PPM1D Is a Potential Target for 17q Gain in Neuroblastoma

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ABSTRACT

Neuroblastomas (NBs) show complex patterns of genetic abnormalities, which may include amplification of the MYCN gene, deletion of 1p, or a gain of DNA at 17q, the last being the most frequent observation in NB tumors. However, the specific genes and the molecular mechanisms responsible for development and progression of NB remain poorly understood. We investigated aberrations of DNA copy number in 25 NB cell lines using comparative genomic hybridization and identified a minimal common region of gain at 17q23. Although gain of distal 17q is the most powerful predictor of adverse outcome currently available for patients with NB, thus far, no potential target genes have been reported for that region. Therefore, we defined the 17q23 amplicon in detail and determined expression levels of 15 genes located within the smallest region of overlap observed among our NB cell lines to identify the most likely target gene(s). Among them, seven (CLTC, VMP1, delta-tubulin, RPS6KB1, FLJ12007, APPBP2, and PPM1D) were consistently overexpressed through increases in regional copy number. Analysis of expression levels of those seven genes in 32 primary NB tumors revealed a significant correlation between higher expression and poorer clinical outcome only with respect to PPM1D. Moreover, down-regulation of PPM1D by transfection of an antisense oligonucleotide suppressed the growth of NB cell lines to a remarkable degree, at least partly by participating in a process leading to apoptotic cell death. Taken together, our results indicate that PPM1D is the most likely target of the 17q23 gain/amplification in NB tumors and may have an important role in the pathogenesis of this disease.

INTRODUCTION

NB, the most common extra-cranial solid tumor in children, is characterized by diverse clinical behavior ranging from spontaneous regression to rapid malignant progression. These differences reflect biological heterogeneity among individual tumors, with the consequence that both prognosis and response to therapy can vary widely from one patient to another (1, 2). In view of this heterogeneity, understanding the characteristics of a given NB tumor is crucial for choosing appropriate therapy. Extensive efforts in the past led to standardization of criteria for diagnosis, staging, and response to treatment known as the INSS (3). However, clinical criteria are not likely to predict disease outcome in a reliable way.

The remarkable variability in clinical course of NB reflects diverse genetic changes acquired by the tumor cells. Among the genomic abnormalities identified in NB tumors to date, some, including amplification of MYCN (4), deletion of 1p (5), gain of 17q (6), and diploidy or tetraploidy (7), are associated with poor prognosis. LOH and FISH analyses have revealed other alterations of subchromosomal regions, e.g., losses at 3p, 4p, 9p, 11q, 14q and gains at 5q and 18q (8, 9). Recent CGH analyses have confirmed cytogenetic abnormalities that were previously reported and also revealed additional genetic aberrations in NB cells (10–12). Despite detection of numerous non-random alterations, to date MYCN is the only gene corresponding to any of the affected chromosomal regions that has been identified. To gain new insights into the pathogenesis of NB and to establish molecular targets for diagnosis and therapy, candidate genes in the altered regions, 1p and 17q in particular, must be identified and investigated.

Tumor cell lines provide valuable resources for gene discovery and functional studies because their molecular and cytogenetic aberrations and biological properties may reflect at least a subset of primary tumors. Many cell lines have been established from NB over the past several years, and detailed knowledge of specific chromosomal aberrations leading to losses, gains, or amplification of particular chromosomal regions in these tumor-derived cells will be instrumental in identifying target genes. We have already carried out extensive CGH studies in various other types of tumor cell lines and have identified genes present in detected amplicons that may be involved in tumorigenesis (13–15).

In the work reported here, we examined 25 NB cell lines by CGH to explore genomic alterations that might affect the development and/or progression of this disease. As in previous studies, we found the most frequent gains at distal 17q. Among the cell lines showing 17q gains, we identified one line in which amplification at 17q23 was detected as a remarkably HLG by CGH and as an HSR by FISH. Gains of distal 17q are observed mainly in advanced stages of NB; this change is considered to be the most powerful genetic predictor of adverse outcome for patients (6, 16). Copy-number gain/amplification of this region, and possible target genes present there, have been reported in breast and gastric cancers (17–21). Taken together, these lines of evidence strongly suggested that 17q23 might harbor one or more genes whose amplification renders them oncogenic, although no potentially significant candidate for NB had yet been identified within this region. Therefore, we carried out further molecular definition of the 17q23 amplicon in NB cell lines by examining expression levels of candidate genes located within the amplicon in cell lines and primary tumors of NB, to identify genes whose products might play important roles in the tumorigenesis of NB.

MATERIALS AND METHODS

NB Cell Lines and Tumors. All human NB cell lines we examined (Table 1) had been established from surgically resected tumors and maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

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The abbreviations used are: NB, neuroblastoma; INSS, International Neuroblastoma Staging System; LOH, loss of heterozygosity; FISH, fluorescence in situ hybridization; CGH, comparative genomic hybridization; HLG, high-level gain; HSR, homogeneously staining region; BAC, bacterial artificial chromosome; PAC, P1-derived artificial chromosome; UCSC, University of California at Santa Cruz; NCI, National Center for Biotechnology Information; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SRO, smallest region of overlap; OPT, oligonucleotide phosphorothioate; NMA, MYCN amplification.
generated from total RNAs of cell lines and primary NB tumors using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions. Real-time quantitative PCR was performed using LightCycler (Roche Diagnostics) with CYBR Green according to the manufacturer’s protocol. Primer sequences for each gene are available on request. The GAPDH gene (Roche Diagnostics) served as an endogenous control, and each sample was normalized on the basis of its GAPDH content. PCR amplification was performed in duplicate for each sample.

**Analysis for TP53 Mutations.** Exons 5–8 of the TP53 gene were analyzed by direct sequencing of their genomic PCR- or reverse transcriptase-PCR-amplified products as described by Smith et al. (27) and Fleckenstein et al. (28), respectively, using an ABI377 sequencer (PE Biosystems, Foster City, CA).

**Transfection with Antisense OPT.** Antisense experiments were performed as previously described (15), with minor modifications. Briefly, we synthesized the following oligonucleotides containing phosphorothioate backbones (OPT; Espec Oligo Service Co., Tsukuba, Japan): PPMID-AS, nucleotides 223–240 of PPMID cDNA (GenBank accession no. NM_003620) in the antisense direction; PPMID-IV, the inverse control for PPMID-AS; PPMID-SC, a scrambled control for PPMID-AS; c-MYC-AS, nucleotides 554–573 of c-MYC cDNA (GenBank accession no. NM_002467; Ref. 29) in the antisense direction; c-MYC-SC, a scrambled control for c-MYC-AS (29); MYCN-AS, nucleotides 175–189 of MYCN cDNA (GenBank accession no. NM_005378; Ref. 30) in the antisense direction; and MYCN-SC, a scrambled control for MYCN-AS (30). The OPT used in this study was delivered into cells using Oligofectamine (Invitrogen) according to the manufacturer’s instructions.

For evaluation of gene expression, 2 × 10^5 cells were plated on a 10-cm dish, transfected, and harvested 24 h later. RNA levels were determined by real-time quantitative RT-PCR. For measurements of viable cells (cell growth) or cell numbers, the day before transfection, 2 × 10^3 or 3 × 10^3 cells were seeded respectively in 96- or 6-well plates. Viable cells were assessed 48, 72 or 96 h after transfection by the trypan-blue exclusion method. For assessing nuclear morphology, cells that had been treated with OPT as the same manner in the analysis of cell numbers were fixed with 4% paraformaldehyde, and then stained with 4',6-diamidino-2-phenylindole. Experiments were repeated three times, and performed in triplicate each time.

**Statistical Analysis.** The Mann-Whitney U test was used to compare the mRNA expression level of each gene among subgroups of primary tumors. Possible correlations between variables of the analyzed tumor samples and gene expression status were tested by the χ^2 or Fisher’s exact test. Kaplan-Meier survival plots were constructed, and log-rank tests were used for comparisons between groups. Survival data were also subjected to Cox proportional-hazards regression analysis. One-way analyses of variance (one-way ANOVA) with subsequent Scheffe’s tests determined the significance of differences in multiple comparisons. P < 0.05 was required for significance in each case.

**RESULTS**

**DNA Copy Number Aberrations Detected by CGH in NB Cell Lines.** An overview of the chromosomal aberrations we detected among 25 NB cell lines is shown in Fig. 1. All lines we tested showed chromosomal imbalances. On average, 9.5 genetic changes were found/cell line, including 5.9 (range, 2–11) gains and 3.4 (range, 1–6) losses. Minimal common regions for the most frequent copy number gains were at 17q23 (25 of 25, 92%), 2p23-p24 (19 of 25, 76%), 1q31-q41 (14 of 25, 56%), 7p11.2-p21 (9 of 25, 36%), 7q22-q36 (9 of 25, 36%), and 8q24.3 (8 of 25, 32%). The most common losses were observed at 1p36.2-pter (16 of 25, 64%) and 11q21-q22 (7 of 25, 28%). Most of the cell lines (22 of 25, 88%) displayed prominent, localized regions of HLG indicative of gene amplification (Table 1, Fig. 1). The chromosomal region at 2p23-p24 showing HLG (14 of 25, 64%) contains MYCN, a gene that is amplified in many NB tumors (4). Another frequent HLG was detected at 17q23 in nine NB cell
The 17q23 amplification detected in NB for the following reasons: (a) distal 17q is the region most frequently involved in NB tumors, as reported elsewhere (10, 11, 16, 31); (b) no potential target gene had yet been identified within this region in NB tumors. To generate a defined map of the 17q23 amplicon, we performed FISH analyses in four of our NB cell lines (MP-N-TS, SJ-N-CG, KP-N-NH, and SMS-N-KAN) that had exhibited remarkable copy number gains (data not shown). Relative positions of BACs on a map of the 17q23 region are indicated in Fig. 2A. Copy numbers, as well as molecular organization of the amplicon, were assessed by analysis of the hybridization patterns on metaphase and interphase chromosomes.

In one cell line (MP-N-TS), 11 BACs (758H09, 619I22, 700K10, 178C03, 767P09, 371B04, 15E18, 106H22, 332H18, 15K02, and 215P18) produced the highest number of signals as HSRs on marker chromosomes (Fig. 2, B and C). Fewer signals were detected with the remaining 9 BACs (142B17, 1081P03, 329E11, 1096F01, 264B14, 136H19, 320E04, 342K02, and 42F20), suggesting that they were located outside the amplicon. The other three cell lines examined by FISH each yielded more than five signals; however, the number of signals in each line was not different among all 20 BACs, except for one increase observed between BAC1096F01 and 106H22 in the SJ-N-CG cell line (Fig. 2, B and C). Therefore, the SRO could be defined between BAC758H09 and 215P18, except for BAC264B14 and 136H19 (SRO I), or else between BAC758H09 and 106H22 (SRO II) if we consider one copy number difference to be significant. The respective sizes of SROs I and II are ~2.2 and 1.8 Mb, according to information in the UCSC database. To avoid the oversight of important target gene(s) for the 17q23 amplification in additional analyses, the SRO I was adopted as a critical region harboring targets.

Expression of Candidate Target Genes within the 17q23 Ampli- cion in NB Cell Lines. Genes activated by copy number increases and involved in the progression of tumors are likely to be located in the SROs of amplicons (14, 19). To determine whether genes amplified at 17q23 were overexpressed in association with amplification, we assessed expression status of transcripts located within SRO I in NB cell lines. On the basis of our map constructed on the basis of the FISH results (Fig. 2B), 15 transcripts, consisting of 10 known genes and five uncharacterized transcripts, were selected from the genome databases archived by UCSC and NCBI (Fig. 2A, Table 2). The expression level in NB cell lines of each transcript, normalized with GAPDH, was divided by the average value found in NB cell lines having normal copy numbers at 17q23 (KP-N-DZ and KP-N-NY, data not shown) and recorded as a fold increase in relative expression level (19). Although the NCBI database predicted some additional transcribed sequences within our SRO, we excluded those from expression screening because their genomic structures and/or expression status suggested that they were unlikely to be real transcripts. Indeed, our preliminary reverse transcriptase-PCR experiments failed to amplify a single product of such predicted sequences (data not shown).

As summarized in Table 2, 7 (CLTC, VMP1, delta-tubulin, RP56kB1, FLJ22087, APPBP2, PPM1D) of 15 transcripts were consistently overexpressed in cell lines exhibiting increased copy numbers at 17q23 (Fig. 2, A and B) compared with lines exhibiting normal copy numbers, strongly suggesting that these transcripts might be
targets for 17q23 gain/amplification in NB (14, 19). On the other hand, none of the other eight transcripts within SRO I was consistently overexpressed in conjunction with copy number gains in the region. Notably, none of the three transcripts located outside SRO II showed consistent overexpression as a consequence of amplification, although one or more of them are positional and functional targets for 17q amplification in breast cancers (33).

Expression of Potential Targets for the 17q23 Amplification in Primary NB Tumors. To identify the most likely target gene(s) among the seven candidates, we additionally determined their expression levels in each of 32 primary NB tumors and compared the expression patterns with clinicopathological data, especially the prognosis of patients. As a screening procedure, we compared living, disease-free patients with those who had died of their tumors, by a nonparametric Mann-Whitney U test. Only PPM1D mRNA expression revealed a significant difference between the two groups (P = 0.0160, Fig. 3A); the other six genes showed none (Fig. 3A). To confirm this result, we considered cases with values of less than or more than the mean expression level of each gene to belong to a low expression group or a high expression group, respectively, and the clinicopathological implications of each gene’s expression level were evaluated by comparisons between the two groups. The clinicopathological factors we analyzed are shown in Table 3. Among the 32 primary NB tumors, only two carried mutations in exons 5–8 of the TP53 gene. In accord with the results of our nonparametric analysis, only PPM1D mRNA expression status showed a statistically significant correlation with patient outcome. Moreover, the PPM1D high expression group showed a significantly poorer outcome compared with the PPM1D low expression group in all patients, as well as in patients who were positive for MYCN amplification (NMA-positive patients; Table 3). In contrast, none of the other six transcripts showed significant correlation between expression level and any clinicopathological factor (data not shown).

Fig. 3B shows Kaplan-Meier survival curves for all 32 NB patients and for the 16 NMA-positive patients. Patients whose tumors showed high PPM1D expression had significantly shorter overall survival times than did patients with low PPM1D expression in their tumors (P = 0.0034, log-rank test; Fig. 3B, left). In addition, a Cox propor-
Among the 16 NMA-positive NB patients, the high PPIM1D at 17q23 in NB, we investigated the effect of overexpressed indicated that p53 activation (34, 35). Because our molecular cytogenetic analyses contribute to the development of human breast cancers by suppressing PPIM1D the 17q23 amplicon (Fig. 2).

NBL cell lines having remarkable gains in the copy number (≥5) within the SRO of the 17q23 amplicon (Fig. 2). 0.00 indicates that expression level is below the lower limit of quantification.

Table 2 Relative expression levels of 15 positional candidate target transcripts for 17q23 amplification in NB cell lines

<table>
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<tr>
<th>Genes/LOCUS</th>
<th>Relative expression level (log 2 ratio)</th>
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<tr>
<td>FLJ20063</td>
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<tr>
<td>CLTC</td>
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<tr>
<td>CGG5147</td>
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<td>GAPDH</td>
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Table 3 Correlation between patient profiles and PPM1D mRNA expression in 32 cases with NB

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<th>high (n=9)</th>
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<th>Pm</th>
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<td>4</td>
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<tr>
<td>≥1 yr</td>
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<td>8</td>
<td>5</td>
<td></td>
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<td>Stage</td>
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<td>3</td>
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<td>9</td>
<td>6</td>
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<tr>
<td>Outcome</td>
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<tr>
<td>All cases</td>
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<td>3</td>
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<td>3</td>
<td>6</td>
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<td>NMA-positive cases</td>
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<tr>
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<td>6</td>
<td>0</td>
<td>0.0114</td>
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<tr>
<td>Dead</td>
<td>10</td>
<td>3</td>
<td>7</td>
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</tbody>
</table>

PPM1D IN NEUROBLASTOMA

![Figure](image)

Table 3. Correlation between patient profiles and PPM1D mRNA expression in 32 cases with NB

- **Age**
  - <1 yr: 19 patients, 15 alive, 4 dead (√)
  - ≥1 yr: 13 patients, 8 alive, 5 dead

- **Stage**
  - 1, 2: 17 patients, 14 alive, 3 dead
  - 3, 4: 15 patients, 9 alive, 6 dead

- **Ploidy**
  - Diploid: 13 patients, 11 alive, 2 dead
  - Others: 17 patients, 12 alive, 5 dead

- **MYCN amplification (NMA)**
  - Negative: 16 patients, 13 alive, 3 dead
  - Positive: 16 patients, 10 alive, 6 dead

- **TrkA expression**
  - Non-low: 16 patients, 13 alive, 3 dead
  - High: 16 patients, 10 alive, 6 dead

- **TP53 mutation**
  - No LOH: 13 patients, 9 alive, 4 dead
  - LOH: 10 patients, 6 alive, 4 dead

- **Wild-type**
  - 30 patients, 22 alive, 8 dead

- **Outcome**
  - All cases: 23 alive, 20 dead, 3 dead

- **NMA-positive cases**
  - Alive: 6 patients, 6 alive, 0 dead
  - Dead: 10 patients, 3 alive, 7 dead

- **Expression level of each gene in each cell line was divided by the average value of that in SK-N-DZ and KP-N-NY cell lines (reference value), which have normal copy number of 17q23, after normalization with GAPDH, and recorded as a fold increase in relative expression level. Fold increase in relative expression levels > 2.0 were considered significant and shown in bold type. Gray background indicates genes/transcript that was overexpressed as a consequence of 17q23 gain/amplification.

- **Inhibition of Cell Growth and Induction of Cell Death by Down-Regulation of PPM1D Expression with Antisense OPT.**
  - PPM1D encodes a protein phosphatase 1D magnesium-dependent, δ isoform, the amplification of which has been reported to contribute to the development of human breast cancers by suppressing p53 activation (34, 35). Because our molecular cytogenetic analyses indicated that PPM1D was likely to be a target for gain/amplification at 17q23 in NB, we investigated the effect of overexpressed PPM1D on NB cell growth/survival by down-regulating this gene with an antisense oligonucleotide (15). MP-N-TS cells, which had exhibited amplification and consequent overexpression of PPM1D, were transfected with an antisense OPT for the PPM1D gene (PPM1D-AS), PPM1D-AS, but not the control OPT (IV or SC) or the transfecting reagent Oligofectamine alone (Mock), induced a decrease in PPM1D mRNA (Fig. 4A). Growth of PPM1D-AS-treated cells was remarkably inhibited compared with control OPT-treated cells (PPM1D-IV, P=0.0034, log-rank test).

- **Correlation between patient profiles and PPM1D mRNA expression in 32 cases with NB**
  - Expression level of each gene in each cell line was divided by the average value of that in SK-N-DZ and KP-N-NY cell lines (reference value), which have normal copy number of 17q23, after normalization with GAPDH, and recorded as a fold increase in relative expression level. Fold increase in relative expression levels > 2.0 were considered significant and shown in bold type. Gray background indicates genes/transcript that was overexpressed as a consequence of 17q23 gain/amplification.

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  - Expression level of each gene in each cell line was divided by the average value of that in SK-N-DZ and KP-N-NY cell lines (reference value), which have normal copy number of 17q23, after normalization with GAPDH, and recorded as a fold increase in relative expression level. Fold increase in relative expression levels > 2.0 were considered significant and shown in bold type. Gray background indicates genes/transcript that was overexpressed as a consequence of 17q23 gain/amplification.
or SC; Fig. 4B) in a time and a dose-dependent manner. Correlations between inhibitory effects on PPM1D expression level and cell growth were observed during our preliminary experiments using several different antisense OPTs targeting different regions of the PPM1D gene, indicating that the effects of our antisense OPTs were exerted specifically through down-regulation of PPM1D expression (data not shown). PPM1D-AS also inhibited growth of CHP134 cells, which have four copies of the PPM1D gene (data not shown).

To investigate additionally the mechanism of PPM1D-AS-induced growth inhibition, we assessed the number of living and dead MP-N-TS cells after treatment with 300 nM OPT or mock-treated cells (Fig. 4C). PPM1D-AS treatment induced a significant increase in dead cells (Fig. 4D) on the viability of MP-N-TS cells at the indicated times after transfection with 300 nM OPT or controls, as determined by direct counting using the trypan-blue exclusion method. The data presented are the means ± SE of three separate experiments. Statistical analysis used the Mann-Whitney U test. a, PPM1D-AS versus PPM1D-IV. All, P < 0.05. e, effect of PPM1D-AS on the numbers of living MP-N-TS cells at the indicated times after transfection with 300 nM OPT or controls, as determined by direct counting using the trypan-blue exclusion method. The data presented are the means ± SE of three separate experiments. Statistical analysis used the Mann-Whitney U test: c, PPM1D-AS versus mock; d, PPM1D-AS versus PPM1D-IV. All, P < 0.05. D, effect of PPM1D-AS on the viability of MP-N-TS cells treated with the indicated concentrations of OPT. Cell viability was determined by WST assay 72 h after transfection (left) or at the indicated times after transfection with 300 nM OPT (right). Percentages were calculated against the absorbance of control cells treated with Oligofectamine alone (mock control). The data presented are the means ± SE of three separate experiments. Statistical analysis used the Mann-Whitney U test: a, PPM1D-AS versus PPM1D-IV; b, PPM1D-AS versus PPM1D-SC. All, P < 0.05. C, effect of PPM1D-AS on the numbers of living MP-N-TS cells at the indicated times after transfection with 300 nM OPT or controls, as determined by direct counting using the trypan-blue exclusion method. The data presented are the means ± SE of three separate experiments. Statistical analysis used the Mann-Whitney U test: c, PPM1D-AS versus mock; d, PPM1D-AS versus PPM1D-IV. All, P < 0.05. E, effect of PPM1D-AS on the viability of MP-N-TS cells treated with the indicated concentrations of OPT. Cell viability was determined by WST assay 72 h after transfection (left) or at the indicated times after transfection with 300 nM OPT (right). Percentages were calculated against the absorbance of control cells treated with Oligofectamine alone (mock control). The data presented are the means ± SE of three separate experiments. Statistical analysis used the Mann-Whitney U test: a, PPM1D-AS versus PPM1D-IV; b, PPM1D-AS versus PPM1D-SC. All, P < 0.05. F, additive effects of PPM1D-AS and c-MYC-AS on the viability of MP-N-TS cells. Cells were treated with combinations of 300 nM each OPT, and cell viability was determined 48 or 72 h after transfection. The same results were observed in CHP134 cells treated with the combination of PPM1D-AS and MYCN-AS (data not shown). Differences among multiple comparisons were analyzed by one-way ANOVA with subsequent Scheffe’s tests: g, c-MYC-SC+PPM1D-AS versus c-MYC-SC; h, c-MYC-SC+PPM1D-AS versus c-MYC-SC+PPM1D-IV; i, c-MYC-AS+PPM1D-AS versus c-MYC-SC+PPM1D-IV; j, c-MYC-AS+PPM1D-AS versus c-MYC-AS+PPM1D-IV; k, c-MYC-AS+PPM1D-AS versus c-MYC-SC+PPM1D-AS. All, P < 0.05.

DISCUSSION

In this study, we have demonstrated that (a) gain of genomic DNA in part of 17q was the most frequent aberration detected by CGH in our panel of 25 NB cell lines; (b) the SRO of 17q gain/amplification was confined to a small portion of 17q in NB cells; (c) among the transcripts located within the SRO, PPM1D was overexpressed through its copy number increase and appeared to be involved in poorer outcomes among patients with NB tumors; and (d) down-regulation of PPM1D expression by antisense OPT inhibited growth and induced cell death in a cell line overexpressing PPM1D. The 17q arm may harbor one or more genes that are rendered oncogenic by copy number gains in NB tumors (31). However, until now no PPM1D IN NEUROBLASTOMA...
potential target genes had ever been identified in NB, although several candidates in that region had been identified positionally and/or functionally in other types of tumors (17–21). Our findings indicate that PPM1D is the most significant candidate as a potential target of distal 17q gain/amplification in NB tumors. Because amplified regions often contain more than two target genes (17–21), we adopted the strategy outlined here for determining the most likely target(s) in this type of neural tumor.

In our CGH analysis of 25 NB cell lines, minimal common regions for the most frequent chromosomal gains were observed at 17q23, 2p23–p24, 1q31-q41, 7p11-p21, 7q22-q36, and 8q24.3, whereas losses were most common at 1p36.2-pter and 11q21-q22. On the whole, our results are consistent with findings of previous studies that examined primary NB tumors, except that we found gains at 1q and 2p and losses at 1p more often in the cell lines (10–12). Such alterations occur more frequently in advanced cases of NB, indicating that genes in those regions may be involved in malignant progression (4, 5, 8, 9). Moreover, the gains at 5p15.3, 11q24 and 20q13.2-q13.3 and the losses at 3p21-pter, 6q24-pter, 9p23-pter, 15q26, 16q24, 17p12, and 18q22-pter that were detected by our CGH analysis (see Fig. 1) also have been observed in advanced NB tumors (12, 36–38). Therefore, our NB cell lines appear to exhibit a pattern of chromosomal alterations that mimics patterns of more advanced primary NB tumors. Because the affected chromosomal segments may include oncogenes or tumor suppressor genes associated with the pathogenesis of NB tumors and because a few target genes already have been identified from the regions in question, cell lines we used in this study can serve as a resource for exploring those targets further.

Bown et al. (6) showed that partial gain of 17q is strongly predictive of poor prognosis in NB, although gain of the whole chromosome is not, indicating that the position of translocation breakpoints on 17q may be important for differential biological/clinical behaviors of NB tumors. Because breakpoints tend to be clustered within the proximal half of 17q, albeit in a variety of positions (31, 32), the dosage of specific gene(s) localized distal to the breakpoints may be critical for progression of NB. Thus, the precise definition of common regions of additional chromosomal material in tumors is an important step toward localizing candidate genes. 17q23.1-qter appeared to be the smallest region of gain on 17q in NB tumors (32, 39). However, the physical size of this region (>26 Mb), as well as relatively high density of genes on 17q, have made identification of target genes difficult. Indeed, only a few genes such as NME1 (17q22; Ref. 40) and survivin (17q25; Ref. 24) have been proposed as possible targets in NB, and none of them has proved to be a true target in this disease. Our CGH and FISH analyses identified the smallest, and remarkably amplified, region on 17q23.2 in NB cell line MP-N-TS. Because (a) proximal and distal sides of the amplified region in MP-N-TS showed normal copy numbers, (b) other cell lines also showed maximal copy numbers in this region, and (c) 17q gain is a highly frequent event in NB cell lines, this amplicon seems to harbor gene(s) that are critical for exerting dosage effects in the progression of this disease. Notably, 17q23 overlaps areas that are frequently gained/amplified in breast (17–20) and gastric (21) cancers in anaplastic meningiomas (41) and in malignant tumors of the peripheral nerve sheath (42). Those observations suggest that some gene(s) located on 17q23 may be responsible for the pathogenesis of those tumors as well as NB. Gene expression profiling of the 17q23 amplicon has been done most precisely in breast cancer, and some of the putative target genes for that disease such as CLTC, RPS6KB1, APPBP2, and TUBX2 are located within our SRO (17–20, 33).

After narrowing the amplicon to a relatively small chromosomal region, we compared the expression level of each positional candidate transcript within the SRO with its copy number in NB cell lines because the common criterion for a putative target gene is that its amplification leads to consistent overexpression (14, 19). Even after this step, however, we still had seven genes as candidates for NB, as in other tumors (17–21). Therefore, we hypothesized that overexpression of those candidate genes might correlate with poor prognosis and/or some other prognostic factor(s) and chose to compare their expression levels in primary NB tumors with clinico pathological data. Of the seven genes in question, only PPM1D expression showed a significant correlation with the prognosis of primary NB (Table 4, Fig. 3). The other six genes might be amplified merely as a consequence of their proximity to PPM1D. However, additional analysis of a larger series of primary NB tumors will be necessary to clarify the roles, if any, of these six genes in the pathogenesis of NB.

PPM1D is rapidly and transiently induced in response to radiation in a wild-type p53-dependent manner and encodes a serine/threonine protein phosphatase (43). Li et al. (35) have demonstrated that overexpression of PPM1D confers two oncogenic phenotypes on cells in culture: attenuation of apoptosis induced by serum starvation and transformation of primary cells in cooperation with RAS. Bulavin et al. (34) also demonstrated that overexpressed PPM1D reduces p53 phosphorylation at Ser33 and Ser46 through inactivation of the p53 mitochondrial-activated protein kinase; abrogates RAS-induced apoptosis; and can (a) partially rescue RAS-overexpressing cells from cell cycle arrest and promote transformation in vitro and (b) expedite tumor formation in vivo after injection of mouse embryonic fibroblasts expressing E1A+RAS into nude mice. Moreover, cells established from Ppm1d−/− mice show decreased proliferation rates (44). Those findings suggest that PPM1D is likely to be a proto-oncogene that can be involved in tumorigenesis in concert with other activated oncogenes and wild-type p53. In keeping with the requirements for assuming an oncogenic function of PPM1D, the cell lines and primary tumors of NB we analyzed in this study showed frequent amplification of MYCN and infrequent mutation of TP53 (Tables 1 and 3). Overexpression of PPM1D through an increase in its copy number may be a good explanation for both the strong link between 17q gain and MYCN amplification and a low frequency of TP53 mutation in NB tumors.

To clarify the functional role of PPM1D in NB, we down-regulated its expression by transfecting antisense OPT into NB cell lines that amplified and overexpressed this gene (Fig. 4A). Down-regulation of PPM1D suppressed cell growth in those experiments, indicating that PPM1D plays an important role in the growth of MP-N-TS (Fig. 4B) and CHIP134 cells (data not shown). The growth inhibitory activity of PPM1D-AS was at least partly correlated with induction of apoptotic cell death (Fig. 4, C–E). Analysis of the phosphorylation status of p53 and of p38 mitogen-activated protein kinase activity in NB tumors will be necessary to clarify the mechanism by which PPM1D contributes to the pathogenesis of NB. Our experiments using combinations of antisense OPTs for PPM1D and c-MYC or MYCN indicated additive inhibitory effects on cell growth (c-MYC; Fig. 4F, MYCN; data not shown), supporting a hypothesis that overexpressed PPM1D confers oncogenic phenotypes on cells by complementing other oncogenes (34, 35). Because some NB tumors having 17q gains show normal copy numbers for MYCN (6), oncogenes other than MYCN may contribute to the development and/or progression of NB.

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