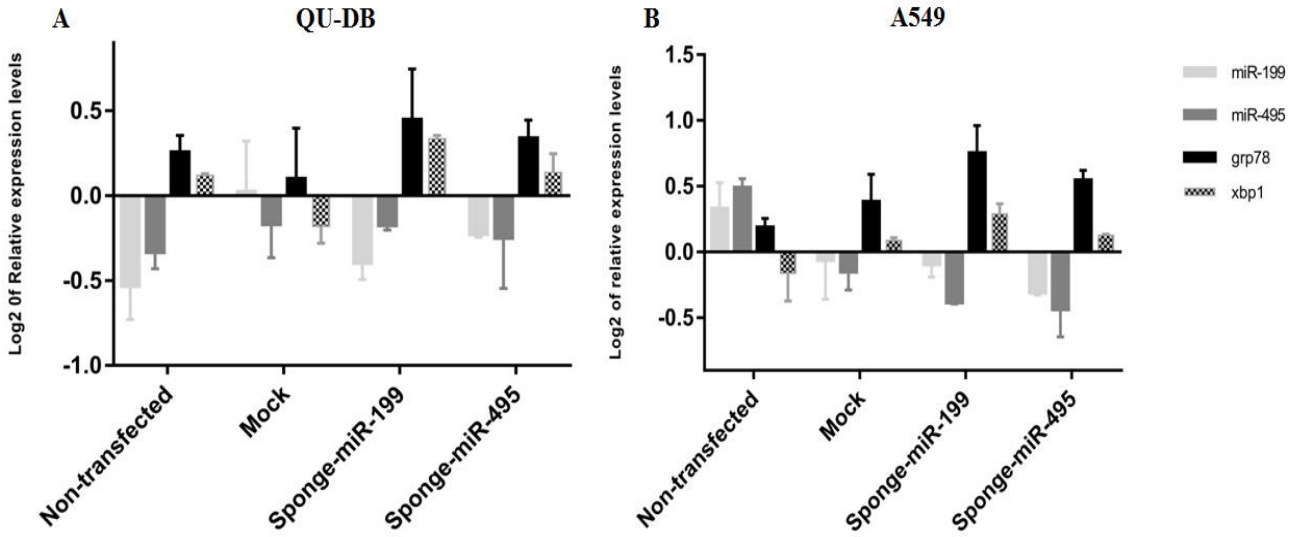


Figure 4: Up-regulation of GRP78 and XBP1s expression levels following introducing the sponges of hsa-miR-199a-5p and hsa-miR-495 to A549 and QU-DB cell lines (P value < 0.05 *).

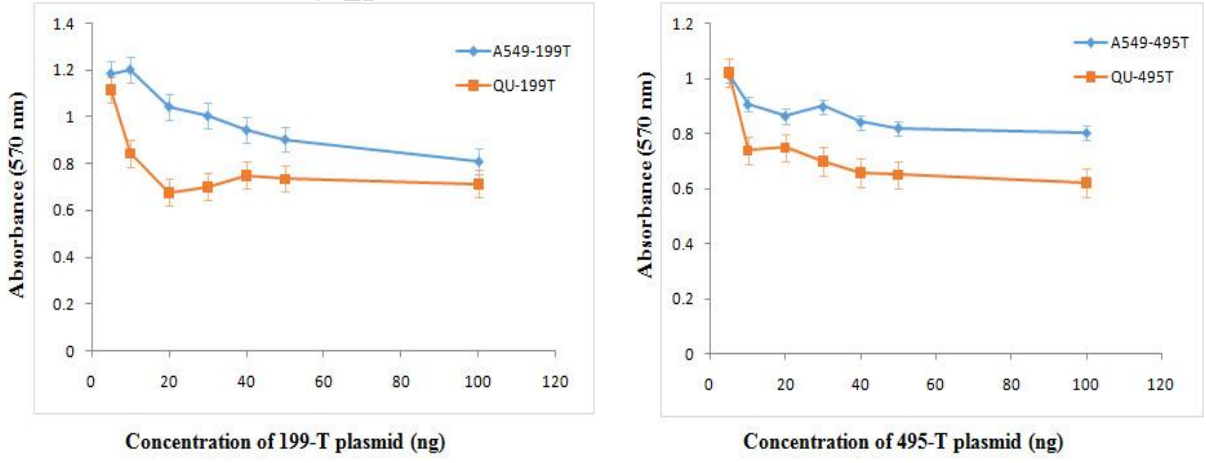


Figure 5: MTT assay showed hsa-miR-199a-5p and hsa-miR-495 decreased cell viability in A549 and QU-DB cells. 199T: recombinant pTracer-SV40 plasmid containing hsa-miR-199a

precursor, 495T: Recombinant pTracer-SV40 plasmid containing hsa-miR-495 precursor sequence. Data are mean± standard error (SE) of three experiments. Unpaired t-tests were used for statistical analysis ($P<0.05$).

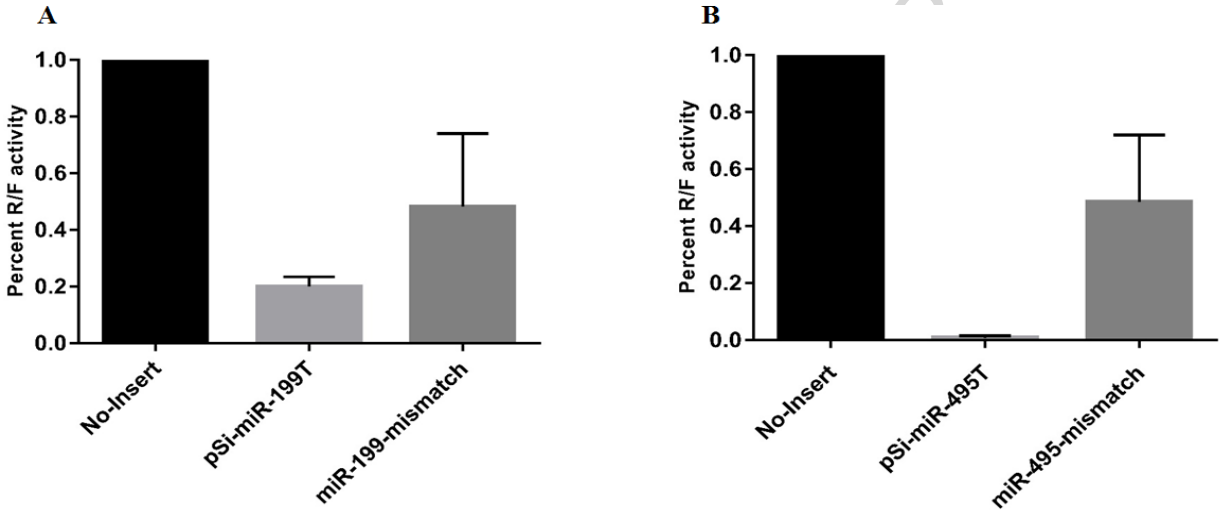


Figure 6: GRP78 transcript has direct interaction with hsa-miR-199a-5p and hsa-miR-495 in transfected HEK-293T (Triplicate, P value: 0.0114 **, Anova test). pSi-miR-199-T represent co-transfection with recombinant vector of psiceck2-wild type 3'-UTR GRP78 and recombinant vector of precursor miRNA-199a; pSi-miR-495-T represent co-transfection with recombinant vector of psiceck2-wild type 3'-UTR GRP78 and recombinant vector of precursor miRNA-495; miR-199-mismatch indicates co-transfection with recombinant vector of psiceck2-mutant 3'-UTR GRP78 and recombinant vector of precursor miRNA-199; miR-495-mismatch indicates co-transfection with recombinant vector of psiceck2-mutant 3'-UTR GRP78 and recombinant vector of precursor miRNA-495.

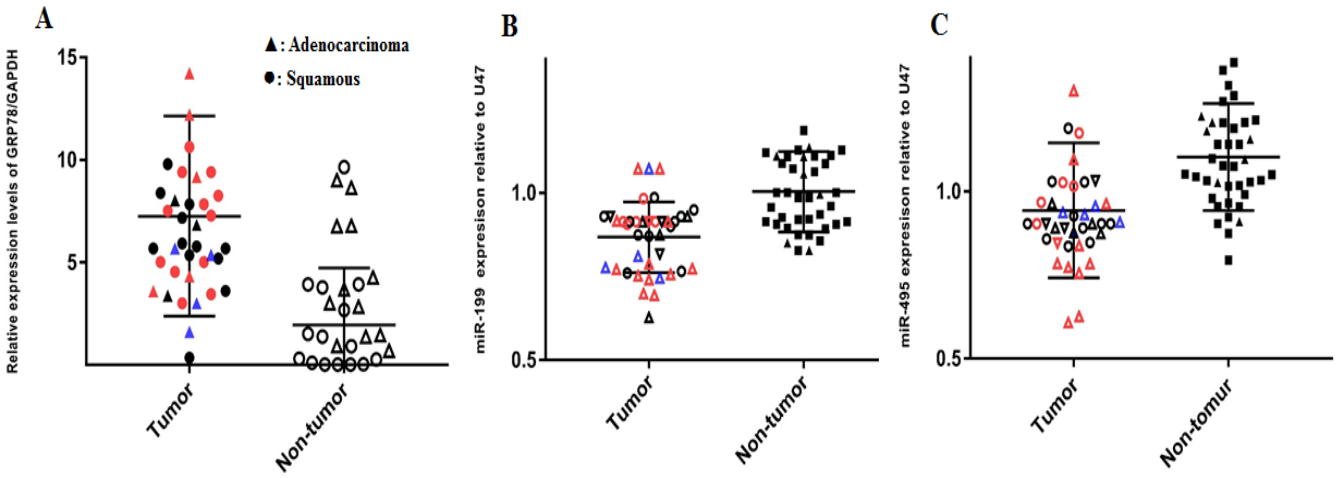


Figure 7: Evaluation of GRP78 as well as hsa-miR-199a-5p and hsa-miR-495 expression levels in 36 pairs of lung cancer samples and their non-tumor counterpart tissues from the same patients (t-test data, P value<0.05***). Triangles and dots represent adenocarcinoma and other types of NSCLC samples, respectively. Red and blue colors are indicating metastatic and low grade samples.

Abbreviations list:

NSCLC	Non-small cell lung cancer
UPR	Unfolded Protein Response
FFPE	Formalin-Fixed Paraffin-Embedded
miRNAs	microRNAs
ER	endoplasmic reticulum
ICD-O	International classification of diseases for oncology
no-RT	no-reverse transcription
REST	relative expression software tool
TME	tumor microenvironment
COPD	chronic obstructive pulmonary disease
hESCs	human embryonic stem cells
HCC	Hepatocellular Carcinoma
miTG	miRNA target gene
Pct	probability of conserved targeting