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DIFFERENTIAL EXPRESSION OF MICRORNAs THAT TARGET GENES INVOLVED IN APOPTOTIC UPR SIGNALING PATHWAY IN NEURONS

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ABSTRACT: The loss of function and progressive death of neurons is the key pathological mechanism that contributes to neurodegenerative diseases (NDDs). ER stress induced apoptotic unfolded protein response (UPR) signaling resulting from misfolded protein accumulation in ER lumen, is a major cause of neuronal death. MicroRNAs (miRNAs), post transcriptional regulators of gene expression are identified as regulators of UPR and apoptosis in various cell types. Altered brain miRNA profiles in NDDs suggest the involvement of miRNA dysregulation in neuronal death. However there are few reports on miRNAs targeting the genes involved in apoptotic UPR signaling in neurons. Hence, we aimed at analyzing the differential expression of predicted miRNAs targeting the important mediators of UPR induced apoptosis like *Perk*, *Ire1α*, *Chop* and *Puma* in ER stress induced neuronal culture. Eighteen miRNAs predicted to target these genes were selected based on miRNA target prediction algorithms. Using real-time PCR analyses, miRNA expressions were examined separately in ER stress induced mouse primary cortical and hippocampal neuronal cultures. From among the 18 predicted miRNAs, seven miRNAs such as miR-24, 27b, 124, 224, 290, 351 and 488 showed statistically significant downregulation among which miR-24, 27b, 224 and 488 decreased to less than 50% of untreated expression in both cortical and hippocampal cultures. Further the levels of target mRNA showed an inverse relationship with the down regulated miRNA levels during ER stress. These results indicate the significant role of miRNAs in regulating ER stress induced UPR pathway in neurons.

KEYWORDS: MicroRNA; Neurodegenerative diseases; Unfolded protein response; Primary neuronal culture; Real-time PCR

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1. INTRODUCTION

Neurodegenerative diseases (NDDs) affect the central nervous system causing the progressive degeneration or death of neurons which include diseases like Alzheimer's (AD), Parkinson's (PD), Huntington's, Prion disease and Amyotrophic Lateral Sclerosis. Apart from genetic mutations, the buildup of toxic proteins due to endoplasmic reticulum (ER) stress, creation of neurotoxic materials arising from mitochondrial dysfunction etc are considered as causes for NDDs all of which eventually lead to apoptosis aiming at protecting the nearby neurons from toxic substances. The ER stress induced apoptotic unfolded protein response (UPR) signaling is suggested to be a major cause for neuronal loss in these diseases. UPR is an adaptive mechanism in response to the accumulation of improperly folded proteins in the ER lumen, which aims at rebalancing protein folding homeostasis. But if the ER functions are severely impaired, the cells fail to recover and in order to protect the organism by eliminating the damaged cell, UPR triggers apoptotic signaling pathways [1, 2]. Sustained UPR activation leading to apoptosis and the presence of specific aberrant proteins arising from perturbations in the ER protein quality control machinery are found in NDDs [3].The UPR is mediated by three ER resident transmembrane sensors: Protein kinase RNA-like ER kinase (*Perk*), Inositol requiring enzyme-1 (*Ire1α*) and Activating transcription factor-6 (*Atf6*). During prolonged ER stress, the apoptotic pathways initiated by these three sensors include (a) the activation of C/EBP homologous protein (*Chop*)/Growth arrest and DNA damage 153 (GADD153), a bZIP transcription factor (b) c-JUN NH2-terminal kinase (JNK) pathway (c) ER associated caspases, all eventually leading to the activation of caspase 3 [4]. The proteins downstream of all the three UPR receptors are known to have proapoptotic role but *Perk* and *Ire1α* are the two principal receptors initiating apoptosis. These sensors are activated during severe ER stress and converge on *Chop* leading to the entry of cells into apoptosis [5]. In neurons, *Chop* induces apoptosis mainly through the activation of *Puma* (p53 upregulated modulator of apoptosis), a BH3 (Bcl-2 homology domain 3)-only BCL2 family member, and the strongly induced proapoptotic gene in neurons during ER stress [6]. *Puma* activates pro-apoptotic Bcl-2 family proteins *Bax* and *Bak*, leading to caspase

activation and ultimately cell death [7, 8]. *Perk-eIf2α-Atf4-Chop* signaling pathway and *Ire1α* mediated induction of proapoptotic JNK signaling through *Ire1α-Traf2-Ask1* pathway are critical for ER stress induced apoptosis in neurons through the induction of *Puma* [9, 10, 11]. *Ire1α* activated *Traf2* pathway in turn activates caspase-12 specific to ER stressed neuronal apoptosis [12]. Thus there is a great interest in the possibility of modulating the activity of *Perk* and *Ire1α* to control the cell fate under ER stress. The miRNAs are endogenous, small (20-22 nucleotides), highly conserved non-coding RNAs which represses gene expression by base pairing at the 3'UTR of their target mRNA. The brain expresses a wide range of miRNAs and their altered expression and dysregulation is a common theme associated with NDDs [13]. But the molecular mechanism behind the pathological association of miRNA dysfunction and its role in regulation of genes involved in neurodegeneration remain largely unknown. miRNAs are key regulators of ER homeostasis and important players in UPR signaling. The miRNA mediated regulation of UPR pathway genes by inhibiting the function of specific mediators of UPR signaling represents a new target for drug therapy for the treatment and prevention of neuronal loss [14, 15]. Depending upon the expression of UPR associated miRNAs, its up-or downregulation, their specific targets and tissue type involved, these miRNAs can act as either adaptive or apoptotic leading to either prosurvival or prodeath effects on the cells [16]. The altered expression of miRNA during ER stress and their effect on cell fate have been studied in various cell types [17]. However, since the miRNA expression and activity is tissue specific, we need to analyze the expression profile of neuron specific and proapoptotic UPR signaling related miRNAs to understand their specific role in neurodegeneration. The miRNA target identification softwares have expanded the possibilities for understanding miRNA-mRNA interaction. In this study using *in silico* analysis the predicted miRNAs that targets *Perk*, *Ire1α*, *Chop* and *Puma* were identified and the expression of these miRNAs and their targets in response to ER stress was studied in primary mouse neuronal culture using real-time PCR analysis.

2. MATERIALS AND METHODS

Materials

Animals and reagents: BALB/c mice were employed in the present study. All procedures performed in studies involving animals were in accordance with the ethical standards of Institutional Animal Ethics Committee, University of Kerala. IAEC-KU-22/2016-17-BT-JKNC2. All the chemicals and reagents used was of analytical or molecular biology grade. The primers used in this study were purchased from Sigma- Aldrich Co. USA and Integrated DNA Technologies (IDT).

2.1 Prediction of miRNAs targeting UPR genes

Online miRNA prediction tools such as TargetScan Mouse v.6.2, microRNA.org, miRWalk2.0 and PicTar were used to select the miRNAs that bind to the 3'UTR region of the target UPR genes *Perk*, *Ire1α*, *Chop* and *Puma*. The list thus obtained was screened to select the miRNAs which were

common to most of the prediction tools, having sequence conservation among vertebrates, with both conserved and poorly conserved binding sites showing 8-mer, 7mer- m8 matches at the target messenger RNAs - miRNA seed region and according to the P_{CT} score, mirSVR score and PicTar score provided by TargetScan, microRNA.org and PicTar respectively. The miRNAs with most favorable scores for efficient 3'UTR binding were selected.

2.2 Primary Mouse Neuronal Cultures

Cortical and hippocampal neurons were dissected out separately from early post natal (P0) mouse and seeded (cell density 5 X 10^5 cells) onto Poly-L-Lysine (Sigma-Aldrich) coated 60mm petridishes, maintained in neurobasal media (Invitrogen) containing B27 supplements (Invitrogen), glutamine (Sigma Aldrich), Penicillin/Streptomycin (Invitrogen) and was maintained at 37^0C under 5% CO_2 and 95% air in CO2 incubator (Eppendrof) [18].

Treatment of cells:Drug treatment was initiated on the 7d of plating. Tunicamycin (Sigma Aldrich) and thapsigargin (Invitrogen) diluted in dimethyl sulphoxide (DMSO) at a final concentration of 3.5µM and 1µM respectively was added directly to the culture medium. The treated culture was maintained at 37^0C under 5% CO_2 and 95% air for 6hrs in order to induce ER stress. Untreated controls and DMSO as vehicle control at a sub lethal concentration of 0.1% in the medium, was maintained throughout the experiment.

2.3 RNA extraction and cDNA synthesis

RNA was purified using High Pure miRNA Isolation Kit (Roche). The high molecular weight RNA and purified small RNAs containing the micro RNAs were eluted separately in 50µl following the manufacturer's instruction. The high molecular weight RNA was checked for its concentration and purity by measuring OD_{260} and $OD_{260/280}$ respectively in Biophotometer (Eppendorf Biophotometer plus) and treated with DNase 1(RNase free) (New England Biolabs) in order to digest DNA contaminants. cDNA was synthesized using Verso cDNA synthesis kit (Thermo Scientific) with oligo(dT) primers following the manufacturer's protocol.

2.4 Detection of spliced *Xbp1* mRNA as a marker of ER stress using semiquantitative PCR analysis

Previously reported primer sequences were used to amplify the spliced and unspliced *Xbp1* mRNA [19]. The 10µl reaction contains 1µl of cDNA (10ng), 2.5µl of 2mM dNTP (0.25mM), 1µl of each primer (10nM each), 1.2µl of 50mM $MgCl_2$ (1.5mM) (Merck), 2µl of 10X Taq reaction buffer (1X), 0.17µl of 3U/µl Taq polymerase (0.5U) (Merck). Following initial incubation at 95^0C for 2min the PCR conditions consisted of 40 cycles at 94^0C for 30s, 62^0C for 30s, 72^0C for 45s with a final incubation at 72^0C for 7min using MJ Mini Personal Thermal Cycler (Biorad). The PCR products were electrophoresed on 3% agarose gel and visualized by ethidium bromide staining. The sizes of amplified unspliced and spliced *Xbp1* mRNA transcripts were 170bp and 144bp respectively.

2.5 Quantitative real-time PCR

The real-time PCR experiments performed in this study followed the *M*inimum *I*nformation for publication of *Q*uantitative real-time PCR *E*xperiments (MIQE) guidelines [20].

2.5.1 Quantification of miRNA expression using real-time PCR

TaqMan probe based real-time PCR was used to measure the expression of individual miRNAs based on S-Poly (T) miRNA assay using a Universal TaqMan probe sequence, Universal reverse primer sequence, and specific oligo(dT) reverse transcription primers and forward primers designed for each miRNA according to Kang *et al* protocol [21] for the quantification of mature miRNAs. The miRNA was polyadenylated and reverse transcribed. The incubations were carried out in MJ Mini Personal Thermal Cycler (Biorad). The reverse transcription products were amplified and quantified using Universal TaqMan probe in Miniopticon Real-Time PCR system (Biorad). The reactions were done in triplicates. The miRNA expression was normalized with SnoRNA-202, showing consistent expression making it a suitable reference gene for normalizing differential expression in our real-time PCR assays. The relative quantity of miRNA was calculated based on $2^{-\Delta\Delta Ct}$ method [22]. The data was expressed as fold change in miRNA level in treated sample with respect to the untreated control.

2.5.2 Quantification of target UPR gene expression using real-time PCR

Primer sequences. The UPR genes *Chop*, *Puma* and *BiP* were amplified using previously reported primers [9, 23]. The real-time PCR primer sequences for *Perk*, *Ire1α* and *β-actin* were designed with online primer designing tool NCBI/Primer BLAST. The sequences were as follows: *Perk:* Forward -5' CCTGACCCATCTGCACTAAT 3', Reverse -5' CATAAATGGCGACCCAGCTT 3' (PCR efficiency: 98.03 % (R^2- 0.987); *Ire1α:* Forward -5' TGTGGTCAAGATGGACTGGC 3', Reverse -5' TCGGAGGAGGTCTCTCACAG 3' (PCR efficiency: 110.17% (R^2- 0.988); *β-actin:* Forward -5' CCGTGAAAAGATGACCCAGATC 3', Reverse -5' CACAGCCTGGATGGCTACGT 3' (PCR efficiency: 100.50% (R^2- 0.996). Real-time PCR amplification was done using VeriQuest Fast SYBR Green qPCR Master Mix with Fluorescein (2X) in Miniopticon Real-Time PCR system (Biorad). The reactions were performed in triplicates containing 1µl of cDNA (10ng/ µl), 0.5µl of each primer (10pmol/ µl) and 5µl 2X SYBR Green qPCR Master Mix in a final volume of 10 µl. Following a 50^0C incubation for 2min and initial denaturation at 95^0C for 5min the PCR profile consisted of 40 cycles of denaturation at 95^0C for 30s and annealing at 60^0C for 30s followed by melt curve analysis at 65^0C gradually increasing 0.5^0C/s to 95^0C with acquisition data at every 1s. The experiment was performed with three biological replicates. The gene expression was normalized to *β-actin* as endogenous control and the relative quantity of mRNA was calculated on the basis of $2^{-\Delta\Delta Ct}$ method. The data was expressed as fold increase in mRNA level in treated sample with respect to the untreated control.

2.6 Statistical analysis

Unpaired Students *t*-test was performed with GraphPad Prism v.5.0 to compare the differences in fold change of mRNA and miRNA levels in the treated samples with respect to untreated controls. P-value of $p<0.05$ was set as the threshold for significance.

3. RESULTS AND DISCUSSION

3.1 miRNAs predicted to target UPR induced apoptotic pathway genes

In order to identify the potential miRNAs that are differentially expressed during ER stress, a list of miRNAs targeting the 3'UTR of UPR regulators such as *Perk* and *Ire1α*, proapoptotic genes such as *Chop* and *Puma*, were selected and compiled using miRNA target prediction algorithms. Based on the selection parameters miRNAs miR-19a, 24, 27b, 124, 144, 148a, 152, 185, 218, 221, 222, 224, 290, 351, 488, 489, 494 and 615 were selected. Our analyses also showed that some of these miRNAs had more than one targets among the selected UPR related genes (Table.1). miR- 489 and miR- 124 were predicted to target the 3' UTRs of *Perk* and *Ire1α* respectively. miR-19a, 224 and 615 targeted the 3'UTR of *Chop* while miR-27b, 221, 222 and 494 targeted the 3'UTR of *Puma.* The miRNAs which had more than one targets included miR-24 which target *Puma, Ire1α* and *Perk ;* miR-218, 290 and 488 target both *Perk* and *Ire1α*;miR-144, 148a and 152 target *Chop* and *Puma* ; miR-185 target *Chop* and *Perk* and miR-351 target both *Puma* and *Ire1α.*

Table.1. miRNAs targeting the selected genes involved in ER stress mediated UPR pathway.

miRNA/Genes	*Perk*	*Ire1α*	*Chop*	*Puma*	mirSVR score (microRNA.org)	Total context score (TargetScan v.6.2)	PicTar score (PicTar)
mmu-miR-19a			✓		-0.0273	-0.09	NP
mmu-miR-24	✓	✓		✓	-0.0155, -04367, -0.0462	NA, -0.75, -0.12,	NA, NA, 5.0440
mmu-miR-27b				✓	-0.2988	-0.10	6.5625
mmu-miR-124		✓			-0.4399	-0.21	3.0222
mmu-miR-144			✓	✓	-0.4994, -0.5399	NA, -0.13	NP, NA
mmu-miR-148a			✓	✓	-0.1489, -0.8139	-0.13, -0.48	NP, 5.7525
mmu-miR-152			✓	✓	-0.1489, -08139	-0.13, -0.48	NP, 5.7619
mmu-miR-185	✓		✓		-0.0055, -0.3529	-0.12, -0.14	NA, NP
mmu-miR-218	✓	✓			-1.1396, -0.009	-0.40, NA	NA, NP
mmu-miR-221				✓	-0.2564	-0.28	6.9755
mmu-miR-222				✓	-0.2656	-0.28	2.2840
mmu-miR-224			✓		-1.1563	-0.23	NP
mmu-miR-290	✓	✓			-0.0348, -0.7491	-0.03, -0.11	NA,NA
mmu-miR-351		✓		✓	-0082, -0.0073	NA, -0.09	NA, NA
mmu-miR-488	✓	✓			-0.2490, -0.1166	-0.08, -0.07	NA, NA
mmu-miR-489	✓				-0.7148	-0.24	NA
mmu-miR-494				✓	-1.2244	-0.14	2.3231
mmu-miR-615			✓		-0.1814	-0.35	NP

miRNA prediction algorithms such as TargetScan Mouse v.6.2, microRNA.org, miRWalk2.0 and PicTar were used to select miRNAs that bind to the 3'UTR region of the target UPR genes *Perk*, *Ire1α*, *Chop* and *Puma*. The obtained list of miRNAs was screened to select 18 miRNAs having sequence conservation among vertebrates, with both conserved and poorly conserved binding sites showing 8-mer, 7mer- m8 matches at the target messenger RNAs - miRNA seed region and according to the mirSVR scores provided by microRNA.org compared with the P_{CT} score and PicTar score provided by TargetScan Mouse v.6.2 and PicTar respectively. miRNAs with significant scores for efficient 3'UTR binding were selected. miRNAs which were common to most of the prediction tools were also included in the list irrespective of their significance of scores. The order of the scores given in the table for each tool is in the order of their binding with *Perk*, *Ire1α*, *Chop* and *Puma.* NP- No predictions for the gene available. NA- No Scores for the gene available.

3.2 Induction of ER stress in cortical and hippocampal culture model

ER stress induction in mice cortical and hippocampal cultures treated with thapsigargin and tunicamycin for six hours was ascertained with ER stress markers, the splicing of *Xbp1* mRNA and *BiP* over expression. The stress induced *Xbp1* mRNA processing evaluated by semiquantitative RT-PCR analysis revealed the accumulation of spliced form of *Xbp1* mRNA when the PCR product was run on 3% agarose gel, while the spliced fragments were absent in untreated controls and DMSO treated cells. Real-time PCR analysis revealed elevated *BiP* expression in drug treated cortical and hippocampal neurons relative to untreated controls and vehicle controls. These results served as an indicator of ER stress and UPR induction in the culture model (Fig.1).

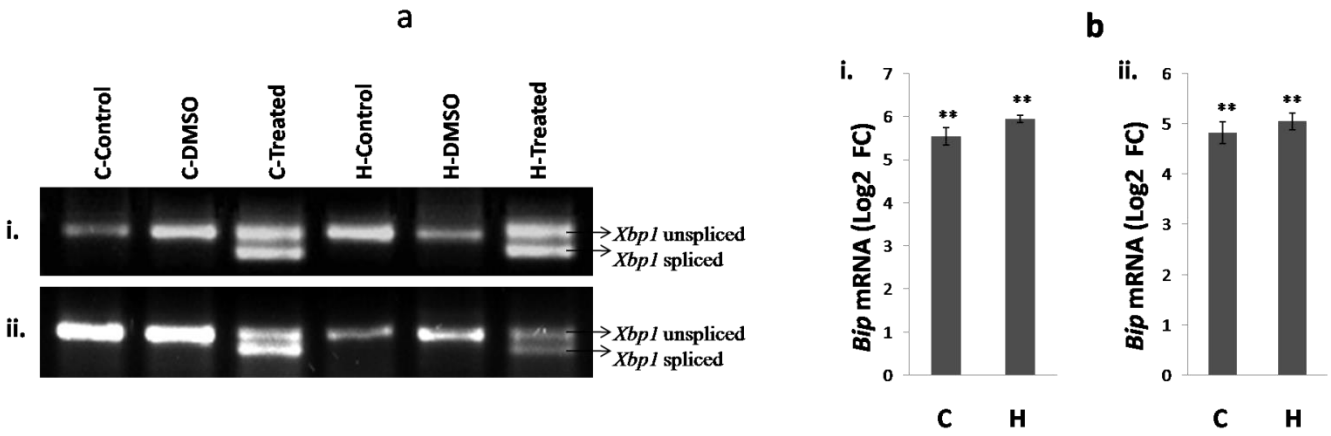

Fig.1. *Xbp1* mRNA splicing and *BiP* overexpression as markers of ER stress.

Cortical (C) and Hippocampal (H) neuronal cells in culture were treated with (**i**) 3.5µM Tunicamycin and (ii) 1µM Thapsigargin for 6hrs. Harvested the cells and (**a**) analyzed the expression of spliced *Xbp1* mRNA by electrophoresis in 3% agarose gel. The spliced *Xbp1* mRNA was considerably expressed after 6hrs of drug stimulation in both cortex and hippocampus which confirmed the induction of ER stress in the culture model. No splicing was observed with untreated controls and DMSO treated vehicle controls. (**b**) analyzed *BiP* expression by real-time PCR analyses. Elevated *BiP* expression noticed in cortical and hippocampal neurons after 6hrs of treatment with the drugs confirmed the development of ER stress in the culture model. The increased fold of *BiP* mRNA levels are normalized to *β-actin* levels and expressed as fold change over untreated controls, n=3, **$p < 0.01$. Error bars represent standard deviation.

3.3 Expression of miRNAs in cortical and hippocampal neuronal culture

The miRNAs which are predicted to regulate *Perk*, *Ire1α*, *Chop* and *Puma* were evaluated by analyzing the changes in miRNA expression in response to the drugs thapsigargin and tunicamycin using real-time PCR analysis (Fig.2). The relative quantitative results demonstrated that out of the 18 miRNAs selected, seven miRNAs, miR-24, 27b, 124, 224, 290, 351 and 488 were significantly

downregulated in both cortical and hippocampal neurons in response to the treatment with ER stress inducing drugs. Out of these, four miRNAs such as miR-24, 27b, 224 and 488 demonstrated over two-fold decrease in its expression in both the cultures. The remaining miRNAs did not show a considerable change in expression in response to the drugs except for the upregulation of miR- 489 and 494 with more than two-fold increase in cortical and hippocampal regions and for miR-148a and 152 in cortical neurons only.

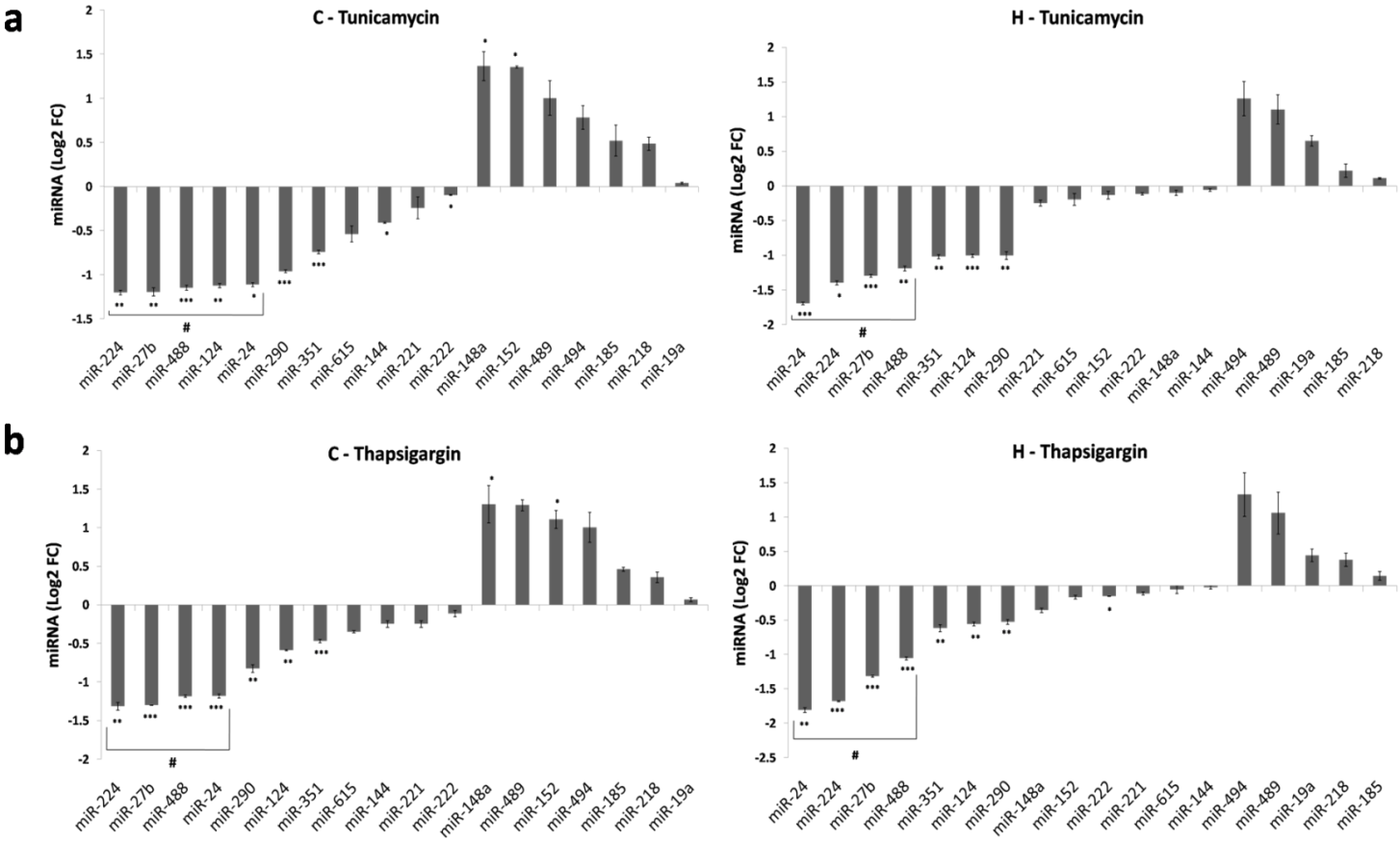

Fig.2. Real-time PCR analyses of miRNAs showing differential expression in ER stress induced cortical and hippocampal neuronal cultures.

ER stress was induced in Cortical (C) and Hippocampal (H) neuronal cultures with (**a**) 3.5µM Tunicamycin and (**b**) 1µM Thapsigargin for 6hrs and real-time quantification of miRNA expression was done as described in the methods. Fold change of each miRNA was calculated with $2^{-\Delta\Delta Ct}$ method, normalized to SnoRNA-202 and represented as fold change over untreated controls. Triplicates were used in the analyses and statistically significant changes in the expression were observed for the miRNAs as shown. miRNAs with a considerable decrease of two-fold in its expression are shown with asterisk (#). *$p<0.05$, **$p < 0.01$, ***$p<0.001$. Error bars represent standard deviation.

3.4 Inverse expression relationship of miRNAs with their target genes in response to ER stress

In order to examine whether the changes in miRNA expression has affected the level of target mRNA, the level of expression of these mRNAs were checked with real-time PCR (Fig.3). The target mRNA levels showed an inverse relationship with the levels of significantly downregulating miRNAs, miR-24, 27b, 124, 224, 290, 351 and 488 in cortical and hippocampal neurons. ER stress inducers enhanced *Perk*, *Ire1α*, *Chop*, *Puma* mRNA levels when compared with untreated cultures with the highest mRNA level for *Chop* gene. *Chop* showed over 100 fold increase in its expression with tunicamycin and over 45 fold increase with thapsigargin in cortical and hippocampal cultures. In cortical neurons *Perk*, *Ire1α* and *Puma* were upregulated over 4 fold with tunicamycin and over 5 fold with thapsigargin. While in hippocampal neurons these genes showed over 5 fold and over 8 fold upregulation with tunicamycin and thapsigargin respectively.

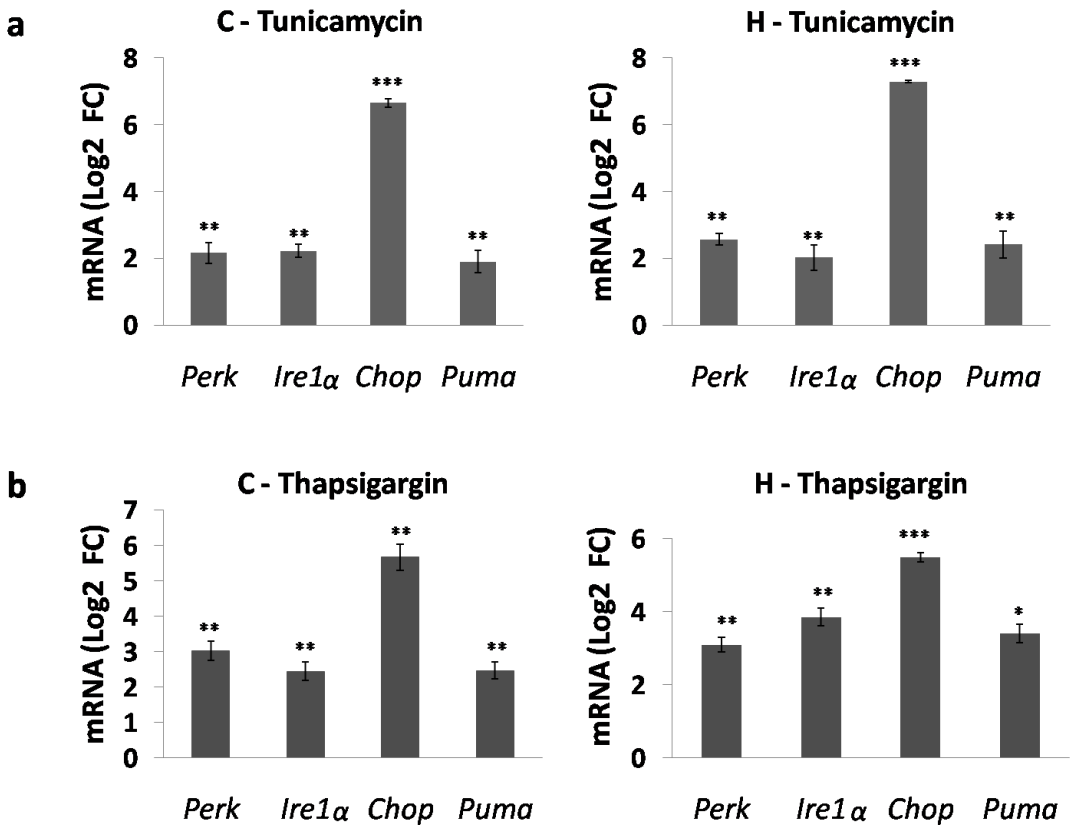

Fig.3. Real-time PCR analyses of UPR genes *Perk, Chop, Puma, Ire1α* showing upregulation in ER stress induced cortical and hippocampal neuronal cultures.

ER stress was induced in Cortical (C) and Hippocampal (H) neuronal cultures with (**a**) 3.5µM Tunicamycin and (**b**) 1µM Thapsigargin for 6hrs and the real-time quantification of mRNA expression was done as described in the methods. Fold change of each mRNA expression was calculated with $2^{-\Delta\Delta Ct}$ method, normalized to *β-actin* and represented as fold change over untreated controls. Triplicates were used in the analyses and statistically significant changes in the expression

were observed for the UPR genes as shown. *p<0.05, **p < 0.01, ***p<0.001. Error bars represent standard deviation.

The molecular mechanism underlying the NDDs is complex and a greater knowledge of the gene regulatory networks will be required to understand the pathophysiology of these diseases. ER stress induced UPR signaling in neurons is important in neuronal damage and is regulated by a number of factors including that mediated by miRNAs. The results presented here showed that in neurons induction of ER stress alters the expression of miRNAs targeting UPR signaling genes and the inverse expression relationship of miRNA-mRNA levels suggests that these genes are subjected to miRNA regulation. In this study out of the 18 selected miRNAs which are predicted to target the UPR pathway genes, we have identified seven novel significantly downregulating miRNAs that respond to ER stress in neurons. Primary cortical and hippocampal neurons were used to analyze the differential expression of miRNAs during ER stress. The cultured neuronal cells were challenged with tunicamycin and thapsigargin, the known chemical inducers of ER stress. Tunicamycin prevents the glycosylation of newly synthesized glycoproteins by inhibiting N-acetylglucosamine transferases while thapsigargin inhibits the Ca2+ ion pump proteins of intracellular membranes located in endoplasmic reticulum there by inducing ER stress. The accumulation of spliced *Xbp1* mRNA and induced expression of *BiP* were observed in the drug treated group confirming the induction of ER stress in the culture model. The 18 miRNAs targeting UPR pathway genes *Perk*, *Ire1α*, *Chop* and *Puma* selected using bioinformatic tools were further analyzed using quantitative real-time PCR in ER stress induced neuronal culture. Specific brain regions are selectively targeted by different NDDs and the miRNA expression and function are specific to different regions of brain. Therefore the expression of each miRNA was analyzed separately in hippocampal and cortical neurons. We showed a statistically significant downregulation of seven miRNAs namely miR-24, 27b, 124, 224, 290, 351 and 488 with most significant under expression for miR-24, 27b, 224 and 488 in both hippocampal and cortical cultures. The expression of certain miRNAs showed variation in their down regulation when analyzed separately in the two brain regions. miR-24 and miR-224 showed a remarkable downregulation in hippocampal neurons than in cortical neurons treated with the drugs. The reduced expression pattern of miRNAs, miR- 124, 290, and 351 levels showed almost similar reduction of nearly 50% in both hippocampal and cortical neurons with tunicamycn while the expression was higher with thapsigargin. The downregulation of miRNAs was accompanied by the upregulation of target mRNAs compared to untreated controls showing an inverse expression relationship between the miRNA-mRNA levels indicating that these miRNAs are possible regulators of their predicted UPR genes. In particular, miR-24 might be a strong candidate responsible for the regulation of UPR pathway with three predicted targets *Perk*, *Ire1α* and *Puma.* It is noteworthy that five of these potent under expressing miRNAs namely miR-24, 124, 290, 351

and 488 target *Ire1α*, the major trigger in ER stress induced apoptosis. A two-fold upregulation, though not significant was expressed by miR-489 and 494 in both the cultures while the levels of miR-148a and 152 were elevated significantly in cortical culture only. The over expressed miRNAs in the study needs further analysis about their targets and role in the regulation of ER stress since the UPR genes are predicted to be their potent targets according to seed sequence analysis. The altered expression of these miRNAs in response to neuronal ER stress is reported for the first time by our study. All of these miRNAs were previously studied to have association with apoptosis either in neurons or in other cell types. Of these, some miRNAs were found to be associated with neurodegenerative condition also. In neurodegenerative brain, the expression profiling studies showed under/over expression of many miRNAs including our selected miRNAs namely miR-19a, 27b, 124, 148a, 185, 221 and 488 [23]. Exosomal miRNAs including miR-24, 27a, 152 and 185 predicted to target the UPR genes showed differential expression in the circulating fluids of patients with AD and PD which can be used as biomarkers for these diseases [24, 25]. When these miRNAs were tested for their expression in ER stressed murine neurons, we observed the downregulation of miR-27b, 24, 124, 488 and upregulation of miR-148a, 152 and 185 which corresponds to their expression pattern in certain brain areas during neurodegenerative conditions. Furthermore the involvement of miR-27b and 124 has been reported in neuronal apoptosis. The administration of miR-27a mimics has been shown to reduce *Puma*, *Noxa* and *Bax* activation in injured cortex and also suppresses apoptotic protease activating factor-1 thereby reducing neuronal apoptosis [26, 27]. miR-124 suppression induces cell apoptosis and accelerates Parkinson's disease development [28]. Although there are no reports on the regulation of neuronal apoptosis by miR-488, it reduces apoptosis in primary osteosarcoma tissues by suppressing *Bim* [29]. miR-24 is also known to suppress *Bim* in cardiomyocytes thus reducing apoptosis [30, 31]. Since ER stress in neurons contributes to neuronal apoptosis, our data showing the ER stress induced downregulation of miR-27b, 24, 124 and 488 and the corresponding upregulation of their proapoptotic UPR targets provides insight into the involvement of these miRNAs in ER stress induced neurodegeneration. The decrease in the expression of miR-351 is shown in mouse embryonic fibroblast in response to ER stress but not yet in neurons [32]. The upregulation of miR-290 in breast cancer cell lines is reported to induce apoptosis [33]. In contrast, in ER stressed neurons the activation of UPR induced apoptosis has been shown to reduce the expression of miR-290. The role of miR-224 has been implicated in neuronal injury by some studies [34]. The abnormal expression observed for these miRNAs revealed their involvement in neuronal UPR induction and the actual mechanism behind their regulation of neuronal apoptosis need to be explored. This study aimed at providing a basis for further experiments that could reveal the mechanism of miRNA regulation of UPR induced neuronal death which helps in the development of RNA-i based therapeutics and for determining molecular markers

associated with NDDs. Overall, our findings report the interplay of the seven miRNAs and their targets in ER stressed neurons indicating that these miRNAs could be acting as negative regulators of their target genes involved in UPR pathway. Hence they may be viewed as potential candidates for further research on miRNA mediated knock down of UPR pathway genes which may reduce the susceptibility of neurons towards apoptosis thereby delaying disease progression.

4. CONCLUSION

RNA-i based gene silencing technology has a great potential for treating many human diseases by targeting only specific genes thus overcoming the limitations of existing medicines. The identification of potential miRNAs with druggable targets in the disease pathway is essential for developing RNA-i based therapeutics. In this study, bioinformatics analysis combined with real-time PCR data reports the differential expression of seven miRNAs in cortical and hippocampal neurons that target genes having potential significance in ER stress mediated apoptosis causing neuronal loss in NDDs.

5. ACKNOWLEDGEMENT

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