Genome-wide Analysis of Gene Expression Associated with MYCN in Human Neuroblastoma\textsuperscript{1,2}

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ABSTRACT

Molecular mechanisms through which MYCN expression contributes to the malignant phenotype of neuroblastoma are unknown. We performed a genome-wide gene expression analysis of 40 well-characterized neuroblastic tumors and 12 cell lines to identify genes and biological pathways associated with MYCN expression. Gene expression was validated by reverse transcription-PCR and immunohistochemistry using tissue arrays. Two hundred twenty-two of 62,839 oligonucleotide probe sets detected expression of genes that were strongly associated with MYCN expression. Differentially expressed genes included examples of known oncogenes, genes associated with neural differentiation, and genes related to cell proliferation. Expression of a subset of these genes was altered after transfection of a neuroblastoma cell line, SK-N-ER, with a MYCN expressing gene construct when protein synthesis was inhibited and have consensus MYCN binding E-box sequences in their promoter regions, suggesting they represent direct targets. Several novel genes/expressed sequences were identified as overexpressed and most likely coamplified with MYCN in a subset of cases. A classification model to identify neuroblastomas with high levels of MYCN expression was developed based on expression profiles. The identification of coexpressed and coamplified genes associated with MYCN overexpression in neuroblastoma suggests biochemical pathways that contribute to the malignant behavior of these tumors and forms a basis for molecular classification.

INTRODUCTION

Neuroblastoma is the most common extracranial malignant solid tumor in infants and children younger than 4 years (1). Prognosis is highly variable and correlates with many biological and clinical features, including age, stage (2), tumor ploidy (3), and other cellular and molecular properties of primary tumors. Outcome remains very poor for stage 4 noninfant patients despite multimodality treatments (4). MYCN amplification occurs in \textasciitilde{}25% of neuroblastomas and can be detected as homogeneously staining regions or double minutes. The size of the amplicon may vary, and genes adjacent to MYCN are frequently amplified (5, 6). Gene amplification is consistently associated with high levels of MYCN expression (7). Numerous studies have demonstrated an association between MYCN gene amplification and aggressive neuroblastoma (8, 9), however, the molecular mechanisms through which MYCN expression governs clinical behavior is unknown (10).

The MYC family consists of five genes (11): MYC (or c-myc); MYCN; MYCL; MYCB; and MYCS, with several variants described for MYCL (12). All MYC genes encode a basic helix-loop-helix leucine zipper protein and are believed to play a role in transcriptional regulation. Two different consensus E-box promoter binding site sequences have been described, CACCGTG (13) and CAGTG (14). MYCN is believed to primarily serve as a transcriptional activator, although down-regulation of some genes has recently been reported (15, 16).

Overexpression of the MYCN protein is weakly transforming, and this oncogenic role is enhanced by cooperating events such as RAS mutation (17). MYCN is also thought to have a role in apoptotic cell death under certain conditions (18, 19). Expression of MYCN in nonamplified neuroblastoma cell lines can induce reentry of quiescent cells into the cell cycle (20), and a correlation has been shown with growth rate, motility, and cell attachment (21). The most compelling experimental evidence for a critical role of MYCN in neuroblastoma is the development of neuroblastoma-like tumors in transgenic mice with neuroectodermal-specific expression of MYCN (22). However, the specific targets of this gene remain unknown, and few studies have been performed to investigate in a comprehensive way the function of MYCN in human tumors (19, 23, 24). We performed a genome-wide gene expression analysis of well-characterized neuroblastic tumors to identify genes and biological pathways associated with high levels of MYCN expression.

MATERIALS AND METHODS

Patient Tumor Samples and Cell Lines. Samples of 40 neuroblastic tumors (Supplemental Table 1) were obtained at the time of surgery and immediately frozen in liquid nitrogen. Three were classified as ganglioneuroma, 3 were stage 4S, 5 stage 2, 8 stage 3, and 21 stage 4. Histological sections of the frozen tissue samples were reviewed, and the areas of interest were manually dissected to provide consistency and avoid nontumoral tissues (>70% tumor cell content). An additional set of 8 neuroblastic tumors (2 ganglioneuromas, 1 stage 2, 1 stage 3, and 4 stage 4 neuroblastomas) were used for validation of the classification algorithm. Twelve neuroblastoma cell lines were also included in the study (Supplemental Table 1; Ref. 25).

Gene Expression Analysis. Total RNA was extracted using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) and purified with the Qiagen RNeasy System (Qiagen, Mississauga, Ontario, Canada), according to the manufacturers’ recommendations. RNA concentration was determined by absorbance at 260 nm, and quality was verified by the integrity of 28S and 18S rRNA after ethidium bromide staining of total RNA samples subjected to 1.2% agarose gel electrophoresis. Total cDNA was synthesized with a T7-polyT primer and reverse transcriptase (Superscript II, Life Technologies, Inc., Carlsbad, CA) before \textit{in vitro} transcription with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY). Labeled nucleic acid target quality was assessed by test 2 arrays and then hybridized (45°C for 16 h) to Affymetrix Human U95 oligonucleotide arrays. After an automated process of washing and staining, absolute values of expression were calculated and normalized from the scanned array data using Affymetrix Microarray Suite 4.0 or 5.0. Gene assignments to probe sets identified as candidate targets of MYCN were identified through the Affymetrix web site\textsuperscript{5} and BLAST search of the ENSEMBL human database.

Vectors, Cell Lines, and Transfection Assays. pMYCN (gift of Dr. Naohiko Ikegaki, Fig. 4A) contains a 1.6-Kb cDNA fragment corresponding to...
exons 2 and 3 of the MYCN gene under control of a CMV immediate early promoter. For transfection, cell lines were grown in RPMI culture medium (SK-N-ER neuroblastoma cell line) or Dulbecco's modified Eagles high glucose culture medium (SK-N-AS and COS-1 cells) with 10% FCS at 37°C to near confluence. Cells (10^6) were transfected with 2 μg of the plasmid (pMYCN or pCMV6) with Lipofectamine (Life Technologies, Inc.) and incubated for 24 h in the absence of serum during the first 12 h. Cells were harvested in the cold and protein lysates prepared by addition of lysis buffer [1% NP40, 150 mM NaCl, 50 mM Tris HCl (pH 8), and 2 mM phenylmethylsulfonyl fluoride]. For some transfections, cycloheximide (20 μg/ml) was added to the culture medium 20 min before transfection and for 24 h (26). Total RNA was extracted using Trizol reagent. Protein concentration was determined by Bio-Rad DC protein assay system (Bio-Rad Laboratories, Hercules, CA).

SDS-PAGE Gel Electrophoresis and Western Blot. A total of 25 μg of each total cell protein extract was loaded on Nu-PAGE polyacrylamide gels (Invitrogen Corporation, Carlsbad, CA), separated by electrophoresis, and transferred to nitrocellulose membranes according to standard procedures. Incubation with anti-MYCN antibody (OncoGene Research, Darmstadt, Germany) and anti-β-tubulin antibody (Sigma-Aldrich Corp., St. Louis, MO) was carried out for 1 h at a dilution of 1:250, followed by hybridization to secondary antibody for 1 h. Anti-RAN antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:500.

cDNA Southern Blotting. Total cDNA was synthesized from 1 μg of RNA by using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA) and amplified by 17 PCR cycles to avoid overamplification of the more abundant cDNA fragments (this number was determined by agarose gel analysis of PCR products after each new cycle). Amplified total cDNA fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane. MYCN probe was prepared by PCR amplification of the insert in pMYCN with digoxigenin-labeled deoxynucleotide triphosphates. Hybridization was carried out overnight at 42°C in a 50% formamide buffer.

Real-Time RT-PCR. Real time RT-PCR was carried out with 100 ng of each total RNA template and 18 μl of a master mix prepared according to the manufacturer (LightCycler Master Kit; Roche, Basel, Switzerland). All reactions were performed in the LightCycler thermocycler with water in place of template as a negative control and a specific RNA used as a positive control. MYCN primers were 5'-CTCAGTACCTCCGGAGAG-3' and 5'-GGCATCCTTTGAGGATC-3'.

Data Analysis. Absolute values of expression for the MYCN gene (based on hybridization to the 35158_at probe set) were used to classify the samples as those with a high level of MYCN expression (>10,000) or low level (<10,000). This cut point was established as described in results below. This probe was also used as an independent variable for the correlation test. Because the genes of interest were skewed such that normality assumptions for parametric tests were not met, we used the Mann-Whitney test that uses rank the gene expression data were skewed such that normality assumptions for probe was also used as an independent variable for the correlation test. Because classification of samples.

RESULTS

Relationship of MYCN RNA Levels to Gene Copy Number and Intracellular Protein Expression. Genome-wide gene expression analysis was performed for 40 neuroblastic tumors and 12 neuroblastoma cell lines using oligonucleotide arrays with 62,839 probe sets (Supplemental Table 1). Relatively high levels of MYCN mRNA were detected in nine of nine MYCN-amplified cell lines and seven of nine MYCN-amplified tumor samples (Fig. 1A). In addition, 4 nonamplified tumor samples had high levels of this mRNA. Validation of MYCN RNA levels was carried out for some samples using LightCycler real-time PCR, and a good correlation with microarray-determined RNA levels was found (Fig. 1B). MYCN gene copy number/cell was confirmed by interphase locus-specific FISH in all six cases for which gene copy number and level of expression were discordant (Fig. 2, A and B). The presence of MYCN protein was determined by immunohistochemistry and immunofluorescence in tumors for which material was available. Nine of 10 tumors (90%) expressing high levels of MYCN mRNA were immunoreactive for nuclear MYCN protein by immunohistochemistry and immunofluorescence (Figs. 1A and 2, C and D). One tumor with high levels of MYCN mRNA (no. 55) did not demonstrate the presence of MYCN protein with either technique, and 1 of 21 tumors with low mRNA level (no. 36) was weakly immunoreactive for nuclear MYCN protein. Most samples with MYCN gene amplification and protein expression have MYCN RNA levels above an inflection between samples NE-NA/2/12 (8,000 FU) and the amplified cell line sample E-A/SK-N-JD (36,000 FU). We therefore decided to use a cut point near this inflection that would separate tumors with both amplification and high relative levels of expression from the remaining samples. Samples with expression above 10,000 FU were defined as having high levels of MYCN (11

The abbreviations used are: RT-PCR, reverse transcription-PCR; AIC, Akaike Information Criteria; FISH, fluorescent in situ hybridization; FU, fluorescent unit.
tumor samples and 9 cell lines) for the purposes of comparison to samples with low level MYCN expression (29 tumors and 3 cell lines).

Identification of Genes with Expression Patterns that Correlate with MYCN. Unsupervised, average-linkage, hierarchical cluster analysis of all tumor samples using 7000 probe sets with the greatest variance (based on the difference between the maximum value and minimum value of at least 3000 FU) demonstrated that samples with high levels of MYCN expression have expression profiles that tended to correlate more strongly with each other than with tumors having low levels of MYCN expression (Fig. 3A). Nine of 11 MYCN-expressing tumors were grouped on the same tertiary node of the dendrogram. However, it should be noted that this observation is based on a...
relatively small sample size, and this correlation was less evident when only stroma poor, stage 4 tumors were included in the analysis (Fig. 3B) and for neuroblastoma cell lines (Fig. 3C).

To identify genes with expression that correlated with \( MYCN \) levels, we compared tumors with and without high levels of \( MYCN \) mRNA as defined above. We used three statistical and mathematical measures to select probe sets with significant, large, relative expression differences between the two groups. Absolute values of expression for every probe set and every tumor sample were compared with that of a representative \( MYCN \) gene probe set (U95A 35158_at) using the Kendall \( \tau \) test to select for those genes with expression patterns that were strongly correlated with \( MYCN \). To detect genes with significant relative quantitative differences in the expression values between tumors with high- and low-level of \( MYCN \) mRNA, we used a Mann-Whitney nonparametric test and set a threshold of at least a 3-fold difference. Two hundred twenty-two of 62,839 probe sets met the strict criteria of \( P < 0.01 \) and at least a 3-fold-change in mean expression (Supplemental Table S3). Seventy-nine probe sets met the criteria for every probe set and every tumor sample were compared with that of a representative \( MYCN \) gene probe set (U95A 35158_at) using the Kendall \( \tau \) test to select for those genes with expression patterns that were strongly correlated with \( MYCN \). To detect genes with significant relative quantitative differences in the expression values between tumors with high- and low-level of \( MYCN \) mRNA, we used a Mann-Whitney nonparametric test and set a threshold of at least a 3-fold difference. Two hundred twenty-two of 62,839 probe sets met the strict criteria of \( P < 0.01 \) and at least a 3-fold-change in mean expression (Supplemental Table S2). Seventy-four probe sets detected genes that were up-regulated and 148 that were down-regulated in tumors with high levels of \( MYCN \) RNA.

We assigned a general biological function to all characterized genes that correlated with \( MYCN \) based on the Gene Ontology web site and literature review. Although a large proportion were of unknown function (63.9%), a number of genes were related to cell proliferation and neural differentiation. Fifteen of 80 partially characterized genes are thought to play a role in neural differentiation or neuronal function and 12 of 15 were down-regulated in tumors with high levels of \( MYCN \). Four \( MYCN \)-associated genes were related to cell cycle regulation or cell proliferation. These findings correlate well with the lack of differentiation and high mitotic-karyorrhectic index that is common for \( MYCN \)-amplified tumors, especially in younger patients (29).

Because most neuroblastomas with \( MYCN \) amplification are poorly differentiated and high stage, we performed the same analysis comparing 8 tumors with high levels of \( MYCN \) expression and 13 tumors with low levels of \( MYCN \) expression that were matched for stroma poor histology and stage 4 disease. Seventy-nine probe sets met the strict criteria of \( P < 0.01 \) for both Mann-Whitney and Kendall tests and 3-fold-change mean expression (Supplemental Table S3). Twenty-two of these probe sets (27.8%) were also identified as differentially expressed in the analysis of all neuroblastomas. Of the 79 genes differentially expressed with \( MYCN \) in stroma poor stage 4 tumors, we were able to assign a general biological function for 43. Three encoded DNA binding or DNA-associated proteins (NR5A1, CRE-Bpa, and H1F5) that are overexpressed with \( MYCN \) expression, as well as the oncogenes \( KIT \) and \( TGFBI \) [encoding a protein induced by transforming growth factor \( \beta \) (30)]. Two cell adhesion-related genes (occludin and protocadherin \( \beta 4 \)) were significantly overexpressed in tumors with low levels of \( MYCN \). Down-regulation in \( MYCN \) tumors of two genes implicated in development and histogenesis (\( HPX-42 \) and \( SIX6 \)) supports the role of \( MYCN \) in primitive cell populations. On the other hand, the genes \( CDCA7 \) and \( LOC166867 \), both with a function of control of the cell cycle and the mitotic spindle checkpoint, are overexpressed in tumors with high levels of \( MYCN \), most likely reflecting the high mitotic rate found in these tumors, whereas FGF9 (one of the genes controlling neural cell proliferation) was down-regulated. The 22 genes that were consistently differentially expressed in comparisons of all tumors and comparisons of stage 4 tumors were mostly uncharacterized, although genes encoding histone H4, synaptic vesicle membrane protein VAT, and the \( MYC \) target gene \( CDCA7 \) were included, along with three genes that are
commonly coamplified with MYCN (DDX1, NCYM, and NAG; Supplemental Tables 2 and 3). Many of the genes associated with neural differentiation and proliferation rate that were identified as differentially expressed in MYCN-related tumors when compared with all others were not as strongly differentially expressed when tumors were matched for histology and stage. These results are not surprising because aggressive tumors are expected to have similar biological attributes regardless of MYCN status.

**MYCN Regulation of Gene Expression in Transfected Cell Lines.** MYCN is believed to function as a DNA binding transcription factor. Therefore, it is likely that some of the gene expression differences we detected in neuroblastic tumors with high levels of MYCN expression are attributable to direct regulation by MYCN; others may be downstream effects or indirect consequences. We used MYCN transfection of neuroblastoma cell lines with low level expression of MYCN to identify early events and direct gene targets. The pMYCN-transfected cell lines demonstrated strong expression of MYCN mRNA by cDNA Southern blotting (Fig. 4D), and MYCN protein was detected by Western blot (Fig. 4B) and immunocytochemistry (Fig. 4C). Strong nuclear immunoreactivity for MYCN was detected in ~15–20% of cells in the pMYCN-transfected cultures and reflects the transfection efficiency. To identify those genes that might be direct targets, we transfected SK-N-ER cells with pMYCN in the presence of cycloheximide and performed expression analysis to identify those genes that are differentially expressed. Under the experimental conditions, MYCN protein is synthesized albeit at a reduced level and protein synthesis is largely inhibited over the 24-h period of incubation. Seventy-three of 222 probe sets identified as differentially expressed in tumor samples also exhibited qualitatively similar changes (>10% increase or decrease) after MYCN transfection in the presence of cycloheximide. Thirty-nine of these exhibited a >50% change and 26 a >100% change. Because only ~20% of cells are transfected, even small changes may be meaningful, but the greater the change the more likely that it is a reproducible (Supplemental Table S2). Regulation in the presence of a protein synthesis inhibitor suggests that these genes could be direct targets of MYCN. As additional support for putative direct interaction by MYCN, we analyzed the DNA sequences corresponding to potential promoter regions of the 222 differentially expressed genes. Four thousand-bp 5’ to the ATG start codon for 139 differentially expressed genes with available sequence were searched for the consensus E-box sequences recognized by MYCN, CACGTG, and CATGTG (14). A consensus binding motif was detected in 110 (79.1%) regions (CACGTG in 41 genes and CATGTG in 95 genes). This frequency was significantly higher than that found in a parallel analysis of the putative promoter regions of 100 randomly selected genes that were not on the list of 222 (χ² test yielded P of 0.0002).

Fifteen of 39 probe sets differentially expressed in tumor samples (>50%) after transfection of SK-N-ER with cycloheximide, and with available sequence, contained an E-box sequence within the 5’ untranslated sequence.

**Cell Context Specificity of MYCN Function.** Physiological gene regulation by MYCN is cell context dependent and requires the presence of cooperating molecules and chromatin structure. These features are expected to vary from cell to cell and within the spectrum of human tumors (20). To gain an understanding of the influence of cell context and to identify MYCN-regulated genes that are not dependent on cellular milieu, we analyzed gene expression in two separate neuroblastoma cell lines that were shown to have low levels of MYCN expression (SK-N-ER, SK-N-AS) along with COS-1 cells. Labeled targets from each cell line 24 h after transfection with either the pMYCN construct or control pCMV6 were hybridized to oligonucleotide arrays. Each transfected cell line demonstrated a relatively cell-specific pattern of differential gene expression with only 12.2% of all probe sets demonstrating the same direction of change (>10% increase or decrease) in expression after MYCN transfection in all three lines. These results suggest that there is a strong cell context influence on MYCN activity with a relatively small number of consistent changes in gene expression identified.

Of the 222 probe sets detecting significant association with MYCN expression for tumors, 29 showed similar directional changes in both neuroblastoma cell lines (SK-N-ER and SK-N-AS cells) with transfection (Supplemental Table S2). Thirteen of 29 genes showed the same directional changes in presence of cycloheximide.

**Expressed Genes in the MYCN Amplicon.** In an effort to identify differentially expressed genes that may be part of the MYCN amplon, we examined all genes and ESTs reported to map to chromosome 2 with expression patterns that strongly correlated (P < 0.01 for Kendall tau correlation test) with MYCN, DDX1, and NAG expression, genes that are often part of the MYCN amplon. The results suggest that several adjacent genes that have not previously been implicated in MYCN amplification (RPS7, ACP1, GREB1, MGC11266, DKFZP566A1524, NSE1, and the uncharacterized gene Hs.110039) are overexpressed and potentially coamplified in some neuroblastomas. An expression map of the region corresponding to neuroblastomas with high levels of MYCN expression (Fig. 5) demonstrates that MYCN is the only gene consistently expressed in all neuroblastomas with 2p amplification and agrees with the findings of others (6, 31). The significance of overexpression of coamplified genes is unknown.

**Molecular Classification of Neuroblastoma.** We evaluated the 222 genes that passed all filters in an attempt to develop a method for classification of neuroblastoma according to MYCN status. Such a classification would be useful in the case of discordant tumors or neuroblastomas that have alterations in MYCN regulated pathways independent of alterations in MYCN status, although it is
**DISCUSSION**

*MYCN* amplification is a well-established clinical marker of aggressive neuroblastoma useful for patient risk stratification. Amplification leads to high levels of gene expression in the majority of cases, and it is assumed that increased *MYCN* levels contribute directly to tumor biology. *MYCN* is a transcriptional regulator, however, few specific in vitro targets have been identified and the mechanisms by which *MYCN* contributes to aggressive tumor biology are not known. We used oligonucleotide arrays with >62,000 probe sets to monitor the effects of *MYCN* in neuroblastoma through analysis of human tumors and cell lines and identified a number of candidate genes that may represent direct and indirect targets of this oncogene. An interesting finding from our analysis that has also been noted in previous studies (8, 32, 33) is that *MYCN* mRNA expression levels did not always coincide with *MYCN* gene copy number (10, 34). This implies that overexpression of *MYCN* occurs in some cases of neuroblastoma without gene amplification and is probably attributable to alterations in transcriptional regulation of *MYCN*, although other possibilities exist (35). The clinical significance of overexpression in the absence of amplification is uncertain. However, in this study, tumors with high levels of *MYCN* expression in the absence of amplification tended to cluster with amplified tumors with increased expression, demonstrating a correlation between level of *MYCN* expression and overall gene expression profile.

It is believed that *MYCN* is able to trigger diverse complex pathways that may contribute to tumor biology, but the exact nature of these pathways has not been clarified. It may be that *MYCN* acts as a master transcriptional regulator controlling other genes that more directly activate or repress critical biological pathways. In fact, some transcriptional regulators (*HTATIP, HTATIP2, DD1X, M11RI1*, and *NCYM*) and oncogenes (*NCYM* and *RAB20*) were found in our study to correlate with the status of expression of *MYCN* in neuroblastoma. Two transcriptional activators with histone acetylase activity (*HTATIP* and *HTATIP2*) were down-regulated in *MYCN*-expressing tumors, and the loss of these genes may contribute to resistance to death-inducing signals and is also implicated in small cell lung carcinoma (36, 37). The oncogenes *NCYM* [capable of transforming cells in culture (38)] and *RAB20* [a positive regulator of *RAS* (39)] were overexpressed in *MYCN*-expressing tumors and could contribute to tumor biology.

Many of the genes that are differentially expressed in neuroblastomas with high levels of *MYCN* are involved in development or neural differentiation, suggesting that overexpression of *MYCN* is associated with altered maturation in progenitor neuroectodermal
cells. For example, HOXC10, a homeobox gene down-regulated in tumors with high levels of MYCN, is normally expressed at high levels in embryonic spinal cord and brain (40, 41), and it is believed to play an important role in development. Homeobox genes may exert a wide spectrum of effects in a variety of organs and body parts during early mammalian development (40). Additional support to the role of MYCN in differentiation comes from the fact that many markers of neural differentiation (PTN, FMNL, DNER, CLU, GTA, NRCAM, ECEL1, and SNPH) were increased in tumors with low MYCN levels, consistent with a greater degree of maturation for these tumors. Periperal myelin protein 22, a marker of Schwann cells (42), and the product of the gene EMP2 that shares 43% amino acid identity with peripheral myelin protein 22 (43) were down-regulated in MYCN-expressing tumors and reflects the stroma poor histology. Two other genes also related to differentiation, CD44 (44) and the receptor LR8 (45), were more highly expressed in tumors without MYCN. Inverse correlation between CD44 expression and MYCN amplification has been reported previously (44). On the other hand, CASPR3 with a potential role in cell recognition within the nervous system (46), STAFF65γ [expressed in the brain (47)], and UNC5C [related to cerebellar development (48)] were more abundant in MYCN-expressing tumors. The differential expression of these genes apparently reflects the primitive cell populations in untreated MYCN-amplified tumors.

Some differentially expressed genes are associated with regulation of cell cycle, cell proliferation, and apoptosis. CDC77 encodes for a cell division cycle-associated protein with neoplastic potential that has been previously identified as a target of MYC (49). CENPE has recently been identified as a target of MYCN (50) and is significantly up-regulated in MYCN-expressing tumors, reflecting the increased proliferation rate present in these tumors. In the same way, CDC2L2 was found to be up-regulated in tumors with MYCN expression, and a role of this cyclin-encoding gene in cell cycle progression has been proposed (51). Of interest, this gene maps at 1p36.3, a region frequently deleted in MYCN-amplified tumors (52, 53), and it has been considered a candidate tumor suppressor in neuroblastoma. On the other hand, PCTAIRE2BP, a molecule associated with a CDC2-like kinase that is expressed in terminally differentiated neurons (54), was down-regulated in MYCN-expressing tumors. Other DNA synthesis and repair-related genes were also up-regulated in tumors expressing MYCN, possibly as a consequence of the increased cell proliferation found in these tumors. One of these genes is UBL1, encoding for sentrin (55), which has been implicated in regulation of human homologous DNA recombination (56), and another one is NSE1, with a function in DNA repair and structural maintenance of chromosomes (57). Several RNAs encoding for proteins that are related to apoptosis and cell death were differentially expressed: EMP2 (58), TEGT (59), and SST (60) were more abundant in tumors with low level of MYCN, and BID was up-regulated. Altered regulation of apoptotic activity may be a key feature of MYCN-induced tumorigenesis.

It is interesting that the gene expression distinctions identified through comparisons of neuroblastomas without regard for stage or histology are not as evident when cases for comparison are controlled for these factors. For example, none of the genes related to neural differentiation showed a strong correlation with MYCN in stage 4 stroma poor tumors, suggesting that non-MYCN-expressing tumors have a similar phenotype to MYCN-overexpressing tumors of the same stage.

Although some of the genes identified in this study have been previously associated with MYCN [clusterin, CENPE (50), DDXI (5, 6), NAG (6)] or to the oncogene MYC [TUBA1 (25), CDC77 (49)], other previously characterized targets of MYCN did not meet the stringent criteria used here. For example, 40 probe sets encoding ribosome associated proteins (RPS3A, RPL18A, RPL9, RPS5, and others) and other genes [PTMA (23), NCL (61), NPM1 (61), and NME1 (62)] that have been associated with MYCN expression in cell lines (61) were not selected in this study because of the requirement for a minimum of 3-fold change, although they demonstrated a statistical correlation with MYCN (P < 0.01 for Kendall tau and Mann-Whitney tests). Different technical platforms could also be a reason for discrepancies. In addition, data from our transfection experiments suggest that cell context could have a dramatic effect on MYCN function. The biological differences between cell and tumor samples could explain the apparent variation in differential expression among experiments.

An important aspect of our study was the identification of genes that are likely to be direct targets of MYCN regulation based on several lines of evidence, including changes in gene expression after MYCN transfection in the presence of cycloheximide, a relationship to MYCN overexpression in human tumors, and the presence of a binding site for the MYCN protein within a potential promoter region. Of the 15 probe sets that met these criteria, PMP22 is related to neural function and differentiation, FAD3S has been implicated in the synthesis of membrane fatty acids, NUMA1 is part of the nuclear mitotic apparatus, and DBY encodes for a RNA helicase. Additional characterization of the remaining genes and their transcriptional regulation will help define a direct role of MYCN in tumorigenesis.

The MYCN amplicon often includes other genes that are overexpressed. One unresolved issue has been to define those genes that are expressed because of coamplification with MYCN and may contribute to the biology of neuroblastomas with 2p amplification. We identified seven genes near MYCN that were strongly overexpressed in a subset of neuroblastomas with high levels of MYCN, some of which had not previously identified in the MYCN amplicon. However, no other gene was found to be highly expressed in every case of neuroblastoma with high levels of MYCN, and none was consistently highly expressed in the absence of MYCN expression.

With the information derived from these studies, we were able to develop methods of molecular classification that use gene expression profiles to identify neuroblastic tumors with high MYCN status. Although these models require independent validation further than the 8 tumors and 12 cell lines samples used in this work, they serve as proof of principle for a clinically relevant molecular subclassification based on gene expression. Defining the molecular profiles of other categories of this heterogeneous disease has a high likelihood of additionally improving our understanding of this enigmatic cancer to refine risk-based therapeutic approaches.

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