Acta Scientific Cancer Biology

Volume 1 Issue 1 June 2017

Research Article

Genetic and Functional Characterization of Cyclopamine Resistant Neuroblastoma Cells

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Received: June 07, 2017; **Published:** August 17, 2017

Abstract

We present a study on neuroblastoma cells, treated with up to six cycles of cyclopamine, an SMO inhibitor of the sonic hedgehog pathway. Several genes involved in apoptosis, cancer stem cell phenotype, and sonic hedgehog pathway regulation were tested for expression before and after treatments. Also, cell proliferation and colony formation in 2D and 3D assay systems were used. The genes related to cancer stem cell phenotypes (CD133 and CD15) seemed to increase their expression after exposition to several treatment cycles, coincident with the idea of neuroblastoma resistance to cyclopamine. MYCN, SMO and BCL-2 equally showed higher expression levels after several cycles of treatment. Cyclopamine treatment of neuroblastoma cells reduced cell proliferation and in vitro tumorigenesis determined by 3D colony formation assays in soft agar. The treatments also induced apoptosis and increased MYCN expression.

As a whole, we may consider cyclopamine a good inhibitor against neuroblastoma along the first stages of the treatment, while resistance to this compound can occur later on. More studies on cyclopamine resistance are needed to better approach to neuroblastoma treatment.

Keywords: Cyclopamine; Neuroblastoma; Sonic Hedgehog; CD133; CD15

Introduction

Neuroblastoma originates from neural crest cells. It is the most frequently diagnosed tumor during the first year of life [1], and currently the most common extra-cranial solid tumor in children, accounting for 15% of deaths associated with paediatric cancer. Each year, 10.2 new cases per million inhabitants are diagnosed in the United States [2]. The mean age at diagnosis is 17 months [3], which shows its high mortality, despite its low incidence. In addition, neuroblastoma is a very heterogeneous and complex disease in which genetic, molecular and cellular characteristics determine whether the tumor will return spontaneously or whether it will metastasize and become resistant to treatment [4]. Therefore, increasing our knowledge about the biochemical basis of the disease could help us to better stratify the risk presented by each patient and to be able to develop better targeted and more effective therapies.

There are indications that the mortality of this tumor depends mainly on three factors: the age of the patient at the time of diagnosis, the clinical stage of the tumor, and the expression levels of MYCN [4].

Therefore, the greater the expression of MYCN in the tumor cells of the patient, the worse the prognosis. In addition, MYCN is one of the target genes of Gli1, the main activator of the sonic hedgehog signalling pathway [5].

It is, therefore, believed that the origin of this tumor could be related to a persistent activation of the sonic hedgehog pathway [6,7]. Consequently, the possibility of using different inhibitors of this pathway as an experimental option of treatment against neuroblastoma, together with the genetic and functional analysis of the cells subjected to treatment, are of importance to further understand the pathobiology of neuroblastoma. One of these compounds is cyclopamine, an inhibitor of Smo, a G-protein coupled receptor that -if mutated- upregulates the sonic hedgehog pathway and promotes cancer [4].

The appearance of resistance to chemotherapy is a frequent reason for tumor recurrence in neuroblastoma, probably due to cancer stem cells. Therefore, we wanted to analyse the gene expression profile and stem cell biological characteristics of neuroblastoma cells in the first 6 cycles of cyclopamine treatment at a concentration that allows the genesis of resistance in those neuroblastoma cells.

Material and Methods

Human Neuroblastoma Cells

The SK-N-DZ cell line, commercialized by ATCC (American Type Culture Collection, VA, USA), was cultured in monolayer with DMEN medium to which 10% fatal bovine serum (FBS) was added, together with 5% non-essential amino acids, 1% penicillin/streptomycin and 0.1% amphotericin B. It was incubated at 37°C in an atmosphere enriched with 5% CO_2. Cell passages were performed with trypsin/EDTA (Gibco) at the time the cells reached 80% confluency.

Cyclopamine Treatment Cycles

In order to make a comparative analysis, four flasks of the SK-N-DZ line were used. Two of them were treated with cyclopamine and the other two non-treated flasks included cyclopamine diluents (DMSO or 100% ethanol). First, 6 treatment cycles were performed with concentrations of 12 µM. DMSO was used as the diluent. Subsequently, the dose of cyclopamine was increased substantially in each cycle (40, 75 and 120 µM) in order to create a resistant cell line to cyclopamine. In these cycles, 100% ethanol was used as a diluent to reach higher concentrations of cyclopamine.

At each cycle, when the cell culture reached a confluence of 60-70%, the culture medium was removed and cyclopamine (or diluent only) and 2.5% FBS medium were added. After 24h, the medium was removed, washed with PBS, and 12 ml of 10% FBS medium were added again. Cells with confluence of 80-90% were trypsinized. A second pass was made when they reached 80-90% confluence again. Taking advantage of this last pass, pellets of 4 million cells were subjected to extraction of RNA.

RNA Extraction and Retrotranscription

Extraction was performed with the AllPrep DNA/RNA/Protein Mini Kit (Qiagen), following the manufacturer's instructions. The amount and purity of RNA was determined by Nanodrop. For retrotranscription, 1 µg of RNA was added, along with 250 µg of random primers, and 2 µl of dNTPs (stock at 5 µM), in a final volume of 12 µl of H_2O-DEPC. An incubation step of 5 min at 65°C was done to eliminate secondary structures. After cooling on ice, 4 µl of 5X buffer and 2 µl of DTT were added and incubated for 2 min at 42°C. Subsequently, 1 unit of Superscript II Reverse Transcriptase was added. After an incubation of 10 min at 25°C, 50 min at 42°C, and 15 min at 70°C, the cDNA was obtained. Finally, 80 µl of H_2O-mQ was added. The cDNA was stored at -20°C.

Quantitative RT-PCR

The study of amplification reactions was performed using an IQ5 Multicolour Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). In each well, 1.25 µl of cDNA, 12.5 µl of Sober Green, 0.5 µl of forward-primer, 0.5 µl of reverse-primer, and 10.25 µl of H_2O-mQ were added. The different genes studied (Table 1) were subjected to the following protocol: an initial step of denaturation at 95°C for 10 min, followed by 40 cycles of amplification consisting of: 30 s at 95°C, 30 s at the melting temperature of each gene, and 30 s at 72°C.

Gene Name	Forward sequence	Reverse sequence	Melting Temp (ºC)
GAPDH	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT	65,7
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	67,05
BAX	ATGGAGCTGCAGAGGATGAT	CCCAAAGTAGGAGAGGAGGC	63,85
BCL-2	GATCCAGGATAACGGAGGCT	GTTGACTTCACTTGTGGCCC	64,4
CD 133.3	TCCGGGTTTTGGATACACCCTA	CTGCAGGTGAAGAGTGCCGTAA	68,25
CD 15	AACTACGAGCGCTTTGTGCC	AGGAGGTGATGTGGACAGCG	67,1
GLI-1	CAAGTGCACGTTTGAAGGCT	CAACCTTCTTGCTCACACATGTAAG	65,4
MYCN	CCCTGAGCGATTCAGATGAT	AATGTGGTGACAGCCTTGGT	64,25
SMO	ACGAGGACGTGGAGGGCTG	CGCACGGTATCGGTAGTTCT	67

Table 1: Primer Sequences for qRT-PCR.

MTT Proliferation Assay

To perform this assay, three 96-well plates were used in order to measure cell survival at 0, 24 and 48h from the beginning of cyclopamine treatment. Each condition was represented 8 times. These were: Control, 1% DMSO (or ethanol) and different concentrations of cyclopamine (1, 2.5, 5, 10, 15, 30, 50 µM) in the MTT performed after the first 6 cycles. First, 7000 cells in 200 µl of medium per well were seeded in each well. The next day the medium was changed and the different conditions were added to the plates for 24 and 48h, and the 0h plate was revealed. The 24h and the 48h plates were revealed after 24h and 48 h of initiation of treatment, respectively.

To develop the plates 50 µl of 2 mg/ml MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] were added and incubation was allowed for 2 - 4h. The cell medium was then removed, 150 µl of DMSO was added, and the absorbance was measured at 550 nm. Finally, a proliferation curve was made with the data obtained. This trial was done three times in order to know the IC50 for treatment with basal cyclopamine, after 6 cycles of cyclopamine, and at the end of the cycle of 120 µM.

In Vitro Tumorigenicity Tested By 3D Colony Formation Assays in Soft Agar

A 2-ml base layer of 0.5% agar with DMEM was prepared on a 6-well plate and allowed to cool at room temperature for 10 - 15 min. Two ml of 0.2% agar, DMEM

and 10,000 cells were then added. It was incubated for 50 min at 37°C for the gel to solidify. Finally, 2 ml of medium were added, followed by incubation for 2 - 3 weeks changing the medium every 2 - 3 days. The experiments were done in triplicate. Once the colonies were formed, they were stained by adding 250 µl of 1/40 violet crystal for 30 - 60 min, and 5 washes were performed with H_2O-mQ. The plates were preserved at 4°C until the colonies formed were counted with the aid of a magnifying glass.

2D Colony Formation Assay

1000 cells were grown in plates for 10 - 14 days. They were stained with 4% paraformaldehyde for 30 min, washed with PBS, 1/40 violet crystal added, and left for 15 min. Finally, they were washed with H_2O-mQ. The assay was performed in triplicate.

Statistical Analysis

For statistical analysis, version 22.0 of SPSS Statistical Software (Chicago, IL, USA) was used. Data were plotted as mean ± standard deviation. P-values < 0.05 were considered statistically significant. The mRNA expression profiles were referenced to their corresponding control. To analyse the possible differences between them, a one-way analysis of variance (ANOVA) was performed. A student t test was performed to analyse the differences between the ability to form colonies on initial agar and after the first treatment cycle, and between the first and third cycle. In addition, a one-way ANOVA was performed to study the number of colonies formed throughout the first three cycles.

Results

Calculation of IC50 For Cyclopamine Treatments

The cell line had an IC50 of 10 µM basal cyclopamine. In Figure 1 the IC50 is reflected for cyclopamine at the end of the first 6 treatment cycles (22 µM at 24h). The MTT of the untreated line remained the same as the baseline

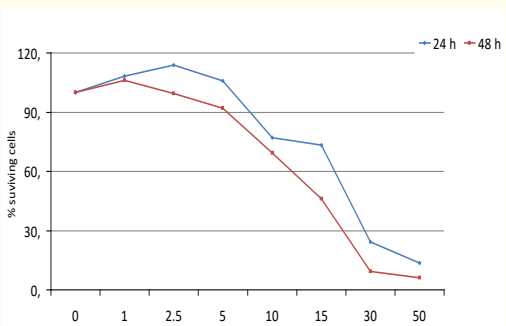

Figure 1: MTT Assay. Cell Viability of Neuroblastoma Cells after 24 h and 48 h of Cyclopamine Treatment.

mRNA Expression of Cancer Genes

The expression profile of BAX, BCL2, CD133, CD15, GLI, MYCN and SMO was analysed during the 6 stages of treatment of the neuroblastoma cell line (Figures 2-5). The action of cyclopamine implied statistically significant changes in the expression profile of each of them except in SMO when analysing gene expression in the different cycles for each gene.

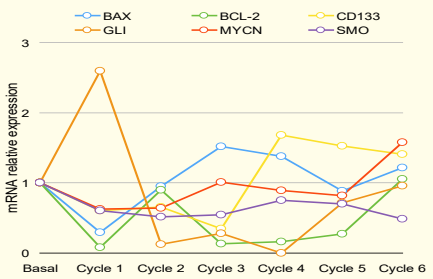

Figure 2: Gene Expression by qRT-PCR after Different Cycles of Cyclopamine Treatment.

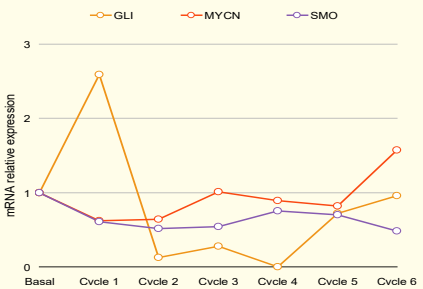

Figure 3: Relative Expression of Gli, MYCN and SMO after Different Cycles of Cyclopamine Treatment.

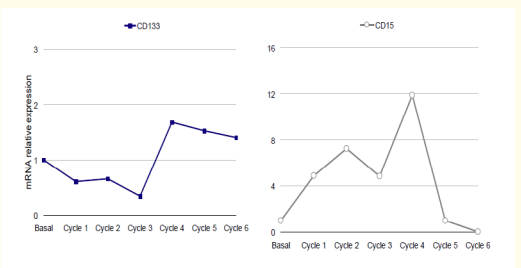

Figure 4: qRT-PCR Expression Results of Cancer Stem Cell Related Genes after Different Cycles of Cyclopamine Treatment.

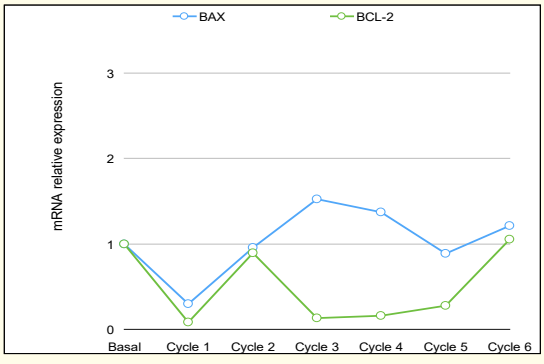

Figure 5: qRT-PCR Expression Results of Apoptosis Related Genes after Different Cycles of Cyclopamine Treatment.

3D Colony Formation Assay in Sof Agar

Statistically significant differences were found between the relative ability to form colonies after the first treatment cycle and the baseline situation when performing a student t test ($p = 0.000$). No statistically significant differences were found in comparing the relative ability to form colonies of cells extracted in the first cycle and those in the third cycle (student $t = 0.587$). However, there was a progressive increase in the relative capacity to form colonies in soft agar as the treatment progressed (Figure 6).

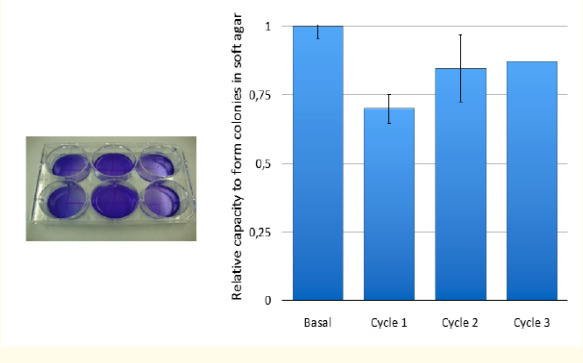

Figure 6: Relative Capacity of the Neuroblastoma Cells to form Colonies in Soft Agar.

2D Colony Formation Assay

There were no statistically significant differences between the number of colonies formed from the treated line and the control line in any of the cycles analysed ($p > 0.05$ in all). The results are shown in Figure 7.

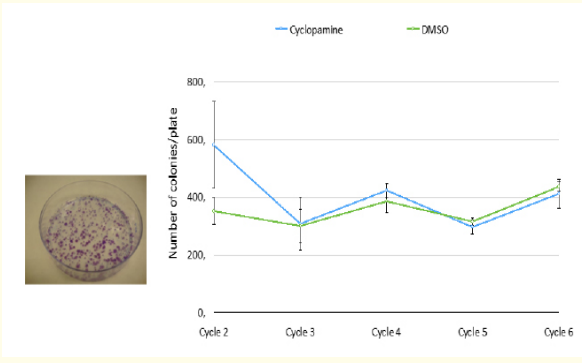

Figure 7: Relative Capacity of the Neuroblastoma Cells to form Colonies in 2D Experimental Assays.

Discussion and Conclusion

In this work, three kinds of assays were chosen to analyse cell phenotypes and cancer stem cell characteristics during the first 6 cycles of exposure of the neuroblastoma cells to cyclopamine: expression of a variety of genes, including those related with cancer stem cell markers; 2D colony formation assays; and 3D soft agar colony formation assays, as an approach to test for tumorigenicity of the neuroblastoma cells *in vitro.*

CD15 and CD133 are membrane markers of stem cells [8,9]. BAX and BCL-2 are genes related to apoptosis [10,11]. Gli and SMO (inhibited by cyclopamine) are part of the sonic hedgehog signalling pathway [12].

Finally, MYCN allows us to evaluate tumor malignancy [5] and is believed to be related to neuroblastoma survival mechanisms [13]. During the first cycles of treatment, we observed that the relative expression of cancer stem cells genes CD15 and CD133 (Figure 4) was reduced respect to controls, suggesting that cyclopamine might have a selective action against cancer stem cells. In fact, this has already been studied by other research groups in brain tumors [14] and tumors of different locations [15,16]. In addition, the decrease of CD133 is similar to the results obtained by Schiapparelli., *et al.* [17]. However, it appears that an increase in the number of cycles made CD15 + cells became more sensitive to treatment. Cyclopamine also decreased Bcl-2 levels favoring a pro-apoptotic cellular state [18]. However, CD15 and CD133 expression (Figure 5) increased progressively with more treatment cycles, which could indicate that cancer stem cells became resistant to the treatment, as demonstrated in the work by Lu., *et al.* [19].

When we analysed the behaviour of the genes involved in the sonic hedgehog pathway (Figures 2 and 3), Gli expression initially decreased, but increased at the end of cycles, which could be related to an emergence of resistance to cyclopamine since Gly is activated by SMO [12]. On the other hand, SMO levels did not vary, which might be explained by cyclopamine inhibition of SMO action by direct binding [20], without altering SMO expression levels, but just its activity on Gli.

The results of the colony formation tests (Figures 6 and 7) are related to those obtained in RT-PCR since the formation of colonies is another characteristic of stem cells. After the first cycle, the relative ability to form colonies in soft agar (Figure 6) was 70%, which agrees with a decrease in the mRNA expression of different stem genes. This value increased progressively until reaching 85% in the third cycle, which could be related to a progressive increase of the resistances. On the other hand, no statistically significant differences were found in the 2D colony formation assays (Figure 7) between the number of colonies formed by the treated line and the control in any of the cycles studied. Possibly, in order to show differences, we must work with higher doses since in this test we have cell anchoring situations.

As a conclusion, we might say that cyclopamine treatment of neuroblastoma cells reduces their cell proliferation and *in vitro* tumorigenesis determined by 3D colony formation assays in soft agar, and also reduces, in the first cycles of treatment, the expression of cancer stem cells related genes, like CD15 and CD133, although the expression of those genes is elevated through further cycles of cyclopamine treatment, meaning that resistance to cyclopamine increases the proportion of cancer stem cells in the neuroblastoma cell line. Equally occurred with Gli and SMO: reduced expression after the first treatment cycles, followed by higher expression at the end of the treatments, meaning that the cells got resistant to cyclopamine. The treatments also induced apoptosis and increased MYCN expression. As a whole, we may consider cyclopamine a good inhibitor against neuroblastoma along the first stages of the treatment, while resistance to this compound can occur later on. Studying the genetic mechanisms of resistance to cyclopamine will help us work on trying to make cells sensitive to this drug again, or to any combination of other drugs with cyclopamine, in order to possibly design better treatments against neuroblastoma.

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