

Neuroblastoma Cell Death Induced by eEF1A2 Knockdown Is Possibly Mediated by the Inhibition of Akt and mTOR Phosphorylation

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ABSTRACT

Background The protein kinase B/mammalian target of the rapamycin (Akt/mTOR) pathway is one of the most potent prosurvival signaling cascades that is constitutively active in neuroblastoma. The eukaryotic translation elongation factor-1, alpha-2 (eEF1A2) protein has been found to activate the Akt/mTOR pathway. However, there is a lack of data on the role of eEF1A2 in neuroblastoma. The present study investigated the effect of eEF1A2 silencing on the viability of neuroblastoma cells and its possible signaling.

Materials and Methods: Human SH-SY5Y neuroblastoma cells were transfected with small interfering RNA (siRNA) against eEF1A2. After 48 h of transfection, cell viability was assessed using an MTT assay. The mRNA expression of p53, Bax, Bcl-2, caspase-3 and members of the phosphoinositide 3-kinases (PI3K)/Akt/mTOR pathway was determined using quantitative real-time RT-PCR (qRT-PCR). The protein expression of Akt and mTOR was measured using Western blot analysis.

Results: eEF1A2 knockdown significantly decreased the viability of neuroblastoma cells. No significant changes were observed on the expression of p53, Bax/Bcl-2 ratio, and caspase-3 mRNAs; however, the upregulated trends were noted for the p53 and Bax/Bcl-2 ratio. eEF1A2 knockdown significantly inhibited the phosphorylation of both Akt and mTOR. Almost all of the class I (PIK3CA, PIK3CB, and PIK3CD) and all of the class II PI3K genes were slightly increased in tumor cells with eEF1A2 knockdown. In addition, a slightly decreased expression of the Akt2, mTORC1, and mTORC2 was observed.

Conclusion: eEF1A2 knockdown induced neuroblastoma cell death, in part through the inhibition of Akt and mTOR, suggesting a potential role of eEF1A2 as a molecular target for neuroblastoma therapy.

Key words: Eukaryotic translation elongation factor-1, alpha-2 (eEF1A2); Neuroblastoma; Small interfering RNA (siRNA); SH-SY5Y cells; Phosphoinositide 3-kinases (PI3K); Akt; mTOR; p53

INTRODUCTION

Neuroblastoma is the most common tumor reported in infants and is the most prevalent childhood extracranial solid tumor¹. It is characterized by clinical variability with a spectrum of diseases ranging from spontaneous regression to

treatment resistance with metastasis and poor prognosis². Most neuroblastoma tumors occur sporadically. The fundamental questions are “Why do many children develop neuroblastoma?”, and “What genetic predisposing factors might be present in these patients?”. At the onset and

progression of neuroblastoma, many oncogenes are deregulated and thus provide an opportunity to overcome this disease³. One such oncogene, which has been found in several tumors and has been reported to express in SH-SY5Y neuroblastoma cells, is the eukaryotic translation elongation factor-1, alpha-2 (*EEF1A2*) gene^{4,5}.

The *EEF1A2* gene encodes eukaryotic translation elongation factor-1, alpha-2 (eEF1A2), a protein that plays an important role in the synthesis of proteins. The overexpression of eEF1A2 has been reported in a variety of solid tumors; such as ovarian and breast cancers⁶⁻⁹. eEF1A1 and eEF1A2 are close isoforms of the eEF1A with eEF1A1 being expressed ubiquitously while eEF1A2 is exclusively expressed in the heart, muscle, and brain¹⁰⁻¹². Both isoforms share a high level of homology (92% identity). The canonical role for these proteins involves binding of amino-acylated tRNA for transport to the ribosomes in the regulation of ribosomal polypeptide elongation¹³. However, eEF1A2 has been found to have a number of non-canonical functions including phosphatidylinositol signaling⁷, anti-apoptotic activity^{14,15}, cytoskeletal modifications¹⁶, and promoting tumor growth through activating the Janus kinase-signal transducer and activator of transcription (JAK/STAT) and phosphoinositide 3-kinases/protein kinase B (PI3K/Akt) signaling¹⁷.

Recent studies have shown that eEF1A2 activates the phosphatidylinositol 3'-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway, which in turn promotes the functional inactivation of the tumor suppressor p53, one of the most frequent events during human tumorigenesis¹⁸. In addition, caspase-3 activity was found significantly increased with the eEF1A2-small interfering RNA (siRNA) treatment, resulting in apoptosis in ovarian tumor cells¹⁹. These data suggest that eEF1A2 plays a role in the neoplastic development and progression. However, its roles in neuroblastoma have not yet been elucidated.

To investigate whether eEF1A2 is involved in the PI3K/Akt/mTOR pro-survival signaling cascade in neuroblastoma, we examined the roles of eEF1A2 by silencing the eEF1A2 protein in human SH-SY5Y neuroblastoma cells using siRNA, and the cell viability, the expression of apoptosis-related genes,

and the expression patterns of the PI3K/Akt/mTOR signaling pathway were investigated.

MATERIALS AND METHODS

Study design

Human SH-SY5Y neuroblastoma cells were divided into two groups: untransfected group and *EEF1A2*-siRNA transfected group. Scrambled siRNA was used as a negative-control group. Western blotting, quantitative real-time reverse transcription polymerase chain reaction, and a cell viability assay were performed in each group.

Cell culture

Human SH-SY5Y neuroblastoma cells were grown in a humidified atmosphere incubator containing 5% CO₂ at 37 °C. Cells were maintained in 1:1 mixture of Eagle's Minimum Essential Medium (MEM) and Nutrient Mixture Ham's F12 medium (Gaithersburg, MD, USA), supplemented with 10% heat-activated fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 1.5 g/L sodium bicarbonate, 100 U/ml penicillin, and 100 µg/ml streptomycin.

siRNA transfection

siRNA against *EEF1A2* and negative-control scrambled siRNA were purchased from Qiagen (ML, USA). Transfection was performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Eugene, OR, USA) mixed with Opti-MEM reduced-serum medium (Gibco, Gaithersburg, MD, USA) according to the manufacturer's instructions. To silence eEF1A2, SH-SY5Y cells were grown in 6-well plates to 60-70% confluence and transfected with siRNA at the final concentrations of 50 pmol. Cells were used for subsequent experiments at 48 h after siRNA transfection. The effectiveness of transfection was assessed using quantitative real-time PCR and Western blotting.

Western blot analysis

SH-SY5Y cells were washed with PBS, trypsinized, and centrifuged at 27,000 RPM for 7 min to collect the pellets. RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1 % SDS, 1% sodium

deoxycholate, 5 mM EDTA, 30 mM Na₂HPO₄, 50 mM NaF) was added into the pellets, mixed, and incubated on ice for 20 min, followed by centrifugation at 12,000 RPM for 20 min. Then, the supernatants were collected. The protein concentrations were determined by using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). 40-60 µg of protein mixed with loading buffer were loaded onto 6-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a PVDF membrane and blocked with 5% nonfat dry milk in 1 x Tris-buffered saline containing 0.1 % Tween-20 for 2 h. The membranes were incubated with primary antibodies as follows: rabbit anti eEF1A2 (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti pan-Akt (Cell Signaling, Danvers, MA, USA), rabbit anti phospho-Akt1 (Cell Signaling), rabbit anti mTOR (Cell Signaling), rabbit anti phospho-mTORC1 (Cell Signaling), and mouse anti β-actin (Sigma-Aldrich) as an internal control. The HRP conjugated anti-rabbit (Abcam, Cambridge, UK) and HRP conjugated anti-mouse IgG (Invitrogen, Eugene, OR, USA) were used as secondary antibodies. Proteins were detected using the ECL chemiluminescence system (Pierce Biotechnology, Rockford, IL, USA). The band intensity was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The targeted protein expression was normalized with β-actin and reported as the relative expression to the respective controls.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from SH-SY5Y cells using PARIS kit (Invitrogen, Eugene, OR, USA). Briefly, cells were harvested in 0.25% trypsin-EDTA, centrifuged, and washed with cold PBS. Disruption buffer was added into the pellets to lyse the nuclei, followed by adding Lysis/Binding solution. RNA samples were eluted using the Elution Solution and stored at -80 °C. The RNA concentrations were measured using Nano-drop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The RNA purity was assessed using the ratio of absorbance at 260 nm and 280 nm. Two-µg total RNA were used for

the reverse transcription using Masterscript Kit and RT-PCR System (5 PRIME, Gaithersburg, MD, USA), according to the manufacturer's protocol. The cDNA was amplified using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA). The housekeeping gene β-actin was used as an internal control. The sequences of the primers for the apoptosis-related genes are as follows: p53: sense, 5'-GGAGTTGTGAGGCGCTGG-3'; antisense, 5'-CACGCACCTCAAAGCTGTTC-3'; Bax: sense, 5'-TGCAGAGGATGATTGCTGAC-3'; antisense, 5'-GAGGACTCCAGCCACAAAGA-3'; Bcl-2: sense, 5'-CAGCTGCACCTGACG-3'; antisense, 5'-ATGCACCTACCCAGC-3'; β-actin: sense, 5'-CATGTACGTTGCTATCCAGGC-3'; antisense, 5'-CTCCTTAATGTACGCACGAT-3'. The primers for the members of the PI3K/Akt/mTOR pathway were the same as those used in our previous study (5). The reaction was performed in Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with triplicates for each sample. The cycling condition was as follows: 3 min for enzyme activation at 95 °C, 40 cycles of 3 s for initial denaturation at 95 °C, and annealing/extension at 60 °C for 1 min. Melting curve analysis was performed to ensure amplification specificity. The transcript levels of the target genes were determined by the comparative threshold cycle (Ct) method.

Cell viability measurement

An MTT assay was used for the cell viability assessment of SH-SY5Y cells. 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) was dissolved in Hank's Balanced Salt Solution (Sigma-Aldrich). Cells were plated onto a 96-well plate at a density of 1 x 10⁴ cells/well and incubated at 37 °C under 5% CO₂ for 24 h. Cells were added with 5 mg/mL of the MTT solution and incubated for 3 h at 37 °C in a humidified atmosphere incubator containing 5% CO₂. Then, medium in each well was replaced with 100 µL of DMSO. The absorbance of the samples was measured at 570 and 690 nm using the BioTek microplate reader with KC4 Synergy HT Software (BioTek, Shanghai,

China). All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 5 (GraphPad software, San Diego, CA). One-way analysis of variance (ANOVA) with Turkey multiple comparison tests was used to test overall statistical significance. Data were presented as mean \pm SEM. Statistical significance was considered as a value of $P < 0.05$.

RESULTS

Effect of eEF1A2 siRNA on the expression of eEF1A1 and eEF1A2

As eEF1A1 is also expressed in developing neurons, and it has a high amino acid sequence similarity with eEF1A2, we first determined the effectiveness and specificity of the eEF1A2 siRNA sequence using quantitative RT-PCR (qRT-PCR) and Western blotting. Transfecting SH-SY5Y cells with eEF1A2 siRNA significantly downregulated the eEF1A2 mRNA expression ($P < 0.05$; Figure 1a). Moreover, the Western blot analysis confirmed the specificity of the siRNA sequence with a significant decrease in the eEF1A2 protein level ($P < 0.01$ compared with untransfected cells; Figure 1b), while the level of the eEF1A1 protein was not affected (Figure 1c).

Effect of eEF1A2 knockdown on cell viability and apoptosis-related genes

Next, we investigated whether the low protein content of eEF1A2 in neuroblastoma cells affected the cell survival. After transfection, the cell viability of SH-SY5Y cells was assessed using an MTT assay. Figure 2a showed that the eEF1A2 knockdown significantly decreased the viability of SH-SY5Y cells ($P < 0.001$ compared with untransfected cells). We also determined whether the decreased survival was associated with changes in the apoptosis-related gene expression. By using qRT-PCR, no significant changes were observed from the expression of p53, Bax/Bcl-2 ratio, and caspase-3 mRNAs (Figures 2b to 2d) although the upregulated trends were notable for p53 and Bax/Bcl-2 ratio.

eEF1A2 knockdown inhibits phosphorylation of Akt and mTOR

As mentioned above that eEF1A2 has been found to activate the PI3K/Akt/mTOR pathway and inactivate p53 in cancer cells (18), we examined the phosphorylation of Akt and mTOR in SH-SY5Y cells using Western blotting. Compared with untransfected cells, the eEF1A2 knockdown significantly inhibited the phosphorylation of both Akt and mTOR ($P < 0.05$; Figure 3).

Effect of eEF1A2 knockdown on the PI3K/Akt/mTOR mRNA expression

To explore the involvement of the PI3K/Akt/mTOR signaling pathway in the eEF1A2 silenced neuroblastoma cells, the expression patterns of the PI3K/Akt/mTOR mRNAs were determined using qRT-PCR. All genes encoding the members of the three human PI3K classes were studied. Class I comprised PIK3CA, PIK3CB, PIK3CD, and PIK3CG genes. Class II consisted of PIK3C2A, PIK3C2B, and PIK3C2G. Class III included PIK3C3. Figure 4a showed that almost all of the class I (PIK3CA, PIK3CB, and PIK3CD) and all of the class II PI3K genes were relatively increased in SH-SY5Y cells with eEF1A2 knockdown, while the expression of the class III gene (PIK3C) was relatively decreased. For the Akt and mTOR genes, the relatively decreased expression was observed for the Akt2, mTORC1, and mTORC2 (Figures 4b and 4c).

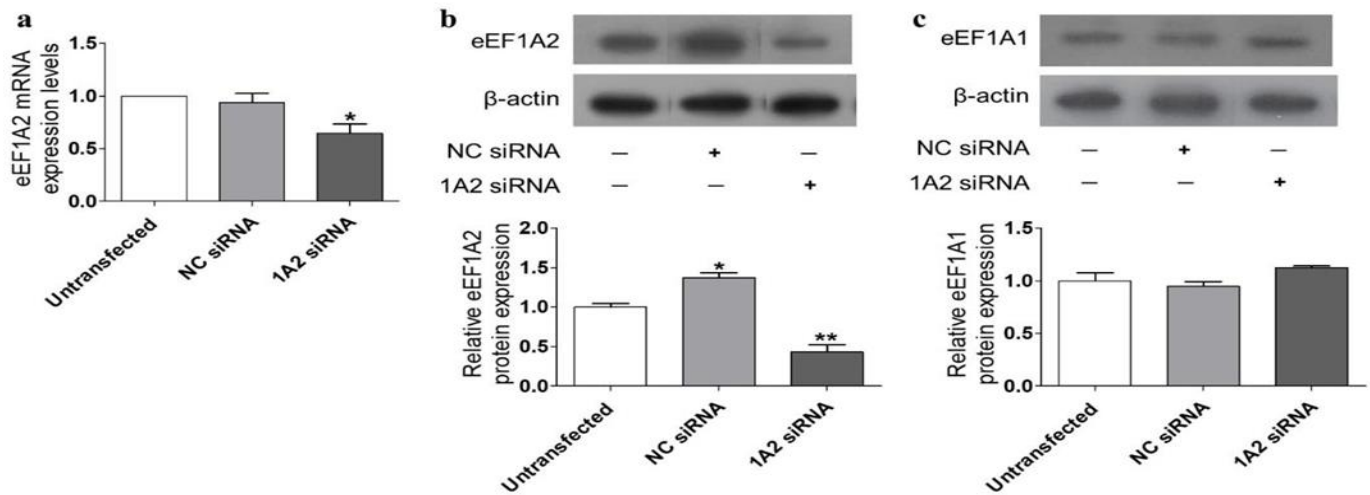


Figure 1. mRNA expression of eEF1A2 and Western blot showing the expression of eEF1A2 and eEF1A1 in SH-SY5Y cells after eEF1A2 siRNA transfection. (a) Expression of eEF1A2 mRNA determined using qRT-PCR. The expression level was normalized to that of β -actin. Data are expressed as mean \pm SEM from three independent experiments. (* $P < 0.05$ versus untransfected cells). (b) eEF1A2 and (c) eEF1A1 protein expression. The density of the bands was normalized with that of β -actin protein. Quantitative data are expressed as mean \pm SEM from three independent experiments (* $P < 0.05$ versus untransfected cells). NC: negative control, 1A2: eEF1A2.

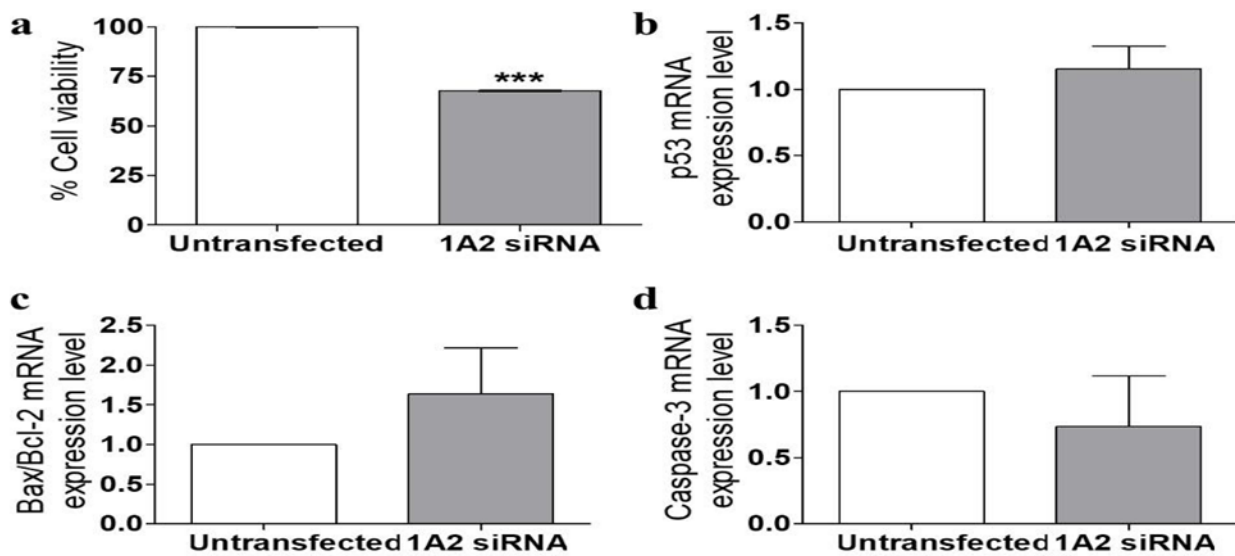


Figure 2. Cell viability of SH-SY5Y cells and mRNA expression of p53, Bax/Bcl-2 and caspase-3 in SH-SY5Y cells after eEF1A2 siRNA transfection. (a) Cell viability determined using an MTT assay. Data are expressed as mean \pm SEM from three independent experiments. (***) $P < 0.001$ versus untransfected cells). (b) p53, (c) Bax/Bcl-2, and (d) caspase-3 mRNA expression determined using qRT-PCR. The expression levels were normalized to the levels of β -actin. Data are expressed as mean \pm SEM from three independent experiments. 1A2: eEF1A2.

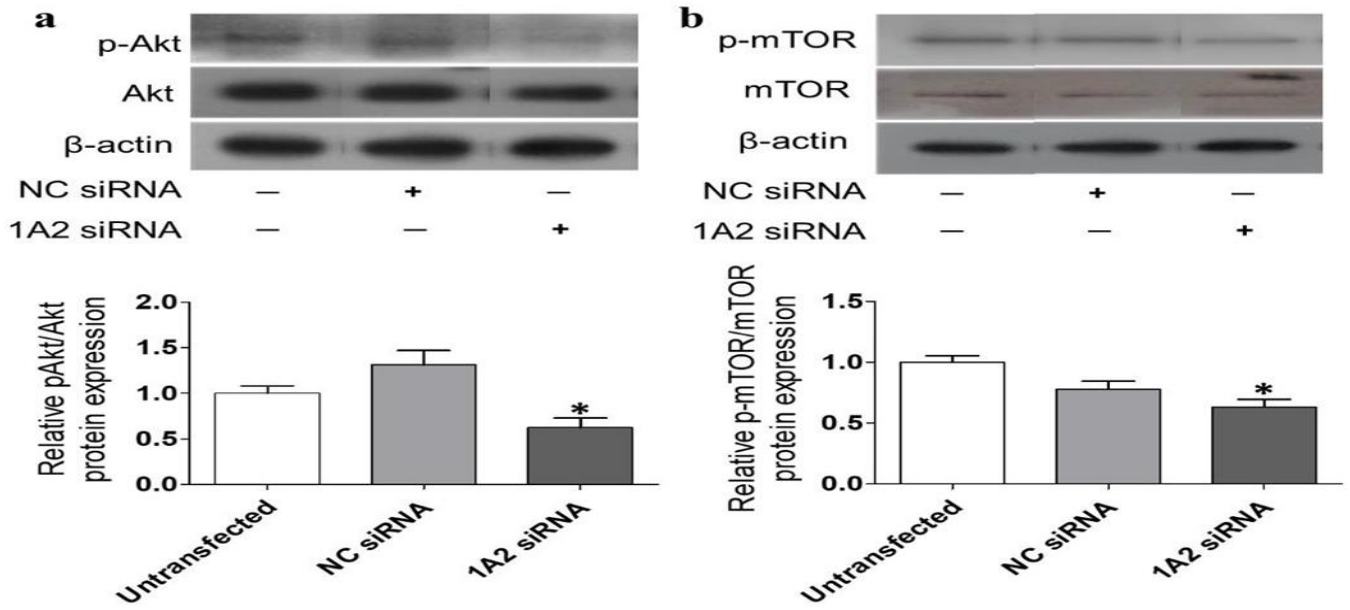


Figure 3. Western blot showing the phosphorylation of (a) Akt and (b) mTOR in SH-SY5Y cells after eEF1A2 siRNA transfection. The density of the bands was normalized with that of β-actin protein. Quantitative data are expressed as mean ± SEM from three independent experiments (*P<0.05 versus untransfected cells). NC: negative control, 1A2: eEF1A2.

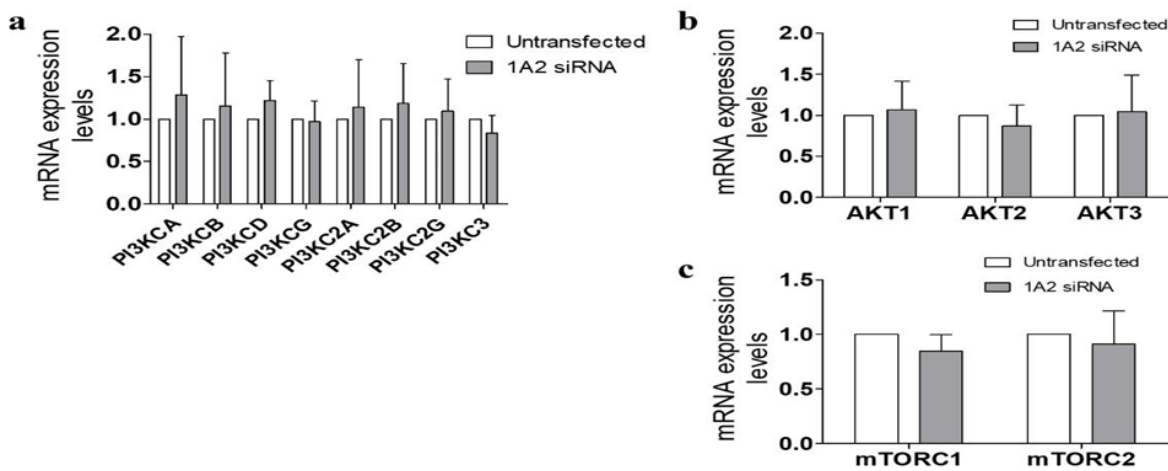


Figure 4. mRNA expression of the PI3K/Akt/mTOR in SH-SY5Y cells after eEF1A2 siRNA transfection. (a) Expression levels of genes of (a) PI3K classes I, II, and III, (b) AKT1, AKT2, and AKT3, (c) mTORC1 and mTORC2. mRNA expression was determined using qRT-PCR. The expression levels were normalized to the levels of β-actin. Data are expressed as mean ± SEM from three independent experiments. 1A2: eEF1A2

DISCUSSION

Our key findings in the present study were: 1) eEF1A2 knockdown increased the death of the SH-SY5Y neuroblastoma cells, and 2) the possible mechanism, underlying the eEF1A2 knockdown-induced cell death, could be mediated through the inhibition of Akt/mTOR phosphorylation. The PI3K/Akt/mTOR pathway, which is a major integrator of cell responses to growth factors and nutrients, and regulating cellular survival, is one of the most potent prosurvival signaling cascades that is constitutively active in neuroblastoma, a MYCN-driven neural crest malignancy²⁰⁻²². As being a master regulator of translation and protein synthesis, the mTOR pathway could be activated by the MYCN protein to drive tumorigenesis²³. eEF1A2 was reported to activate the PI3K/Akt/mTOR pathway in the breast cancer cell line⁷ and hepatocellular carcinoma cells¹⁸, suggesting its potential role in tumor development and progression. Thus, effective inhibition of the Akt/mTOR functions, probably via eEF1A2 silencing, may represent a potential strategy for therapeutics in neuroblastoma.

The Akt kinase family consists of three members: Akt1, Akt2 and Akt3. Although all three isoforms are structurally homologous and have similar mechanisms of activation, they exhibit distinct features. Akt activation in neuroblastoma has been shown to be a predictor of malignancy²⁴ and chemoresistance²⁵. Nevertheless, our qRT-PCR results did not show a significant change in the mRNA expression of Akt1, Akt2 and Akt3, although an expected decreased trend was observed for Akt2. A similar decreasing trend was also observed for mTORC1 and mTORC2 mRNAs. Previous studies have supported that change in the expression of the Akt isoforms in human malignancies is a much more frequent event than their gene amplification, indicating transcriptional regulation of Akt during tumorigenesis²⁶. Among the three Akt isoforms, Akt2 is more important in cancer development and progression^{26,27}. Previous studies have shown that N-myc acts as a crucial downstream effector of PI3K/Akt in neuroblastoma angiogenesis²⁸, and its expression is mainly regulated by Akt2²⁹.

Our findings that eEF1A2 knockdown inhibited Akt phosphorylation, with a modest downregulation of Akt2 mRNA, may indicate the important role of the eEF1A2/Akt signaling in neuroblastoma development and progression.

Recent studies have demonstrated that PI3K-mTORC2 but not PI3K-mTORC1 regulates neuroblastoma aggressiveness³⁰. In addition, inhibition of both mTORC1 and mTORC2 signaling decreases cell viability and suppresses growth in neuroblastoma cells by inducing cell cycle arrest and cell apoptosis³¹. The decrease in the viability of SH-SY5Y cells in our present study may in part be mediated through dysregulations in protein synthesis, which is indicated by inhibition of mTOR phosphorylation, with modest downregulated mTORC1 and mTORC2 mRNAs, as a result of the eEF1A2 knockdown.

A slight upregulation of almost all of class I (PIK3CA, PIK3CB, and PIK3CD) and all of the class II PI3K genes found in the eEF1A2-silenced cells may imply an upstream compensatory mechanism as a result of the functional inactivation of their downstream Akt/mTOR. PIK3C2B has been implicated in neuroblastoma tumorigenesis, and an inhibition of PIK3C2B has been shown to inhibit the early stage of neuroblastoma formation³². Our results suggested that eEF1A2 knockdown might mediate the PI3K signaling at its downstream effectors, rather than the PI3K itself.

Neuroblastoma exhibits the two opposing pathways of aberrant mTOR signaling that mediates growth and proliferation, and active p53 that mediates growth arrest and apoptosis. Previous findings demonstrated a molecular mechanism linking the p53 function with mTOR suppression³³. An increased Bax/Bcl-2 ratio in cancer cells has also been found to be associated with the elevated levels of p53, and p53-mediated cell cycle arrest and apoptosis can be mediated through a caspase-3-independent pathway³⁴. These previous data support our present findings that show a positive correlation of p53 and Bax/Bcl-2 ratio but negatively with the caspase-3 mRNA expression in the eEF1A2-silenced cells. Therefore, this

observation warrants further investigations on the protein expression level.

In addition, it was observed in the present study that although eEF1A2 was silenced, the close isoform eEF1A1 remained expressed in the siRNA transfected cells. Studies have shown that *EEF1A2* appears to be a single-copy gene, whereas numerous pseudogenes for *EEF1A1* exist in the human genome³⁵. The mutually exclusively expression of the two isoforms in neuroblastoma cells suggests their functionally equivalent roles in protein synthesis, which may explain the statistical insignificance in our qRT-PCR experiments. An additional limitation of our study is that the knocking down did not completely abolish the expression of eEF1A2 protein. As a result, it may influence the protein synthesis of other cellular signaling pathways that affect the survival of neuroblastoma cells. Knock-out mouse models are required for precise loss-of-function effects. However, our results suggest that tumor maintenance may be mediated by eEF1A2.

CONCLUSION

In summary, the present study demonstrated a novel role of eEF1A2 in neuroblastoma cells. Silencing the eEF1A2 protein induces neuroblastoma cell death, which is possibly mediated through the inhibition of Akt and mTOR phosphorylation. The results also emphasized the PI3K/Akt/mTOR pathway as therapeutic target in neuroblastoma. Our results may shed light on the role of eEF1A2 as a potentially molecular target for neuroblastoma therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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