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Original article

Identification of a Guanine-Rich-Oligonucleotides (GRO) in the Promoter of the MYCN Gene which Inhibits Neuroblastoma Growth

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Abstract

Neuroblastoma is the most common solid tumor of childhood malignancy. The biological properties vary from indolent to aggressive depending on how different genes play their role in transforming neural crest cells leading to spontaneous regression to an unfavorable outcome. Guanine-Rich Oligonucleotides (GRO) are being tested as a potential target for an anti-cancer drug. In this study, we have identified GROs at the promoter regions of the MYCN oncogene (MYCN-15) and it formed quadruplex in-vitro shown by circular dichroism (CD). Then three neuroblastoma cell lines were used to explore the effect of MYCN-15 on different biological properties: cellular growth, differentiation, and death. The neuroblastoma cell lines SH-SY5Y and SK-N-AS have a single copy of the MYCN gene, whereas the SK-N-BE2 cell line has multiple copies (n-Myc amplified). MYCN-15-induced cellular differentiation in both SK-N-AS and SH-SY5Y cells was followed by cell death in the SH-SY5Y cell line only. MTT [3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide] assay revealed, that there was modest growth inhibition in both SH-SY5Y (~58%) and SK-N-BE2 (~38%) cells when treated with MYCN-15 oligonucleotide. There were no significant changes in survivin protein expression in both SK-N-AS and SH-SY5Y cells when treated with MYCN-15 oligonucleotides. Taken together, MYCN-15 can be used as an essential target oligonucleotide for treating human neuroblastoma.

Keywords: ***Guanine-Rich Oligonucleotides (GRO), Quadruplex, Neuroblastoma, MYCN, surviving***

Introduction

The neural crest-derived tumor, neuroblastoma accounts for 7 to 10% of all childhood cancers worldwide. Induction of neuronal differentiation has been considered a novel strategy to treat a certain group of neuroblastomata with aggressive behavior [1]. In aggressive cases of neuroblastoma, the survival rate is 40%. Normally, the neural crest cells are involved in the development of the nervous system, but if there is a change in biology (mutation of the gene/genes, abnormal expression of proteins), a consequence that results would be neuroblastoma [2]. Clinically, the symptoms and signs of this cancer vary from person to person. The symptoms usually appear from an early age, even at birth, and there is a high frequency of the spreading of the disease at diagnosis. There are certain subsets of neuroblastoma that tend to spontaneous resolution in infancy. For example, some stage 4S cases (spread only in the bone marrow) diagnosed with neuroblastoma at birth, disappear after a few months of birth [3]. A wide variety of genetic alterations are associated with different stages of neuroblastoma, including but not limited to, Alk, TrkA, DBNF, GDNF, MYCN, and MYCN. High expression of MYCN and amplification of the MYCN gene are associated with unfavorable groups of neuroblastoma [4]. The low expression of MYCN and the single copy of the MYCN gene are associated with a favorable outcome of the disease [5]. Neuronal differentiation refers to the spontaneous maturation of cancer cells. The exact mechanism of certain subsets of neuroblastoma regression is yet to be known in detail, but certain evidence suggests that self-regression happens through the process of differentiation of the cancer cells. The role of the deprivation of the growth factor (neurotrophic growth factor), loss of telomerase, and some other genetics (DNA change) and epigenetics (change of the phosphate group of the DNA) changes have also been proposed [6].

Normally, DNA forms a double helix, which is made up of two polynucleotide strands that spiral around an imaginary axis. Throughout the genome, there are a lot of GC (Guanine and Cytosine) rich regions, especially in the promoter region of the genes and telomeres (end of the chromosome) [7]. These oligonucleotides of GC-rich regions often naturally form a quadruplex (four-stranded) rather than a double helix in a three-dimensional structure. The ability of the oligonucleotides for encoding genomic G-quadruplex forming sequences has been shown in the silencing of the genes that regulate cancer formation and cellular maturation/differentiation [8].

The translation of the nucleotide sequence to the amino acid sequence is handled by the cell through means of the ribosomes [9]. n-Myc and IAP (Inhibitor of Apoptosis Protein) are genes that regulate genetic expression, synthesize proteins (MCYN and

MYCN protein respectively), and play an important role in the transformation of neuroblastoma [10].

The MYC family gene expression is mostly associated with the proliferation of cell populations. N-Myc was discovered as a proto-oncogene, but due to genetic alteration, this gene transforms into an oncogene. A part of the Myc family of proto-oncogenes, N-Myc is essential for neurogenesis and thus plays an important role in the development of the nervous system. N-Myc proteins (MYCN) are included in cellular processes such as neurogenesis, cell growth, apoptosis, differentiation, and the repression of genes that block cellular proliferation [11]. It also stimulates the expression of genes that control cell size and growth, such as those that encode ribosomal proteins, translation factors, and metabolic enzymes [12]. Amplification of the MCYN gene is considered a bad prognosis in human neuroblastoma [13].

Survivin is a member of the IAP (Inhibitor of Apoptosis Protein) family genes, expressed in most cancer cells but not in normal cells. It is highly expressed in the S and G2M phases of the cell cycle [14]. Its expression levels correlate with more aggressive disease and poor clinical outcomes there is a high level of protein expression in unfavorable groups (Stage 3 & 4) of neuroblastoma and a low level of expression in favorable groups (Stage 1, 2 & 4S) [3].

This experiment wishes to investigate the effect of quadruplex forming DNA/Oligonucleotides at the promoter region of the MYCN gene on childhood common cancer neuroblastoma. It is asked if the Guanine-Rich Oligonucleotide (GRO) sequence at the promoter regions of oncogenes, MYCN Oligonucleotide, forms a quadruple helix rather than a double helix and whether there is a response in neuronal differentiation and growth.

Materials and Methods

Identification of Quadruplex:

The QGRS program from *http://bioinformatics.ramapo.edu/QGRS/analyze.php* web page was used. The promoter region of the MYCN gene (Homo sapiens MYCN proto-oncogene, bHLH transcription factor (MYCN), transcript variant 1, NCBI Reference Sequence: NM_001293228.2) was identified and copied into the QGRS program to identify several putative G-rich oligonucleotides (G-quadruplex) and one sequence with a high probability (high G score) was chosen to perform our experiment. The oligonucleotides extrapolated from the MYCN gene promoter region named MYCN-15. An artificially mutated version of the oligos, mMYCN-15, was used as an internal control.

Circular Dichroism Spectroscopy:

MYCN-15 and mMYCN-15 oligonucleotides were annealed by boiling for 5 minutes, adding a physiological buffer to final concentrations of 20 mmol/L KH_2PO_4 dibasic, 120 mmol/L KCl, 5 mmol/L $MgCl_2$, and slowly cooled down to room temperature. Annealed ODNs were dissolved in a physiologic buffer at a concentration giving an absorbance at 260 nm of 0.8 (about 5 µmol/L). Structures of oligonucleotides in solution were determined by circular dichroism (CD) spectroscopy. Spectra were recorded on a Jasco-810 spectropolarimeter (Jasco), using a quartz cell of 1 mm optical path length, an instrument scanning speed of 200 nm/min, and a response time of 2 seconds, and over a wavelength range of 340 to 220 nm. The spectra were representative of 3 average scans taken at 25°C and were baseline corrected signal contributions due to buffer.

Cell Culture:

The human neuroblastoma cell line SH-SY5Y (CRL-2266), SK-N-AS, SK-N-SH, and SK-N-BE2 were obtained from ATCC (Manassas, VA, United States) and maintained in DMEM supplemented with 10% FBS, Penicillin Streptomycin (100 U/ml; 100 mg/ml), and 2mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO2/95% air. The semi-confluence culture was washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin-EDTA solution, reseeded as 1 × 105 cells/ml of DMEM containing 10% FBS or 1% FBS or 1% BSA, and used for differentiation experiments after overnight incubation. neuroblastoma cells were incubated with the absence or presence of G-quadruplex Oligonucleotides for the appropriate duration of the experiment.

Cell Differentiation and/or Growth Inhibition Imaging:

Neuroblastoma cells SH-SY5Y and SK-N-SH cells were plated at 300 cells per 60 mm tissue culture dish. The following day, different concentrations of MYCN-15 (0, 2.5, 5, 7.5, and 10 µM) were added directly to the media. The cells were incubated in the presence of the oligonucleotides for 5 days. Cells will then be harvested and the dead cells and live cells with trypan blue exclusion were counted every day by light microscopy. Before counting, the cells were photographed for neurite extension and flattening of cells, which represented the cellular differentiation. Each experiment was repeated, and the data was validated.

Western Blot Analysis:

After being treated with indicated concentrations of MYCN-15 oligo or mMYCN-15 for 4 days, the cells were harvested by scraping with media, then pelletized and washed twice with PBS. Then, the cells were exposed to RIPA buffer (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.251% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS): Thermo Fisher Scientific, United States) supplemented with protease and phosphatase inhibitors cocktail (Thermo Fisher Scientific, United States) and incubated for 30 min on ice. The lysates were centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatants were collected as total cell lysate. Protein concentration was determined by the BCA method (Kit). Proteins (30 µg) were separated into 6–12% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Bellerica, MA, United States). The membrane was washed with Tris-buffered saline (TBS; 10 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.5% (v/v) Tween 20 (TBST) followed by blocking with 5% (v/v) non-fat dried milk solution prepared in TBST and then incubated overnight with primary antibodies at 4°C. The antibodies used were survivin and β-actin. After this, the membrane was exposed to secondary antibodies conjugated to horseradish peroxidase for 2~3 h at room temperature and further washed three times with TBST. Immunoreactivity was detected by the luminol-based chemiluminescence (ECL) system. Equal protein loading was assessed by the expression level of β-actin. The densitometric analysis was performed using ImageJ (National Institute of Health, Bethesda, MD, United States) software.

MTT Analysis:

The cells were seeded into 96-well plates (Corning, Lowell, MA, USA) at 5000 Cells/well. Twenty-four hours later, the cells were seeded in different concentrations of MYCN-15 and a control oligo mMYCN-15 dissolved in water was added to the medium directly for an additional 96 hours. MTT dye (5 mg/mL, Sigma, Saint Louis, MO, USA) was added to each well. After four-hour addition of MTT reagent cells were lysed (10% SDS in 0.1N HCl) by a lysis buffer and the optical density was measured at 570 nm on a multi-well plate reader. Background absorbance of the medium in the absence of cells were subtracted. All samples were assayed in triplicate, and the mean for each experiment was calculated. Results were expressed as a percentage of control, which was considered to be 100%.

Statistical Analysis:

Data was expressed as mean ± standard error of the mean (SEM). The significance level of treatment effects was determined by MTT assay using a Coefficient-one-tailed test followed by the student's t-test.

Results

1. Identification of G-quadruplex in MYCN gene promoter:
Quadfinder:(*http://bioinformatics.ramapo.edu/QGRS/index.php*)
As shown in Figure #1, there are many G-quadruplex sequences present in the MYCN promoter region. The sequence with the highest G-Score was selected to investigate in the experiment (MYCN-15). For the controls, G-nucleotides were randomly selected, replacing them with non-Guanine substitutes (Cytosine, Adenine, or Thymine), and named mMYCN-15.

Selected G-quadruplex sequence for experiments

1. MYCN15: 5'- GGGC GGGA Quad finder GGG -3'
2. mMYCN15: 5'- TTTC CCCA AAGA CGA -3'

Fig. 1: The promoter of MYCN gene run into the QGRS program and the putative G-rich oligonucleotides (G-quadruplex) were recommended with the G-score.

2. MYCN-15 nucleotide sequence but not mutant mMYCN-15 forms parallel Guanine-Quadruplex.
Next, it was investigated whether these selected oligonucleotide forms the Quadruplex conformation in aqueous solution. The nucleotide sequences were run through a circular dichroism (CD) Spectroscopy. To characterize the secondary structure of MYCN-15 and mMYCN-15, ODNs (Optical Density Nucleotides) were analyzed by CD spectroscopy. MYCN-15 formed a quadruplex in physiological buffer, represented by a peak absorbance at 260 nm and a trough absorbance at 240 nm (Fig. 2). The mutant mMYCN-15 sequence did not form a quadruplex in solution as they did not form a peak at 260 nm.

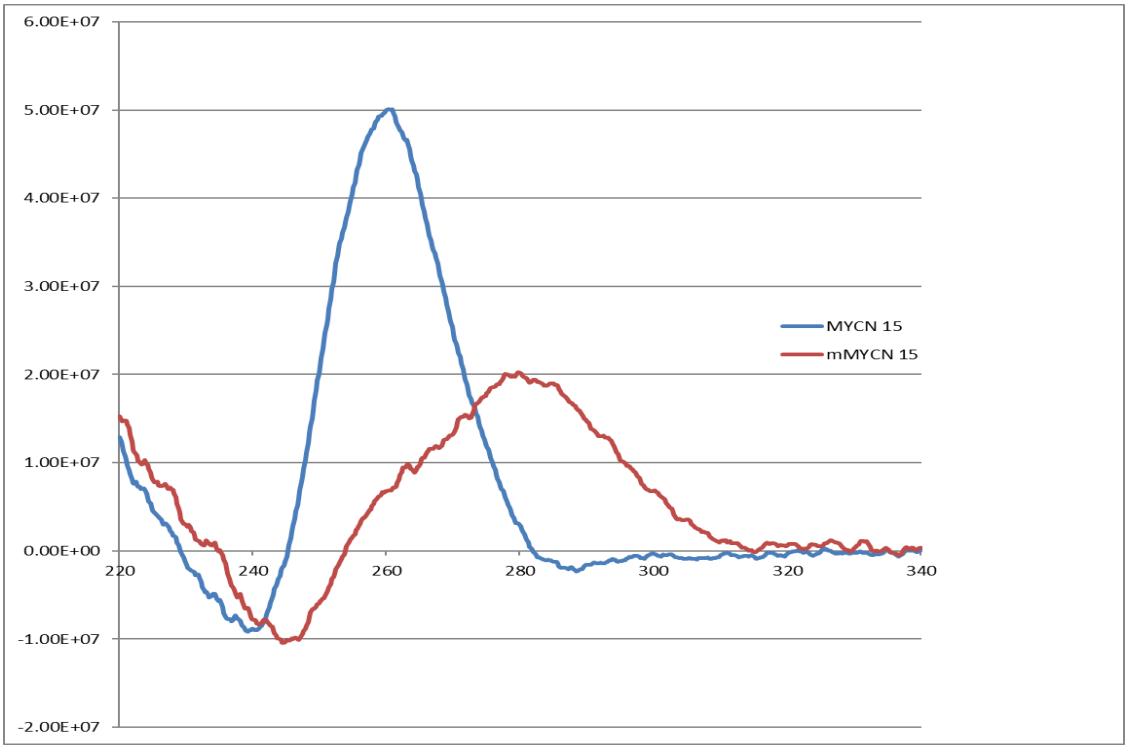

Fig. 2: Circular Dichroism (CD) Spectroscopy: MYCN-15, not the mMYCN-15 oligonucleotides represented by a peak absorbance at 260 nm forms a quadruplex in solution.

3. Quadruplex forming oligo MYCN-15 induces cell differentiation in human neuroblastoma.
The effect of the quadruplex forming oligonucleotides on different biological properties of neuroblastoma cell line SK-N-SH and SH-SY5Y were tested. SK-N-SH is a neuroblastoma cell line that displays predominantly epithelial morphology and grows in adherent culture. (Fig. 3). Treatment with 13 Cis-retinoic acid used as a control, caused these cells to differentiate and adopt a neuronal phenotype (data not shown), characterized by flattening of cells. SH-SY5Y cells are derived from SK-N-SH neuroblastoma cells and are neuronal subtype.

Oligo MYCN-15 induced cellular differentiation by flattening the cells (Fig. 3). 13-cis Retinoic Acid (data not shown) was used as a positive control. SH-SY5Y cells, which are derived from SK-N-SH and retain only neuronal properties, induce differentiation by extending their neurites (not shown) followed by decrease in number when treated with 10 μM of MYCN-15 oligonucleotides (Fig. 3) for 5 days.

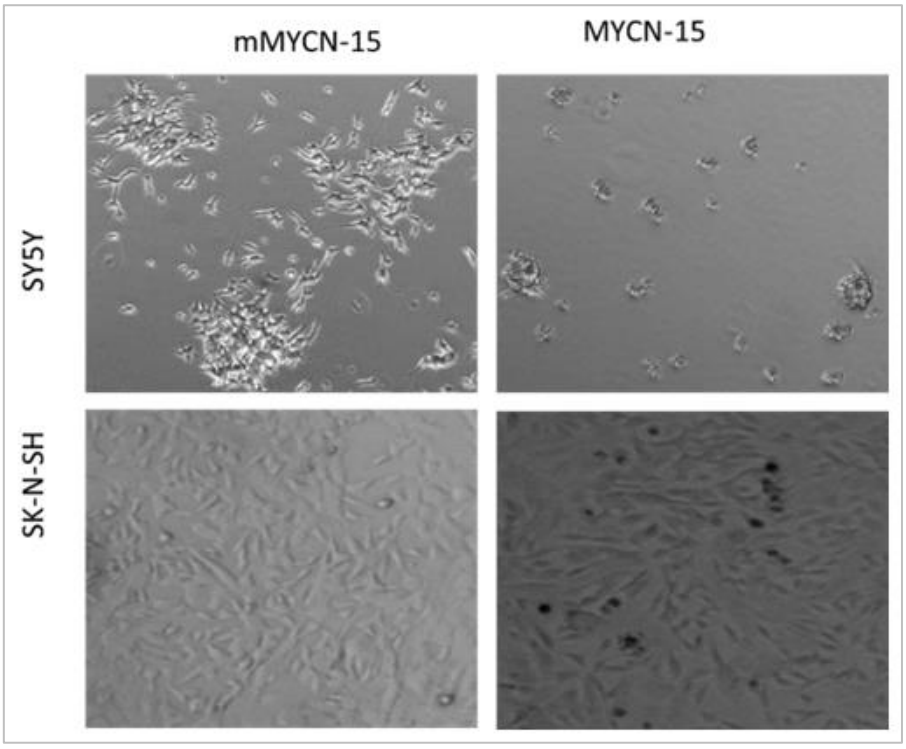

Fig. 3: SH-SY5Y and SK-N-SH neuroblastoma cells were treated with 10 μM of MYCN-15 oligonucleotides for 5 days. SH-SY5Y cells extended their neurites followed by cell death (Upper-right), and SK-N-SH neuroblastoma cells became flattening (Lower-right).

4. MTT assay revealed MYCN-15 oligonucleotide are sensitive to neuroblastoma cells.

To substantiate the above morphological changes observed in neuroblastoma cells by MYCN-15 an, the potential growth inhibition by MTT assay was investigated. The MTT assay represents the mitochondrial activity of the cells that is corresponding to the number of cells eventually indicates the growth of the cellular population. SH-SY5Y neuroblastoma cells have one copy of the MYCN gene and favorable biology, on the other hand, SK-N-BE2 neuroblastoma cell has multiple copies of MYCN gene (n-MYC amplified) and carries unfavorable biology [15]. MYCN-15 oligonucleotides had limited growth inhibition in both SH-SY5Y, and SK-N-BE2 cells, (~55% vs ~40%) (Fig. 4) when treated for 5 days. This data indicated that MYCN-15 oligonucleotides inhibited cellular growth both in the SH-SY5Y, and SK-N-BE2 neuroblastoma cell lines.

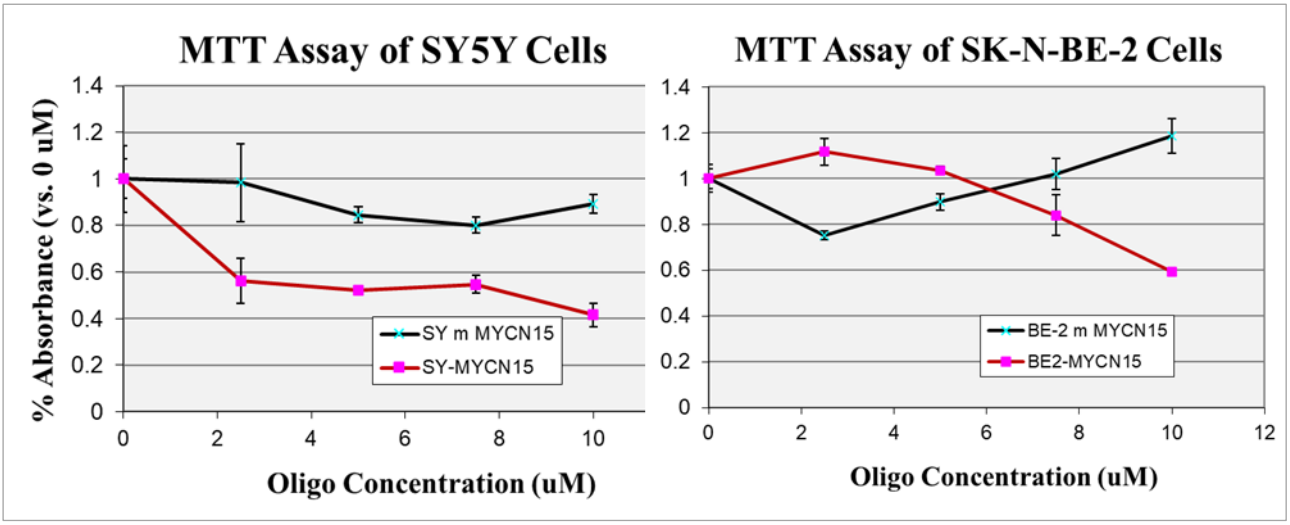

Fig. 4: MTT assay of SH-SY5Y, and SK-N-BE2 neuroblastoma cells. The cells were plated into 96-well plates, treated with different concentrations of oligonucleotides and assay was done on day 5. The cell sensitivity exerted by MYCN-15 and mMYCN-15 oligonucleotides in SH-SY5Y (left), and in SK-N-BE2 (right) cells, were shown.

5. The expression of survivin protein is not affected by MYCN-15 oligonucleotides

Multiple levels of evidence suggested that the G-quadruplex in a given gene's promoter region inhibits the transcription machinery by strand invasion leading to downregulation of protein expression of that gene. So, next it was investigated whether MYCN-15 oligonucleotide has the ability to down-regulate the survivin gene expression. Figure 5, showed the bands of protein expression in SK-N-AS cells treated with MYCN15, mMYCN15, SVN-23, and mSVN-23 oligonucleotides. The first row of bands represents survivin expression while the second row of bands represents β-Actin expression (control). The relative expression of survivin in SK-N-AS cells is shown in Figure 5 (left). The were no significant changes of relative survivin expression in cells treated with MYCN-15 and mMYCN oligonucleotides both in SK-N-AS and SH-SY5Y cells However, when treated with SVN-23 and mSVN-23 oligonucleotides (positive control), the expression of survivin was down regulated significantly in both neuroblastoma cell line.

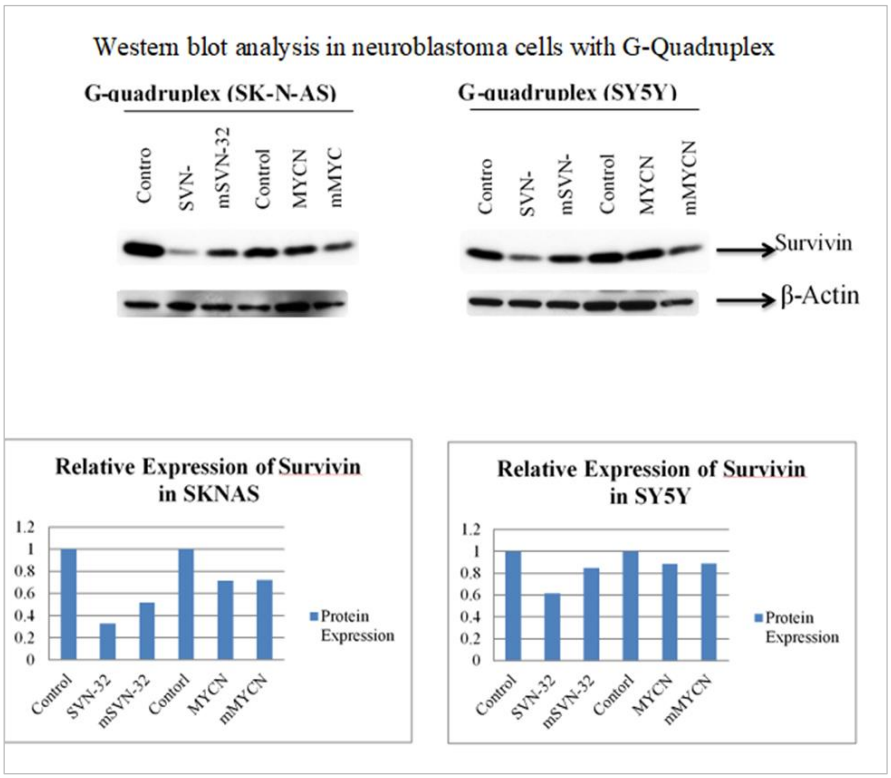

Fig. 5: SK-N-AS and SH-SY5Y neuroblastoma cells were treated with 10μM of oligonucleotides and subjected to western blotting with antibody against survivin and β-actin. Survivin expression was downregulated when treated with SVN-32 oligonucleotide (positive control) but no significant changes of survivin expression when treated with MYCN-15 oligos in both cell lines. Relative survivin protein expression normalized with β-actin was shown.

Discussion and Conclusion

Purpose of Experiment: G-rich Quadruplex forming DNA sequence are present throughout the genome specially telomeric region and promoter region of certain genes. The quadruplex forming sequence in the promoter region of certain oncogene has also been shown slowing the growth of several cancer cells [17]. The purpose of this experiment is whether the G-quadruplex forming sequences in the promoter regions of the MYCN gene can be used to target the unfavorable biological groups of embryonal childhood tumor, neuroblastoma [18]. Certainly, our results in this study rationalize the potential treatment of human neuroblastoma with this putative G-quadruplex sequence, MYCN-15.

Major Findings: For the first time, we were able to identify and validate the structural presence of the G-rich DNA sequences in the promoter region of the MYCN oncogene and they formed quadruplex as evident by CD analysis. Human neuroblastoma cell lines were treated (SK-N-SH, SH-SY5Y, SK-N-AS, SK-N-BE2) with MYCN-15. The unique results for these neuroblastoma cells are (a) SH-SY5Y (neuronal type) cells derived from SK-N-SH neuroblastoma cells mixed (epithelial & neuronal) type cells and retain only neuroblast cells [15]. It was shown for the first time that MYCN-15 G-rich oligo induced differentiation in SK-N-SH by flattening the cell morphology and SH-SY5Y cells by extending the neurites. Ultimately, these cells underwent growth arrest and perhaps cellular death as well (~55 % sensitive, by MTT analysis) (b) SK-N-BE2 cells, which carries multiple copies of N-Myc genes (N-Myc amplified) also sensitive to MYCN-15 (~40%). Lastly, it has been shown that the survivin protein expression is not changed significantly when treated with MYCN-15, irrespective of the status of the n-Myc copy numbers.

Support of Research hypothesis by data: Our data has supported the first part of our working hypothesis. We have shown clearly that G-rich sequences in the promoter region of the MYCN gene changed their normal canonical conformation, double-helix to non-canonical quadruplex conformation (Fig. 2). It has also been evidently proven in our second part of the hypothesis that there were neuronal differentiation and/or cell death by flattening of cells (SN-N-SH) and extension of the neurites followed by cellular morphology suggestive of cell death (SH-SY5Y) (Fig. 3, 4). This differentiation and or cell death of neuroblastoma cells are validated by MTT assay which represents a smaller number of cells in MYCN-15 treated cells.

Comparison with other research: The concept of targeting G-quadruplex as a therapeutic strategy was first developed for telomeric DNA and telomerase inhibition. Studies of several independent laboratories aimed at understanding the repeats in telomere have shown that neuroblastoma with high telomerase activity had a poor prognosis unfavorable biology and advanced stage of neuroblastoma [19]. Amplification of MYCN is associated with adverse outcome in neuroblastoma, although MYCN expression without multiplication is shown to be beneficial [20]. Down-regulation of MYCN gene expression has been shown to induce neuronal differentiation in neuroblastoma [3]. Together, these findings indicate that the MYCN gene signaling pathways are important in maintaining an undifferentiated phenotype and that the inhibition of MYCN gene could contribute to less aggressive neuroblastoma.

Explanation of Findings: A mutated version of G-rich oligonucleotides m-MYCN-15 (Table 1) has been treated by randomly replacing most of the guanine with non-guanine oligos to use as an internal control. m-MYCN-15 did not form Quadruplex as evident by CD analysis (Fig. 2). Interestingly when the neuroblastoma cells were treated with these control oligos, a certain level (~12%) of sensitivity in SH-SY5Y neuroblastoma cells was observed as well in MTT analysis (Fig 4). One of the explanations could be that these mutant oligoes serve as an antisense or SiRNA of other prominent genes important for regulation of neuroblastoma biology [21]. The survivin expression is not changed when targeting the MYCN gene with MYCN-15. However, the expression of the

survivin protein is modestly (~70%) reduced in SK-N-AS and ~40% in SH-SY5Y cells when treated with another control oligos (SVN-32). Considering the fact that both the MYCN and survivin genes are important in regulating neuroblastoma biology, our finding suggests that MYCN gene might work downstream of survivin gene and/or different pathways to regulate the neuroblastoma biology.

Recommendation for future research: Our study as well as other investigator has shown that the MYCN gene is highly expressed in SK-N-AS, SH-SY5Y and SK-N-BE neuroblastoma cells [22].We also showed before that survivin, an anti-apoptosis gene is highly expressed in unfavorable groups of neuroblastomas [4]. This study shows that when MYCN-15 G-quadruplex oligonucleotide is used, there is growth arrest/ cell death both in irrespective of the n-MYC copy status and without significant changes of survivin protein expression. This will lead us a window of opportunity to investigate whether survivin is upstream of the MYCN signal transduction or whether there is an independent pathway [23]. As we have identified this unique oligo, this G-rich oligonucleotide will be conjugated with fluorescent (GFP/RFP) and the neuroblastoma cells will be treated to validate their uptake by the cells in future.

It is concluded that G-quadruplex forming sequences in the MYCN promoter region could be a potential candidate for the treatment of common childhood solid tumor, neuroblastoma.

Ethics approval and consent to participate

"Not applicable"

List of abbreviations

GRO: Guanine-Rich-Oligonucleotides,
CD: Circular Dichroism,
MTT: [3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide].

Conflicts of Interest

"The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper."

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Authors' contributions

MAI: planning, designing, organizing, executing & data accusation, and major contribution in writing
FAS: Execution of experiments, data interpretation and major contribution in writing.
SDT: Experimental supervision and data analysis
TM: Scientific comments, literature search.
TA: Scientific comments, literature search
DMM: Overall supervision and significant scientific comments and direction in writing.

"All authors read and approved the final manuscript."

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