Effects of MYCN Antisense Oligonucleotide Administration on Tumorigenesis in a Murine Model of Neuroblastoma

Catherine A. Burkhart, Andy J. Cheng, Janice Madafaglio, Maria Kavallaris, Mario Mili, Glenn M. Marshall, William A. Weiss, Levon M. Khachigian, Murray D. Norris, Michelle Haber

Background: Human MYCN (hMYCN) oncogene amplification is a powerful predictor of treatment failure in childhood neuroblastoma, and dysregulation of hMYCN protein expression appears to be critically involved in the pathogenesis of this disease. We used hMYCN antisense (AS) oligonucleotides to investigate, both in vitro and in vivo, the therapeutic potential of inhibiting hMYCN expression. Methods: We transiently transfected human neuroblastoma IMR-32 cells, which have an amplified hMYCN gene, with fluorescently labeled hMYCN AS or scrambled (SCR) control oligonucleotides and used fluorescence-activated cell sorting to enrich for cell populations containing different levels of the oligonucleotides. We used fluorescence immunocytochemistry or reverse transcription polymerase chain reaction to assay gene expression levels and trypan blue exclusion to assay for cell populations containing different levels of the oligonucleotides. We used fluorescence immunocytochemistry or reverse transcription polymerase chain reaction to assay gene expression levels and trypan blue exclusion to assay growth inhibition in the cell populations. We examined the effects of continuous treatment for 6 weeks with AS or SCR oligonucleotides via subcutaneously implanted microosmotic pumps on tumor growth in a transgenic mouse model of hMYCN-induced neuroblastoma (n = 20 mice per group). All statistical tests were two-sided. Results: IMR-32 cells treated with AS oligonucleotides had approximately half as much hMYCN protein and cell proliferation as either SCR oligonucleotide–transfected or mock-transfected controls; the differences were statistically significant. Transgenic mice treated with AS oligonucleotides had lower tumor incidence and statistically significantly lower tumor mass than SCR-treated or untreated control mice. Compared with control treatments, AS oligonucleotide treatment in vitro and in vivo was associated with decreased expression of hMYCN and putative hMYCN target genes but not with that of closely related genes. Several AS oligonucleotide–treated mice developed tumors contralateral to the site of oligonucleotide administration, whereas SCR oligonucleotide–treated or untreated mice displayed bilateral tumor growth. Conclusions: Decreased expression of hMYCN protein is achievable with the use of AS oligonucleotide treatment, even in the presence of hMYCN oncogene amplification. Antisense strategies targeting the hMYCN oncogene in vivo decrease mouse neuroblastoma tumorigenesis. Investigation of their clinical effect in children with neuroblastoma is warranted. [J Natl Cancer Inst 2003;95:1394–1403]

Neuroblastoma, a tumor that arises from the sympathetic nervous system, is the most common solid tumor of infancy (1). One of the key predictors of poor outcome in this disease is amplification of the human MYCN (hMYCN) oncogene (1), which occurs in 25%–30% of neuroblastomas. Such amplification is associated with advanced-stage disease, rapid tumor progression, and a survival rate of less than 15%, despite advances in treatment strategies (1–3). Although initial studies indicated that hMYCN protein could cooperate with the mutant oncoprotein Ha-ras in neoplastic transformation in vitro (4), a more definitive role for the hMYCN oncogene in neuroblastoma tumorigenesis was confirmed by the development of a transgenic mouse model in which expression of the hMYCN oncogene was targeted to mouse neural crest cells with the use of a tyrosine hydroxylase promoter (5). Neuroblastoma tumorigenesis in these mice is associated with transgene dose, and the tumors that develop in these mice are similar to human neuroblastomas with respect to their locations and those of their metastases, their histology, syntenic chromosomal gains and losses, and amplification of the hMYCN oncogene in many of the tumors (5–7).

Although these data suggest a direct role for the hMYCN oncoprotein in neuroblastoma pathogenesis, the mechanism by which hMYCN contributes to both the development of this disease and its poor prognosis is still unclear. The hMYCN oncoprotein functions as a transcriptional regulator (8) and thus may influence tumorigenesis and patient survival by regulating the expression of key genes involved in the neuroblastoma malignant phenotype. MYCN regulates the expression of genes that encode the multidrug resistance–associated protein 1 (MRP1) and ornithine decarboxylase (ODC). MRP1 is an ATP-dependent drug efflux pump that confers resistance to many of the drugs used in the treatment of neuroblastoma (1,9,10). We previously demonstrated a strong correlation between hMYCN and MRP1 gene expression in vitro (11,12), in murine neuroblastoma tumors (7), and in primary neuroblastoma samples (13). In addition, we have shown that MRP1 expression is a powerful independent prognostic indicator of this disease (14). ODC is the rate-limiting enzyme in the production of polyamines (15). Although polyamines, and therefore ODC, are essential for normal cell proliferation, increased ODC activity can induce cellular transformation in vitro (16), and high ODC levels are associated

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with a variety of tumors, including those of the brain and prostate (17,18). Several studies (8,19) have identified ODC as a target gene for the hMYCN oncprotein.

A targeted molecular approach to therapy has proven to be promising for the treatment of specific cancers (20). However, the pharmacologic inactivation of oncogenes such as hMYCN must be achieved in the presence of a background of high-level overexpression associated with gene amplification that is characteristic of the most aggressive forms of this disease. We therefore examined whether we could specifically target hMYCN by using an antisense phosphorothioate oligonucleotide approach, both in vitro and in vivo.

**MATERIALS AND METHODS**

**Phosphorothioate Oligonucleotides**

All oligonucleotides used for transfection were obtained from Gene Link (Tarrytown, NY), contained phosphorothioate linkages at the first two and last three internucleotide bonds, and were fluorescently labeled with fluorescein isothiocyanate (FITC). The hMYCN antisense (AS) oligonucleotide (5'-GAT CATGCCCGGCAT-3') was complementary to the first five codons of the hMYCN oncogene (21): the scrambled (SCR) control oligonucleotide (5'-TGATCCCGGAGTCA-3') contained the same base sequence as hMYCN AS, but the order of those bases was random. We also used a phosphorothioate mismatch AS control oligonucleotide (5'-GATtATGCatGGC AT-3') that contained the same base sequence as the hMYCN AS oligonucleotide with the exception of three internal base mismatches. The identical hMYCN AS and SCR oligonucleotides were used for treatment of animals, except that they were not fluorescently labeled.

**Cell Culture and Transfection**

The hMYCN-amplified neuroblastoma cell line IMR-32 (22), which contains approximately 25 copies of the hMYCN oncogene per haploid genome, was maintained in Dulbecco’s modified Eagle medium (Invitrogen, Auckland, New Zealand) containing 10% fetal calf serum (FCS) (Trace Scientific, Clayton, Victoria, Australia) at 37°C and 5% CO₂. For individual experiments involving detection of a specific protein following a particular transfection treatment, cells were plated in 10 replicate wells of a 96-well plate at 2 x 10⁴ cells/well. Twenty-four hours after plating, the cells were transfected with either hMYCN AS, mismatched AS, SCR oligonucleotides (1 μM), or an equivalent volume of saline (mock-transfected control), complexed with seven equivalents of ExGen500 transfection reagent (MBI Fermentas, Hanover, MD) in Opti-MEM medium (Life Technologies, Grand Island, NY) supplemented with 5% FCS, according to the instructions provided with the ExGen500 reagent. Three hours after transfection, FCS was added to the cells at a final concentration of 10% and the cells were incubated for 24 hours. Each individual transfection experiment was repeated on at least two independent occasions.

**Flow Cytometry and Cell Sorting**

Transfected cells from replicate wells were pooled, resuspended in culture medium, and centrifuged at 100g for 5 minutes. The supernatant was aspirated and the remaining cell pellet was resuspended in 500 μL of phosphate-buffered saline (PBS)/5% FCS. Cells were sorted with the use of a FACSVantage SE flow cytometer (BD, Franklin Lakes, NJ). The viable cell population was gated for sorting, and cells with fluorescence intensity representing the highest 30% of the total gated population were isolated to enrich for successfully transfected cells.

**Fluorescence Immunocytochemistry**

Transfected cells (2 x 10⁴), following sorting where indicated, were centrifuged onto duplicate glass slides at 80g for 10 minutes with the use of a Cytospin 3 (Shandon Scientific, Astmoor, Runcorn, Cheshire, U.K.) and fixed for 10 minutes in ice-cold acetone. Air-dried slides were rinsed in PBS and incubated with 20% normal goat serum (ICN Pharmaceuticals, Aurora, OH)/PBS for 1 hour at room temperature to block nonspecific antibody binding. Slides were incubated for 1 hour at room temperature with the monoclonal anti-MYCN antibody NCMII 100 (1:40 dilution; Oncogene Research Products, San Diego, CA), washed three times in PBS for 5 minutes, and then incubated with a Cy3-conjugated goat anti-mouse antibody (1:2000 dilution; Amersham, Little Chalfont, Buckinghamshire, U.K.) for 1 hour at room temperature. The slides were washed three times in PBS for 5 minutes and counterstained with DAPI II (Vysis, Downers Grove, IL), and the cells were visualized by standard fluorescence microscopy with the use of an Axiosplan 2 microscope (Zeiss, Oberkochen, Germany). Images were captured using a Sensicam Charged Coupled Device (CCD) camera (PCO Imaging, Kelheim, Bavaria, Germany).

To ensure accurate comparisons between treatment groups and within experiments, we used the same exposure times to capture images of all cells. Exposure times were initially optimized for each experiment by capturing images of untransfected IMR-32 cells stained with the NCMII 100 antibody to detect hMYCN protein. We used identical staining protocols to detect MRP1, ODC, and MRP2 proteins in transfected cells. MRP1 staining was performed with the use of a rat monoclonal anti-human MRP1 antibody (MRP1, 1:20 dilution; Alexis Biochemicals, Carlsbad, CA) followed by incubation with a Cy3-conjugated goat anti-rabbit antibody (1:1000 dilution; BD); ODC was detected with the use of a rabbit anti-human ODC polyclonal antibody (1:200 dilution; ICN Pharmaceuticals) followed by incubation with a Cy3-conjugated goat anti-rabbit antibody (1:2000 dilution; Amersham). MRP2 was detected with the use of a mouse anti-human MRP2 monoclonal antibody (1:20 dilution; Alexis Biochemicals) followed by incubation with a Cy3-conjugated goat anti-mouse antibody (1:1000 dilution; Amersham). Individual cells from captured images were viewed and traced on a high-resolution computer monitor, and the mean fluorescence intensity and area of 100 cells for each staining were quantitated, as previously described (23), with the use of Image-Pro v4.5 software (Media Cybernetics, Silver Spring, MD).

**Growth Inhibition Assay**

Following a 24-hour exposure of cells to hMYCN AS oligonucleotides, SCR oligonucleotides, or ExGen500 transfection reagent alone, the transfection medium was removed from the cells and replaced with Dulbecco’s modified Eagle medium supplemented with 10% FCS, and the cells were cultured for an additional 48 hours. Cell viability was determined by the trypan blue exclusion assay, and viable cells were counted with the use of a hemocytometer. Experiments were performed on three separate occasions.
Transgenic Mice and In Vivo AS Treatment

The generation of the hMYCN transgenic mouse model, which is based on targeted expression of the hMYCN oncogene to mouse neuroectodermal cells via the mouse tyrosine hydroxylase promoter, has been described previously (5). The transgenic mouse line was backcrossed for four generations to 129SVter mice (Animal Resources Centre, Perth, Australia) and maintained as an inbred line. Tumor formation in these transgenic mice is dependent on transgene dosage, with 100% of homozygous MYCN mice (MYCN"++") and approximately 30% of hemizygous MYCN mice (MYCN"+/-") developing tumors with average latencies of approximately 7 and 15 weeks, respectively (7). A cohort of 60 MYCN transgenic mice (21 MYCN"++" mice and 39 MYCN"+/-" mice) was divided into three groups (n = 20 mice per group), each of which consisted of seven MYCN"++" mice and 13 MYCN"+/-" mice. Two groups of mice were treated continuously for 6 weeks with MYCN AS or SCR oligonucleotides with the use of subcutaneously implanted microsomatic pumps that delivered a 5-mM solution of the oligonucleotide at a rate of 0.5 μL/hour (Alza Corporation, Palo Alto, CA), as previously described (24,25). In all cases, treatment began when the mice were 4 weeks old, which is before these mice develop palpable tumors. Briefly, the mice were anesthetized by intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine, and the pumps were implanted into their flanks. The third group of mice was left untreated so that we could follow the normal course of tumor development in this cohort. All mice were assessed for tumor formation until they were 20 weeks old by subjecting them to thrice-weekly abdominal palpations that were performed by experienced veterinary and technical staff who were blinded to the oligonucleotide treatments the mice received. The mice were killed when a palpable tumor was detected, and the tumors were resected, measured, and snap-frozen in liquid nitrogen for subsequent molecular characterization. All surviving mice were killed when they were 20 weeks old and were dissected and examined for evidence of tumor development. One of the MYCN"+/-" mice treated with SCR oligonucleotides died at 10 weeks of age from complications of surgery without evidence of tumor formation. Because this death occurred well before the mean age of tumor development in MYCN"+/-" mice, this animal was excluded from the subsequent analysis. Tumor mass was calculated from the measured size of the tumor as previously described (26) and, in those cases where secondary thoracic tumors occurred, total tumor burden was calculated as the sum of the weights of the individual tumors. All experimental procedures involving transgenic mice were approved by the University of New South Wales Animal Care and Ethics Committee and were conducted under the Animal Research Act, 1985 (New South Wales, Australia) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

DNA Isolation and Genotyping

Genomic DNA was isolated from mouse-tail tips harvested when animals were 2–3 weeks of age according to the Chelex 100 (Bio-Rad Laboratories, Hercules, CA) DNA isolation method, which does not involve the use of proteinase K (27). The genotypes of MYCN transgenic mice were determined by real-time polymerase chain reaction (PCR) with the use of oligonucleotide primers and probes specific for the hMYCN transgene and the mouse β-actin gene, as previously described (7). The sequences of the primers and probes are listed in Table 1. The primers and probe for the mouse β-actin gene were designed to also amplify actin pseudogenes so that we could control for genomic instability by evaluating the copy number using different reference points within the genome. We performed primer-limiting experiments for each primer set to ensure that no competition between primer pairs occurred during co-amplification of the transgene and the control genes. Real-time PCR reactions contained mouse-tail DNA (10 ng) and 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), as well as primers (β-actin forward at 500 nM, β-actin reverse at 300 nM, hMYCN forward at 400 nM, hMYCN reverse at 500 nM) and probes for mouse β-actin and hMYCN at a final concentration of 200 nM. The PCR reaction consisted of an initial incubation at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 58 °C for 1 minute. PCR data were collected with the use of a Prism 7700 Sequence Detection System (Applied Biosystems). To determine the hMYCN transgene dosage, we used the ΔΔCt method, a comparative technique that involves determining the Ct value, defined as the cycle number a particular sample achieves when it crosses a set fluorescence threshold intensity, normalized to the β-actin control Ct value in each case and expressed relative to a calibrator (28).

RNA Isolation and Gene Expression Analysis

We used guanidinium thiocyanate–phenol–chloroform extraction to isolate total cytoplasmic RNA from transfected IMR-32 cells and from the murine tumors, and we used 1 μg of RNA, random hexanucleotide primers, and Moloney murine leukemia virus reverse transcriptase to synthesize cDNAs, as previously described (14). With the exception of a single tumor from an MYCN"++" AS-treated mouse, all tumor samples yielded high-quality RNA that was suitable for PCR analysis. The mouse β2-microglobulin gene was used as an internal control for the reverse transcription PCR (RT-PCR) as previously described (12,14). hMYCN, murine MYCN (mMYCN), human ODC, murine ODC, human c-myc, and murine MRP1 gene expression was determined by real-time PCR using the method described in the previous section (or for human c-myc, a Pre-Development Assay Reagent Kit; Applied Biosystems) and annealing temperatures of 59 °C (hMYCN, mMYCN, and murine MRP1) or 60 °C (human c-myc, human ODC, and murine ODC). For real-time PCR experiments, the level of target gene expression was determined using the ΔΔCt method. Expression of the murine mdr1a, mdr1b, MRP2, c-myc, and L-myc genes was determined by 30 cycles (35 cycles for MRP2) of conventional PCR using 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final reaction volume of 25 μL. Following a 10-minute incubation at 94 °C, each cycle consisted of 45 seconds at 94 °C, 45 seconds at 59 °C, and 90 seconds at 72 °C. The primer and probe sequences used for expression analysis are listed in Table 1. All analyses of gene expression were performed on at least three separate occasions.

Statistical Analysis

Results are expressed as mean values with 95% confidence intervals (CIs). For experiments involving two groups of mice, comparisons were made using two-tailed, unpaired Student’s t tests. For experiments involving three groups of mice, one-way analysis of variance was performed, and when that analysis re-
revealed a statistically significant difference among the groups, pairwise differences between groups were determined with the use of Fisher’s protected least significant difference (PLSD) correction. $P$ values of less than .05 were considered statistically significant. Linear regression analysis of the association between hMYCN gene expression and that of its target genes was performed on the entire cohort of murine abdominal tumors that had yielded high-quality RNA for analysis ($n = 30$), irrespective of their treatment group assignment, such that each point in an individual regression analysis represented a single mouse. All data were analyzed with the use of StatView 4.1 software (Abacus Concepts, Berkeley, CA).

RESULTS

Effects of AS Oligonucleotide Administration on In Vitro Expression of hMYCN Protein and Its Target Genes

We examined whether we could modulate hMYCN expression in neuroblastoma cells by transfecting the cells with a 15-mer AS phosphorothioate oligonucleotide that targeted the ATG start site of the coding sequence of hMYCN. Previous studies (21,24,29,30) demonstrated that treatment of neuroepithelioma cells with this hMYCN AS oligonucleotide was associated with a decrease in hMYCN protein levels. However, these neuroepithelioma cells lack hMYCN gene amplification and display only low-level basal expression of the oncogene (21,24). To examine the effects of this oligonucleotide on high-level hMYCN protein expression, we used the human neuroblastoma IMR-32 cells, in which the hMYCN gene is amplified and overexpressed (22). We transiently transfected IMR-32 cells with FITC-labeled hMYCN AS or SCR control oligonucleotides and used an antibody to detect hMYCN protein 24 hours later (Fig. 1, A). Only cells with this hMYCN AS oligonucleotide were associated with a markedly less hMYCN protein (visualized by red fluorescence).

Table 1. Oligonucleotide primers and probes used for DNA and RNA analyses

<table>
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<tr>
<th>Assay</th>
<th>Genes</th>
<th>Primer and probe sequences*</th>
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<tr>
<td>Real-time PCR</td>
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<td></td>
<td></td>
<td>5'-TAGCC(A/C)CGCTCGGTCAAGG-3'</td>
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<td></td>
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<td>5'-GGGACGCTTGTACCTAGCTATG-3'</td>
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<td>Human MYCN transgene/cDNA</td>
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<td>5'-TGAGAGAAGGAAAGCTTCTAGTGGGAGG-3'</td>
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<td>5'-GGAGGACGCTCGGTCAAGG-3'</td>
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<td></td>
<td>Murine ODC</td>
<td>Primers: 5'-GGGACGCTTGTACCTAGCTATG-3'</td>
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<td></td>
<td>Murine β2-microglobulin</td>
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<td>5'-GGGACGCTTGTACCTAGCTATG-3'</td>
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<td>5'-GGGACGCTTGTACCTAGCTATG-3'</td>
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*All probes were used at a final concentration of 200 nM. Primer concentrations for all real-time polymerase chain reactions (PCRs) were optimized by primer-limiting experiments. The concentration of each primer for all conventional PCR reactions was 1.25 μM.
than cells that had taken up high levels of the SCR control oligonucleotide. We determined the mean hMYCN fluorescence intensity of 100 randomly selected transfected cells in each treatment group and found that AS oligonucleotide–treated cells had statistically significantly less MYCN-associated fluorescence (mean = 23.6 arbitrary units [AU]) than SCR oligonucleotide–treated cells (mean = 47.4 AU; difference = 23.8 AU, 95% CI = 19.8 to 27.9 AU; P < .001 versus AS oligonucleotide–treated cells, Fisher’s PLSD) or mock-transfected cells (mean = 52.0 AU; difference = 28.4 AU, 95% CI = 25.1 to 31.7; P < .001 versus AS oligonucleotide–treated cells, Fisher’s PLSD) (Fig. 1, B). We also observed a small but statistically significant difference in MYCN fluorescence intensity between SCR-treated cells and mock-transfected control cells (mean difference = 4.6 AU, 95% CI = 0.6 to 8.6; P = .017, Fisher’s PLSD), such that SCR-treated cells had less hMYCN fluorescence than mock-transfected cells. To confirm that the decrease in hMYCN expression in the AS-treated cells was specific for the AS oligonucleotide, we transfected cells with a mismatch AS control oligonucleotide, which contained the same base sequence as the hMYCN AS oligonucleotide with the exception of three internal base mismatches, and found no detectable difference in fluorescence intensity between cells treated with this oligonucleotide and cells treated with SCR oligonucleotide (data not shown).

Given the relatively low transfection efficiency in these experiments, we next examined the effects of AS oligonucleotides on hMYCN expression in cell populations that were specifically enriched for successfully transfected cells. Thus, following transient transfection, IMR-32 cells were subjected to fluorescence-activated cell sorting to isolate cell populations exhibiting either low levels of the transfected oligonucleotide (i.e., cells displaying the bottom 30% of the range of fluorescence intensity values) or high levels of the transfected oligonucleotide (i.e., cells displaying the top 30%) (Fig. 2). In cells containing low levels of the hMYCN AS oligonucleotide, levels of staining for hMYCN, MRP1, and ODC proteins were similar to levels observed in cells containing high levels of the SCR oligonucleotide (Fig. 2, A–C). However, hMYCN, MRP1, and ODC protein levels were lower in cells containing high levels of the hMYCN AS oligonucleotide than in cells containing either high levels of the SCR oligonucleotide or low levels of the hMYCN AS oligonucleotide. To examine whether these apparent differences were associated with an overall decrease in transcription in response to the AS oligonucleotide treatment, we examined the levels of MRP2, an ATP-binding cassette transporter protein that is closely related to MRP1. Expression of MRP2 is not associated with neuroblastoma outcome and is not modulated by increased expression of hMYCN in a tetracycline-inducible system (Haber M, Gilbert J, Smith J, Norris MD: unpublished observations). We observed no apparent differences in MRP2 staining between the AS- and SCR-treated cells (Fig. 2, D).

We also examined the AS- and SCR-treated cells containing high levels of transfected oligonucleotide for expression of c-myc, an myc family member closely related to hMYCN. In these experiments, we used real-time PCR rather than immunocytochemistry to examine c-myc RNA levels because the c-myc protein is expressed at low levels in IMR-32 cells. We found that AS-transfected cells had statistically significantly more c-myc RNA expression (mean ΔΔCt = 43.0) than mock-transfected cells (mean ΔΔCt = 8.0; difference = 35.0, 95% CI = 13.3 to 56.8; P = .002, Fisher’s PLSD) or SCR-transfected cells (mean ΔΔCt = 4.9; difference = 38.2, 95% CI = 15.5 to 60.8; P = .001, Fisher’s PLSD) (Fig. 3, A). By contrast, real-time PCR analysis revealed that AS-transfected cells had statistically significantly less ODC RNA expression (mean ΔΔCt = 24.5) than either mock-transfected cells (mean ΔΔCt = 44.5; difference = 20.0, 95% CI = 1.1 to 38.9; P = .016, Fisher’s PLSD) or SCR-transfected cells (mean ΔΔCt = 42.7; difference = 18.2, 95% CI = 4.2 to 32.2; P = .024, Fisher’s PLSD), a result that paralleled our immunocytochemical findings regarding both hMYCN and ODC expression in these treated cells. We observed no statistically significant difference in c-myc (P = .66, Fisher’s PLSD) or ODC (P = .77, Fisher’s PLSD) RNA expression between the mock-transfected and SCR-transfected control cells.

Concomitant with a decrease in hMYCN protein expression, IMR-32 cells transfected with AS oligonucleotides exhibited
less cell proliferation (mean = 21.9 × 10^3 cells) than SCR-transfected (mean = 38.3 × 10^3 cells; difference = 16.4 × 10^3 cells, 95% CI = 5.4 × 10^3 to 27.4 × 10^3 cells; P = .004, Fisher’s PLSD) or mock-transfected cells (mean = 39.1 × 10^3 cells; difference = 17.1 × 10^3 cells, 95% CI = 7.8 × 10^3 to 26.4 × 10^3 cells; P = .003, Fisher’s PLSD) (Fig. 3, B). However, there was no statistically significant difference in mean cell viability between hMYCN AS-transfected cells (mean viability = 74.1%) and SCR-transfected cells (mean viability = 75.3%; difference = -1.2%, 95% CI = -10.5% to 8.1%; P = .74, Student’s t test), indicating that the decrease in cell number was associated with the specific growth inhibitory effects of the AS oligonucleotide rather than with nonspecific toxic effects of the antisense treatment.

Effects of In Vivo hMYCN AS Treatment on Mouse Neuroblastoma Tumorigenesis

To examine the effect of decreased hMYCN expression on neuroblastoma tumorigenesis, we treated 4-week-old homozygous (hMYCN+/+) and hemizygous (hMYCN+/−) transgenic mice continuously for 6 weeks with either hMYCN AS or SCR control oligonucleotides (n = 20 mice per group). The oligonucleotides were delivered with the use of subcutaneously implanted microosmotic pumps. We followed the AS-treated, SCR-treated, and untreated mice for tumor formation until they were 20 weeks old, an age by which approximately 95% of MYCN+/+ and approximately 30% of MYCN+/− mice will have a palpable tumor. A total of 10 (26.3%) of the 38 hMYCN+/− mice who reached the experimental endpoints and all 21 (100%) of the 21 hMYCN+/+ mice developed tumors. All 31 of these mice developed abdominal tumors (11 from the untreated group, 12 from the SCR oligonucleotide–treated group, and eight from the AS oligonucleotide–treated group). Secondary thoracic tumors were also evident in three AS-treated, six SCR-treated, and four untreated mice, and no thoracic tumors occurred in mice that did not bear abdominal tumors.

hMYCN+/− mice treated with hMYCN AS oligonucleotides had a tumor incidence of approximately 8% (95% CI = 1% to 34%; 1 of 13 mice), which was fivefold less than that of SCR-treated hMYCN+/− mice (tumor incidence = 42%, 95% CI = 20% to 68%; 5 of 12 mice) and fourfold less than that of untreated hMYCN+/− mice (tumor incidence = 31%, 95% CI =
13% to 58%; 4 of 13 mice) (data not shown). Although hMYCN AS treatment did not reduce the tumor incidence in MYCN+/+ mice, we observed that the mean total tumor mass for all AS-treated mice (mean tumor mass = 115.9 mg) was statistically significantly less than that for the SCR-treated mice (mean tumor mass = 393.9 mg; difference = 278.1 mg, 95% CI = 85.6 to 470.5 mg; P = .012, Fisher’s PLSD) or for untreated mice (mean tumor mass = 367.8 mg; difference = 251.9 mg, 95% CI = 39.8 to 464.1 mg; P = .024, Fisher’s PLSD) (Fig. 4, A).

There were no statistically significant differences in mean tumor latency between the AS-treated mice (6.4 weeks, 95% CI = 5.9 to 7.0 weeks), SCR-treated mice (6.8 weeks, 95% CI = 6.3 to 7.3 weeks), and untreated mice (6.5 weeks, 95% CI = 5.8 to 7.2 weeks; P = .54, one-way analysis of variance).

We observed several cases of unilateral tumor development (relative to the midline of the mouse) in AS-treated mice (three of eight mice); in each case, the tumor site was contralateral to the location of the implanted pump (Fig. 4, B). By contrast, SCR-treated and untreated control mice displayed uniformly bilateral tumor growth (Fig. 4, B). These results suggest a local effect of hMYCN AS oligonucleotide treatment.

Expression of MYCN, MRP1, and ODC in Murine Tumors

We and others (8,11,12,14,19) have previously shown that the MRP1 and ODC genes appear to be transcriptional targets of the hMYCN oncogene in human neuroblastoma cells. To determine whether a similar relationship would hold in the murine neuroblastoma tumors, we examined the association between expression of the human MYCN transgene and expression of the mouse homologues of the human MRP1 or ODC genes among the entire cohort of murine abdominal tumors. Consistent with our previous findings in primary human neuroblastoma tumors, we observed a statistically significant correlation between...
hMYCN and MRP1 expression (Pearson’s $r = .811; P<.001$) (Fig. 5, A) and between hMYCN and ODC expression (Pearson’s $r = .740; P<.001$) in these tumors (Fig. 5, B). By contrast, linear regression analysis revealed no statistically significant correlation between expression of the hMYCN transgene and expression of either mdr1a or mdr1b (Fig. 5, C and D), the murine homologues of the human multidrug resistance gene mdr1 (31). These results are consistent with data from studies (11,32,33) of human neuroblastoma tumors and cell lines indicating that mdr1 expression does not correlate with hMYCN expression and does not appear to be a transcriptional target of hMYCN.

To examine whether the unilateral tumor development we observed only in mice treated with AS oligonucleotides was associated with specific changes in gene expression, we compared expression of the hMYCN transgene and murine MRP1 and ODC genes in the unilateral and bilateral tumors from AS-treated mice (Fig. 6, A). We found that the unilateral tumors expressed statistically significantly less human and murine MYCN RNA and murine MRP1 and ODC RNA than the bilateral tumors. This difference in gene expression was not due to an overall decrease in transcription in the unilateral tumors because expression of murine MRP2, mdr1a, and mdr1b genes, whose expression is not regulated by hMYCN expression, was not lower in these tumors than in the bilateral tumors (Fig. 6, B). We similarly found that expression of the mouse genes c-myc and L-myc, both myc family members closely related to hMYCN, was not lower in unilateral tumors compared with bilateral tumors (Fig. 6, C). These results are consistent with our in vitro findings and suggest that treatment with the AS oligonucleotide was specific for hMYCN.

**DISCUSSION**

In this study, we found that antisense inhibition of the hMYCN oncogene in vivo was associated with decreases in tumor incidence and tumor mass as well as with an unusual pattern of unilateral tumor growth. Gene expression analysis of the unilateral tumors confirmed that AS oligonucleotide treatment was associated with a decrease in the expression of hMYCN and its putative target genes but not of genes not targeted by hMYCN. The observed effects of antisense treatment were specific to the inactivation of hMYCN protein because treatment did not affect the expression of closely related members of the myc and ATP-binding cassette transporter gene families. We observed that MYCN AS oligonucleotide treatment, both in vitro and in vivo, was associated with a statistically significant increase in c-myc expression, which is consistent with earlier studies that demonstrated negative cross-regulation of these two oncogenes (34,35).

The targeting of the hMYCN gene to mouse neural crest cells provides direct evidence of the critical contribution of this oncogene to the malignant phenotype of neuroblastoma and highlights the potential of hMYCN as a candidate for antisense therapy. This animal model mirrors the biology and presentation of human neuroblastoma (5). We have recently shown that the hMYCN gene undergoes amplification in the majority of tumors in these animals, further highlighting the clinical relevance of this model (7). Given the frequency of oncogene amplification in a number of different cancer types, effective inhibition of these highly expressed target genes has important clinical implications. The results of this study indicate that inactivation of hMYCN protein is not only possible against a background of
gene amplification but is also associated with a marked suppression of the malignant phenotype.

In recent years there have been an increasing number of clinical trials involving antisense therapies directed at various genes whose expression is associated with cancer, including those encodingraf-1 (36,37), protein kinase C-α (38,39), and bcl-2 (40,41). Such treatments inhibit gene expression by blocking translation or splicing of RNA or by causing degradation of the target RNA through an RNase H–mediated mechanism (42). The therapeutic inhibition of the hMYCN protein is an appealing strategy because the expression of this gene is highly restricted during embryogenesis and almost completely absent in adult tissues (43). Thus, hMYCN protein inactivation in nonmalignant cells is not expected to be associated with adverse side effects. Indeed, we observed no toxicities following the continuous administration of AS oligonucleotides to the mice (data not shown). In addition, results of a recent study (44) suggest that long-term gene inactivation in tumors may not be necessary to achieve the desired pharmacologic effects, even when the target oncogene is expressed in critical cellular pathways of normal cells. These investigators used a conditional transgenic mouse model of c-myc–induced osteogenic sarcoma to demonstrate that transient c-myc oncogene inactivation was sufficient to suppress tumor growth in these mice. The tumor cells lost their malignant potential, and further expression of the oncogene induced an apoptotic response rather than causing tumor regrowth.

Our results raise the possibility that hMYCN antisense therapy may ultimately be clinically useful in the treatment of childhood neuroblastoma. Our demonstration that AS treatment is associated with decreased expression of both hMYCN protein and its target genes, especially MRP1, suggests that tumors may become more sensitive to chemotherapeutic agents when these drugs are given in conjunction with hMYCN antisense treatment. Combination drug and antisense therapy directed at bcl-2 has already shown promising results in clinical trials for the treatment of melanoma (40). In addition, we previously demonstrated human neuroblastoma cells stably transfected with MYCN antisense constructs have an increased sensitivity to chemotherapeutic agents that are MRP1 substrates (12). Therefore, treatments involving hMYCN-targeted therapies may be more effective than currently available treatments for this highly aggressive and chemoresistant disease.

**REFERENCES**


NOTES

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