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Clinical Cancer Research

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Imaging, Diagnosis, Prognosis

Array-Based Comparative Genomic Hybridization Identifies *CDK4* and *FOXM1* Alterations as Independent Predictors of Survival in Malignant Peripheral Nerve Sheath Tumor

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Abstract

Purpose: Malignant peripheral nerve sheath tumors (MPNST) are highly aggressive sarcomas with variable patient survival and few known prognostically relevant genomic biomarkers. To identify survival-associated genomic biomarkers, we performed high-resolution array-based comparative genomic hybridization (aCGH) on a large set of MPNSTs.

Experimental Design: Candidate gene alterations identified by aCGH in 38 MPNSTs were validated at the DNA, RNA, and protein levels on these same tumors and an independent set of 87 MPNST specimens.

Results: aCGH revealed highly complex copy number alterations, including both previously reported and completely novel loci. Four regions of copy number gain were associated with poor patient survival. Candidate genes in these regions include *SOX5* (12p12.1), *NOL1* and *MLF2* (12p13.31), *FOXM1* and *FKBP1* (12p13.33), and *CDK4* and *TSPAN31* (12q14.1). Alterations of these candidate genes and several others of interest (*ERBB2, MYC and TP53)* were confirmed by at least 1 complementary methodology, including DNA and mRNA quantitative real-time PCR, mRNA expression profiling, and tissue microarray-based fluorescence *in situ* hybridization and immunohistochemistry. Multivariate analysis showed that *CDK4* gain/amplification and increased FOXM1 protein expression were the most significant independent predictors for poor survival in MPNST patients ($P < 0.05$).

Conclusions: Our study provides new and independently confirmed candidate genes that could serve as genomic biomarkers for overall survival in MPNST patients. *Clin Cancer Res; 17(7); 1924–34. ©2011 AACR.*

Introduction

Malignant peripheral nerve sheath tumors (MPNST) are highly aggressive and frequently lethal Schwann cell-derived neoplasms associated with poor survival (1–5). Roughly half of MPNSTs develop sporadically and the other half arise in the setting of neurofibromatosis type 1 (NF1). The 5-year survival rate for NF1-associated MPNST patients is reportedly half of that for sporadic cases (21% vs. 42%; ref 5). However, other studies suggest that a diagnosis of NF1 does not affect survival (6). Clinicopathologic factors, such as tumor grade and anatomic site reportedly influence MPNST patient outcome, although the clinical course remains highly variable and difficult to predict in individual patients.

Traditional cytogenetic studies have reported that MPNSTs have remarkably complex karyotypes with a wide spectrum of chromosomal alterations including translocations, duplications, and numerical gains and losses, making it difficult to discern which of these alterations are biologically relevant (7–9). Unfortunately, no single alteration

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Translational Relevance

Malignant peripheral nerve sheath tumor (MPNST) is an aggressive malignancy with considerable biological variability, a variable and often lethal clinical course, and limited response to current therapeutic approaches. A major impediment to more effective disease management has been a lack of available prognostic or predictive biomarkers. Through initial identification of several survival-associated copy number alterations, followed by confirmatory assays and a comprehensive multivariate analysis, our present study demonstrates that *CDK4* gain/amplification and increased FOXM1 protein expression are significant independent predictors for poor survival in MPNST patients. If confirmed in additional prospective studies, these biomarkers may provide clinically useful information for managing patients with this aggressive malignancy. Interestingly, a previous report has demonstrated that the multi-kinase inhibitor sorafenib can inhibit MPNST growth, suggesting the potential of CDK4 as a therapeutic target and also as a potential predictive biomarker of response to sorafenib therapy.

has consistently proven to be diagnostically or prognostically useful across multiple studies. For example, alterations on chromosome 17 (where the *NF1* gene is located) are expected, given that MPNSTs are highly over-represented among NF1 patients. However, *NF1* losses are not always encountered (8–10), suggesting that other tumorigenic pathways also exist. A major limitation of traditional cytogenetic studies, however, has been their limited resolution of ~10 Mb. This has complicated the discovery of specific, recurring genetic alterations involving small regions.

High-resolution genome-wide DNA microarray analyses have identified many novel tumor-specific microdeletions and amplifications in leukemia, lung, and ovarian cancers (11–13). To date, a similar approach applied to MPNSTs has been limited to few studies with either less comprehensive or lower resolution array platforms (~1 Mb; refs 14–16). In the present study, we have analyzed a larger cohort of MPNSTs utilizing both Affymetrix and Nimblegen array-based comparative genome hybridization (aCGH) platforms (0.25–2.5 kb of resolution with whole genome coverage) to identify recurrent DNA copy number alterations (CNA) that are associated with reduced patient survival, independent of traditional clinical and pathological parameters in patients with this extremely heterogeneous tumor. The results from this study provide a short list of candidate genes with CNAs which may prove clinically useful as prognostic biomarkers for patients with MPNST.

Materials and Methods

Patients and specimens

A cohort of 38 patients with histologically confirmed MPNST was included in this study for microarray analysis. Among the 38 patients, 23 (60%) individuals carried a previous diagnosis of NF1 and 15 (40%) were sporadic cases. At the time of last follow-up, 14 (37%) cases had documented metastases, whereas 16 (42%) had no evidence of metastatic disease. Recurrence data were unavailable for 8 of the cases. The majority (33/38) of cases subjected to genomic analysis was primary tumors, although 5 cases of recurrent tumors were also included. A full accounting of clinical and pathological data is provided in Supplemental Table T1. DNAs and RNAs were extracted from frozen tumors, using commercial reagents (QIAamp DNA Mini Kit, Qiagen; Trizol reagent, Invitrogen) and following manufacturers' standard protocols. These same specimens were previously utilized for gene expression profiling (17). For biomarker validation studies, another independent set of 121 formalin-fixed paraffin-embedded MPNST tumors represented in a tissue microarray (TMA) was utilized; 87 of these patients had clinical annotation and follow-up data (18). Of these 87 annotated cases, 25 (29%) were NF1-associated and 62 (71%) were sporadic MPNSTs. A total of 68 primary, 12 locally recurrent, and 7 metastatic tumors were represented. The majority of the tumors (78/87) were high grade and 9/87 were classified as low grade. Supplemental Table T2 provides additional data regarding the validation set. All tissue specimens and clinical data from these patients were collected and utilized in accordance with Institutional Review Board–approved protocols.

Array-based comparative genome hybridization

The Affymetrix Genome-Wide 500K SNP Mapping array set was employed to detect genomic alterations in the set of 38 MPNSTs. For aCGH analysis, relative copy number values of the MPNSTs were generated using a reference set of 37 nonmalignant DNA samples derived from 22 males and 15 females. To identify meaningful CNAs, 2 DNA copy number analysis tools, Partek Genomics Suite (PARTEK, www.partek.com) and Copy Number Analyzer for GeneChip (CNAG, www.genome.umin.jp; ref 19) were applied to the aCGH data. CNAs identified with each of the 2 tools were compared with identify recurrent regions with minimal overlap. Physical positions of CNAs were annotated according to human genome build 35 (www.ncbi.nlm.nih.gov). All CNAs were filtered against the human normal copy number variation (CNV) database (http://projects.tcag.ca/variation) prior to further analysis. CNAs identified on the 500K SNP platform were also confirmed in a subset of 8 patients where patient-matched tumor and germline DNAs were available, using a dual-color high-density NimbleGen 135K aCGH platform (see Supplemental Text for details).

Quantitative real-time PCR

SYBR Green chemistry was utilized in quantitative real-time PCR (qRT-PCR) assays; qRT-PCR primer sequences are listed in Supplemental Table T3. The qRT-PCR was

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performed in 10-μL reactions containing 10 ng of cDNA or genomic DNA and gene-specific primers each at 200 nmol/L of final concentration, as previously described (20), on the ABI 7900HT system with the following program: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C, 56°C, and 72°C for 15 , 30 , and 30 seconds, respectively. All sets of primers yielded a single peak in dissociation curve analysis and had an efficiency of greater than 0.9.

Immunohistochemistry

Fresh TMA sections were obtained from the Washington University/Siteman Cancer Center Tissue Procurement Core Facility, and immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase complex technique as described previously (21). The primary antibodies included FOXM1 (Santa Cruz; Cat. #: sc-502; 1:200 dilution), MYC (Zymed; Cat. #: 9E10; 1:200 dilution), SOX5 (Abcam; Cat. #: ab26041; 1:500 dilution), and p53 (Biogenex; Cat. #: AM240-5M; 1:200 dilution). Evaluation of each core on the TMA was made independently by 2 pathologists (L.S., A.P.), and the final score for each tumor was derived from the average score of replicate cores on the arrays (see Supplemental Text). CDK4 TMA IHC was also performed, but was not interpretable because of poor staining quality of this antibody reagent on the TMA spots.

FISH

FISH was performed on TMA slides as previously described (22, 23). Freshly cut slides were subjected to dual-color hybridization with the following probe pairings: centromere enumerating probe 12 (CEP12)/*CDK4* (12q14.1), CEP17/*HER2* (*ERBB2*; 17q21.1), and CEP8/*MYC* (8q24.21), respectively. Five of the probes were obtained commercially and were labeled with SpectrumGreen (CEP8, CEP12, and CEP17) or SpectrumOrange (*MYC* and *ERBB2*) fluorochromes (Vysis). The *CDK4* locus was targeted with a locally developed probe, utilizing the RP11-571M6 human BAC clone as previously published (24; see Supplemental Text).

Statistical analysis

Each of the minimally overlapping genomic regions was tested for association with clinical variables, for which the Kaplan–Meier model was used for survival analysis. Differential expression of candidate genes was detected between patients with and without CNAs where positive correlations with survival were seen using the Mann–Whitney *U* test. Survival probability was first estimated using the Breslow test (univariate Cox regression) for each of the genomic and relevant clinical variables, and then the significant predictors in univariate models were entered into multivariate models. The stepwise selection method was utilized to build the most significant models. The cut-off *P* value was set as 0.05. All computations were performed in SAS (v9.1.3).

Results

Characterization of MPNST genomic alterations

As shown by high-resolution aCGH (Fig. 1) and further quantified by calculation of the "breakpoint index" (a surrogate measure of genomic instability calculated based upon the total number of independently amplified and deleted genomic segments identified in a tumor based on aCGH analysis, see ref. 13), MPNSTs demonstrate a highly complex and disrupted genome. MPNST genomes had higher overall complexity (mean "breakpoint index" = 346) compared with similarly characterized genomes of other tumor types: leukemia (mean = 88; ref 25), ovarian cancer (median = 95; ref 13), and pilocytic astrocytoma (mean = 111; ref 20). The indices were, however, similar to lymphoma (mean = 374; ref 26). The percentage of the total genome affected by CNAs was also calculated to assess genome complexity of individual tumors. This ranged widely from 0.2% to 97% (median 34%) among the 38 MPNSTs (Fig. 1C). This observation no doubt reflects the pathological diversity of tumors characterized in this cohort, although a direct association between reduced survival time and overall high genome complexity (defined here as $\geq$ 34% of the genome affected by CNA) did not quite reach statistical significance (Fig. 2A, $P = 0.071$).

Many of the genomic loci associated with recurrent CNAs harbor genes previously implicated in MPNST biology and were observed with frequencies comparable to those reported in previous studies (14; Supplemental Table T4). For example, 19 of 38 (50%) cases had deletion at the NF1 locus. Interestingly, however, this event was independent of clinical diagnosis as 13 of 23 NF1 patients (56%) as well as 6 of 15 sporadic cases (40%) demonstrated deletions. Several other recurrent CNAs of genes not previously associated with MPNSTs were also identified. These included gains of *PDCD1* at 2p25.3 (47% of cases), *SOX5* at 12p12.1 (34% of cases), *FOXM1* at 12p13.33 (29% of cases), and *NOL1* at 12p13.31 (29% of cases) as well as losses of *PSIP1/p75* at 9p22.3 (50% of cases; ref 27), *PTPRD* at 9p23 to 9p24.1 (47% of cases; ref 28), and *PTEN* at 10q23.31 (34% cases). Although it is likely that NF1-associated and sporadic MPNSTs arise in the context of different genetic backgrounds, we found that none of these nor any other loci identified in this analysis was present with statistical significance between NF1-associated and sporadic MPNSTs.

Identification of survival-associated genomic alterations

Minimally overlapping regions of genome alteration (1,564 copy number gains and 252 copy number losses) identified using 2 alternate segment calling algorithms were correlated with clinical variables. Each genome region evaluated contained at least 1 annotated gene and recurred in greater than 10% of 38 tumors. A detection frequency of 10% (4 tumors) was chosen as a minimum cut-off to provide sufficient statistical power given

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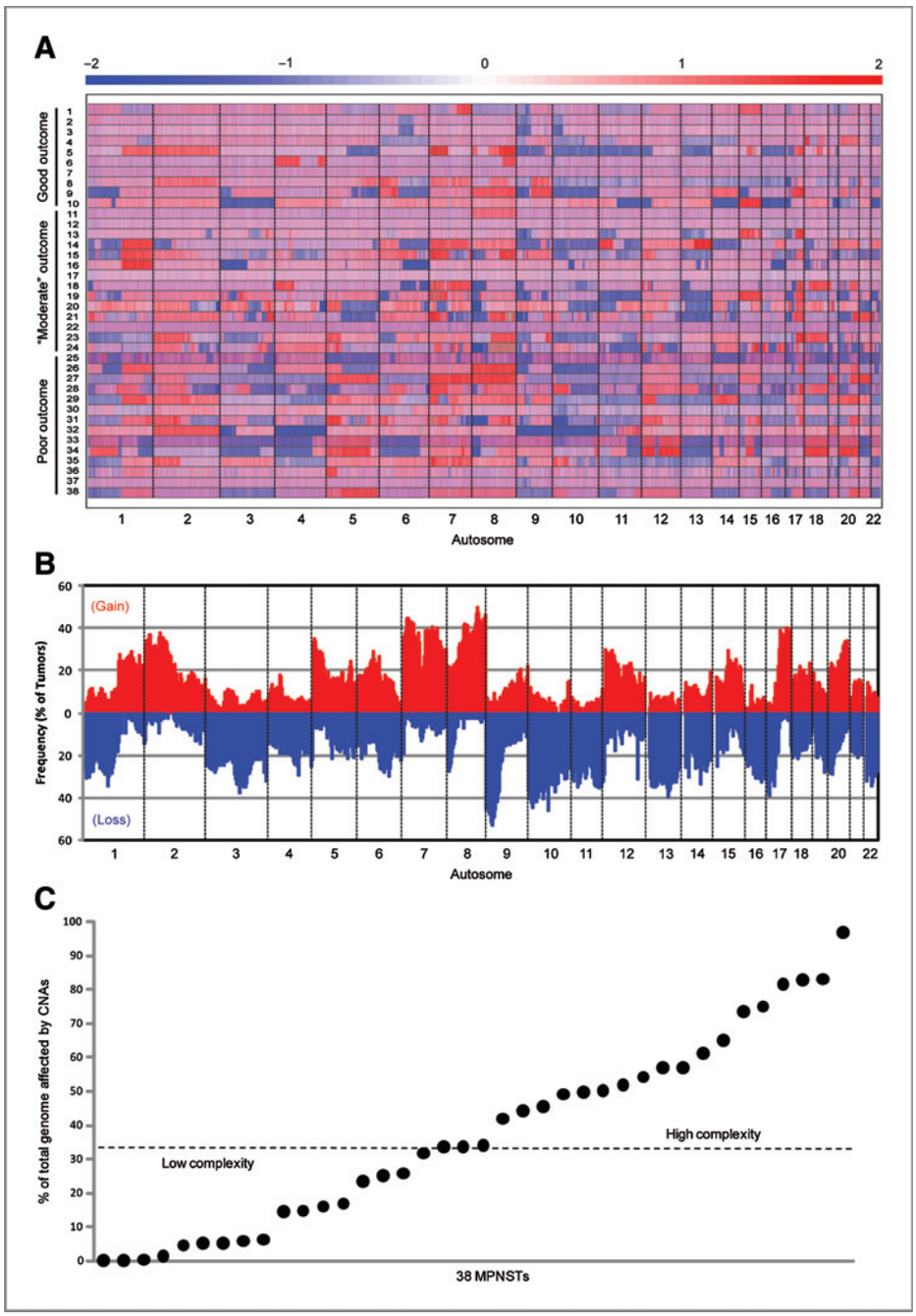

Figure 1. Demonstration of high complexity genome alterations in 38 MPNSTs, defined by PARTEK Genomic Segmentation algorithm in aCGH analysis. A, heat map of SNP-level copy number data. Copy number loss is shown in blue and gain in red on each of 22 autosomal chromosomes. A 2-color bar at the top of the map indicates the data range in log2 ratio. "Good outcome" is defined as survival more than 24 months, "moderate outcome" as survival 13 to 24 months (or "indetermined" as lost to follow-up while alive, with recorded survival <24 months), and "poor outcome" as survival of 12 months or less. Note that in general, patients with "poor outcome" had more CNVs than patients with "good outcome." B, frequency of individual CNAs, across all autosomal chromosomes in 38 MPNST patients. Blue represents copy number losses and red denotes copy number gains. C, distribution of percentage of the total genome affected by CNAs among 38 MPNSTs analyzed by aCGH. Calculation of "percentage" is based upon NCBI Build 35, May 2004 Assembly (hg17) for human genome size (2864255932 bp for autosome).

the sample size and recognizing the already extreme diversity of this tumor type. Using either Mann–Whitney *U* test or chi-square test, no significant association was observed between these regions and clinical variables such as age, gender, tumor status (i.e., primary or recurrent), anatomic location and grade, metastasis, recurrence, or NF1 association. Using the Kaplan–Meier model, however, 36 minimally overlapping regions with copy number gains demonstrated significant associations with decreased survival times (Supplemental Table T5). No regions with copy number losses were statistically associated with patient survival.

When ranked by both frequency and *P* value, the most significant region of copy number gain was a 173 kb minimally overlapping region at 12p12.1 that includes only a portion of the *SOX5* gene (Fig. 2B). This region demonstrated copy number gains in 13 tumors, but with variability in the size of the locus affected (Fig. 3A). Three other genomic regions associated with survival were also identified (Fig. 2C and E), where differential mRNA expres-

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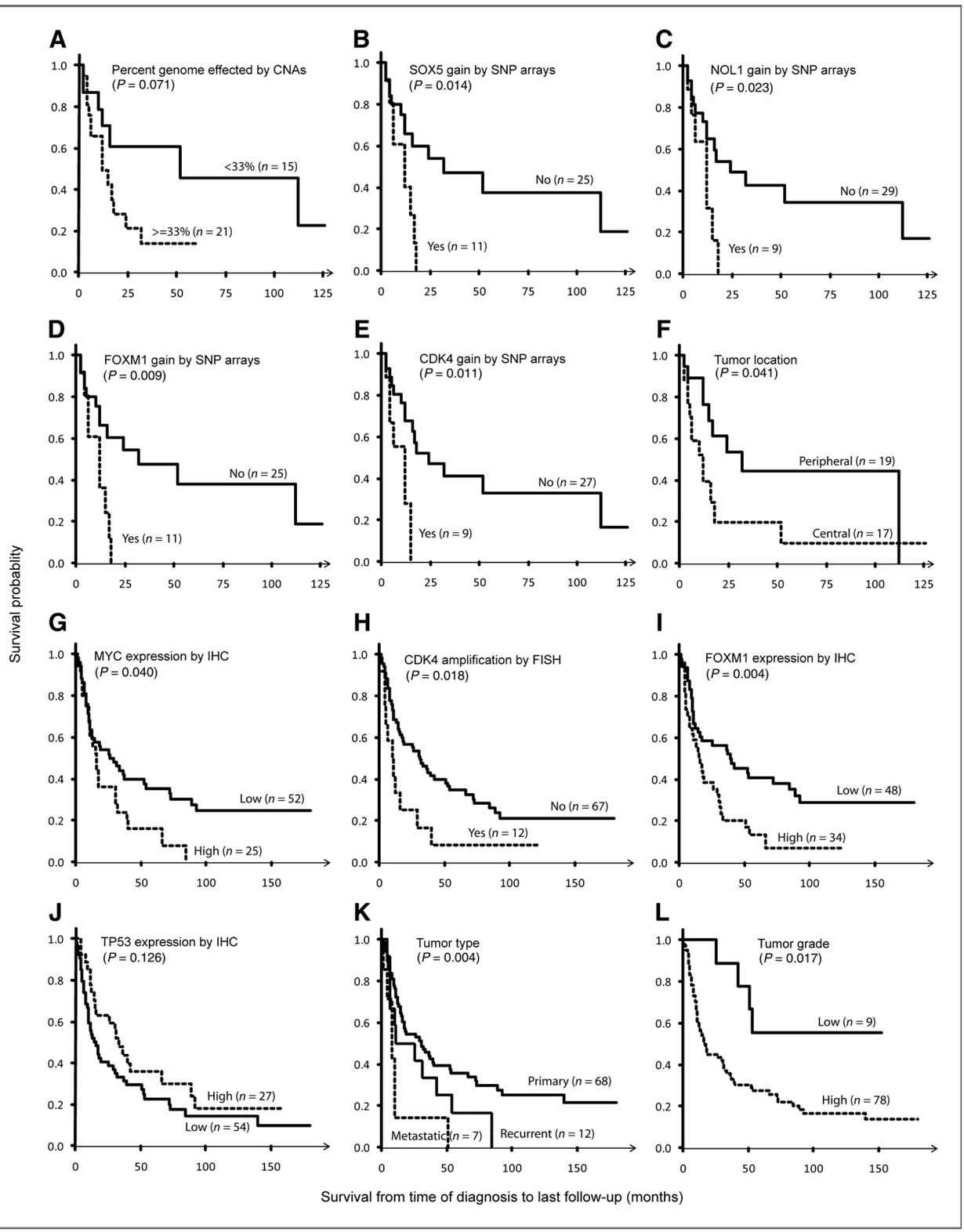

Figure 2. Kaplan–Meier survival curves in MPNST patients of 2 independent cohorts, stratified by specific genome CNAs or clinical variables, as indicated in : A–F, initial set of 38 MPNSTs; G–L, confirmation set of 87 MPNSTs. Less than the total number of tumors in each cohort was analyzed when individual biomarker or clinical data were not available.

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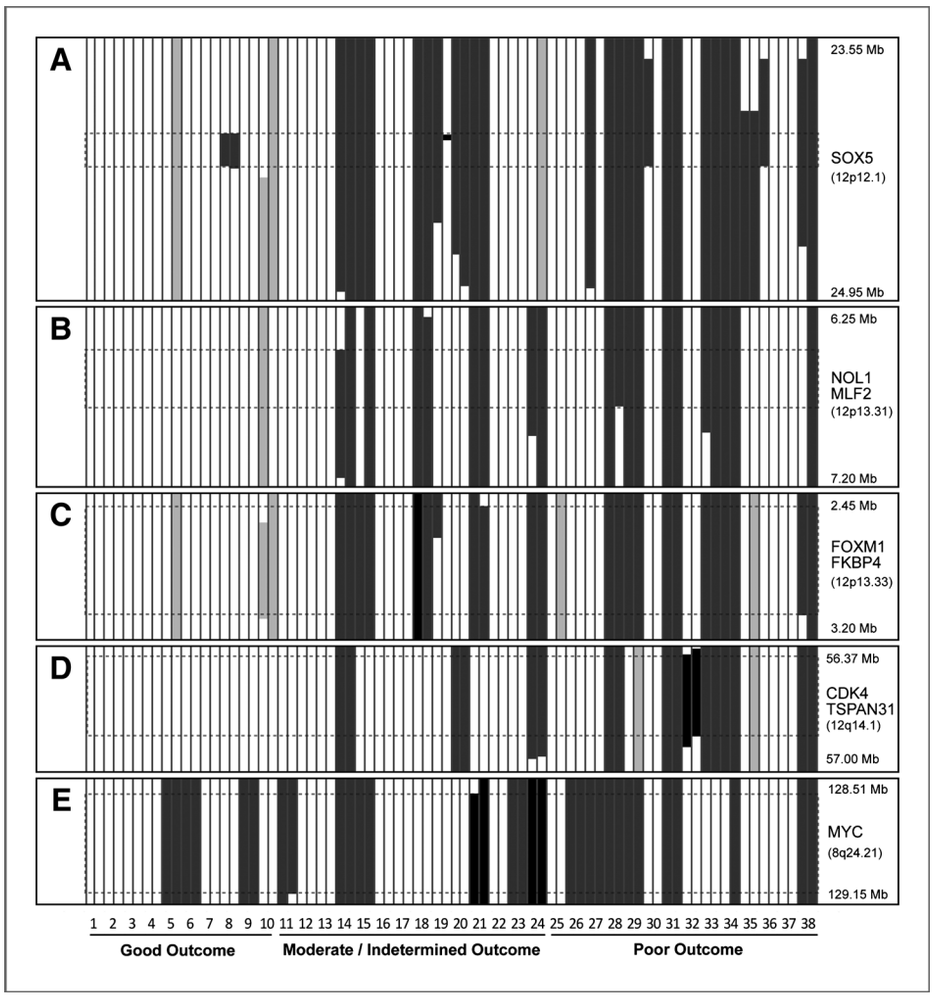

Figure 3. Five minimally overlapping regions of copy number gain identified by cross-comparison of segmentation data using 2 independent algorithms in copy number analysis of a set of 38 MPNSTs. For each of 38 tumors, 2 segments are shown in adjacent columns: left—segmentation detected by Hidden Markov Model and right—segmentation detected by Genomic Segmentation algorithm. Categories of CNAs are designated by a gray scale from white (neutral), light-gray (deletion), and dark-gray (low copy number gain) to black (high-level DNA amplification). The dotted lines in each of the 5 regions indicate the minimally overlapping region in cross-comparison. The genomic location is shown at the top and bottom within each region, along with representative genes at specific cytobands. Patients outcome: "good" as survival more than 24 months, "moderate" as survival 13 to 24 months (or "indetermined" as lost to follow-up while alive, with recorded survival <24 months), and "poor" as survival of 12 months or less.

sion was seen in at least 1 overlapping gene for each of the regions, based on previously published expression profiling data of the same tumors (Table 1; ref 17). The sizes of these 3 regions were 265 kb at 12p13.31, 551 kb at 12p13.33, and 420 kb at 12q14.1, and included the entire coding sequence of *NOL1/MLF2* (9 cases; 25%), *FOXM1/FKBP4* (11 cases; 31%), and *CDK4/TSPAN31* (9 cases; 25%), respectively (Fig. 3B–D). In comparison, the 8q24.21 locus harboring *MYC*, a gene frequently amplified in neuroectodermal tumors, was also amplified in 13 (34%) MPNSTs (Fig. 3E) and was associated with a trend toward decreased survival ($P > 0.05$). As described below, this association reached statistical significance on subsequent confirmation experiments that evaluated *MYC* protein expression (Fig. 2G).

Confirmation of copy number alterations associated with survival

To confirm results obtained from aCGH data sets, qRT-PCR was performed to quantify DNA copy number for 7 specific candidate genes using the same 38 MPNST DNAs employed for aCGH analysis. DNA qRT-PCR results confirmed associations between decreased survival and DNA copy number gains for all 7 genes (Table 1). Using the same MPNST RNAs from our previously reported gene expression microarray study (17), qRT-PCR analysis confirmed differential expression for 5 of the 7 genes (*NOL1*, *MLF2*, *FOXM1*, *FKBP4*, and *CDK4*), whereas *SOX5* and *TSPAN31* showed no differential expression (Table 1). Given that only a portion of the *SOX5* gene was involved in the amplified region, we questioned whether this amplification reflected a qualitative rearrangement of the locus, rather than *SOX5* amplification. However, sequencing of RNAs from tumors that exhibited *SOX5* amplification using both 3′ and 5′ RACE (rapid amplification of cDNA ends) failed to identify a novel fusion gene transcript in these tumors (data not shown).

We also employed an independent cohort of 121 patients tumors (87 with clinical follow-up) to construct a TMA and evaluate gene amplification and protein expression of several candidate genes (*CDK4*, *FOXM1*, and *SOX5*) that were identified and confirmed by the genomic approaches described above. For comparison, we also examined several other previously reported biomarkers

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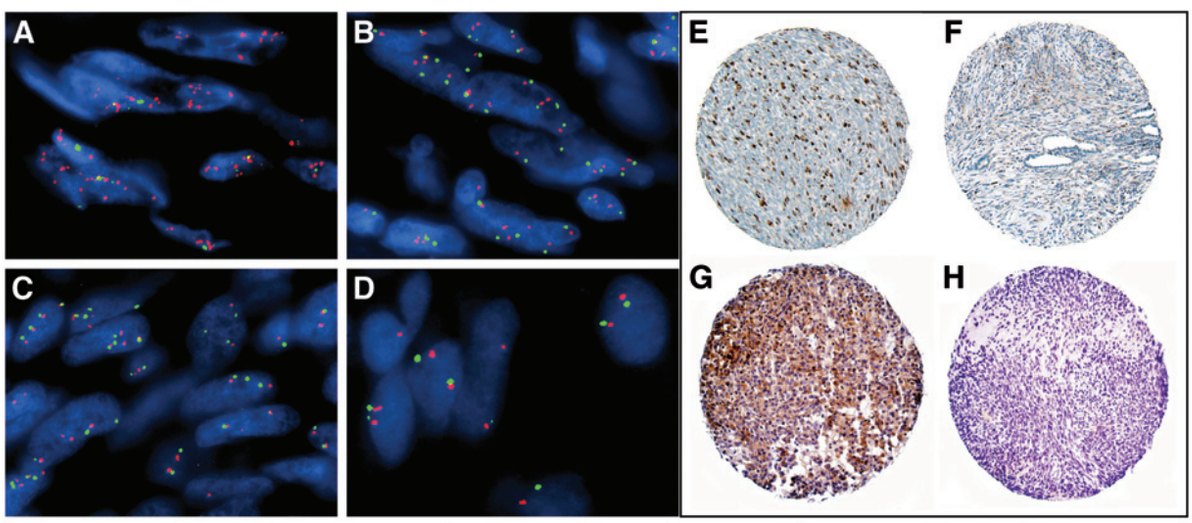
Table 1. Validation summary on potential survival marker genes

Markers	Copy number gains (losses) are associated to survival in 38 MPNSTs?	Differential mRNA expression exists in arrays between tumors with and without CNAs?	CNAs identified by aCGH are confirmed by qRT-PCR in same MPNSTs?	Differential mRNA expression is confirmed by qRT-PCR in same MPNSTs?	Gene amplification in FISH is associated with survival of 87 MPNSTs?	High protein expression in IHC is associated with survival of 87 MPNSTs?
SOX5	**Significant**	No	**Significant**	Not significant	n/a	Not significant
NOL1	**Significant**	**Yes**	**Significant**	**Significant**	n/a	n/a
MLF2	**Significant**	**Yes**	**Significant**	**Significant**	n/a	n/a
FOXM1	**Significant**	**Yes**	**Significant**	**Significant**	n/a	**Significant**
FKBP4	**Significant**	**Yes**	**Significant**	**Significant**	n/a	n/a
CDK4	**Significant**	**Yes**	**Significant**	**Significant**	**Significant**	n/a
TSPAN31	**Significant**	**Yes**	**Significant**	Not significant	n/a	n/a
ERBB1	Not significant	No	n/a	n/a	Not significant	n/a
MYC	Not significant	**Yes**	n/a	n/a	Not significant	**Significant**
TP53	Not significant	No	n/a	n/a	n/a	Not significant

of survival (ERBB2, MYC, and TP53) in this same cohort. Using Kaplan–Meier survival analysis, *CDK4* amplification in 12 of 79 (15%) cases (scored as both true gene amplification and high-level polysomies/gains by FISH analysis) and increased protein expression of FOXM1 and MYC were statistically associated with significantly shorter overall survival in this cohort (Figs. 2G–I and 4). In contrast, *MYC* and *ERBB2* gene amplification as well as TP53



CDK4 Copy-number changes in FISH (A–D)			FOXM1 protein expression in IHC (E, F)			MYC protein expression in IHC (G, H)		
CDK4 status	# of cases	Classes	FOXM1 scores	# of cases	Classes	MYC staining	# of cases	Classes
Amplification	2	*CDK4* amplification	+++	12	High expression	Negative	52	MYC expression (–)
High-level polysomies	10		++	22		Nuclear +	3	MYC expression (+)
General polysomies	34	*CDK4* non-amplification	+	12	Low expression	Cytoplasm +	22	
Normal	33		0	36				
Total # of cases*	79		Total # of cases*	82		Total # of cases*	77	

* Only counts on cases with evaluable tissue cores in 87 patients who had complete survival data availble for further analysis.

Figure 4. A–D, representative TMA FISH analysis of *CDK4* copy number gain (red, CDK4 probe; green, chromosome 12 probe). A, *CDK4* gene amplification; B, high-level polysomy 12; C, polysomy 12; D, normal copy numbers of *CDK4* and chromosome 12. For survival analysis, A and B were grouped into a single category of *CDK4* amplification, with C and D classified as *CDK4* nonamplification. E–H, representative TMA IHC analysis of FOXM1 (E, F) and MYC (G, H) protein expression. High expression (E, G; scores of +++ and ++) versus low or negative expression (F, H; scores of + and –) for each antibody is shown.

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(Fig. 2J) and SOX5 protein immunoreactivity were not associated with overall survival.

Finally, a multivariate analysis was carried out to determine whether any of the genomic biomarkers identified could serve as independent predictors for survival (Table 2). Among all genomic and clinical variables examined, only 4 CNA regions (representatively named as *CDK4*, *FOXM1*, *NOL1*, and *SOX5*) and anatomic site (Fig. 2F) showed significant associations with survival time by univariate regression analysis in the 38 MPNSTs. In addition, *CDK4* gene amplification by FISH analysis, FOXM1 and MYC protein expression, tumor type (i.e., primary, recurrent, or metastatic; Fig. 2K) and tumor grade (Fig. 2L) also demonstrated statistically significant association with overall survival. In multivariate analysis, however, the most significant survival predictor was *CDK4* copy number gain by aCGH in the 38 patient cohort and corresponding *CDK4* gene amplification by FISH in the 87-patient cohort. Tumor anatomic site in the 38 patient cohort, and high FOXM1 protein expression and tumor type in the 87-patient cohort were also independently significant in multivariate analyses.

Discussion

MPNST is an aggressive malignancy with considerable biological variability and limited response to current therapeutic approaches. This phenomenon is well demonstrated by the clinical heterogeneity of patient outcome in the current study, with overall survival ranging from less than a month to over 13 years. A major impediment to more effective disease management has been a lack of available prognostic or predictive biomarkers. While other sarcomas exhibit characteristic translocations that lead to signature fusion transcripts (29), a definitive set of clinically relevant genomic alterations has been difficult to identify for MPNST, likely because of the large number of gross structural genomic aberrations identified in individual tumors.

Our current study provides one of the first global, high-resolution genomic views of MPNST with independent validation of several biologically relevant genes. Not surprisingly, the overall level of genomic complexity is higher than previously observed by conventional cytogenetic analyses. Identifying the presumably small number of biologically relevant genomic alterations among a large number of secondary changes resulting from genomic instability remains a daunting task. For this reason, it was reassuring that analysis of these highly complex data sets nevertheless identified a number of loci already known to be important in MPNST tumorigenesis and malignant progression. These included losses of *NF1* on 17q11.2 and *CDKN2A* on 9p21, as well as common gains on 7p, 8q, 12, and 17q. However, a relatively larger sample size, the use of a high-resolution genomic platform, and correlation with clinical follow-up data in this study identified a number of novel genes whose copy number gain could serve as clinically useful prognostic markers. In contrast to single gene, high-level amplification that is seen at *MYC* and *EGFR* loci in some tumor types, aCGH analysis typically identifies larger genomic regions exhibiting more subtle copy number increases. This may be attributed to both the limited dynamic range of microarray-based technology as well as biological heterogeneity of the tumors themselves.

Interestingly, some of the genome CNAs that we identified by aCGH were discordant when measured at the level of gene transcription and/or protein expression. For example, *SOX5* copy number gains were associated with diminished survival times, but increased mRNA and protein levels were not. There are a number of explanations for such discrepancies, including the possibility of translocations that result in biologically meaningful alterations unassociated with increased expression. Although sequencing of *SOX5* transcripts in tumors with amplification at this locus was performed in this study, our inability to identify chimeric transcripts by this targeted sequencing approach does not entirely exclude this possibility. Conversely, while *MYC* protein and RNA expression levels correlated with survival, we did not observe a similar relationship at the genome level, as determined by either FISH or aCGH. This suggests that mechanisms of increased expression at the transcriptional level may be more important for this particular gene. These repeated observations underscore the need for a comprehensive evaluation of all candidate biomarkers at the genome, transcriptome, and protein levels in independent sample sets, particularly as they are being considered as prognostic tools.

For example, in the recent literature, p53 expression has been proposed as an important predictor of survival time in MPNST patients (30, 31). Our data, however, did not confirm this finding at the level of protein expression, gene expression, or genome deletion. Although 50% of MPNSTs had deletions at the *TP53* locus in our present study, differential mRNA expression between *TP53* deleted and nondeleted tumors was not seen ($P > 0.05$) and *TP53* deletion itself did not stratify MPNST patients by survival. Furthermore, in the second cohort of 87 MPNSTs, p53 immunoreactivity was not statistically associated with survival time. One reason for this discrepancy between our current study and previous studies could reflect the presence of p53 substitution mutations that would obviously not be detected by aCGH but that could affect protein expression and stability. However, the analytical and interpretive techniques applied to IHC in the current study were similar to those of previous reports and as such, this discrepancy is more difficult to explain. In contrast, we did identify recurrent copy number gains involving loci at 12p13.31 and 12p13.33 that were associated with survival on univariate analyses. Although these 2 regions did not remain statistically significant in our primary cohort of 38 MPNST patients by multivariate analysis, we were able to subsequently demonstrate concurrent mRNA and protein overexpression of *FOXM1* in this region. In a second larger cohort of 87 MPNSTs, FOXM1 over-expression by IHC was associated with poor survival, a significance which was retained on multivariate analysis. FOXM1 is a transcription

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Table 2. Multivariate analysis of both clinical and genomic variables for independent survival predictors in MPNST patients

		aCGH test cohort ($N = 36$)					TMA validation cohort ($N = 87$)				
Parameter		***n***	**Median survival**	***P*[a]**	**HR[b]**	***P*[b]**	***n***	**Median survival**	***P*[a]**	**HR[b]**	***P*[b]**
Location	Central	17	12 (5–18)	0.052	2.82 (1.15–6.92)	0.024	32	27 (12–39)	0.879		
	Peripheral	19	32 (15–112)				55	19 (11–52)			
Tumor type	Primary	32	16 (12–52)	0.653			68	30 (16–52)	0.045	1.41 (1.02–1.94)	0.038
	Recurrent	4	12 (6–30)				12	18 (6–42)			
	Metastatic						7	8 (4–10)			
Grade	Low	5	18 (5–31)	0.429			9	16 (11–31)	0.026		
	High	31	15 (12–32)				78	14 (6–124)			
Metastasis	Yes	13	15 (12–32)	0.223							
	No	15	10 (6–60)								
Recurrence	Yes	20	24 (10–112)	0.248			20	28 (10–72)	0.555		
	No	16	15 (12–32)				67	19 (12–37)			
NF1 associated	Yes	22	18 (6–52)	0.919			25	33 (11–92)	0.402		
	No	14	15 (12–112)				62	17 (12–39)			
Age	≤20	4	42 (10–52)	0.126			15	83 (15–132)	0.124		
	21–30	7	5 (4–112)				17	26 (6–54)			
	31–40	12	15 (6–48)				20	15 (5–142)			
	>40	13	12 (4–24)				35	16 (4–140)			
Gender	Male	16	12 (10–112)	0.281			39	19 (10–42)	0.468		
	Female	20	18 (15–52)				48	28 (13–51)			
CDK4 gain	Yes	9	12 (4–15)	0.021	4.22 (1.43–12.44)	0.009					
	No	27	24 (12–112)								
CDK4 amplification	Yes						12	10 (5–16)	0.023	2.04 (1.03–4.04)	0.041
	No						67	31 (16–51)			
FOXM1 gain	Yes	11	12 (6–17)	0.016							
	No	25	32 (12–112)								
FOXM1 expression	High						34	15 (8–29)	0.005	1.93 (1.12–3.31)	0.017
	Low						48	39 (14–84)			
NOL1 gain	Yes	9	12 (4–15)	0.034							
	No	27	24 (12–112)								
SOX5 gain	Yes	11	12 (6–17)	0.024							
	No	25	32 (12–112)								
SOX5 expression	High						23	18 (11–42)	0.553		
	Low						37	14 (9–31)			
MYC gain	Yes	16	15 (6–52)	0.459							
	No	20	24 (12–112)								
MYC expression	High						25	16 (10–30)	0.045		
	Low						52	28 (12–54)			
TP53 expression	High						27	33 (15–66)	0.126		
	Low						54	15 (10–29)			

NOTE: Numbers in parenthesis are 95% CI.

[a]Univariate Breslow method.

[b]Multivariate Cox regression.

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factor that is only expressed in proliferating cells and has critical functions in tumor development and progression (32, 33). Expression has been associated with enhanced invasion and metastasis in pancreatic and prostate cancers (32, 34). Increased expression of FOXM1 also correlates with increased tumorigenicity of cultured glioma cells and in human glioblastomas, its expression levels are inversely correlated with patient survival (35). Our current data suggest that increased FOXM1 protein expression is a significant predictor of poor survival in MPNST patients.

Finally, of the numerous candidate loci and genes identified in this study, *CDK4* appears to be the most clinically and biologically significant. This gene was initially identified as a region at 12q14.1 that demonstrated recurrent copy number gains associated with patient survival. Of the 12 genes within this locus, only *CDK4* demonstrated concordant copy number gains and mRNA overexpression by qRT-PCR. Furthermore, by FISH analysis in an independent patient cohort, we found a significant association between *CDK4* amplification (15% of tumors) and decreased survival in a multivariate analysis. Studies have demonstrated amplification of the *CDK4* region in many human cancers (36–38), a finding variably associated with tumorigenesis and/or survival. This 12q amplicon has long been recognized for its role in sarcoma tumorigenesis. Interestingly, however, in contrast to what has been reported in dedifferentiated liposarcoma (39), MDM2 coamplification with CDK4 was uncommon in MPNSTs analyzed in this study. To our knowledge, our study is the first to provide specific evidence for a significant association between *CDK4* amplification and poor patient survival in MPNSTs. Interestingly, a previous report has demonstrated that the multi-kinase inhibitor sorafenib can inhibit MPNST growth, in part through hypophosphorylation of pRB at CDK4-specific sites (40), suggesting the potential of CDK4 as a therapeutic target and also as a potential predictive biomarker of response to sorafenib therapy.

In conclusion, *CDK4* gain/amplification and increased FOXM1 protein expression were the most significant independent predictors for poor survival in MPNST patients after accounting for well established clinicopathologic prognostic variables in MPNSTs. If confirmed in prospective studies, these prognostic and potentially predictive biomarkers may provide clinically useful information for managing patients with this aggressive malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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