MYC Messenger RNA Expression Predicts Survival Outcome in Childhood Primitive Neuroectodermal Tumor/ Medulloblastoma[1]

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ABSTRACT

Purpose and Experimental Design: **Cerebellar primitive neuroectodermal tumors/medulloblastomas (PNET/MB) are the most common malignant brain tumors in childhood. To identify PNET/MB biological prognostic factors that define a patient group with a sufficiently good prognosis to permit a reduction in treatment intensity, we determined the expression levels of *MYC* mRNA in fresh frozen tumor samples from 26 PNET/MB patients using semiquantitative reverse transcription-PCR.**

Results: ***MYC*** **mRNA expression levels in primary PNET/MB showed a wide range with a 22-fold difference between the highest and lowest values and did not correlate with *MYC* gene amplification. *MYC* mRNA expression was an independent significant prognostic factor for progression-free survival outcome and was more predictive than standard clinical factors. The combination of low *MYC* mRNA expression and high *TrkC* mRNA expression identified a good outcome group of PNET/MB patients ($n = 7$) with 100% progression-free survival after a median follow-up time of 55 months (range, 15–91 months). Three of these seven good outcome patients survived without radiotherapy.**

Conclusions: **Low *MYC* mRNA expression is a powerful independent predictor of favorable clinical outcome in PNET/MB. Assessment of *MYC* mRNA levels is feasible and may be incorporated in prospective PNET/MB clinical trials to aid in treatment planning for patients with PNET/MB on confirmation of our results in larger studies.**

INTRODUCTION

CNS[3] PNET, including PNET/MB, are the most common malignant brain tumors in children and constitute 20–25% of all pediatric brain tumors (1). Because of the high risk of leptomeningeal dissemination, standard postoperative treatment for PNETs includes not only local radiotherapy but also craniospinal radiotherapy and chemotherapy (2, 3). Such treatment causes long-term morbidity including endocrine and growth disturbances, as well as neurocognitive dysfunction, which is particularly severe in young children (4–9).

One strategy to reduce treatment-related long-term effects in patients at the greatest risk for severe radiation-induced brain injury is to delay radiotherapy through the use of prolonged chemotherapy (10, 11). Nearly two decades ago, van Eys *et al.* (12) used multiagent chemotherapy including mechlorethamine, vincristine, procarbazine, and prednisone as primary therapy after surgery for infants with malignant brain tumors. Only those patients with treatment failure and tumor progression received radiotherapy. Six of 12 infants with PNET/MB treated between 1976 and 1988 remain tumor free without having received radiotherapy and have been followed for a median time of 10.6 years (range, 6.2–15.2 years; Ref. 13). Cognitive functions in this group were within the normal ranges, stable across annual assessments, and clearly better than those in children who received radiation therapy. In older PNET/MB patients, one strategy to reduce treatment-related long-term effects is to reduce craniospinal radiation doses. Goldwein *et al.* (14) used 18 Gy of radiation to the craniospinal axis, a posterior fossa boost to 50.4–55.8 Gy, and chemotherapy to treat PNET/MB patients between 18 and 60 months of age. Seven of 10 patients survived with a median follow-up of 6.3 years, and the mean intelligence quotient of these patients was within the normal ranges.

Clinical prognostic factors for PNETs have been identified over the last two decades (15–19). These include metastatic stage, extent of tumor resection, tumor location, and age and are currently used to distinguish a high-risk group of patients (metastatic stage ≥ 1, residual tumor bulk ≥ 1.5 cm^2, age < 3 years, supratentorial tumor location) from a standard-risk group. However, investigators have appropriately avoided the use of the term "good risk" or "good outcome" because published survival rates in the 50–60% range do not justify this term. At the present time, clinical prognostic factors do not identify a "good

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[3] The abbreviations used are: CNS, central nervous system; PNET, primitive neuroectodermal tumor; MB, medulloblastoma; RT, reverse transcription; FBS, fetal bovine serum; NB, neuroblastoma.

Table 1 Clinical characteristics of 78 PNET/MB patients diagnosed at the Children's Hospital of Philadelphia between 1988 and 1998

	Patients included in the present study (n = 26)	Patients not included in the present study (n = 52)	P (Fisher's exact tests)
Gender			0.8
Male	17 (65%)	36 (69%)	
Female	9 (35%)	16 (31%)	
Age at diagnosis			0.6
<3 yrs	8 (31%)	13 (25%)	
≥3 yrs	18 (69%)	39 (75%)	
Metastatic stage			0.6
M_{1-3}	7 (27%)	10 (19%)	
M_0	19 (73%)	42 (81%)	
Surgery			0.7
Gross total resection	20 (77%)	43 (83%)	
Partial resection	6 (23%)	8 (15%)	
Biopsy	0 (0%)	1 (2%)	
Therapy			0.2
XRT ≥ 50 Gy + chemo[a]	20 (77%)	45 (86%)	
XRT ≥ 50 Gy alone	2 (8%)	0 (0%)	
Chemo alone	4 (15%)	6 (12%)	
No chemo + no XRT ≥ 50 Gy	0 (0%)	1 (2%)	

[a] XRT, local radiation therapy; chemo, chemotherapy.

outcome" group of PNET/MB patients that can be treated with a substantially less toxic treatment strategy compared with standard treatment. Because it is unlikely that new clinical prognostic factors will be found, biological prognostic factors must be identified to further improve PNET/MB prognostic systems.

Several biological factors have been studied as candidate prognostic factors in PNETs. Glial fibrillary acidic protein (*GFAP*) expression (20), *HER2/HER4* coexpression (21), Ki-67 (MIB-1) proliferation index (22), and mitotic index (23) have been analyzed in multivariable analysis and found to have independent predictive value. When hazard ratios were reported, the prognostic impact of these factors was similar or less than that of clinical factors. By contrast, the predictive value of *TrkC* mRNA expression has been shown to exceed that of all clinical factors (24, 25).

MYC (c-*myc*) has been the subject of several published studies directed at identifying biological prognostic markers in human PNET/MB. Most show that *MYC* gene amplification is uncommon in PNET/MB, with an incidence of ~8% in primary tumors (26–31). The incidence of *MYC* gene amplification in PNET/MB cell lines and xenografts may be higher and ranges up to 67% (32, 33), suggesting that *MYC* gene amplification correlates with cell line establishment and tumorigenicity. *MYC* gene amplification has been suggested as an indicator of poor prognosis in case reports (29, 34, 35) and in a recently published study of 29 PNET/MB patients (26). In aggregate, however, published studies suggest that the frequency of *MYC* amplification is not sufficiently high to provide prognostic information of greater value than clinical variables.

Mechanisms to activate *MYC* other than gene amplification are well recognized in various solid tumors. They include retroviral insertional mutagenesis, chromosomal translocation, somatic mutations, or activation by transcription factors (reviewed in Ref. 36). Transcriptional regulation of *MYC* may involve the *adenomatous polyposis coli (APC)* and β-*catenin* (*CTNNB1*) pathways, which may be involved in PNET pathogenesis. A subset of PNETs is associated with Turcot's syndrome (37), which is the association of colonic cancer with primary brain tumors and is characterized by germ-line mutations in the *APC* gene. Whereas mutations of *APC* have not been detected in sporadic PNETs (38, 39), approximately 5% of sporadic PNETs have been reported to contain mutations in a second Wingless/Wnt pathway member, β-*catenin* (40, 41). Mutations in either *APC* or β-*catenin* act to stabilize β-*catenin* protein. Stabilized β-*catenin* protein accumulates and translocates into the nucleus, where it complexes with *Tcf4* and up-regulates the transcription of *MYC* as well as other targets (42, 43). In a recent study, aberrant nuclear β-*catenin* staining has been demonstrated in 18% of sporadic PNET/MB and in one PNET/MB from patient with Turcot's syndrome (41). Therefore, mechanisms other than gene amplification may activate *MYC* transcription in PNET/MB. However, expression levels of *MYC* transcripts have not been reported for a representative group of PNET/MB.

To study the biological significance of *MYC* in PNET, we measured *MYC* mRNA expression levels in 6 PNET cell lines and 26 primary PNET/MB by semiquantitative RT-PCR and compared *MYC* mRNA expression levels with *MYC* gene copy number, clinical and biological variables, and survival outcomes.

PATIENTS AND METHODS

Patients and Therapy. Seventy-eight patients were diagnosed with a PNET/MB at the Children's Hospital of Philadelphia between January 1988 and December 1998. All diagnoses were confirmed by histological assessment of a tumor specimen obtained at surgery by one neuropathologist (L. B. R.). Adequate frozen tumor tissue to perform RT-PCR was available from 26 PNET/MB patients. Tumor samples were snap-frozen in liquid nitrogen in the operating room and then

stored at −80°C until further analysis. The demographic and treatment characteristics of the 26 study patients are summarized in Table 1. They are comparable with those of the 52 PNET/MB patients not included in the present study due to lack of frozen tumor tissue. Therefore, the subset of patients included in the present study can be considered representative. The median age at diagnosis for these PNET/MB patients was 6.4 years (range, 0.3–14.8 years). Seventeen (65%) PNET/MB patients were male, and nine (35%) were female. Evidence of leptomeningeal metastasis (M_{1-3}) was present in 7 (27%) PNET/MB patients, and 19 (73%) patients were M_0. Tumor location was cerebellar in all patients. Twenty (77%) patients had ≥90% surgical tumor resection, six (23%) patients had 50–90% resection of the tumor, and no patient had <50% of the tumor resected. Postoperative therapy included radiation and/or chemotherapy. Twenty patients had combined radiotherapy and chemotherapy, two patients had radiotherapy alone, and four infants had chemotherapy alone. Chemotherapy was administered to 15 patients according to a previously described protocol including vincristine, lomustine, and cisplatin (44), to 7 younger children according to infant brain tumor protocols (10), and to 2 patients according to other regimens. Median follow-up for the 15 patients who remain alive and progression free at the time of this report was 62 months (range, 15–105 months). Approval to link laboratory data to clinical data has been obtained by the Institutional Review Board.

Tumor Cells. DAOY and PFSK human PNET cells were purchased from American Type Culture Collection (Manassas, VA). D341, D425, and D458 human PNET cells were a kind gift from Dr. Henry Friedman (Duke University, Durham, NC). UW228-2 human PNET cells were a kind gift from Dr. John R. Silber (University of Washington, Seattle, WA). DAOY, D341, D425, and D458 cells were cultured in Richter's Zinc Option medium/10% FBS; PFSK cells were cultured in RPMI 1640/10% FBS; UW228-2 cells were cultured in DMEM/10% FBS. All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Isolation of RNA and Semiquantitative RT-PCR. Tumor tissue was disrupted with a sterile disposable tissue grinder (Sage Products Inc., Crystal Lake, IL). After tissue homogenization in guanidinium isothiocyanate-containing buffer, total RNA was isolated using a silica gel-based method according to procedures recommended by the manufacturer (RNeasy; Qiagen Inc., Valencia, CA). This included DNase digestion of the purified RNA. The yield of RNA from the various samples was calculated by spectrophotometry. RT reactions were carried out using 1 μg of total RNA in a total volume of 20 μl containing 150 ng of random hexamers, 0.5 mM deoxynucleotide triphosphates, 10 mM DTT, and 200 units of SuperScript II reverse transcriptase (all from Life Technologies, Inc., Gaithersburg, MD) in 20 mM Tris-HCl (pH 8.4) with 50 mM KCl and 2.5 mM $MgCl_2$. Total RNA was denatured at 70°C for 10 min and immediately chilled on ice. First-strand cDNAs were obtained after 10 min at 23°C and 50 min at 42°C. The reaction was terminated at 70°C (15 min). Two units of RNase H (Life Technologies, Inc.) were added to each RT reaction followed by incubation at 37°C for 20 min.

The method used for semiquantitative RT-PCR has been described previously (45, 46). In brief, PCR was carried out in a final volume of 20 μl containing 1 unit of Taq Gold polymerase, 100 μM deoxynucleotide triphosphates, 0.4 μM of each primer, buffer consisting of 50 mM KCl and 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 2 μl of the RT product as template. PCR primers were designed to bracket cDNA sequences that cross an intron-exon boundary in genomic DNA. Primer sequences specific for *MYC* (c-*myc*) were 5′-TGGTCTTCCCCTACCCTCTCAAC-3′ (sense) and 5′-GATCCAGACTCTGACCTTTTGCC-3′ (antisense) with an expected PCR product size of 265 bp. *MYC* primers were biotinylated at their 5′ ends. Semiquantitation was accomplished by comparison to signal generated by multiplexed primers to amplify *GAPD* in each reaction (sense, 5′-CATCAAGAAGGTGGTGAAGC-3′; antisense, 5′-GAGCTTGACAAAGTGGTCGT-3′; product size, 160 bp). Due to the high expression of *GAPD*, primers were mixed at a ratio of 1:199 biotinylated:unbiotinylated to ensure that all signals remained within a linear dynamic range. Amplification was performed on a PTC-100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA). The samples were initially denatured at 95°C for 12 min, followed by 18 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. The final cycle was followed by a 5-min extension step at 72°C. The absence of contaminants was routinely checked by RT-PCR assays of negative control samples (H_2O control or no reverse transcriptase added). Ten μl of each PCR sample were analyzed in parallel with a biotinylated molecular weight marker (Amersham, Arlington Heights, IL) on a nondenaturing 6% polyacrylamide gel. DNA was electrotransferred to a nylon membrane and immobilized by UV cross-linking. Detection of biotin-labeled DNA was performed by a chemiluminescent detection system (Tropix, Bedford, MA). To provide a semiquantitative assessment of RT-PCR-studies, we performed densitometric analysis of transcript signals using the NIH Image program (United States NIH).[4] Expression levels of the target transcripts were normalized by the use of the internal standard *GAPD*. The analysis of RT-PCR was blind to all clinical data.

Isolation of Genomic DNA and Semiquantitative PCR. Frozen tumor tissue adequate to isolate genomic DNA and perform PCR was available from 11 PNET/MB patients. Genomic DNA was isolated using the QIAmp DNA Mini kit (Qiagen Inc.) according to procedures recommended by the manufacturer. In brief, tumor tissue was disrupted with a sterile disposable tissue grinder (Sage Products Inc.), lysed with proteinase K, and incubated with RNase A. Genomic DNA was then purified using a silica gel-based method. The yield of DNA from the various samples was calculated by spectrophotometry. The method used for semiquantitative PCR was similar to that used for RT-PCR described above, with the following difference: biotinylated *GAPD* primers were used instead of a 1:199 biotinylated:unbiotinylated *GAPD* primer mixture. Moreover, the cycle number was 20 instead of 18. Cell lines and tumors with *MYC*/*GAPD* signal ratios of >1.5 were considered to be indicative of *MYC* gene amplification, as confirmed through analyses of cell lines of known *MYC* copy number.

[4] http://rsb.info.nih.gov/nih-image/.

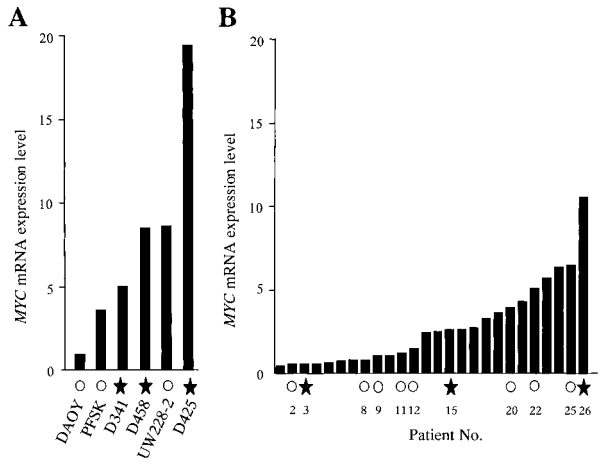

Fig. 1 mRNA expression levels of *MYC* in PNET cell lines (*A*) and primary PNET/MB (*B*) in comparison with *MYC* gene amplification (★, present; ○, absent). *MYC* mRNA expression was determined by semiquantitative RT-PCR with *GAPD* as internal standard.

***TrkC* mRNA Expression and *GFAP* Expression.** *TrkC* mRNA expression, assessed by *in situ* hybridization, was available for 19 of the 26 patients in the present study and has been published previously (25). *GFAP* expression, assessed by immunohistochemistry, was published previously (20). Information on seven additional cases has been added in the present study.

Statistical Analysis. Clinical characteristics of included and excluded patients were compared using Fisher's exact tests. Relative risk of progression or death was calculated by univariate and multivariable analysis using Cox regression models (47). Progression-free survival and overall survival were determined by Kaplan-Meier analysis (48), and differences between survival curves were calculated using the Mantel log-rank test (49) to confirm the results based on Cox regression models.

RESULTS

***MYC* Expression in Human PNET Cell Lines and Primary PNET/MB.** PNET cell lines showed a wide range of *MYC* mRNA expression, with the lowest expression in DAOY PNET cells (normalized expression level = 0.85) and the highest expression in D425 PNET cells (normalized expression level = 19.33; Fig. 1*A*). These results, which are consistent with Northern blot results published previously by others (50), validate the methods used in this study. We also used semiquantitative PCR to identify *MYC* gene amplification. D425, D341, and D458 PNET cells had high *MYC:GAPD* signal ratios (4.6, 4.1, and 2.4, respectively), indicating *MYC* gene amplification. UW228-2, PFSK, and DAOY had low *MYC:GAPD* signals (0.27, 0.38, and 0.54, respectively), indicating single copy numbers. These results are consistent with Southern blot results for these cell lines published previously (33, 50). Moreover, we were able to detect 3-fold *MYC* gene amplification in the PNET cell line D283 (data not shown), indicating a high sensitivity of the assay. In DAOY, PFSK, and UW288-2 PNET cell lines that do not have *MYC* gene amplification, *MYC* mRNA expression levels showed a 10-fold difference, indicating that mechanisms other than *MYC* gene amplification can contribute to increased *MYC* mRNA expression.

PNET/MB primary tumors showed also a large range of *MYC* mRNA expression. The median normalized *MYC* mRNA expression level in primary PNET/MB was 2.5 (range, 0.5–10.6) with a 22-fold difference between the highest value and the lowest value (Fig. 1*B*). Log-transformed *MYC* mRNA expression levels showed a normal distribution pattern. Tumors with *MYC* mRNA expression $\geq$ the median (*i.e.,* 2.5) were defined as PNET/MB with high *MYC* mRNA expression, whereas those with *MYC* mRNA expression levels <2.5 were defined as PNET/MB with low *MYC* mRNA expression.

To compare *MYC* mRNA expression levels with *MYC* gene amplification in primary PNET/MB, we selected 11 PNET/MB with sufficient frozen tissue to isolate genomic DNA and performed PCR. We found that *MYC* gene amplification was present in 3 of 11 PNET/MB. Similar to the PNET cell lines, *MYC* mRNA expression was highly variable in the PNET/MB with no *MYC* gene amplification and showed an 11.6-fold difference between the highest and lowest values (Fig. 1*B*).

***MYC* mRNA Expression and Survival.** Table 2 summarizes *MYC* mRNA expression, *TrkC* mRNA expression, *GFAP* expression, patient characteristics, treatment, and survival outcomes. Ten of 26 patients died as a result of progressive disease, and 1 patient is alive with progressive disease. There were no significant correlations between *MYC* mRNA expression levels and metastatic stage, age, or gender. A *t* test revealed that *MYC* mRNA expression was independent of *TrkC* mRNA expression ($P = 0.60$) and *GFAP* expression.

Univariate Cox regression analysis using *MYC* mRNA expression as a categorical variable showed that *MYC* mRNA expression was a significant prognostic factor for predicting progression-free survival (hazard ratio, 10.89; $P = 0.024$) and overall survival outcome (hazard ratio, 8.29; $P = 0.047$). When compared with the effect of metastatic stage, age, gender, extent of resection, therapy, *TrkC* expression, and *GFAP* expression, *MYC* mRNA expression was the most robust prognostic factor followed by *TrkC* mRNA expression (Table 3). Five-year overall and progression-free survival of the 26 patients are summarized in Table 4. Multivariable Cox regression analysis with inclusion of the clinical factors metastatic stage, age, and gender revealed that *MYC* mRNA expression remained a significant prognostic factor for progression-free survival (hazard ratio, 14.32; $P = 0.013$) and overall survival outcome (hazard ratio, 10.59; $P = 0.034$). The cumulative survival curves in the groups with high and low levels of *MYC* mRNA expression are shown in Fig. 2. The 5-year cumulative progression-free survival rate of the group with low levels of *MYC* mRNA expression was 92% and was significantly better than the 38% progression-free survival rate of the group with a high level of *MYC* mRNA expression (log-rank; $P = 0.0048$).

MYC gene amplification seemed not to be a predictor of tumor progression in the present study. Two of three patients (patients 3 and 26) whose PNET/MB showed evidence of *MYC* gene amplification have been followed for 70 months and are tumor free. However, the number of PNET/MB evaluated for *MYC* gene amplification was too small to include this variable in Cox regression models.

Information on *TrkC* mRNA expression was available for

Table 2 Patient characteristics, treatment, and survival outcomes compared with laboratory variables of primary PNET/MB specimens

Patient no.	Sex	Age (yrs)	M stage	Tumor resection	Chemotherapy	XRT (Gy) local/cs[a]	Follow-up (mo)	Outcome	*MYC*	*TrkC*	*GFAP*
1	M	7.2	**M_3**	**50–90%**	**No**	56/36	17	Dead of PD	0.47	**Low**	−
2	M	12.1	M_0	≥90%	Yes	56/36	91	Alive, no PD	0.56	High	−
3	M	10.7	**M_3**	**50–90%**	Yes	54/36	70	Alive, no PD	0.58	n.d.	−
4	M	12.9	M_0	**50–90%**	Yes	54/36	34	Alive, no PD	0.59	n.d.	−
5	M	12.4	**M_3**	≥90%	Yes	56/36	52	Alive, no PD	0.62	n.d.	−
6	M	8.5	M_0	≥90%	Yes	56/36	90	Alive, no PD	0.76	High	+
7	F	**0.6**	M_0	≥90%	Yes	**0**	15	Alive, no PD	0.8	High	−
8	M	3.5	M_0	≥90%	Yes	52/23	46	Alive, no PD	0.83	High	−
9	M	7.6	M_0	≥90%	Yes	56/23	38	Alive, no PD	1.04	n.d.	−
10	F	10.9	**M_3**	≥90%	Yes	55/23	47	Alive, no PD	1.07	High	−
11	M	**1.4**	M_0	≥90%	Yes	**0**	62	Alive, no PD	1.27	High	−
12	M	6.4	M_0	≥90%	Yes	60/36	105	Alive, no PD	1.5	n.d.	+
13	F	**0.3**	**M_1**	**50–90%**	Yes	**0**	55	Alive, no PD	2.48	High	−
14	F	6.7	M_0	≥90%	Yes	56/40	122	Dead of PD	**2.53**	**Low**	−
15	M	7.6	M_0	≥90%	Yes	60/40	75	Dead of PD	**2.61**	High	−
16	M	9.7	M_0	**50–90%**	**No**	54/35	21	Dead of PD	**2.64**	High	−
17	M	**1.8**	M_0	≥90%	Yes	50/18	6	Dead of PD	**2.76**	High	−
18	M	**1.3**	M_0	≥90%	Yes	**0**	11	Dead of PD	**3.31**	n.d.	−
19	M	**1.5**	**M_3**	≥90%	Yes	56/36	90	Alive, no PD	**3.64**	High	−
20	M	**1.8**	**M_3**	≥90%	Yes	52/36	15	Dead of PD	**3.94**	**Low**	−
21	F	3.9	M_0	≥90%	Yes	55/36	37	Dead of PD	**4.34**	**Low**	+
22	M	8.6	M_0	≥90%	Yes	56/36	65	Dead of PD	**5.09**	High	−
23	F	**2.7**	M_0	**50–90%**	Yes	54/36	38	Alive, PD	**5.73**	n.d.	+
24	F	14.8	M_0	≥90%	Yes	54/36	19	Dead of PD	**6.38**	**Low**	−
25	F	6.4	M_0	≥90%	Yes	54/36	90	Alive, no PD	**6.52**	High	−
26	F	6.8	M_0	≥90%	Yes	56/36	70	Alive, no PD	**10.57**	High	−

[a] XRT, radiation therapy; *MYC*, *MYC* mRNA expression determined by semiquantitative RT-PCR; *TrkC*, *TrkC* mRNA expression determined by *in situ* hybridization; *GFAP*, glial fibrillary acidic protein expression determined by immunohistochemistry; PD, progressive disease; n.d., not detected.

[b] Bold type indicates clinical and biological risk factors.

Table 3 Univariate and multivariable analysis of clinical and laboratory variables and progression-free survival in 26 patients with PNET/MB

	Univariate analysis		Multivariable analysis[c]	
Variable[a]	Hazard ratio (95% CI)[b]	*P*	Hazard ratio (95% CI)	*P*
MYC mRNA expression	10.89 (1.37–86.38)	0.024	14.32 (1.74–118.07)	0.013
TrkC mRNA expression	3.78 (0.92–15.51)	0.065		
Age	2.35 (0.65–8.51)	0.19	1.87 (0.32–11.00)	0.49
Surgery	2.41 (0.60–9.75)	0.22		
Therapy	2.40 (0.59–9.83)	0.22		
Gender	1.99 (0.42–9.39)	0.39	3.75 (0.68–20.48)	0.13
GFAP expression	1.23 (0.27–6.10)	0.76		
Metastatic stage	0.88 (0.19–4.18)	0.88	0.45 (0.06–3.28)	0.43

[a] The variables were compared in the following ways: *MYC* mRNA expression, ≥2.5 *versus* <2.5 normalized expression level; *TrkC* mRNA expression, <1.7 *versus* ≥1.7 antisense: sense signal ratio; age, <3 years *versus* ≥3 years; surgery, extent of resection <90% *versus* ≥90%; therapy, local radiation therapy or chemotherapy alone *versus* local radiation therapy + chemotherapy; gender, male *versus* female; *GFAP* expression, present *versus* absent. M stage (metastatic stage), M_{1-3} *versus* M_0.

[b] CI, confidence interval.

[c] Multivariable analysis was performed after the comparison for *MYC* mRNA expression was adjusted for metastatic stage, age, and gender.

19 of 26 PNET/MB patients. Despite the small sample size and limited number of clinical events, *TrkC* mRNA expression was a prognostic factor with a hazard ratio approaching statistical significance (hazard ratio, 3.78; $P = 0.065$). *GFAP* expression was not a prognostic factor in the present study. Whereas two of four patients (patients 6 and 12) whose PNET/MB was positive for *GFAP* were tumor free after 90 and 105 months of follow-up, two other patients (patients 21 and 23) had tumor progression. Univariate Cox regression analysis resulted in a nonsignificant hazard ratio of 1.23 ($P = 0.76$).

Prognostic Model Combining *MYC* and *TrkC* mRNA Expression. The combination of *MYC* mRNA expression and *TrkC* mRNA expression identified three risk groups of patients (Fig. 3). Children with PNET/MB expressing high *TrkC* mRNA and low *MYC* mRNA levels ($n = 7$) had the best survival outcome, with a progression-free survival rate of 100% after a

Table 4 Survival and progression-free survival in 26 patients with PNET/MB

Variable	% 5-year PFS (95% CI)[a]	% 5-year survival (95% CI)
All patients	69 (47–83)	72 (50–86)
Gender		
Female (n = 8)	71 (26–92)	71 (26–92)
Male (n = 18)	58 (31–78)	72 (46–87)
Age at diagnosis		
<3 yrs (n = 8)	50 (15–77)	60 (19–85)
≥3 yrs (n = 18)	69 (39–86)	69 (39–86)
Metastatic stage		
M_0 (n = 19)	62 (35–80)	74 (48–88)
$M_{1–3}$ (n = 7)	67 (19–90)	67 (19–90)
Surgery		
≥90% resection (n = 20)	67 (40–84)	74 (49–88)
≥50% but <90% resection (n = 6)	50 (11–80)	67 (19–90)
Therapy		
XRT ≥50 Gy + chemo (n = 20)	68 (41–84)	72 (44–88)
XRT ≥50 Gy or chemo alone (n = 6)	50 (11–80)	50 (11–80)
MYC mRNA expression		
MYC ≥2.5 (n = 13)	38 (14–63)	45 (18–69)
MYC <2.5 (n = 13)	92 (57–99)	92 (54–99)
TrkC mRNA expression		
TrkC ≥1.7 (n = 14)	75 (39–92)	85 (52–96)
TrkC <1.7 (n = 5)	0	0
Antigen expression		
No glial (n = 22)	64 (38–82)	72 (48–86)
Glial (n = 4)	50 (6–84)	75 (13–96)

[a] PFS, progression-free survival; CI, confidence interval; XRT, local radiation therapy; chemo, chemotherapy.

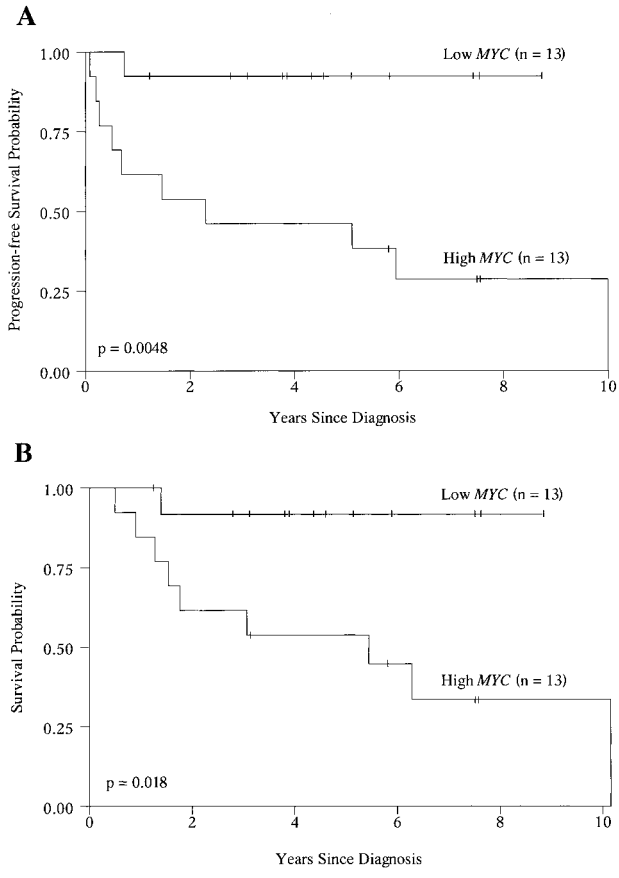

Fig. 2 *MYC* mRNA. The Kaplan-Meier curves show the probability of progression-free survival (*A*) and overall survival (*B*) in terms of the level of expression of *MYC* mRNA. The survival curves were analyzed by the log-rank test.

median follow-up time of 55 months (range, 15–91 months). Of note, three of seven patients with good outcome survived without radiotherapy. Children with PNET/MB expressing high *TrkC* mRNA and high *MYC* mRNA levels (n = 7) had an intermediate progression-free survival outcome (71% at 5 years), whereas the group with low *TrkC* mRNA expression (n = 5) had the worst progression-free survival outcome (20% at 5 years) regardless of *MYC* mRNA expression. The prognostic model using *MYC* and *TrkC* mRNA expression was superior to known clinical factors in identifying a good outcome group of patients. Of 10 "standard-risk" patients (age at diagnosis ≥ 3 years, no evidence of metastatic disease, and ≥90% tumor resection), 5 (50%) died because of progressive disease.

DISCUSSION

The *MYC* proto-oncogene, a member of a family of highly related genes that includes *MYCN* and *MYCL*, encodes a nuclear phosphoprotein involved in the transcription of genes central to regulating the cell cycle (51), cellular proliferation (52), apoptosis (53), and embryonic development and differentiation (54). *MYC* proteins form heterodimers with *Max*, which is also a helix-loop-helix leucine zipper protein. *MYC/Max* heterodimers bind to specific DNA sequences located in the transcriptional control region of target genes and alter the transcription of these target genes by transactivation or transrepression.

MYC expression is normally tightly regulated throughout the cell cycle but may become deregulated or activated, contributing to malignant transformation (55). Dysregulation of *MYC* has been implicated in the pathogenesis of a variety of human neoplasms, including Burkitt's lymphoma (reviewed in Ref. 56), breast carcinoma (57), colon adenocarcinoma (58), lung carcinoma (59), and prostate carcinoma (60). One important mechanism of *MYC* dysregulation results from gene amplification. *MYC* gene amplification has been identified in various cancers, including lung carcinoma (61) and breast carcinoma (62), and the presence of *MYC* gene amplification has been reported to correlate with unfavorable prognosis in breast carcinoma (63, 64). In childhood NB, amplification of *MYCN*, a *MYC* family member, occurs in about 25% of primary tumors and is strongly associated with advanced-stage disease, rapid tumor progression, and poor prognosis (65–67).

We found that there was a wide range of *MYC* mRNA expression and that *MYC* mRNA expression did not correlate with the presence of *MYC* gene amplification in PNET cell lines or primary PNET/MB. This finding is consistent with that of Bruggers *et al.* (50), who reported similar *MYC* mRNA expression levels in D425 cells (100-fold *MYC* amplification) compared with D283 cells (3-fold *MYC* amplification) and higher *MYC* mRNA expression levels in PFSK cells (single-copy *MYC*) compared with DAOY cells (single-copy *MYC*). Dispar-

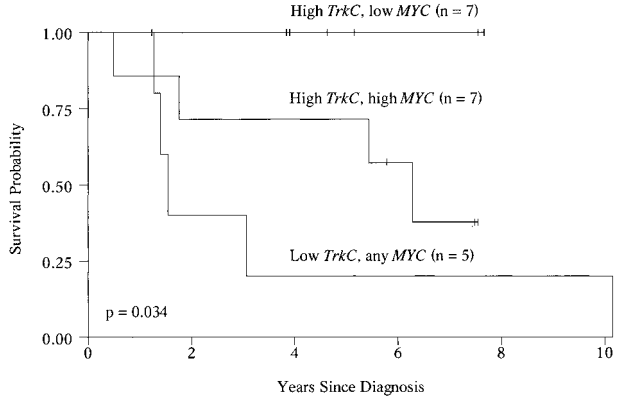

Fig. 3 Cumulative survival curves of patients with PNET/MB, according to expression levels of both *MYC* mRNA and *TrkC* mRNA. Survival was better in the group with high levels of *TrkC* mRNA expression and low levels of *MYC* mRNA expression than in the group with high levels of *TrkC* mRNA expression and high levels of *MYC* mRNA expression ($P = 0.066$).

ity between *MYC* gene copy number and *MYC* mRNA expression levels indicates the presence of alternative mechanisms to gene amplification in activating *MYC* and has been also reported for other cancers, including breast and colorectal carcinomas (58, 68).

In the present study, high levels of *MYC* mRNA expression correlated strongly with an unfavorable survival outcome in PNET/MB patients. *MYC* mRNA expression was more predictive than standard clinical factors that did not reach statistical significance on univariate analysis because of the relatively small samples size (26) and the limited number of clinical events (11). *MYC* mRNA expression levels did not correlate with metastatic stage, age, or gender of PNET patients. In multivariable analysis, correcting for these factors, the hazard ratio for *MYC* mRNA expression remained significant, indicating that *MYC* mRNA is of independent prognostic significance. Accordingly, *MYC* mRNA expression represents one of four individual biological prognostic factors we have identified in PNETs (20, 22, 25). Of these, *TrkC* mRNA and *MYC* mRNA expression seem to be the most potent prognostic factors when hazard ratios are compared and when the results of independent research groups are considered. Kim *et al.* (24) determined *TrkC* mRNA expression in 42 PNET/MB patients by Northern blot analysis and found that tumors with high *TrkC* mRNA expression were less likely to progress than tumors with low *TrkC* mRNA expression. Herms *et al.* (31) determined *MYC* expression in 72 PNET/MB by *in situ* hybridization, and they found that 30 patients with *MYC* mRNA-expressing PNETs/MBs had a worse survival outcome than did 42 patients whose tumors did not express *MYC* mRNA using their assay. In the present study, *MYC* mRNA expression was independent of *TrkC* mRNA expression and *GFAP* expression, suggesting that further analysis of these expression patterns be performed.

It is well known that the mechanisms regulating tumorigenesis are multifactorial. Therefore, it is unlikely that any single biological prognostic factor will be sufficiently robust to optimally segregate all PNET patients into risk-based strata. Accordingly, multiple factors of biological relevance are more likely to provide a more accurate system of defining risk groups. In the present study, we demonstrate this by combining *MYC* and *TrkC* mRNA expression. The group with low *MYC* and high *TrkC* mRNA expression had a 100% five-year progression-free survival outcome after a median follow-up time of 55 months (range, 15–91 months). Of note, three patients in this group were infants. Although the number of patients is too small to draw firm conclusions, it is conceivable that in this age group, where neurotoxic consequences of radiation therapy are greatest, a good outcome group can be identified by biological factors and potentially treated and cured with chemotherapy alone.

CNS PNETs and NB share clinical and biological similarities. They are both embryonal tumors derived from primitive neuroectodermal cells. They both occur predominantly in early childhood and represent the most common extracranial and intracranial neoplasms, respectively, in this age group. We now show that the combination of *MYC* mRNA expression and *TrkC* mRNA expression may be used in a prognostic model of CNS PNET/MB similar to the combination of *MYCN* gene amplification and *TrkA* mRNA expression in NB. Combination of *TrkA* mRNA expression and *MYCN* amplification identifies a low-risk group of NB patients (high *TrkA* expression and normal *MYCN*), an intermediate-risk group of NB patients (low *TrkA* expression and normal *MYCN*), and a high-risk group of NB patients (low *TrkA* expression and amplified *MYCN*; Ref. 69). Other well-established prognostic markers in NB are histological characteristics (70), deletions of chromosome 1p (71, 72), and gains of chromosome 17q (73). The most appropriate therapy for NB patients is determined not only by the use of clinical factors but also through the use of prognostication models including biological features (74, 75).

We anticipate that biological prognostic factors, in addition to clinical factors, will similarly define risk groups and help direct therapy decisions for children with PNET/MB. We therefore strongly recommend prospectively assessing *MYC* and *TrkC* mRNA expression levels in future PNET/MB clinical trials in an effort to validate this model for future incorporation in risk classification systems for clinical use.

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