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c-Myc Promoter Activation in Medulloblastoma

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Abstract

The c-myc oncogene is commonly activated in medulloblastoma. Genomic amplification is a well-documented cause of c-myc activation but does not account for all cases of c-myc activation. In this study, we sought other means by which c-myc is overexpressed in medulloblastoma. Twelve medulloblastoma or PNET cell lines were screened for c-myc genomic amplification, mRNA levels, and protein levels. Two medulloblastoma lines, D283 Med and D721 Med, were identified that expressed c-myc mRNA and protein at high levels without genomic amplification. The c-myc gene’s regulatory sequences were normal in those cell lines. However, specific regions of the promoter, independent of the c-myc lines, D283 Med and D721 Med, were identified that expressed c-myc amplification, mRNA levels, and protein levels. Twelve medulloblastoma other means by which c-myc is overexpressed in medulloblastoma. We sought to identify additional mechanisms by which c-myc is activated in medulloblastomas. Tumor cell lines were identified as having high c-myc expression, independent of genomic amplification, and the mechanism of activation was examined using sequencing and studies of promoter activity. The identification of another means besides genomic amplification by which c-myc is activated in these cell lines provides another important clue in how medulloblastomas develop.

Introduction

Medulloblastoma is one of the most common pediatric brain malignancies, affecting roughly one in 200,000 United States children each year (1). Genetic alterations have been identified in medulloblastomas, including some tumor cell lines with genomic amplification of the c-myc oncogene (2). Myc family members are transcription factors that activate cell cycle progression, block cellular differentiation, and are implicated in many human malignancies (3). For patients with medulloblastomas, the expression of c-myc mRNA by the tumor correlates with poor survival (4–6), suggesting that high c-myc expression produces a more aggressive tumor phenotype.

Genomic amplification of c-myc occurs frequently through double-minute chromosomes and is found in ~5–10% of primary tumors (4, 7). However, the number of medulloblastoma cases with pathological levels of c-myc protein leading to poor survival cannot be explained by genomic amplification alone. On the basis of previous studies, an estimated one-third of medulloblastomas have a genomic amplification-independent means of c-myc activation that produces high protein levels (4, 8).

Various means of amplification-independent c-myc activation have been identified in other human cancers such as the chromosomal translocations in Burkitt’s lymphoma that constitutively activate mRNA expression (9). Protein levels of c-myc in multiple myeloma are increased 20-fold by a mutation in the 5’ untranslated internal ribosome entry segment (10). Other mutations have been demonstrated in mucosa-associated lymphoid tissue lymphomas at the myc intron factor binding site, removing a negative regulatory sequence

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(11). In colon cancer, APCβ or β-catenin mutations activate the c-myc promoter at a β-catenin/Tcf-4 transcription factor binding site (12). APC and β-catenin mutations have been found in a small fraction of medulloblastomas (13–15), and nuclear β-catenin staining was reported in 18% of medulloblastomas studied (16). Therefore, genomic amplification-independent activation of c-myc is a likely mechanism for tumor progression in many medulloblastomas.

We sought to identify additional mechanisms by which c-myc is activated in medulloblastomas. Tumor cell lines were identified as having high c-myc expression, independent of genomic amplification, and the mechanism of activation was examined using sequencing and studies of promoter activity. The identification of another means besides genomic amplification by which c-myc is activated in these cell lines provides another important clue in how medulloblastomas develop.

Materials and Methods

Cell Lines. Ten medulloblastoma cell lines [D283 Med; (17), D341 Med, D425 Med (18), D487 Med, D556 Med, D851 Med, D721 Med, MHH-Med-1 (19), MHH-Med-4 (19), and MHH-Med-5 (19) and two PNET cell lines JPFSK-1 (20) and SK-PN-DW (21)] were used in this study. Of these cell lines, MHH-Med-4, PFSK-1, and SK-PN-DW grew adherent to the plastic culture flasks, whereas the rest of the cell lines grew in suspension. All cell lines were grown in zinco-optima media supplemented with 10% fetal bovine serum and antibiotics.

FISH Analysis. The genomic amplification status of c-myc in the 12 medulloblastoma and PNET cell lines was determined using FISH as described previously (22). Briefly, the Vysis LSI c-myc probe (8q24.12-q24.13) was used according to the manufacturer’s instructions (Vysis, Inc., Downers Grove, IL). The CEP11 probe (11p11.1-11q11) was used as a control.

Analysis of c-myc Expression. Northern blot analysis was performed using total RNA isolated from the 12 cell lines and processed as described earlier (23). A radioactively labeled 795-bp PCR product specific for c-myc was used as the probe.

For immunoblot analysis, total cell lysates were prepared and protein levels were quantified using the Bradford assay. Equal amounts of protein from each sample were resolved by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. Detection of c-myc was performed by using the mouse monoclonal clone 9E10 antibody (OncoGene Research Products, La Jolla, CA) at a dilution of 5 μg/ml as the primary antibody and horseradish peroxidase-conjugated goat antimouse immunoglobulin (Pierce Biotechnology, Rockford, IL) as the secondary antibody. Bound antibody was visualized by chemiluminescence with the use of the SuperSignal West Pico substrate (Pierce Biotechnology). The molecular weights were determined by prestained standards.

Plasmid Constructs. The Xmn I-PvuII fragment of the c-myc promoter region subchained upstream of a minimal promoter and the firefly luciferase reporter gene in the pBV-Luc plasmid (12) was kindly supplied by Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). This fragment contains P1 and P2, the major sites of transcription of c-myc. Various c-myc promoter nested deletions (fragments 1–6) and fragments (fragments 7–19) were either used directly or derived from a previous c-myc promoter study (12). The pTOPFLASH and pFOPFLASH reporter constructs (also supplied by B. Vogelstein) contained either an optimized Tcf binding site or a mutated Tcf binding site (24). The β-catenin mutant construct, pC7-neo-β-catenin S33Y, S373, and S453 was kindly provided by Y. Hagiwara (25).

The abbreviations used are: APC, adenomatous polyposis coli; FISH, fluorescent in situ hybridization; SAGE, Serial Analysis of Gene Expression.
used in this study has been described previously (24). The Renilla luciferase reporter gene (pRL-CMV) was obtained from Promega (Madison, WI) and was used as a control for transfection efficiencies.

DNA Transfection and Luciferase Assays. The 12 medulloblastoma or PNET cell lines grown in 24-well plates were transfected in triplicate with 3 μg of the various plasmid constructs using either Lipofection (Life Technologies, Inc., Bethesda, MD) or trans-Fast (Promega). The cells were cotransfected with 250 ng of pRL-CMV as a control for transfection efficiency. Twenty-four h after transfection, the firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) as suggested by the manufacturer and an FB12 luminometer (Zyuls Instruments, Oak Ridge, TN). Firefly luciferase activity was normalized to 10^7 Renilla luciferase activity units.

c-Myc Mutation Screening. A 640-bp region of the c-myc promoter 630 bp upstream and 10 bp downstream of P1 and exons 1–3 was screened for mutations in the 12 medulloblastoma cell lines by sequencing of PCR-amplified and purified genomic DNA, using manual sequencing with P32-labeled dideoxy terminators (USB, Cleveland, OH).

Site-directed Mutagenesis. Transcription factor binding sites were defined by several online databases found online, including Transfac 3.4, and by literature searches. Specific mutations were introduced into the c-myc promoter construct fragment 17 (Fig. 1A) at putative binding sites for known transcriptional activators of c-myc using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) that uses mutagenic primers in a PCR-based method. The binding sites for known transcription factors were replaced by mutations (summarized in Fig. 1B) previously published to be most effective in decreasing trans-activation activity.

Results

Ten medulloblastoma and two PNET cell lines were analyzed for genomic amplification of the c-myc oncogene. Five of the 12 cell lines exhibited genomic amplification of c-myc (Table 1). Representative interphase nuclei of a cell line without genomic amplification and one with genomic amplification are shown in Fig. 2. Genomic copy number for c-myc in the cell lines with genomic amplification was over 25 copies/cell, typically 50–100 copies in double-minute chromosomal aberrations. We also confirmed that D283 Med had between two to six tandem copies of c-myc (8). Although primary tissues from the original tumors were not available, genomic amplification of c-myc had previously been identified in xenografts of D283Med, D341Med, and D425 Med (2).

Two medulloblastoma cell lines, D283 Med (17) and D721 Med, were identified without c-myc genomic amplification but with high c-myc mRNA and protein expression (Table 1). We reasoned that the increased levels of both c-myc mRNA and c-myc protein were likely from increased promoter activity or increased mRNA stability. The promoter and all three exons of c-myc were screened in both cell lines for mutations, but none were found. In addition, the myc intron factor binding site (11) was screened for mutations in D283 Med, as well as in 8 primary medulloblastomas, but no mutations were identified. The promoter and coding regions of the primary tumors were normal as well. Because the promoter and coding regions of D283 Med and D721 Med were normal and there was no genomic amplification, then it was likely that trans-acting promoter activation was responsible for the elevated mRNA levels.

Transactivation of the c-myc promoter in the 12 medulloblastoma and PNET cell lines was initially investigated using the entire c-myc promoter, spanning from nucleotides −2433 to +354 relative to the major transcriptional initiation site P1, placed upstream of a firefly luciferase reporter gene (Fig. 3A). The c-myc promoter activity was 10-fold higher in D283 Med and 20-fold higher in D721 Med when compared with the average promoter activity in the other 10 medulloblastoma and PNET cell lines, suggesting that the elevated mRNA and protein levels in these cell lines were because of enhanced transcriptional activity. A series of nested deletions and fragments of the c-myc promoter (Fig. 1A) were then used to map the region of the c-myc promoter that was activated in these two cell lines.

The c-myc promoter activity remained high for the nested deletion constructs fragments 2–5 and was lost for fragment 6 in both D283 Med and D721 Med (Fig. 3, B and C). In D283 Med, two independent regions of the c-myc promoter were activated. The smallest transacted regions that induced activation of the c-myc promoter were the constructs fragment 9 (−1224 to −1095 relative to P2) and fragment 17 (−268 to +90 relative to P2). Regions smaller than the 130-bp fragment 9 resulted in substantial loss of transcriptional activity (data not shown). In D721 Med, the smallest transacted c-myc promoter construct that still exhibited activity was fragment 16 containing nucleotides −90 to +90 relative to the major transcriptional start site, P2 (Fig. 3C), suggesting that the mechanism of c-myc promoter activation was different in the two medulloblastoma cell lines. Other cell lines, D581Med, PFSK-1, and SK-PN-DW, were transfected with fragments 1–4, 7, 8, 13, 14, and 15; these fragments induced only low levels of activation of the c-myc promoter in these cell lines (results

not shown). Because fragments 1–4 contain fragment 17, it can be deduced that fragment 17 is specifically activated in D283Med and D721Med and not in the other lines tested.

The c-myc promoter region fragment 9 contains a β-catenin/Tcf binding site (CCTTTGATT) and disruption of the APC pathway has been implicated in medulloblastoma pathogenesis. We therefore tested to see if the c-myc promoter was activated by β-catenin and Tcf-4 in D283 Med. There was no activation of an optimized Tcf binding site placed upstream of the luciferase reporter (Fig. 3D). Activation of this binding site was observed in the colorectal carcinoma cell line, Hct 116 (Fig. 3D), which has a mutant β-catenin (24).

Co-transfection with a mutant activated β-catenin showed enhanced transcriptional activity (Fig. 3D) showing that no other component of this pathway was lacking in D283 Med. Therefore, activation of c-myc by the APC pathway was ruled out in D283 Med.

Fragment 17 was screened for binding sites of known transcriptional activators of c-myc as well as other oncogenes. Site-directed mutagenesis and deletion of these sites (Fig. 1B) were performed to reproduce mutations and deletions that had been previously found to diminish promoter activity. However, the mutation and deletion of these sites did not result in the complete ablation of activity of fragment 17 in D283 Med and in D721 Med (data not shown), indicating that additional transcription factors not tested in this study or multiple transcription factors are responsible for c-myc activation from these promoter regions.

**Table 1** c-myc expression in medulloblastoma and PNET cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Genomic copy number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transcripts/cell&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Quantitative PCR&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Northern&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Western&lt;sup&gt;e&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>D283 Med</td>
<td>2-6</td>
<td>59</td>
<td>164</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>D341 Med</td>
<td>&gt;25</td>
<td>223</td>
<td>792</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>D425 Med</td>
<td>&gt;25</td>
<td>262</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>D487 Med</td>
<td>&gt;25</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D556 Med</td>
<td>&gt;25</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>22</td>
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<tr>
<td>D661 Med</td>
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</tr>
<tr>
<td>D781 Med</td>
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<td>2</td>
<td>4</td>
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<td>+</td>
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<tr>
<td>MHH-Med-4</td>
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<td>3</td>
<td>1.2</td>
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<tr>
<td>MHH-PNET-5&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>2</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
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</table>

<sup>a</sup> The genomic copy number of c-myc was estimated using FISH.

<sup>b</sup> SAGE data for some medulloblastoma cell lines are available (http://www.ncbi.nlm.nih.gov/SAGE/), and the c-myc transcript numbers/cell was calculated assuming 200,000 transcripts/cell.

<sup>c</sup> Relative values based on total Sybr Green I fluorescence, using myc-specific primers and a LightCycler real-time PCR machine (Roche).

<sup>d</sup> Northern analysis was used to estimate the messenger RNA levels of c-myc.

<sup>e</sup> A monoclonal antibody specific for c-myc was used to assess the protein levels of c-myc.

<sup>f</sup> Primitive neuroectodermal tumors.

**Discussion**

The overexpression of c-myc was one of the first acquired genomic alterations found in medulloblastoma (2) and has been studied extensively because of its role in several types of human cancer. Although genomic amplification and point mutations of c-myc provide obvious evidence of its role in several cancers, upstream modes of pathological c-myc activation are more difficult to demonstrate. The role of APC and β-catenin/Tcf-4 activation of c-myc in colon cancer was clearly demonstrated using SAGE and promoter deletions (12). APC and β-catenin are also implicated in medulloblastoma and possibly in the activation of c-myc in this tumor type (13, 15, 16, 25).

In this study, using medulloblastoma cell lines as an experimental model, we tried to determine the means of c-myc activation. One-third of the cell lines studied had genomic amplification of c-myc (Table 1). An independent means of c-myc activation was found in 2 of 12 cell lines, but somewhat surprisingly was not because of β-catenin. Although there are a few β-catenin (13) or even more rare APC mutations in medulloblastoma (15), they do not account for all genomic amplification-independent activation of the c-myc promoter. Therefore, we sought to identify upstream transactivators of c-myc in these two cell lines by searching for consensus binding sites of known transcription factors.
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transcription factors. We subsequently tested their involvement in c-Myc overexpression by mutating or deleting these sites (Fig. 1D) and using a luciferase-based reporter assay to assess c-Myc promoter activity.

One limitation with these studies is that they were necessarily performed using established cell lines. Although cell lines can retain the original causative mutations, there may be dramatic selection for certain subsets of mutations. This appears to be the case for these cell lines, where 33% had c-myc genomic amplification, compared with a reported 5% of 77 primary tumors studied by the Children’s Oncology Group (22). On the basis of the low frequency of APC or β-catenin mutations in medulloblastoma tumors, it is maybe not surprising that in a small set of cell lines, there was no activation of the β-catenin binding region in the c-myc promoter. There may also be a selection bias against cell lines with this mode of activation.

Our studies have identified two different regions in the c-myc promoter that are activated in a subset of medulloblastoma cell lines. Analysis of the SAGE profile of D283 Med did not, however, reveal a highly expressed transcription factor known to bind to its activated region (data not shown), nor did site-directed mutagenesis pinpoint a single responsible transcription factor as was done with β-catenin binding sites in colon cancer. Although this reverse progression through a pathogenic pathway for medulloblastoma did not reveal a new causative mutation, it did show that multiple mechanisms are responsible for c-myc activation. The protein binding sites in these activated regions might also provide clues to additional pathways that activate c-myc in medulloblastoma. The data also suggest that because multiple mechanisms activate c-myc that the protein, rather than an upstream activator, is a better target for therapeutic intervention against c-myc’s malignant effects in aggressive medulloblastomas.

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References


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