Exogenous expression of miRNA-3613-3p causes APAF1 downregulation and affects several proteins involved in apoptosis in BE(2)-C human neuroblastoma cells

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Abstract. MicroRNAs (miRNAs) are a class of small non-coding RNAs involved in post-transcriptional gene regulation. Furthermore, dysregulation of miRNA expression is an important factor in the pathogenesis of neuroblastoma. Our previous study identified that overexpression of monocyte chemoattractant protein-induced protein 1 protein led to a significant downregulation of a novel miRNA molecule, miRNA-3613-3p. In the present study, the potential involvement of miRNA-3613-3p in the cell biology of neuroblastoma was investigated. It was identified that the expression of miRNA-3613-3p varies among a range of human neuroblastoma cell lines. As the delineation of the functions of a miRNA requires the identification of its target genes, seven putative mRNAs that may be regulated by miRNA-3613-3p were selected. Furthermore, it was identified that overexpression of miRNA-3613-3p causes significant downregulation of several genes exhibiting tumor suppressive potential [encoding apoptotic protease-activating factor 1 (APAF1), Dicer, DNA fragmentation factor subunit β, von Hippel-Lindau protein and neurofibromin 1] in BE(2)-C human neuroblastoma cells. *APAF1* mRNA was the most significantly decreased transcript in the cells with miRNA-3613-3p overexpression. In accordance with the aforementioned results, the downregulation of cleaved caspase-9 and lack of activation of executive caspases in BE(2)-C cells following miRNA-3613-3p overexpression was observed. The results of the present study suggest a potential underlying molecular mechanism of apoptosis inhibition via *APAF1* downregulation in human neuroblastoma BE(2)-C cells with miRNA-3613-3p overexpression.

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Introduction

Neuroblastoma is the most common type of extracranial solid tumor in children. It arises from cells of sympathoadrenal lineage from the neural crest, which migrate away from their place of origin during embryogenesis. Primary tumors typically occur in the adrenal glands, chest, head, neck and pelvis (1). The clinical presentation of neuroblastoma varies from spontaneous regression to rapid progression despite multimodal therapies. This depends on the age of the patient at diagnosis, as well as genetic prognostic factors, with the most important being N-Myc (*MYCN*) gene amplification, which occurs in 25% of tumors (2). Annually, >600 novel cases of neuroblastoma are recognized in the USA, with ~50% of the patients being diagnosed at the advanced stages of the disease (1). The outcome of therapy of high-risk neuroblastoma cases remains poor, with the long-term survival rate being <50% (3).

Recently, assessments of serum microRNAs (miRNAs) have developed into promising non-invasive diagnostic tools for patients, including those with neuroblastoma (4). miRNAs are small non-coding RNA molecules that function as negative regulators of gene expression at the post-transcriptional level. The expression of >60% of protein-coding genes is considered to be regulated by miRNAs. One transcript can be recognized by numerous miRNAs, whereas one miRNA is able to regulate a number of mRNAs. Therefore, the expression of miRNAs must be tightly regulated to ensure normal growth and development of an organism (5). Dysregulation of miRNA expression contributes to tumor progression in numerous types of cancer, including neuroblastoma (5). Certain miRNA molecules, such as miRNA-34a and miRNA-17-92, have been described as potent tumor suppressors and oncogenes in neuroblastoma, respectively (6). This is due to their involvement in the regulation of the tumor phenotype via control of cellular processes, including proliferation, differentiation and apoptosis (6). Although the function of several individual miRNA molecules has been elucidated in neuroblastoma (5), the functions of numerous miRNAs require experimental delineation.

miRNA-3613-3p is a novel miRNA molecule, which attracted interest in our previous study during screening for differentially expressed miRNAs in BE(2)-C neuroblastoma

cells with monocyte chemoattractant protein-induced protein 1 (*MCPIP1*) overexpression (7). It was identified that the overexpression of MCPIP1 causes significant downregulation of miRNA-3613-3p expression in BE(2)-C cells as well as the upregulation of several putative target genes of miRNA-3613-3p (7). To date, the expression of miRNA-3613-3p has been demonstrated by several other groups (8-13). However, the effect of miRNA-3613-3p overexpression on cell viability has only been studied in one type of cell. Zhang *et al* (14) identified that miRNA-3613-3p led to a significant downregulation of cell proliferation via negative regulation of genes encoding cyclin-dependent kinase 1, NUF2 NDC80 kinetochore complex component, baculoviral inhibitor of apoptosis protein repeat-containing 5, ZW10-interacting kinetochore protein and SPC24 NDC80 kinetochore complex component in human HepG2 hepatoblastoma cells. Furthermore, miRNA-3613-3p was identified among five miRNA molecules as a central regulatory factor in RNA interference-dependent control of p53 expression in the aforementioned cell line (15). Additionally, the expression of miRNA-3613-3p was low in the hepatocytes and serum of patients with acute viral hepatitis caused by hepatitis B virus and was associated with possible downregulation of signal transducer and activator of transcription 3 expression due to specific binding with miRNA-3613-3p (13). Another premise of involvement of miRNA-3616-3p in cancer is its potential biomarker value in adenocarcinoma (11) and the enrichment of the complementary strand of the mature miRNA-3613-3p in exosomes derived from a colon cancer cell line (9). Deregulated expression of miRNA-3613-3p has also been identified in several other pathological states (8,10,12). Nevertheless, the involvement of miRNA-3613-3p in the pathogenesis of neuroblastoma remains to be elucidated.

On the basis of our previous study (7), it was decided to investigate the potential functions of miRNA-3613-3p in neuroblastoma. First, the differential expression of miRNA-3613-3p was assessed in a range of human neuroblastoma cell lines. Furthermore, the seven putative target genes of miRNA-3613-3p were investigated using extensive bioinformatics analysis. In order to verify the interaction between miRNA-3613-3p and its predicted target mRNAs, three steps were performed. The expression of the seven proposed target genes was investigated in BE(2)-C human neuroblastoma cells transfected with miRNA-3613-3p mimic using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression of the selected putative target genes was analyzed at the protein level using western blotting. The predicted binding sites in the apoptotic protease-activating factor 1 (*APAF1*) 3' untranslated region (3'UTR) were verified using a luciferase reporter assay. Additionally, the effect of enforced miRNA-3613-3p expression on BE(2)-C human neuroblastoma cell viability. was assessed. Furthermore, the expression and activation levels of several proteins involved in apoptosis in BE(2)-C cells overexpressing miRNA-3613-3p were investigated.

Materials and methods

Cell culture. Seven human neuroblastoma cell lines, BE(2)-C [American Type Culture Collection (ATCC), Manassas, VA, USA], Kelly [Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Leibniz, Germany], IMR-32 (ATCC), SK-N-SH (ATCC), CHP-134 [European Collection of Authenticated Cell Cultures (ECACC), Porton Down, UK], LAN-1 (DSMZ) and LAN-5 (DSMZ), and a prostate cancer cell line PC3 (ECACC) were cultured at 37°C in an incubator with 5% CO_2. Cells were routinely screened for *Mycoplasma* contamination and all tests were negative according to a MycoAlert™ *Mycoplasma* Detection kit (Lonza Group, Ltd., Basel, Switzerland). LAN-1 cells were cultured in a 1:1 mixture of Eagle's minimal essential medium and Ham's F12 medium (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). BE(2)-C cells were cultured in a 1:1 mixture of Eagle's minimal essential medium and Ham's F12 medium supplemented with non-essential amino acids (NEAA), 1 mM sodium pyruvate and 10% fetal bovine serum. Kelly and CHP-134 cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck KGaA) with 10% fetal bovine serum. IMR-32 and SK-N-SH cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 1% NEAA solution and 1 mM sodium pyruvate. LAN-5 cells were cultured in RPMI-1640 supplemented with 20% fetal bovine serum. The PC3 cell line was cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA) with 10% fetal bovine serum. All neuroblastoma cell culture media were additionally supplemented with 50 μg/ml gentamicin (Sigma-Aldrich; Merck KGaA), whereas the PC3 cell culture was supplemented with 40 μg/ml gentamicin.

Generation of the genetic construct. A fragment of *APAF1* (NCBI accession no. NM_013229.2) 3'UTR containing putative binding sites for miRNA-3613-3p was amplified from BE(2)-C cells by PCR using primers with recognition sites for *Xho*I and *Sal*I restriction enzymes added at the 5' end (forward, 5'-ACCTGCTCGAGAAATTGGTATTTTAATACTG-3' and reverse, 5'-AGTAAGTCGACAGCAAGACTCTGTCTCCAA-3'). For the amplification of the 3'UTR fragment DyNAzyme II DNA Polymerase (Thermo Fisher Scientific, Inc.) was used following the manufacturer's protocol. The thermocycling conditions were: Pre-incubation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec; final elongation at 72°C for 10 min. Next, the products were separated by agarose gel electrophoresis using a 1% gel, visualized using SimplySafe™ (Eurx, Gdańsk, Poland) and isolated using the Gel-Out kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. Isolated products and pmiRGlo plasmid vector (E1330; Promega Corporation, Madison, WI, USA) were digested using *Xho*I and *Sal*I restriction enzymes. Following digestion, the pmiRGlo plasmid was additionally dephosphorylated using calf intestinal alkaline phosphatase. Next, DNA was purified using a QIAquick Nucleotide Removal kit (Qiagen GmbH, Hilden, Germany). The *APAF1* 3'UTR fragment was incorporated into the pmiRGlo plasmid vector using T4 DNA ligase. The genetic construct obtained was verified using DNA sequencing (Genomed, Warszawa, Poland).

Cell transfection with miRNA-3613-3p mimic. miRNA-3613-3p mimic (cat. no. 4464066) and scrambled oligonucleotide (cat.

no. 4464058), lacking complementarity to sequences in the human transcriptome, as a negative control were purchased from Ambion; Thermo Fisher Scientific, Inc. For enforced overexpression of miRNA-3613-3p, BE(2)-C cells were seeded at a density of 3×10^4 cells/cm^2 in complete medium as afore-mentioned. After 24 h of culture, the medium was changed. miRNA mimic or negative control and Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) were diluted in Opti-MEM™ (Gibco; Thermo Fisher Scientific, Inc.), and added to the cell culture at final concentrations of 30, 40 and 50 nM. Transfected cells were passaged after 24 h. For prolonged overexpression of miRNA-3613-3p, miRNA-3613-3p mimic or negative control and Lipofectamine 2000 diluted in Opti-MEM were added to the transfected cells culture after 48 h. Overexpression of miRNA-3613-3p was verified using RT-qPCR.

LAN-1 cell transfection with miRNA-3613-3p inhibitors. miRNA-3613-3p inhibitor (cat. no. 4464084) and corre-sponding negative control (cat. no. 4464076) were purchased from Ambion; Thermo Fisher Scientific, Inc. For the silencing of miRNA-3613-3p, LAN-1 neuroblastoma cells were seeded at a density of 2.6×10^4 cells/cm^2 in complete medium as aforementioned. After 24 h of culture, the medium was changed. The miRNA-3613-3p inhibitor, negative control and Lipofectamine 2000 transfection agent were diluted in Opti-MEM. The inhibitor and negative control were added to the cell culture at a final concentration of 30 nM. Cells were collected 48 h after transfection.

Kelly cell transfection with genetic constructs containing an expression cassette for wild-type or mutated MCPIP1 protein. Plasmid vectors used to obtain enforced expression of MCPIP1 protein were as described previously (16). The transfection procedure for Kelly cells was as described in our previous study (17). Overexpression of wild-type or mutated MCPIP1 protein was verified by western blotting.

RNA isolation. RNA was extracted from cells using TRI-REAGENT® (Lab Empire, Rzeszów, Poland) according to the manufacturer's protocol. The concentration and purity of the isolated RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The integrity of the RNA was verified by agarose gel electro-phoresis using a 1% gel.

mRNA RT-qPCR. For each sample, 500 ng of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, using a GenAmp thermocycler (PerkinElmer, Inc., Waltham, MA, USA). qPCR was performed using an Eco thermocycler (Illumina, San Diego, CA, USA) with KAPA SYBR® FAST qPCR Master mix (Kapa Biosystems, Inc., Wilmington, MA, USA). The thermocycling conditions were: Pre-incubation at 95˚C for 10 min; 40 cycles of denatur-ation at 95˚C for 15 sec, and annealing and elongation at 60˚C for 30 sec; a melting cycle at 95˚C for 15 sec, 55˚C for 15 sec and 95˚C for 15 sec. The cDNA used for qPCR was diluted 50-fold, and the reference gene 40S ribosomal protein S13 (*RPS13*) was used. The primers for *DICER*, *APAF1*, DNA fragmentation factor subunit β (*DFFB*), neurofibromin 1 (*NF1*),

retinoic acid-related orphan receptor α (*RORA*), kinesin family member 3A (*KIF3A*) and *RPS13* used in these experiments were described previously (7). The sequences of von Hippel-Lindau protein (*VHL*) primers were as follows: Forward, 5'-TCCACAGCTACCGAGGTCA-3' and reverse, 5'-GGCAAA AATAGGCTGTCCGT-3'. All experiments were performed in triplicate at least three times. For quantification of the relative mRNA level, the ΔΔCq method was used (18).

miRNA RT-qPCR. RT of miRNAs was performed using miRCURY LNA™ Universal RT microRNA PCR (Exiqon; Qiagen GmbH). For each reaction, 100 ng total RNA was used. miRNA RT-qPCR experiments were performed using miRCURY LNA microRNA PCR and ExiLENT SYBR Green master mix (Exiqon; Qiagen GmbH), according to the manu-facturer's protocol. For the PCR, the cDNA was diluted 80-fold and U6 small nuclear RNA (snRNA) was used as the reference. Primers for miRNA-3613-3p (cat. no. 2100646) and the refer-ence U6 snRNA (cat. no. 203907) were provided by Exiqon; Qiagen GmbH. All RT-qPCR experiments were performed in triplicate at least three times, and the ΔΔCq method (18) was used to quantify the relative level of miRNA-3613-3p.

Protein extraction and western blot analysis. Protein extrac-tion was performed using TRI-REAGENT, according to the manufacturer's protocol. Protein concentration was assessed using the bicinchoninic acid method (19). Protein extracts were resolved using SDS-PAGE (8, 12 or 15% gel, depending on the protein weight). Subsequently, the western blotting procedure was performed as described previously (20). The following primary antibodies purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) were used: Anti-DICER rabbit mono-clonal antibody (mAb) (cat. no. 5362, 1:1,000); anti-APAF1 rabbit mAb (cat. no. 8723, 1:1,000); anti-caspase-9 mouse mAb (cat. no. 9508, 1:1,000); anti-cleaved caspase-9 rabbit mAb (cat. no. 7237, 1:10,00); anti-caspase-7 rabbit mAb (cat. no. 12827, 1:1,000); anti-cleaved caspase-7 rabbit mAb (cat. no. 8438, 1:1,000); anti-poly(ADP-ribose) polymerase (PARP) rabbit mAb (cat. no. 9542, 1:1,000); anti-caspase-3 rabbit mAb (cat. no. 9665, 1:1,000); anti-cleaved caspase-3 rabbit mAb (cat. no. 9664, 1:1,000); and anti-α-tubulin rabbit mAb (cat. no. 2144, 1:1,000). Anti-rabbit IgG goat mAb (cat. no. 7074, 1:2,000; Cell Signaling Technology, Inc.) and anti-mouse IgG rabbit polyclonal antibody (cat. no. A-9044, 1:40,000; Sigma-Aldrich; Merck KGaA) conju-gated to horseradish peroxidase were used as the secondary antibodies. The relative protein levels were determined using densitometric scanning of immunoreactive bands using Quantity One software (version 4.6.9; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to α-tubulin levels.

Cell viability assay. BE(2)-C cells were cultured in 96-well plates and transfected with miRNA-3613-3p mimic or nega-tive control. After 48 h, the transfection with miRNA-3613-3p mimic or negative control was repeated as aforementioned. Cellular ATP levels were assessed using an ATPlite Luminescence ATP Detection assay system (PerkinElmer, Inc.), according to the manufacturer's protocol. Luminescence signals were determined using an Infinite M200 luminescence reader (Tecan Group, Ltd., Mannedorf, Switzerland). The experiment was performed three times.

Luciferase reporter assay. BE(2)-C cells were seeded in the 96-well plates and cultured in complete medium for 24 h. Next, the cells were co-transfected with 40 nM miRNA-3613-3p mimic or negative control, and 300 ng pmiRGlo plasmid vector with the cloned fragment of APAF1 3'UTR or empty pmiRGlo plasmid vector. Luciferase activity was assessed using the Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol 48 h after co-transfection. Determination of luminescence signals from firefly and *Renilla* luciferases was performed using an Infinite M200 luminescence reader. The experiment was performed three times.

Bioinformatics analysis. Visualization of the precursor form of the miRNA-3613-3p was generated using the Mfold algorithm (unafold.rna.albany.edu/?q=mfold/download-mfold). Additionally, miRNA-3613-3p was subjected to analysis using the miRPath version 3.0 web server algorithm (21). Biological processes potentially regulated by the miRNA were examined using microT-CDS (22). For the Kyoto Encyclopedia or Genes and Genomes (www.genome.jp/kegg) pathway enrichment analysis, P<0.05 was considered to indicate a statistically significant difference and the threshold for microT-CDS was 0.8. Furthermore, the predicted target genes of miRNA-3613-3p were determined using the miRWalk2.0 database (23), and the enriched functional patterns from the PANTHER (www.pantherdb.org/about.jsp) and Gene Ontology algorithms (geneontology.org) were analyzed. Furthermore, the visualization of miRNA-mRNA duplexes and calculation of free energies of each binding was performed using the RNAhybrid tool (24). In addition, the difference in the free energy of each base-pairing interaction between miRNA and mRNA was determined using the PITA algorithm (25). In order to determine the cross-species conservation of putative binding sites of miRNA-3613-3p, the TargetScan algorithm was used (26).

Statistical analysis. Data are presented as the mean ± standard error of the mean. All experiments were performed at least three times. Experiments were performed on Kelly cells transfected with an empty vector, wild-type or the mutated ribonuclease domain-deprived MCPIP1, BE(2)-C cells transfected with negative control or miRNA-3613-3p mimic and LAN-1 cells transfected with negative control or miRNA-3613-3p inhibitor. Pairwise t-tests were performed to compare the differences between the means of the NC vs. 3613-3p. For comparison of more than two means, one-way analysis of variance with Tukey's post hoc test implemented in Statistica (version 13; StatSoft, Inc., Tulsa, OK, USA) was performed. P<0.05 was considered to indicate a statistically significant difference.

Results

miRNA-3613-3p is differentially expressed in a range of human neuroblastoma cell lines. The expression levels of miRNA-3613-3p were determined in seven human neuroblastoma cell lines and a prostate cancer cell line PC3, which was used as a positive control, as the expression of miRNA-3613-3p in the PC3 cell line has been identified previously (27-29). In total, six of the neuroblastoma cell lines analyzed were characterized by *MYCN* amplification [Kelly, CHP-134, IMR-32,

LAN-1, LAN-5 and BE(2)-C], whereas SK-N-SH exhibited only two copies of the oncogene. The highest endogenous expression of miRNA-3613-3p was observed in LAN-1 cells, whereby the mean level of miRNA-3613-3p was increased ~6-fold compared with that in PC3 prostate cancer cells (Fig. 1A). In Kelly, IMR-32 and LAN-5 cell lines, the mean expression level of miRNA-3613-3p was only slightly higher (between 1.5- and 2-fold increased) compared with that in PC3 cells (Fig. 1A). In three human neuroblastoma cell lines, SK-N-SH, CHP-134 and BE(2)-C cells, the expression levels of miRNA-3613-3p were decreased compared with that in PC3 cells. The lowest mean expression of miRNA-3613-3p was observed in BE(2)-C cells, with a 60% decrease compared with PC3 cells.

In our previous study, it was identified that enforced expression of the ribonuclease MCPIP1 caused significant downregulation of miRNA-3616-3p in human neuroblastoma BE(2)-C cells (7). To investigate this observation further, the expression of miRNA-3613-3p was determined in another neuroblastoma cell line with high *MYCN* amplification, Kelly, with enforced MCPIP1 expression. The mean levels of miRNA-3613-3p were significantly decreased in cells with enforced MCPIP1 expression (50% decreased), compared with the control cells (Fig. 1B). However, in Kelly cells overexpressing the mutated form of MCPIP1 protein, deprived of ribonucleolytic activity, no significant differences were observed in miRNA-3613-3p expression (Fig. 1B). These results indicated that the precursor form of miRNA-3613-3p may be cleaved directly by the MCPIP1 protein. Visualization of the precursor form of miRNA-3613-3p uncovered a characteristic pyrimidine-purine-pyrimidine loop sequence (CAU) (Fig. 1C) that has been identified in all MCPIP1 target genes (30).

miRNA-3613-3p potentially regulates pathways involved in neuroblastoma pathogenesis. As the differential gene expression across the cell lines suggests a potential involvement of miRNA-3613-3p in neuroblastoma pathogenesis, miRNA-3613-3p was investigated by bioinformatics analysis using the DIANA TOOLS-miRPath algorithm (21) to determine its possible function in this process. KEGG pathway analysis revealed 44 pathways that may be regulated by miRNA-3613-3p (all P<0.05). Following a thorough review of the literature, 16 biological processes involved in growth, metastasis or chemoresistance of neuroblastoma associated with miRNA-3613-3p were identified (Fig. 2A). The most important and statistically significant signaling pathways were as follows: Hippo signaling pathway, ubiquitin-mediated proteolysis, Wnt signaling pathway, forkhead box O signaling pathway and transforming growth factor β (TGFβ) signaling pathway (Fig. 2A). Furthermore, miRNA-3613-3p was predicted to be involved in various cellular processes including proteoglycan turnover in cancer, tumorigenesis-associated transcriptional dysregulation and regulation of cellular signaling in cancer (Fig. 2A).

Additionally, the predicted targets of miRNA-3613-3p identified on the miRWalk2.0 database were investigated using bioinformatics functional analysis using Gene Ontology and PANTHER algorithms. The examination showed a substantial overlap with the aforementioned KEGG pathway analysis. According to the Gene Ontology algorithm, the majority of the putative miRNA-3613-3p target genes regulate
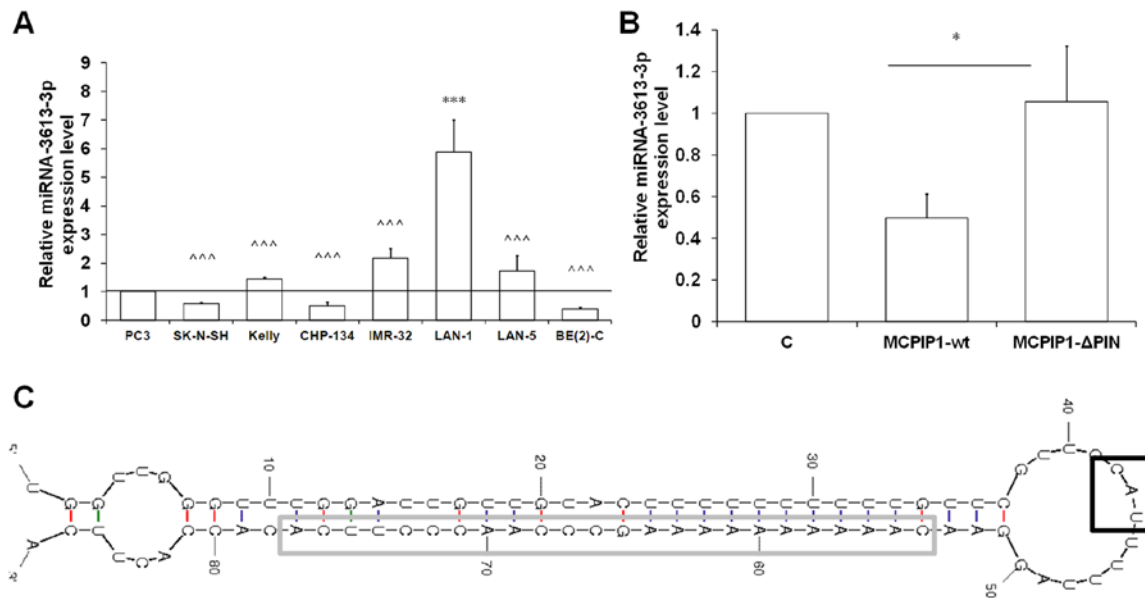
Figure 1. miRNA-3613-3p is differentially expressed in a range of human neuroblastoma cell lines. (A) miRNA-3613-3p expression levels in seven human neuroblastoma cell lines and PC3 prostate cancer cell line determined using RT-qPCR and U6 snRNA as the reference gene. (B) miRNA-3613-3p expression level in Kelly human neuroblastoma cells with enforced expression of the wild-type (MCPIP1-wt) or mutated (MCPIP1-ΔPIN) form of MCPIP1 protein assessed by RT-qPCR. U6 snRNA was used as the reference gene. Results are presented as the mean ± standard error of the mean. To compare more than two means, analysis of variance was performed [(A) F(1,7)=15.0046, P=0.000006; (B) F(1,2)=7.7228, P=0.0291]. (C) Pre-miRNA-3613-3p secondary structure. The CAU sequence is shown in-frame. *P<0.05 and ***P<0.001 vs. control; ^^^P<0.001 vs. LAN-1. miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; snRNA, small nuclear RNA; MCPIP1, monocyte chemoattractant protein-induced protein 1; C, control; MCPIP1-wt, wild-type MCPIP1; MCPIP1-ΔPIN, mutated ribonuclease domain-deprived MCPIP1.

tumorigenesis; however, they may also be involved in apoptosis and p53 pathway regulation (Fig. 2B). Bioinformatics functional analysis using PANTHER algorithm revealed a potential involvement of miRNA-3613-3p in the regulation of several signaling pathways important in neuroblastoma pathogenesis, such as the Wnt, TGFβ or protein kinase B (Akt) signaling pathways (Fig. 2B).

Bioinformatics analysis of predicted miRNA-3613-3p target genes. The identification of mRNAs that interact with a miRNA of interest usually starts by searching multiple databases for predicted miRNA target genes. The algorithms developed for prediction of miRNA-mRNA interactions take into account the miRNA complementarity to the 3'UTR of the transcript. However, certain algorithms additionally use conservation comparison, such as TargetScan or DIANA-microT, whereas others consider binding site accessibility (e.g. PITA and rna22) (31). In our previous study (7), eight putative target genes of miRNA-3613-3p were proposed. In the present study, miRNA-3613-3p was resubjected to thorough bioinformatics analysis using different algorithms and seven of the originally proposed predicted target genes were selected by identifying those that were present in all databases with high probability values, and are involved in neuroblastoma pathogenesis. The genes selected were as follows: *APAF1*, *DFFB*, *DICER*, *NF1*, *RORA*, *KIF3A* and *VHL*. Additionally, visualization of miRNA-mRNA duplexes and analysis of the free energy of their binding were performed. Putative binding sites with the free energy of the binding <-20 kcal/mol (1 kcal=4.184 kJ) were identified for all seven predicted target genes (Fig. 3A and B) (7). Furthermore, the free energies calculated for estimated binding between *DFFB* or *NF1*

transcripts and miRNA-3613-3p were <-25 kcal/mol (7). The visualization of complementarity between miRNA-3613-3p and the predicted binding sites at the target mRNA 3'UTRs allowed the differentiation of well-defined seed regions at the 5' end of miRNA-3613-3p for all the putative target genes with the exception of *DICER*. However, the fitting of the *DICER* transcript and miRNA-3613-3p contained a distinct 3'-supplementary site (Fig. 3A).

Another important factor of miRNA-mediated regulation of gene expression is binding site accessibility, which depends on the secondary structure of a target RNA (31). The difference in the free energy of the base-pairing interactions between miRNA-3613-3p and the mRNAs, and within the mRNA sequence itself, were calculated and are presented as the PITA score (Table I). The general consensus is that predicted targets with PITA scores <-10 kcal/mol are functional (genie.weizmann.ac.il/pubs/mir07/mir07_notes.html). Such differences in free energy of base pairing interactions between miRNAs and mRNAs, and within the target sequence were identified for *DFFB*, *NF1* and *RORA* (Table I). Although the effect of miRNA-mediated gene silencing depends on the miRNA concentration, even sites with PITA scores >-10 kcal/mol may be functional in cells with high expression of a certain miRNA (29). Therefore, it was decided not to exclude any of the predicted target genes from further experimental verification on the basis of the PITA score.

Enforced expression of miRNA-3613-3p causes a significant decrease in the expression of several predicted target genes. Human BE(2)-C neuroblastoma cells were transfected with miRNA-3613-3p mimic to increase the level of miRNA-3613-3p. This cell line was selected for transfection
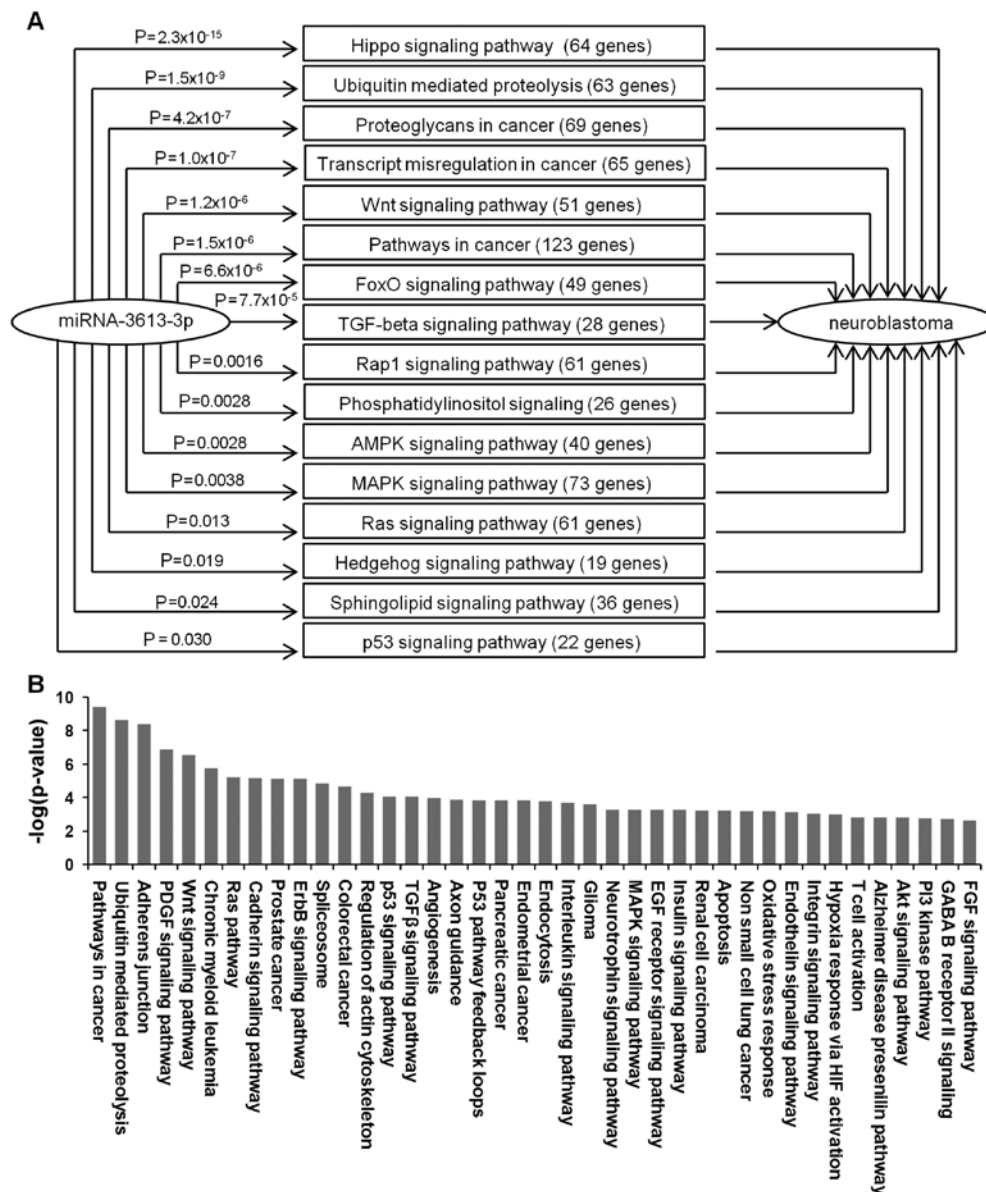
Figure 2. Bioinformatic analysis of the potential involvement of miRNA-3613-3p in neuroblastoma pathogenesis. (A) Potential Kyoto Encyclopedia of Genes and Genomes miRNA-3613-3p target pathways identified through literature research with involvement in neuroblastoma growth, metastasis or chemoresistance. (B) Bioinformatic functional analysis of the processes regulated by predicted miRNA-3613-3p target genes generated using Gene Ontology and the PANTHER algorithm. miRNA, microRNA; FoxO, forkhead box O; TGF, transforming growth factor; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase; FGF, fibroblast growth factor; GABA, γ-aminobutyric acid; PI3 kinase, phosphoinositide 3-kinase; Akt, protein kinase B; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

with miRNA mimic as it expressed the lowest endogenous level of miRNA-3613-3p (Fig. 1A). As miRNA-mediated gene silencing depends on the concentration of a certain miRNA in the cell (32), three different concentrations of the miRNA mimic were used for transfection (30, 40 and 50 nM). The mean level of enforced expression in BE(2)-C cells transfected with the 40 nM concentration of the mimic was increased ~6-fold compared with that in the cells transfected with miRNA mimic at the 30 nM concentration (Fig. 3C). Of note, the mean expression level of miRNA-3613-3p in the cells transfected with the highest concentration of the mimic (50 nM) was lower compared with cells transfected with the two other miRNA-3613-3p mimic concentrations (30 and 40 nM) (Fig. 3C). This phenomenon may have been caused by the toxic effect of high concentrations of mimic in the cells.

Therefore, the results obtained for the cells transfected with 50 nM mimic should be interpreted with caution.

The first approach to verify the aforementioned predicted target genes of miRNA-3613-3p was to assess their expression levels in BE(2)-C cells transfected with miRNA-3613-3p mimic. In cells transfected with the lowest concentration of mimic, a decrease in mRNA levels to 80% was observed only for *APAF1* compared with the control cells transfected with a scrambled oligonucleotide (Fig. 3D). The mean levels of expression of other putative target genes (*DICER*, *DFFB*, *VHL*, *KIF3A*, *RORA* and *NF1*) remained unchanged compared with the control cells (Fig. 3D). BE(2)-C cells transfected with 40 nM miRNA mimic were characterized by a significant downregulation in 4/7 predicted target genes, namely *DFFB*, *APAF1*, *VHL* and *NF1* to between 65 and 75% of
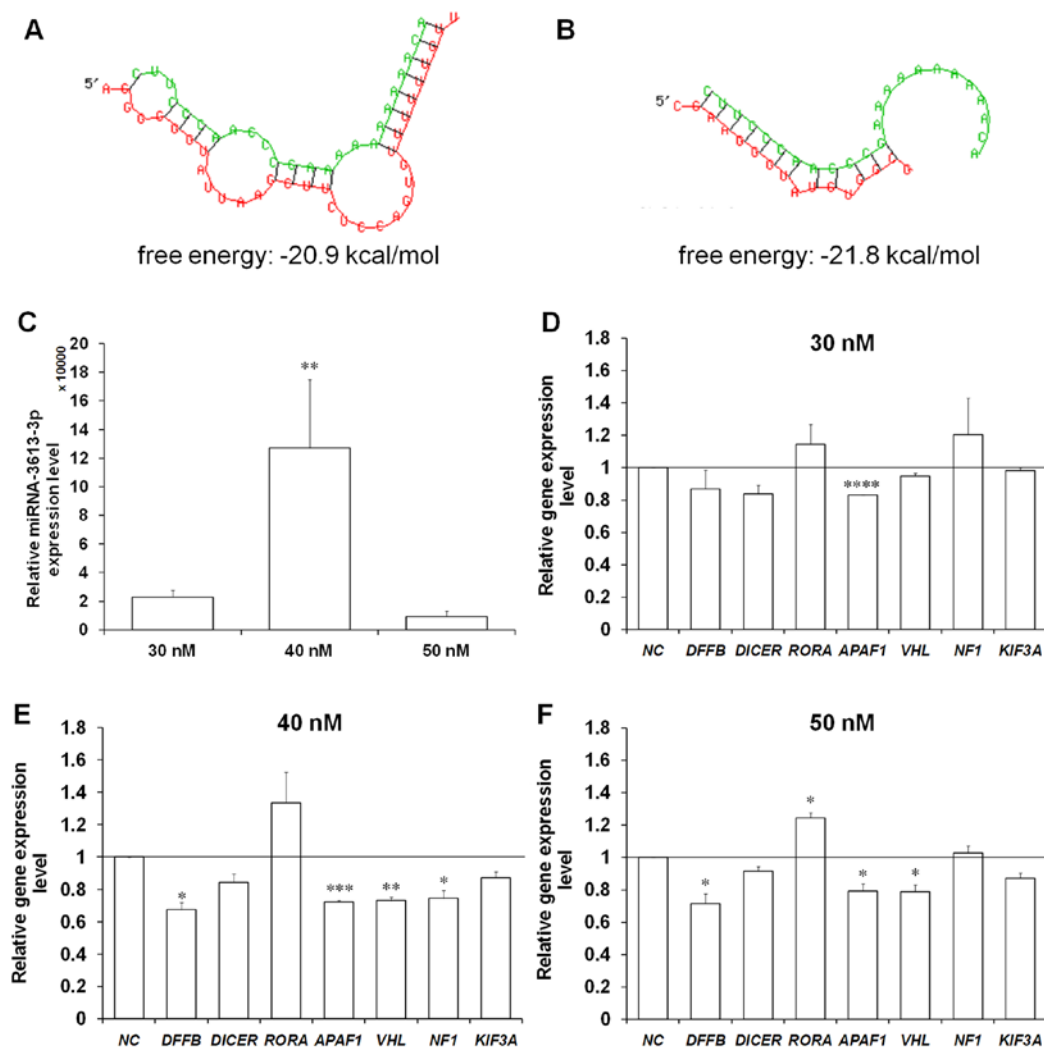
Figure 3. Enforced expression of miRNA-3613-3p causes a significant decrease in the expression of certain of its predicted target genes in the BE(2)-C human neuroblastoma cell line. The visualization of complementarity regions of miRNA-3613-3p and (A) *DICER* or (B) *APAF1* mRNA, and calculation of free energy of the binding. The miRNA sequence is indicated in green and the target mRNA sequence is indicated in red. (C) Expression levels of miRNA-3613-3p in the transfected cells measured using RT-qPCR with U6 small nuclear RNA used as the reference. Expression levels of seven predicted miRNA-3613-3p target genes assessed using RT-qPCR in BE(2)-C cells transfected with miRNA-3613-3p mimic at (D) 30, (E) 40 and (F) 50 nM. 40S ribosomal protein S13 was used as the reference gene. Results are presented as the mean ± standard error of the mean. To compare more than two means, analysis of variance was performed [(A) $F_{(1,3)}=9.36334$, $P=0.01111$]. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ vs. NC. miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control (scrambled oligonucleotide); *DFFB*, DNA fragmentation factor subunit β; RORA, retinoic acid-related orphan receptor α; *APAF1*, apoptotic protease-activating factor 1; *VHL*, von Hippel-Lindau protein; *NF1*, neurofibromin 1; *KIF3A*, kinesin family member 3A.

control levels (Fig. 3E). As the mean overexpression of the miRNA in cells transfected with 40 nM mimic was increased 6-fold compared with cells transfected with the 30 nM group (Fig. 3C), it was hypothesized that an increased level of enforced miRNA-3613-3p expression leads to more potent silencing of the putative target genes. Although the level of miRNA-3613-3p in the cells transfected with 50 nM miRNA mimic was decreased compared with the cells transfected with 30 nM miRNA mimic (Fig. 3C), a significant decrease in the mean expression levels of *DFFB*, *APAF1* and *VHL*, to 80% of the control, was observed (Fig. 3F).

To examine further the possible regulatory mechanisms by miRNA-3613-3p on the predicted target genes, the relative protein levels of two of the aforementioned seven putative targets were determined. *APAF1* was selected as its gene expression was significantly decreased in the cells transfected with miRNA mimic at all concentrations (Fig. 3D-F).

Furthermore, the relative protein levels of *DICER* in the transfected cells were determined, as it has a crucial function in miRNA biogenesis (33). Examination of the possible binding between miRNA-3613-3p and *APAF1* or *DICER* mRNAs uncovered putative binding sites with low free energies in the base pairing of the two transcripts (Fig. 3A and B). No significant alterations were identified in the protein levels of APAF1 and DICER in cells transfected with the mimic at 30 nM compared with in the control cells (Fig. 4A). Western blot analysis of APAF1 levels in the cells transfected with 40 nM miRNA-3613-3p mimic revealed that its signal was downregulated to between 70 and 80% of that of the control (Fig. 4B). Similarly, DICER protein expression was downregulated only in cells transfected with 40 nM mimic (Fig. 4B). These results confirmed that a higher concentration of miRNA-3613-3p in BE(2)-C neuroblastoma cells provided more potent silencing of the predicted target genes at the protein level.
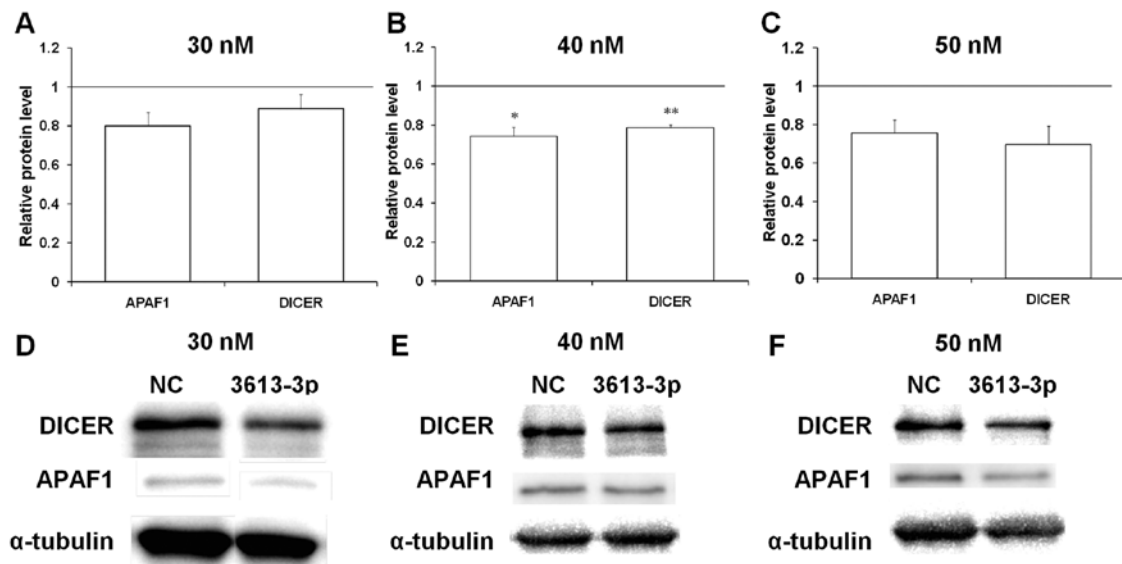
Figure 4. Enforced expression of miRNA-3613-3p causes a significant decrease in APAF1 and DICER protein levels in human neuroblastoma BE(2)-C cells. Relative APAF1 and DICER levels in BE(2)-C cells transfected with miRNA-3613-3p mimic at (A) 30, (B) 40 and (C) 50 nM were determined by western blotting. α-tubulin was used as the reference protein. Results are presented as the mean ± standard error of the mean. Representative images of DICER, APAF1 and the α-tubulin levels in cells transfected with miRNA-3613-3p mimic at (D) 30, (E) 40 nM and (F) 50 nM. *P<0.05 and **P<0.01 vs. NC. miRNA, microRNA; APAF1, apoptotic protease-activating factor 1; NC, negative control.
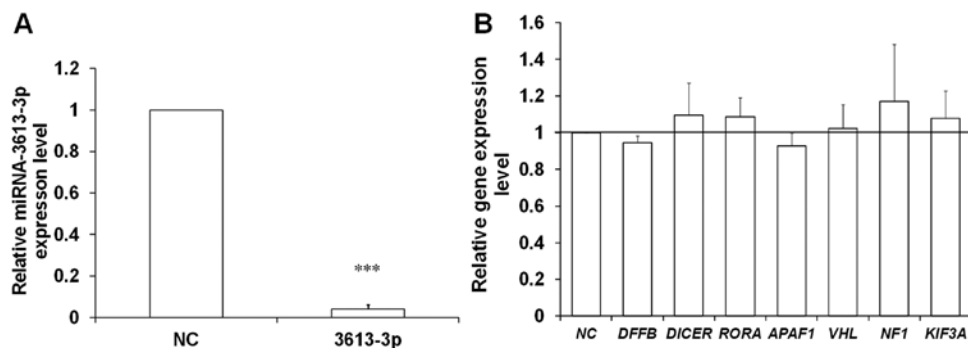

Figure 5. Downregulation of miRNA-3613-3p does not affect expression of predicted target genes of the miRNA investigated. (A) Relative miRNA-3613-3p expression levels in LAN-1 cells transfected with miRNA-3613-3p inhibitor determined using RT-qPCR with U6 small nuclear RNA used as the reference. (B) Expression levels of the predicted target genes of the miRNA investigated in LAN-1 cells transfected with miRNA-3613-3p inhibitor determined using RT-qPCR with 40S ribosomal protein S13 used as a reference gene (B). All experiments were performed three times in triplicate. Results are presented as the mean ± standard error of the mean. ***P<0.001 vs. NC. miRNA, microRNA; RT-qPCR, reverse transcription-polymerase chain reaction; NC, negative control; *DFFB*, DNA fragmentation factor subunit β; *RORA*, retinoic acid-related orphan receptor α; *APAF1*, apoptotic protease-activating factor 1; *VHL*, von Hippel-Lindau protein; *NF1*, neurofibromin 1; *KIF3A*, kinesin family member 3A.

On the basis of the aforementioned results, *APAF1* was selected as the most likely target gene of miRNA-3613-3p and subjected to further verification. The expression of this gene was decreased upon enforced expression of miRNA-3613-3p in all tested conditions at the mRNA and protein levels (Figs. 3D-F and 4).

Downregulation of miRNA-3613-3p in LAN-1 neuroblastoma cells does not affect the expression of putative target genes. Additionally, in order to obtain the downregulation of the miRNA investigated, LAN-1 neuroblastoma cells were transfected with miRNA-3613-3p inhibitor. This cell line was selected for the transfection with the inhibitors owing to its having the highest endogenous miRNA-3613-3p expression (Fig. 1A). The level of silencing achieved of the miRNA investigated was significantly potent, at ~7% of the control (Fig. 5A).

To examine further the effect of the altered miRNA-3613-3p levels on the putative target gene expression, the mRNA levels of all seven predicted target transcripts were assessed in LAN-1 cells transfected with the inhibitors. As expected, the expression of *DFFB*, *APAF1*, *VHL* and *NF1* genes was not decreased in this model. In addition, an increase in the expression of the putative miRNA-3613-3p target genes was not observed (Fig. 5B). However, the endogenous level of miRNA-3613-3p in LAN-1 neuroblastoma cells was four orders of magnitude lower compared with in BE(2)-C cells transfected with the mimic. Furthermore, the decrease in the predicted target genes in the BE(2)-C cells upon upregulation of the miRNA investigated was ~30% compared with the control (Fig. 3D-F). Therefore, the lack of enhanced putative miRNA-3613-3p target gene expression in LAN-1 cells transfected with the inhibitors was not unforeseen.
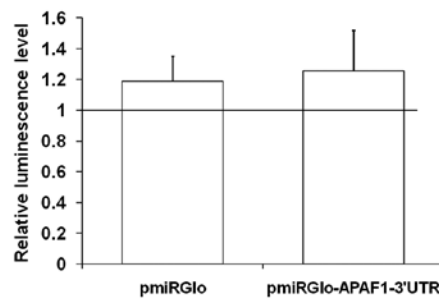
Figure 6. miRNA-3613-3p does not directly target predicted binding sites in the *APAF1* 3'UTR of BE(2)-C cells. (A) Putative binding sites for miRNA-3613-3p in the *APAF1* 3'UTR. (B) Relative luciferase activity was determined in BE(2)-C cells co-transfected with miRNA-3613-3p mimic or NC, and pmiRGlo or pmiRGlo-APAF1-3'UTR. Results are presented as the mean ± standard error of the mean. miRNA, microRNA; *APAF1*, apoptotic protease-activating factor 1; UTR, untranslated region; NC, negative control.
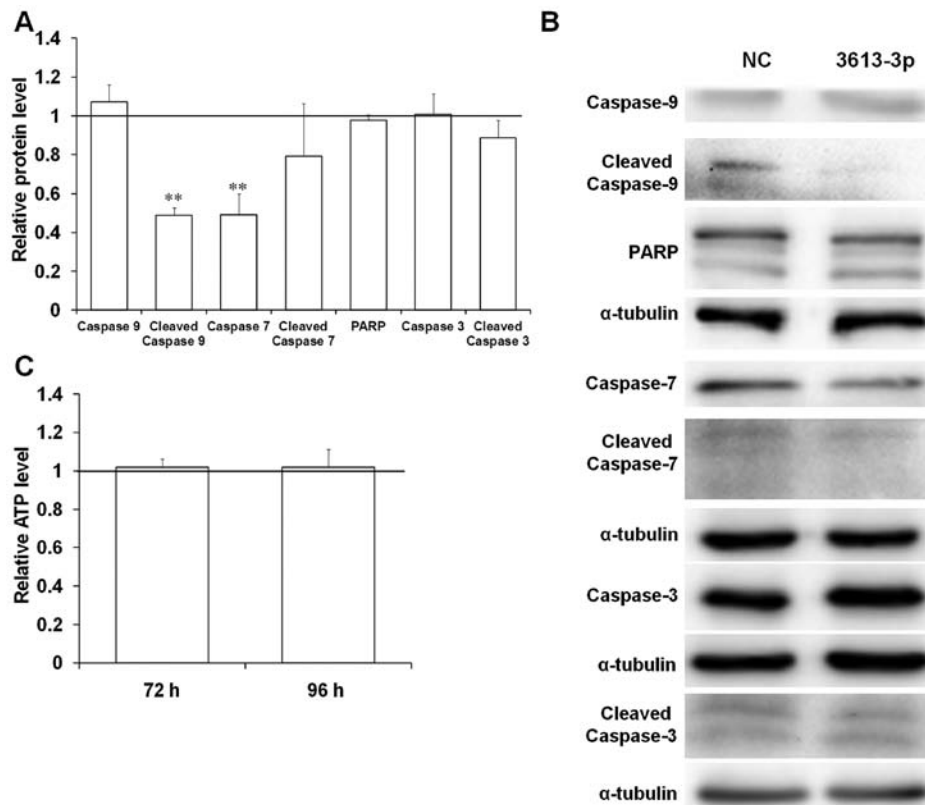

Figure 7. Enforced expression of miRNA-3613-3p causes a decrease in the expression or activation of selected proteins involved in apoptosis in the BE(2)-C human neuroblastoma cell line. (A) Relative levels of selected proteins involved in the apoptosis pathway were determined in BE(2)-C human neuroblastoma cells with miRNA-3613-3p overexpression after 96 h using western blot analysis. α-tubulin was used as the reference protein. (B) Representative images of caspase-9, cleaved caspase-9, caspase-7, cleaved caspase-7, PARP, caspase-3, cleaved caspase-3 and α-tubulin levels in BE(2)-C cells with miRNA-3613-3p overexpression after 96 h. (C) Relative ATP level in BE(2)-C cells with ectopic expression of miRNA-3613-3p after 72 and 96 h. Results are presented as the mean ± standard error of the mean. **P<0.01 vs. NC. miRNA, microRNA; PARP, poly(ADP-ribose) polymerase; NC negative control.

Putative binding sites for miRNA-3613-3p predicted in the APAF1 sequence are not functional. Three putative binding sites for miRNA-3613-3p were predicted in the *APAF1* 3'UTR (Fig. 6A). The free energies of the binding between miRNA-3613-3p and *APAF1* mRNA varied between -8.5 and -20 kcal/mol (Fig. 6A). Additionally, the differences between the free energies of the base-pairing interactions between miRNA-3613-3p and *APAF1* mRNA, and within the mRNA, were calculated using the PITA algorithm. The analysis revealed lower free energies of the miRNA-mRNA binding compared with base-pairing interactions within the mRNA sequence, although for binding site 1, the difference was minimal (Fig. 6A). Taking into account the fact that cross-species conservation is usually observed for functional miRNA-binding sites (31), the conservation of the three predicted binding sites was investigated. Bioinformatics analysis revealed that predicted binding sites 1 and 3, but not 2, are conserved across species (Fig. 6A).

For experimental verification of the interaction between the putative binding sites in the *APAF1* 3'UTR sequence and miRNA-3613-3p, luciferase reporter assays were performed. It was identified that miRNA-3613-3p did not affect the luciferase activity in cells transfected with the pmiRGlo vector containing the predicted binding sites of miRNA-3613-3p cloned from the *APAF1* 3'UTR (Fig. 6B). This result provides evidence for the lack of specific binding of miRNA-3613-3p to the predicted binding sites in the *APAF1* 3'UTR. Therefore, the observed downregulation of *APAF1* expression following miRNA-3613-3p overexpression (Figs. 3D-F and 4) may not have been caused by the direct interactions between the *APAF1* and miRNA-3613-3p sequences.

Enforced expression of miRNA-3613-3p affects the levels and activation of selected proteins involved in apoptosis in the BE(2)-C human neuroblastoma cell line. As the APAF1 protein serves a crucial function in the triggering of caspase cascades via the intracellular apoptotic pathway (34), the levels and activation of the important proteins involved in this process was investigated in cells with miRNA-3613-3p overexpression. The western blot analysis results revealed that the protein expression of PARP, caspase-3, cleaved caspase-3 and caspase-9 remained unchanged in cells transfected with miRNA mimic (Fig. 7A and B). However, the relative protein levels of caspase-7 significantly decreased to 50% of the control group upon miRNA-3613-3p overexpression (Fig. 7A and B). APAF1 protein facilitates proteolytic cleavage and activation of caspase-9 following the release of cytochrome *c* from mitochondria (34). As the cells transfected with miRNA-3613-3p mimic were characterized by a decrease in *APAF1* expression levels, it was expected that the activation of the caspase-9 protein would be affected. A significant downregulation in cleaved caspase-9 expression in the cells with miRNA-3613-3p overexpression to 50% of the control was observed (Fig. 7A and B). Furthermore, the level of inactive caspase-9 remained unchanged in cells with miRNA-3613-3p overexpression (Fig. 7A and B). Thus, miRNA-3613-3p may have disrupted caspase-9 activation via APAF1 downregulation.

Apoptosis activation in cells serves a crucial function in the regulation of cellular growth and survival. Measurement

Table I. Bioinformatics analysis of putative binding sites of target mRNAs.

Gene	No. of sites	PITA score
APAF1	55	-7.4
DFFB	53	-11.53
DICER	151	-7.78
NF1	247	-14.9
RORA	225	-11.88
KIF3A	127	-9.37
VHL	56	-9.16

The number of sites is the count of putative binding sites with a seed size ≥8 nucleotides long. The PITA score represents the overall effect of all binding sites combined together on the given UTR. The score is a $\Delta\Delta G$ value in kcal/mol between the base-pairing interactions within the mRNA and within the miRNA-mRNA duplex. *APAF1*, apoptotic protease-activating factor 1; *DFFB*, DNA fragmentation factor subunit β; *NF1*, neurofibromin 1; *RORA*, retinoic acid-related orphan receptor α; *KIF3A*, kinesin family member 3A; *VHL*, von Hippel-Lindau protein; miRNA, microRNA.

of cellular ATP levels is a sensitive and precise method for the assessment of cell viability (35). Therefore, the ATP content in the cells transfected with miRNA-3613-3p mimic was determined to investigate whether miRNA-3613-3p overexpression affects the viability of BE(2)-C cells. The transfection of 40 nM miRNA-3613-3p mimic did not significantly change the ATP level in cells after 72 and 96 h (Fig. 7C). This result indicates that miRNA-3613-3p overexpression does not affect the viability and growth of BE(2)-C cells.

Discussion

Patients with low- and intermediate-risk neuroblastoma may be successfully treated with surgical resection and neo-adjuvant chemotherapy. However, the treatment of high-risk neuroblastoma cases requires aggressive multimodal therapies, including chemotherapy, abscission of the tumor, radiotherapy, 13-*cis*-retinoic acid-induced cellular differentiation and immunotherapy with anti-GD2 ganglioside antibodies (1). Despite the application of the aforementioned treatment strategies, the event-free survival rate of patients with high-risk neuroblastoma remains 50% (3). Thus, there is a requirement for the development of novel therapeutic strategies.

miRNA molecules are considered a potential source of novel clinical tools in neuroblastoma (36,37). Differential miRNA expression in distinct subtypes of neuroblastoma as well as the development of miRNA microarrays has enabled the use of miRNA expression patterns as specific biomarkers for disease risk stratification (36). Furthermore, miRNA molecules are present in bodily fluids, including plasma, serum and saliva. Assessment of circulating miRNA expression patterns revealed significant differences between healthy individuals and patients with neuroblastoma (37). The lack of invasiveness of the mode of collection of circulating miRNA samples makes them a likely novel class of precise biomarkers (37). In addition, a number of individual miRNAs have been identified to function

as potent oncogenes or tumor suppressors, which provides opportunities for the development of miRNA-based therapies (5). One of the strategies is to restore the normal levels of tumor-suppressive miRNA using miRNA mimics, chemically modified oligonucleotides that have the sequence of the naturally occurring miRNA molecule (37). Targeted delivery of miRNA-34a mimic to neuroblastoma cells was demonstrated to inhibit tumor growth *in vivo* (38). Another potential strategy for miRNA-based therapies is silencing of specific oncogenic miRNA via antagomirs, chemically modified oligonucleotides that have complementary sequences to an endogenous miRNA (39). Injection of miRNA-17-5p antagomirs leads to complete neuroblastoma tumor regression *in vivo* in 30% of cases (40). With the increasing possibility of the clinical application of miRNAs in neuroblastoma treatment, it is important to understand the involvement of miRNA-dependent gene regulation networks in disease pathogenesis.

In our previous study, it was demonstrated that MCPIP1 ribonuclease overexpression significantly inhibits the growth and proliferation rate of BE(2)-C human neuroblastoma cells (41). Application of miRNA microarrays allowed for the delineation of miRNA expression patterns in BE(2)-C cells following MCPIP1 overexpression. Of note, the most significantly downregulated miRNA upon MCPIP1 overexpression was a novel miRNA, miRNA-3613-3p (7), which, to the best of our knowledge, had not been previously investigated in neuroblastoma. Deregulation of miRNA-3613-3p expression in Kelly and BE(2)-C cells with MCPIP1 overexpression, characterized by inhibition of pro-proliferative pathways, suggests its involvement in the regulation of neuroblastoma cell biology. Additionally, it was identified that the overexpression of MCPIP1 protein causes the downregulation of the most potent oncogene in neuroblastoma, *MYCN*, in two highly tumorigenic cell lines, BE(2)-C and Kelly, as well as inhibition of the Akt/mammalian target of rapamycin signaling pathway (17). Of note, the PANTHER algorithm revealed the potential involvement of miRNA-3613-3p in the regulation of the Akt signaling pathway. Further evidence of the involvement of miRNA-3613-3p in neuroblastoma cell biology is the differential expression of miRNA-3613-3p demonstrated in our study in a variety of human neuroblastoma cell lines. Of note, the cell lines characterized by increased miRNA-3613-3p expression (LAN-1, LAN-5, IMR-32 and Kelly), compared with the PC3 cell line, are N-type neuroblastoma cells (42). They adhere poorly to the cell culture plate and exhibit a tendency to aggregate. Furthermore, the cell lines with the lowest endogenous expression of miRNA-3613-3p [BE(2)-C and CHP-134] are I-type neuroblastoma cells, identified by marked adhesion to the cell culture plate and extensive migration (43,44). Thus, there may be an association between the expression level of miRNA-3613-3p and neuroblastoma cell phenotype. Comparison of the expression of the miRNA investigated in neuroblastoma cell lines and the normal cells of the same origin would be worthwhile and may shed more light on the possible oncogenic function of miRNA-3613-3p in the pathogenesis of this type of cancer. Neuroblastoma is an embryonal tumor that arises from the sympathoadrenal cells in the neural crest of the embryo. Owing to technical difficulties and ethical considerations, human embryonic neural crest cells are not available for research purposes. Deriving neural

crest cells from human induced pluripotent stem cells appears to be a promising alternative and is currently being developed by several research groups (45).

An extensive search of the databases and analysis using various bioinformatics software allowed us to select seven putative target genes of miRNA-3613-3p. Of note, all the predicted target genes that were downregulated in cells with miRNA-3613-3p overexpression (*APAF1*, *DFFB*, *DICER*, *VHL* and *NF1*) exhibit a tumor-suppressor potential. *APAF1* encodes a protein crucial for the activation of the caspase cascade in the programmed cell death intracellular pathway (34). *DFFB*, another potential miRNA-3613-3p target gene, encodes a nuclease involved in DNA fragmentation during apoptosis (46). As one of the hallmarks of cancer is resistance to programmed cell death, pro-apoptotic proteins exhibit tumor-suppressive potential in numerous types of cancer (47). Additionally, APAF1 was identified to be a suppressor of another type of tumor of neuroectodermal origin: Melanoma (48). Furthermore, *DICER*, a putative target gene of miRNA-3613-3p, encodes a key ribonuclease involved in miRNA biogenesis, which supports the finding of global miRNA downregulation in the pathogenesis of neuroblastoma (5). Furthermore, a low expression of this gene was identified to be a prognostic factor for stage 4 neuroblastoma (33). *VHL* serves a function in neuronal cell differentiation (49). In addition, the expression level of this gene could serve as a biomarker in neuroblastoma, and its downregulation points to a high-risk subtype of the disease (50). *NF1* encodes a negative regulator of the mitogen-activated protein kinase signaling pathway and is an important prognostic factor of retinoic acid therapy outcome (51). Additionally, BE(2)-C cells with MCPIP1 protein overexpression and miRNA-3613-3p downregulation are characterized by significant increases in *APAF1* and *DFFB* levels at the transcriptional and translational levels (7). These results support the data from the present study that demonstrated the downregulation of the expression of the aforementioned genes in BE(2)-C cells transfected with miRNA-3613-3p mimic, and may indicate an oncogenic function of miRNA-3613-3p. However, owing to high heterogeneity of neuroblastoma cell lines, it may be difficult to draw general conclusions concerning the function of the gene in the pathogenesis of this type of cancer. Silencing of the miRNA investigated in another neuroblastoma cell line, LAN-1, did not lead to an increase in expression of any of the seven predicted target genes. This may suggest that the action of miRNA-3613-3p depends strongly on the cellular context.

Of the seven putative target genes of miRNA-3613-3p, the downregulation of *APAF1* in cells with miRNA-3613-3p overexpression was the most significant compared with control cells. Despite the presence of the three putative binding sites for miRNA-3613-3p at the 3'UTR of *APAF1* mRNA, the reporter gene assay did not signal an interaction between the predicted sequences in the transcript and miRNA-3613-3p. Nevertheless, the *APAF1* gene may be regulated by miRNA-3613-3p through binding sites at the 5'UTR or coding sequence in the transcript. Alternatively, the downregulation of the gene in cells with miRNA-3613-3p overexpression may be the result of miRNA-3613-3p targeting an activator of *APAF1*, subsequently downregulating *APAF1* expression indirectly.

The protein product of the *APAF1* gene serves a key function in the activation of the caspase cascade in the intracellular apoptotic pathway. Following release from the mitochondria, cytochrome *c* forms a complex with the APAF1 oligomer. This interaction allows caspase-9 to be cleaved and activated, which consequently leads to the proteolytic activation of executive caspases, such as caspases-7 and -3, resulting in apoptosis (34). Analysis of the expression and activation of several proteins involved in programmed cell death in cells transfected with the miRNA-3613-3p mimic produced notable results. A significant decrease was observed in the level of activated cleaved caspase-9 in human BE(2)-C neuroblastoma cells. This is in accordance with the aforementioned decrease in the expression of the activator of this caspase, APAF1 protein, in cells transfected with the miRNA-3613-3p mimic. Inhibition of caspase-9 proteolysis in cells with ectopic miRNA-3613-3p expression may limit the possibility of activating the apoptosis process. Furthermore, in cells with miRNA-3613-3p overexpression, no alterations in the levels and activation of the executive caspase-3 and PARP protein, responsible for DNA fragmentation during the last phase of the apoptosis process (52), were identified. This confirms further a lack of activation of the programmed cell death process following miRNA-3613-3p overexpression in human BE(2)-C neuroblastoma cells.

In conclusion, the results of the present study identified that miRNA-3613-3p may directly or indirectly regulate the expression of several genes with tumor suppressor potential (*APAF1*, *DFFB*, *NF1*, *VHL* and *DICER*) in human neuroblastoma cells. The most likely target gene of miRNA-3613-3p appears to be *APAF1*; however, this interaction might not be direct. Transfection with miRNA mimic did not result in the activation of BE(2)-C cell apoptosis after 96 h. However, it may inhibit this process by lowering caspase-9 proteolysis via downregulation of APAF1 protein. Additionally, it was identified that enforced expression of miRNA-3613-3p does not affect the viability of BE(2)-C cells. The results obtained indicate a possible tumor-promoting function of miRNA-3613-3p in BE(2)-C neuroblastoma cells.

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Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Authors' contributions

IN, EB and MD performed experiments and acquired data; IN interpreted the data; IN designed experiments and drafted the manuscript; HR and IH edited the manuscript. All authors approved the final content for journal submission and publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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