Identification of p53 as a strong predictor of survival for patients with malignant peripheral nerve sheath tumors

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The purpose of this study was to identify new prognostic biomarkers with clinical impact in malignant peripheral nerve sheath tumor (MPNST), a highly aggressive malignancy for which no consensus therapy exists besides surgery. We have used tissue microarrays (TMAs) to assess in situ expression of 14 cell-cycle-regulating proteins in 64 well-characterized MPNST patients: 36 sporadic and 28 with neurofibromatosis type 1 (NF1). We developed a new software application for evaluation and logistics of the TMA images and performed a literature survey of cell cycle proteins in MPNST. For NF1-associated patients, there was a clear association between nuclear expression of p53 and poor survival ($p = 0.004$). Among the other proteins analyzed, we also found significant associations between survival and clinical variables, but none were as strong as that for p53. For the total series of MPNSTs, p53 was shown to be an independent predictor of survival, and patients without remission,

with tumor size larger than 8 cm, and with positive p53 expression had a 60 times greater risk of dying within the first 5 years compared with the remaining patients ($p = 0.000002$). This is the most comprehensive study of in situ protein expression in MPNST so far, and expressed p53 was found to be a strong surrogate marker for outcome. Patients in complete remission with a primary p53-positive MPNST diagnosis may be considered in a high-risk subgroup and candidates for adjuvant treatment. *Neuro-Oncology* 11, 514–528, 2009 (Posted to *Neuro-Oncology* [serial online], Doc. D08-00271, January 30, 2009. URL <http://neuro-oncology.dukejournals.org>; DOI: 10.1215/15228517-2008-127)

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Malignant peripheral nerve sheath tumor (MPNST) is a rare tumor type with an incidence of 0.001% in the general population.^{1,2} For individuals with neurofibromatosis type 1 (NF1), which is caused by a germline mutation of the *NF1* gene, the lifetime risk for obtaining MPNST is 6%–13%.^{3,4} The 5-year survival rate for this group of MPNST patients has been reported to be only half the survival

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rate for the sporadic MPNST cases.^{1,3,5-8} It is still unclear whether this is due to a difference in the molecular phenotype between sporadic and NF1-associated MPNST. Thus, there is a need for informative prognostic and predictive markers for this malignancy.

In general, MPNSTs display a complex and highly variable karyotype, but some recurrent genetic aberrations have been reported.^{9,10} Loss of proximal regions of chromosome arm 17q, which includes the *NF1* gene, is found in most MPNSTs, as well as in plexiform neurofibromas, which are considered precursor lesions to MPNST in NF1-associated patients.¹¹⁻¹⁵ Mutations of the *NF1* gene are found in both NF1-associated and sporadic MPNSTs.¹⁶ We have previously reported copy number gain in the distal part of 17q, which harbors antiapoptotic and proliferative genes such as *BIRC5* and *TOP2A*.^{12,17-19} Another frequently observed chromosomal aberration is deletion of 9p21, reflecting loss or large rearrangements of the cyclin-dependent kinase (CDK) inhibitor 2A gene (*CDKN2A*).²⁰⁻²³ This gene encodes the two nonhomologous isoforms, p14^{ARF} and p16^{INK4a}, both with important effects on cell cycle progression (Fig. 1).

Previous studies have addressed the possibility of using protein expression levels of some of the central cell cycle components as prognostic markers in MPNST,²⁴⁻³⁰ but little is known about how and to what extent these proteins contribute to the development of NF1-associated and sporadic MPNST. The clinical impact of different levels of protein expression remains mostly unknown. One reason for the limited knowledge is the low incidence rate of MPNST, making it difficult to obtain sufficiently large cohorts for proper evaluation of prognostic factors. Furthermore, the number of proteins analyzed has been limited, and larger numbers of proteins should be included in a single study to provide a more comprehensive picture of the complex regulation of the cell cycle. Finally, since there is no consensus treatment for MPNST, some patients have received radio- or chemotherapy prior to tumor resection, which complicates the interpretation of the impact of clinical and biomolecular features. In this study, we have used an immuno-

histochemical approach to analyze the expression of 14 cell-cycle-related proteins in a tissue microarray (TMA) that contains a joint series of Norwegian and Swedish MPNSTs ($n = 64$) for which long-term clinical follow-up data were available. A new software application was developed for visualization, scoring, and storage of the scanned TMA images.

Materials and Methods

Patients

MPNSTs from 64 patients (31 women and 33 men) were included in this study. The samples were collected at tumor orthopedic centers at Lund University Hospital (Lund, Sweden) and the Norwegian Radium Hospital (Oslo, Norway) during 1980–2002. Twenty-eight of these patients had NF1, and 36 were sporadic cases. The median age at diagnosis for these two groups was 24 and 54 years, respectively. Histopathologic classification and grading were reviewed by sarcoma reference pathologists following published guidelines.^{10,31,32} The tumors of nine patients were low grade and those of 52 were high grade; the tumor grade was unknown in the remaining three cases. At latest follow-up, 39 patients had died after 1–225 months (median 19), 23 were alive 7–369 months (median 122) after diagnosis, and two patients were lost to follow-up. The clinical data are summarized in Table 1.

The study was approved by the regional ethics committee for medical research of the South-Eastern Norway Regional Health Authority and by local ethics committees at Lund University.

Tissue Microarray

The basic TMA technology was originally described in 1998.³³ The TMA block was initially constructed by transferring 79 cylindrical tissue cores (0.6 mm diameter) from formalin-fixed and paraffin-embedded tumor samples into a recipient paraffin block.¹⁸ Later, 27 cores

Fig. 1. Schematic overview of the cell cycle components investigated in this study. The percentages of malignant peripheral nerve sheath tumors with positive expression are indicated for each protein. Oncogenic proteins are shown in green and tumor suppressor proteins in red.

Table 1. Clinical data for patients with malignant peripheral nerve sheath tumor ($n = 64$)

Patient ID	Sex	Age at Diagnosis	Neurofibromatosis Disease	Grade	Status ^a	Follow-up (Months)	Tumor Size (cm)
001	F	20	Sporadic	High	DFD	40	4
002	F	20	Sporadic	High	ANED	112	2
003	M	23	NF1	High	DFD	27	6
004	F	35	Sporadic	High	DFD	5	NA
005	M	62	Sporadic	High	ANED	142	3
006	M	45	Sporadic	High	DFD	43	8
007	M	30	NF1	High	ANED	203	7
008	F	15	NF1	High	DFD	7	10
009	M	28	Sporadic	High	DFD	67	NA
010	M	41	NF1	High	DFD	9	10
011	F	17	NF1	High	DFD	17	6
012	F	23	NF1	High	DFD	1	NA
013	M	26	NF1	Low	DFD	78	4
014	F	77	Sporadic	High	DNED	133	10
015	F	85	Sporadic	High	DFD	1	30
016	M	26	Sporadic	NA	ANED	122	2
017	F	80	Sporadic	Low	DNED	130	6
018	M	46	NF1	High	DFD	2	40
019	F	70	Sporadic	Low	DFD	42	16
020	F	19	NF1	Low	AWD	182	11
021	F	18	NF1	High	DFD	15	7
022	M	21	Sporadic	High	DFD	6	8
023	M	64	Sporadic	Low	ANED	116	20
024	M	41	NF1	High	ANED	122	6
025	M	74	Sporadic	NA	DNED	51	8
026	M	22	Sporadic	High	DFD	20	14
027	F	63	NF1	High	DNED	225	4
028	F	56	Sporadic	High	DFD	32	13
029	M	33	NF1	High	DFD	15	8
032	F	77	Sporadic	High	DFD	12	20
034	M	15	NF1	Low	ANED	138	8
035	M	42	Sporadic	High	DFD	12	7
036	F	20	Sporadic	High	ANED	115	5
037	F	34	NF1	High	DFD	16	15
038	F	71	NF1	High	DNED	100	10
039	M	50	Sporadic	High	DFD	47	3
040	F	50	Sporadic	Low	ANED	122	4
041	F	33	NF1	High	ANED	98	18
042	M	68	Sporadic	High	DFD	5	10
043	M	14	NF1	High	AWD	105	10
044	M	17	NF1	High	DFD	5	12
045	M	21	NF1	High	ANED	60	2
046	M	45	NF1	NA	ANED	125	6
047	F	25	NF1	High	ANED	49	9
048	F	35	NF1	High	DFD	12	25
049	M	27	Sporadic	High	ANED	46	7
050	M	30	Sporadic	High	ANED	133	13
051	F	73	Sporadic	High	DFD	8	6
052	F	37	Sporadic	High	DFD	81	7
053	F	15	NF1	High	DFD	19	12
054	M	62	Sporadic	High	NA	NA	10
055	F	63	Sporadic	High	DFD	46	8

(continued)

Table 1. Clinical data for patients with malignant peripheral nerve sheath tumor ($n = 64$) (*continued*)

Patient ID	Sex	Age at Diagnosis	Neurofibromatosis Disease	Grade	Status ^a	Follow-up (Months)	Tumor Size (cm)
056	M	24	NF1	High	ANED	185	16
057	F	24	NF1	High	DFD	32	10
058	F	26	NF1	High	NA	NA	11
060	M	20	NF1	High	ANED	369	6
061	F	59	Sporadic	High	ANED	142	4
103	F	60	Sporadic	High	DFD	14	12
104	M	83	Sporadic	High	AWD	7	13
105	M	13	Sporadic	High	ANED	102	5
109	F	15	Sporadic	Low	ANED	60	4
115	M	54	Sporadic	High	DFD	19	16
117	M	79	Sporadic	High	DNED	6	8
119	M	54	Sporadic	Low	DNED	65	10

Abbreviation: NA, not available.

^aStatus at last follow-up: DFD, dead from disease; ANED, alive, no evidence of disease; DNED, dead; no evidence of disease; AWD, alive with disease.

were added to the same TMA that we present here, including one core from each of two benign neurofibromas, as well as two cores from a single plexiform neurofibroma. The TMA block also contained 30 cores from normal tissues and other cancer types that were used as staining controls. At least one core from each of the 64 MPNSTs was included in the TMA, and for 21 tumors, two to five parallel cores were included. For the *in situ* protein expression analyses, 5- μ m-thick sections of the TMA block were adhered onto Superfrost Plus microscope slides (Menzel GmbH & Co. KG, Braunschweig, Germany).

In Situ Protein Expression Analyses

In situ protein expression was probed by incubating the TMA slides with selected primary antibodies listed in Supplementary Table 1. Sections of the TMA block were deparaffinized in xylene (Merck, Whitehouse Station, NJ, USA) and by rinsing twice in 100% ethanol, followed by 96% and 70%, and then in water. Antigen retrieval was performed by heating in a microwave oven at 850 W for 5 min and then 100 W for 15 min, and then immersion in one of the following buffers: 10 mM sodium citrate (pH 6.0), 1 mM EDTA (pH 8.0), or 10 mM Tris, 1 mM EDTA (pH 9.0), depending on the primary antibody used (see Supplementary Table 1). The buffers also contained 0.05% Tween 20 (all reagents were from Sigma-Aldrich, St. Louis, MO, USA). Staining was performed according to the protocol for DAKO EnVision+ using the reagents supplied with the K5007 kit (Dako, Glostrup, Denmark). Briefly, this included blocking of endogenous peroxidase activity for 5 min before incubation with the primary antibody of choice for 30 min at room temperature. A negative control experiment was provided by omitting the primary antibody for one slide. Then, the secondary antibody, conjugated with horseradish-peroxidase-labeled polymer, was applied and incubated for 30 min. Staining was completed by incubation for 5 min with 3,3'-diaminobenzidine (DAB), which results in a brown

precipitate at the antigen site. Excess DAB was rinsed off before the slides were counterstained with hematoxylin and dehydrated in increasing concentrations of ethanol and xylene. Finally, glass cover slips were mounted with Depex glue (Chemiteknikk, Oslo, Norway).

The TMA sections were scanned at $\times 400$ magnification into digital high-resolution images using the NanoZoomer Digital Pathology (Hamamatsu Photonics K.K., Hamamatsu, Japan).

TMA Software

A new software application, TMA-ImageAnalyzer, was developed for processing the scanned TMA slides and as infrastructure for the images and scoring results from the individual tissue cores and antibodies. A representation of the graphical user interface of the software is shown in Fig. 2. The software was a beta release of a product developed in the Department of Medical Informatics, Rikshospitalet University Hospital, Oslo, Norway. A commercial version will be made available through Room4 Group Ltd. (East Sussex, UK).

Scoring of Immunohistochemistry Results

The expression of the 14 cell-cycle-related proteins was scored independently by two researchers by visual inspection of the immunohistochemical staining of the TMAs. For nuclear protein expression, we categorized the samples into five groups according to the percentage of nuclei with positive staining: 0%, 1%–5%, 6%–10%, 11%–50%, and $>51\%$. For the statistical analyses, we grouped the samples with staining less than 5% as negative and more than 5% as positive. For cytoplasmic protein expression, moderate and strong staining was considered positive. If a number of samples were taken from one tumor, the scoring was considered positive if at least one of these samples was stained positively on the microarray. The interobserver variability was 13% on average. Before reevaluation of these cases, a pathologist

Fig. 2. Illustration of the graphical user interface of the TMA-ImageAnalyzer software. The proteins investigated are listed on the left. The five columns on the left show the staining results in different malignant peripheral nerve sheath tumors according to the scoring category to which they have been assigned, while the right column shows control tissue; the number of samples in each category is shown at the top.

was consulted to establish the boundaries between positive and negative samples.

Western Blot Analyses for Quality Control of Antibodies

Western blot analyses³⁴ of the antibodies were performed as a quality control to confirm that they bind to proteins with the anticipated molecular masses. This was done using 12 cell line extracts as protein sources, as previously described.²³

Statistical Analyses

The statistical significance of the differences in protein expression observed between groups of patients was calculated using Fisher's exact test. The bivariate correlation for coexpression of proteins was described using the Spearman correlation coefficient. Disease-specific and disease-free survival curves were analyzed using the Kaplan-Meier method, and the Breslow test was used to compare the equality of the survival functions. Finally, we used Cox regression for multivariate analyses to determine the parameters with the greatest impact on the survival. All statistical analyses were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA). For our hypothesis testing, we report *p*-values lower than 0.05, but the true significance of these findings should be viewed in light of the number of tests performed in each analysis. For the 14 proteins, the Bonferroni corrected significance level should be 0.0036.

Results

In Situ Expression of Cell Cycle Proteins in MPNST

The scoring results from the in situ protein expression analysis of the MPNSTs are illustrated in Fig. 3. The detailed results for nuclear and cytoplasmic staining for each antibody are shown in Table 2, and our staining remarks are presented in Supplementary Table 1. Results for each patient group (with and without NF1) are shown in detail in Table 3. The largest difference between NF1-associated and sporadic MPNSTs was found for staining of nuclear p27^{Kip1} (n-p27^{Kip1}) and cytoplasmic p27^{Kip1} (c-p27^{Kip1}) (*p* = 0.03).

The Western blot quality controls for each antibody confirmed binding to protein bands of the expected size in at least one of the cell lines. For a few antibodies, we also observed additional bands that we have not analyzed further (Table 4). We expected to see a variety of protein isoforms in different cancer tissues depending on posttranslational modifications, alternative splicing events, partial degradation, and variable degrees of oligomerization, and in the following immunohistochemical analyses, we have assumed that all antibodies are specific for one protein.

Correlations among Proteins

We calculated the bivariate correlation for all the in situ expression results and found that c-CDK4 and c-p14^{ARF} had the highest Spearman correlation factor (*r* = 0.56, *p* = 0.00002). Among the sporadic MPNST samples,

Fig. 3. Examples of immunohistochemical staining in malignant peripheral nerve sheath tumors using antibodies against four proteins: p53, CDK4, cyclin D1, and p14^{ARF}. For each protein, a negative sample, nuclear expression, cytoplasmic expression, and positive control tissue are shown (from left to right). The positive tissues (right column) are colorectal cancer (top right) and malignant melanoma (all others). *For CDK4, there is positive cytoplasmic staining in the sample with negative nuclei.

n-p21^{Cip1} and n-cyclin D1 correlated best ($r = 0.61$, $p = 0.0001$), and for the NF1-associated samples, n-MDM2 and n-p14^{ARF} had the highest correlation ($r = 0.69$, $p = 0.00006$). All significant correlations are presented in Table 5.

Tumor Heterogeneity

For 21 patients, the TMA contained between two and five parallel samples taken from different parts of the same tumor. Comparisons of the immunohistochemical scoring results for these samples provide information about the heterogeneity of the protein expression within one particular tumor. The proteins CDK2, p53, MDM2, and Ki67 displayed the largest variation within individual tumors, with opposite results (positive in one core, negative in another) found in 7–9 of the 21 tumors in question. In contrast, RB1, p14^{ARF}, p21^{Cip1}, cyclin D1, cyclin D3, and CDK4 showed variation in two or fewer of the 21 tumors. For the following calculations, a tumor was considered positive if at least one of the parallels was scored as positive.

Protein Expression in MPNST Related to Patient Survival

Table 6 lists the significance values for all clinical parameters and selected proteins in relation to 5-year disease-specific survival. Table 7 lists the test statistics for NF1-associated and sporadic tumors separately. Kaplan-Meier plots comparing the survival curves related to clinical parameters and the most significant immunohistochemical results are shown in Fig. 4. Our data show that the 5-year survival is close to 50% for both NF1-associated patients and sporadic MPNST patients (Fig. 4A). As expected, complete remission after initial surgery, tumor size, and tumor grade were significantly associated with survival (Fig. 4B–D). Among the proteins, patients with p53-positive tumors had a strikingly poorer 5-year survival compared with the group with p53-negative tumors ($p = 0.008$; Fig. 4E; Table 6), an association that was particularly strong for the NF1-associated patients ($p = 0.004$; Table 7). Furthermore, if the nuclear expression of p53 is stratified into five levels according to the percentage of positive nuclei, the survival rate decreases with increasing amounts of p53 ($p = 0.002$ for the trend; Fig. 4F). The association between p53 and survival holds even if patients that

Table 2. Numbers of cases with different expression levels of cell cycle proteins in malignant peripheral nerve sheath tumor

Antigen	Negative		Positive		51%–100%	Positive Expression (%)
	0%	1%–5%	6%–10%	11%–50%		
Nuclear expression						
p14 ^{ARF}	1	5	18	22	16	90
p16 ^{INK4a}	30	8	4	13	7	39
p18 ^{INK4c}	21	11	13	6	0	37
p21 ^{Cip1}	54	1	5	1	1	11
p27 ^{Kip1}	40	9	7	4	1	20
p53	7	12	19	19	4	69
MDM2	0	4	18	29	10	93
Cyclin D1	26	18	10	8	0	29
Cyclin D3	41	6	6	5	4	24
Cyclin E1	0	4	6	16	35	93
CDK2	40	1	18	1	0	32
CDK4	0	3	7	33	17	95
Ki67	13	11	12	17	9	61
RB1	3	3	6	24	25	90
Antigen	Negative	Moderate	Strong	Positive Expression (%)		
Cytoplasmic expression						
p14 ^{ARF}	8	31	23	87		
p18 ^{INK4c}	15	27	5	68		
p27 ^{Kip1}	56	6	0	10		
p53	10	23	28	84		
MDM2	8	31	22	87		
Cyclin D1	52	11	0	17		
Cyclin E1	1	23	38	98		
CDK4	13	28	19	78		

were not in complete remission are excluded ($p = 0.03$, $n = 40$; Fig. 4G), and if patients that had metastatic cancer at the time of the initial diagnosis are excluded ($p = 0.03$; $n = 54$; data not shown). A positive c-p14^{ARF} is also correlated with poor survival ($p = 0.04$; Table 6). For n-cyclin D1, we found a correlation with good prognosis in the group of sporadic MPNST patients ($p = 0.011$), but not among the NF1 patients (Table 7).

No essential differences were seen when we used alternative clinical end points, such as disease-free survival or time to first event (data not shown).

A multivariate Cox regression was performed with the three proteins and the three clinical variables that were shown to affect the disease-specific survival in the Kaplan-Meier plots (Table 6). The parameter “metastasis at time of diagnosis” was omitted since all eight cases with metastases were included in the group without complete remission. These analyses revealed complete remission as the strongest predictor of overall survival ($p = 0.000012$), followed by n-p53 ($p = 0.014$) and tumor size ($p = 0.021$), whereas tumor grade, c-p53, and c-p14^{ARF} did not provide additional information. For patients with all three characteristics combined—no remission, large tumor size, and positive p53

expression—the relative risk of dying within 5 years was 60 times higher (95% confidence interval, 11–329; $p = 0.000002$; $n = 58$).

Discussion

To our knowledge, the present study is the most comprehensive immunohistochemical investigation of cell-cycle-related proteins in MPNST. Previous studies have often been limited by the number of antibodies considered, the number of tumor samples included, or both (Table 8). We also provided long and reliable follow-up data for patients still alive and included several quality control steps to ensure optimal immunohistochemical staining for each antibody. The histopathology of all the MPNST tissue cores on the TMA was confirmed by an expert soft tissue tumor pathologist, and at least two persons independently scored each antibody. All the staining results were scanned into digital high-resolution images, making any discrepancies easy to evaluate on a computer screen using the software TMA-Image-Analyzer.

Table 3. Frequencies of positive in situ expression of cell-cycle-related proteins in NF1-associated malignant peripheral nerve sheath tumor (MPNSTs), sporadic MPNSTs, and benign neurofibromas

Antigen	NF1 (%) (n = 28)	Sporadic (%) (n = 34)	p-Value	Neurofibroma (n = 3)
Nuclear expression				
RB1	96	85	NS	3/3
CDK4	93	97	NS	3/3
Cyclin E1	92	94	NS	2/3
p53	63	74	NS	2/3
Ki67	50	71	0.12	0/3
CDK2	29	34	NS	1/3
p14 ^{ARF}	26	30	NS	3/3
p16 ^{INK4A}	25	50	0.07	1/3
Cyclin D1	21	35	NS	1/3
Cyclin D3	18	29	NS	0/3
p18 ^{INK4c}	9	10	NS	0/3
p27 ^{Kip1}	7	29	0.05 *	0/3
p21 ^{Cip1}	7	15	NS	0/3
MDM2	96	91	NS	3/3
Cytoplasmic expression				
Cyclin E1	96	100	NS	2/3
p14 ^{ARF}	89	85	NS	1/3
p53	85	82	NS	2/3
p18 ^{INK4c}	65	71	NS	3/3
p27 ^{Kip1}	0	18	0.03 *	1/3
CDK4	78	79	NS	2/3
MDM2	15	38	0.05 *	2/3
Cyclin D1	14	20	NS	0/3

Abbreviation: NS, not significant.

p-Values for the comparisons between NF1-associated and sporadic MPNST patients are calculated using Fisher's exact test.

*Statistically significant.

Protein Interdependence and Interpretation of In Situ Data

In contrast to earlier reports,^{26,28,35} we found no significant difference in the distribution of p53 or Ki67 when we compared the high- and low-grade MPNSTs. This could be related to the absence of a universally accepted grading system for sarcomas.³⁶ The Scandinavian grading system is primarily based on Broder's grading of malignant tumors.³² None of our three benign neurofibroma samples expressed Ki67, whereas more than half of the MPNSTs had positive expression (Tables 2 and 3). Although expression of p53 is expected to be lower in neurofibromas, we were not able to distinguish them from MPNSTs in our data based on the small number of neurofibromas (Table 3).³⁰

A large variation is reported for p53 expression in MPNST (Table 8). Our nuclear scoring results are within the range of these previous studies, and we also observed cytoplasmic expression. The large variation among different studies could be explained by the use of different antibodies in various concentrations, as well as different cutoff levels for scoring.

A common reason for accumulation of p53 in cancer cells is mutation in the *TP53* gene. For MPNST, there are conflicting data regarding the influence of *TP53*

mutations. A handful of studies, each with only four to seven cases, report up to 75% mutations or deletions in *TP53*,^{37–39} and a higher frequency is generally reported for sporadic cases compared with NF1-associated MPNST. In contrast, in a series of 16 MPNSTs, including 11 from NF1-associated patients and 5 sporadic MPNST patients, we did not detect any mutations in the coding exons 2–11 of *TP53*.⁴⁰ Our findings are supported by other studies (range, 5–32 cases) reporting infrequent mutations.^{27,30,41–43} The stability of p53 is also linked to the negative feedback loop involving MDM2.⁴⁴ Among the 18 of our samples that were negative for p53, only one was positive for MDM2. In tumors with both p53 and MDM2 expression, p14^{ARF} is a candidate for suppression of MDM2 activity.⁴⁵ We found p14^{ARF} expressed in all samples that also expressed MDM2.

Heterogeneity

The large intratumor variation of protein expression for CDK2, p53, MDM2, and Ki67 suggests that events leading to altered expression of these genes, such as mutations, deletions, amplifications, or epigenetic modifications, occur late in tumor development.

The most homogeneous expression patterns were seen for RB1, p14^{ARF}, p21^{Cip1}, cyclin D1, cyclin D3, and

Table 4. Estimated molecular mass of the major bands observed on Western blots of protein extracts from 12 cell lines

Antigen	Cell Line											
	LOVO (Colon) ^a	HTC116 (Colon)	HT-29 (Colon)	SW480 (Colon)	Tera-2 (Testis)	NTERA-2 (Breast)	2102Ep (Testis)	Melanoma (Skin)	HT-1080 (Connective Tissue)	PC-3 (Prostate)	DU 145 (Prostate)	MCF7 (Breast)
p14 ^{ARF}		36	36	36						14	14	
p16 ^{INK4A}	29, 70	29, 70	29, 70	29, 70	16, 29, 70	29, 70	16, 29, 70	29, 70	29, 70	29, 70	16, 29, 70	29, 70
p18 ^{INK4c}	27, 60	27, 60	27, 60	27, 60	27, 60	27, 60	27, 60	27, 60	27, 60	18, 27, 60	27, 60	27, 60
p21 ^{Cip1}	21	21						21	21		17	21
p27 ^{Kip1}	27, 24, 21	27, 24, 21	27, 24, 21	27	27	27	27	27	27	27	27	27
p53		53		53	53	53	53	53	53, 44	53, 44	53, 44	
MDM2 ^b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cyclin D1	33	33		33			33	33	33	33	33	33
Cyclin D3		33	33	33			33	33	33	33	33	33
Cyclin E1	47	47, 70, 40	40		47	47	47	47, 40	47	47, 40, 70	47	47
CDK2	34, 32	34, 32	34, 32	34, 32	34, 32	34, 32	34, 32	34, 32	34, 32	34, 32	34, 32	34, 32
CDK4	34, 25, 24	34, 45, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24	34, 25, 24
Ki67		170		230, 200, 170		345, 20	345, 20	230, 17	230, 200, 170	395, 345, 200	395, 345, 230, 200, 170	
RB1	65	106, 65, 63	65	106, 65	106, 63	106, 65	65	106, 65	106, 65	106, 65		106

Abbreviation: ND, not done.

^aOriginal source of cancer cell line.

^bFor MDM2 we found two bands of 76 and 90 kDa in a set of malignant peripheral nerve sheath tumors (results not shown).

Table 5. Bivariate correlation among the analyzed proteins

Patient group	Antigen 1	Antigen 2	r-Value	p-Value
NF1 and sporadic	c-p53	c-CDK4	0.52	0.00002
NF1 and sporadic	c-p53	c-p14 ^{ARF}	0.54	0.00001
NF1 and sporadic	c-CDK4	c-p14 ^{ARF}	0.56	0.000002
NF1 and sporadic	n-Cyclin D3	n-p21 ^{Cip1}	0.51	0.00002
NF1 and sporadic	n-Cyclin D1	n-p16 ^{INK4A}	0.44	0.0003
NF1 and sporadic	n-p53	c-MDM2	0.34	0.008
Sporadic	n-p21 ^{Cip1}	n-Cyclin D1	0.61	0.0001
Sporadic	n-Cyclin D3	c-Cyclin D1	0.61	0.0006
Sporadic	c-p18 ^{INK4c}	c-Cyclin D3	0.50	0.009
Sporadic	c-Cyclin D1	c-MDM2	0.50	0.003
NF1	n-p18 ^{INK4c}	n-Ki67	0.55	0.004
NF1	n-MDM2	n-p14 ^{ARF}	0.69	0.00006

Table 6. Prognostic factors for 5-year survival in malignant peripheral nerve sheath tumors

Parameter	Survival % (SD)	Number of Cases	p-Value ^a	HR ^b	95% CI ^b	p-Value ^b
Metastasis at time of diagnosis						
No	57 (6.8)	54	1×10^{-9} *			
Yes	13 (12)	8				
Complete remission						
Yes	62 (7.5)	42	2×10^{-6} *	8.1	3.2–21	0.000012*
No	14 (9.4)	14				
Grade						
Low	89 (11)	9	0.015*			0.12
High	42 (7.0)	50				
Tumor size						
<8 cm	64 (8.6)	32	0.009*	2.8	1.2–6.9	0.02*
>8 cm	37 (9.3)	27				
Location						
Extremities	67 (8.2)	34	0.10			
Nonextremities	36 (9.6)	25				
Sex						
Female	43 (9.0)	30	0.35			
Male	58 (8.9)	32				
Hereditary						
Sporadic	50 (8.6)	35	0.87			
NF1	52 (9.6)	27				
Age (years)						
<20	57 (13)	14	0.46			
21–30	64 (13)	14				
31–40	33 (19)	6				
>40	45 (9.6)	28				
n-p53						
Positive	39 (7.6)	41	0.008*	6.4	1.5–29	0.014*
Negative	83 (9.1)	18				
c-p53						
Positive	43 (7.1)	49	0.007*			0.13
Negative	100	10				
c-p14 ^{ARF}						
Positive	47 (7.0)	52	0.04*			0.46
Negative	88 (12)	8				

Abbreviations: SD, standard deviation; HR, hazard ratio; CI, confidence interval.

^aUnivariate *p*-Values (Breslow test).^bMultivariate *p*-Values (Cox regression; *n* = 47).

*Statistically significant.

Table 7. Prognostic factors for 5-year survival of patients with neurofibromatosis type 1 (NF1)-associated malignant peripheral nerve sheath tumors (MPNSTs) or sporadic MPNSTs

Parameter	NF1-Associated Patients			Sporadic MPNST Patients		
	% Survival (SD)	Number of Cases	p-Value ^a	% Survival (SD)	Number of Cases	p-Value ^a
Metastasis at time of diagnosis						
No	58 (10)	24	$2 \times 10^{-9*}$	55 (9.3)	30	0.0006*
Yes	0	3		20 (18)	5	
Complete remission						
Yes	67 (10)	21	0.00015*	57 (11)	21	0.012*
No	0	6		25 (15)	8	
Tumor size						
<8 cm	67 (14)	12	0.13	63 (11)	20	0.03*
>8 cm	43 (13)	14		31 (13)	13	
Grade						
Low	100	3	0.13	83 (15)	6	0.06
High	44 (10)	23		38 (9.6)	27	
Location						
Extremities	69 (13)	13	0.33	65 (11)	21	0.25
Nonextremities	36 (13)	14		36 (15)	11	
Sex						
Female	39 (14)	13	0.35	47 (12)	17	0.73
Male	64 (13)	14		53 (12)	18	
n-p53						
Positive	29 (11)	17	0.004*	46 (10)	24	0.30
Negative	89 (11)	9		76 (15)	9	
n-Cyclin D1 ³						
Positive	20 (18)	5	0.12 ^b	81 (12)	11	0.011 ^{b*}
Negative	59 (11)	22		39 (11)	22	
c-p53						
Positive	41 (11)	22	0.07	44 (9.6)	27	0.05*
Negative	100	4		100	6	

^aUnivariate p-Values (Breslow test).^bn-Cyclin D1 expression had opposite effect on survival for sporadic MPNST patients and NF1-associated patients.*Statistically significant: $p < 0.05$.

CDK4. Events affecting the expression of these proteins therefore most likely occur prior to, or early in, the development of MPNST.

Protein Expression in MPNST Related to Patient Survival

We found that especially the NF1-associated patients with accumulation of nuclear p53 had poor survival ($p = 0.004$; Table 7). The Cox regression analysis identified p53 as the strongest predictor of poor survival for the entire patient group, including the sporadic cases (Table 6). Positive c-p53 expression was also associated with reduced survival ($p = 0.01$), and there were no disease-specific deaths within the group with negative cytoplasmic staining. However, since only nine samples were negative for c-p53, this parameter reached a significance of only $p = 0.13$ in the multivariate analysis. The association between p53 expression and poor patient survival in MPNST has to our knowledge been reported only in one previous study of 28 cases.²⁴ In addition, a difference

between the NF1-associated patients and the sporadic MPNST patients regarding p53 expression and time of recurrence has also been reported.²⁶ For p16^{INK4a}, there seems to be a tendency for lower expression with increasingly aggressive phenotypes; one study reported positive immunoreactivity for p16^{INK4a} in virtually all neurofibromas and in almost as many plexiform neurofibromas,²⁸ whereas the frequency of MPNST with positive staining for p16^{INK4a} is severely reduced (Table 8). This can be explained in part by deletions of the *CDKN2A* gene, which have been reported by us and others.^{20–22} Furthermore, p16^{INK4a} immunoreactivity is typically observed in those that do not have homozygous deletions of the *CDKN2A* gene.^{23,41} There was only one disease-related death among the eight patients with negative c-p14^{ARF} expression ($p = 0.04$; Fig. 4G), an alternative transcript of the *CDKN2A* gene. We found that expression of both n-p27^{KIP1} and c-p27^{KIP1} was significantly lower in NF1-associated MPNSTs than in sporadic cases (Table 3); in fact, no tumors with cytoplasmic staining could be found among the NF1-associated MPNSTs. A cor-

Fig. 4. Disease-specific survival curves for neurofibromatosis type 1 association (A), complete remission after the first surgery (B), tumor size (C), tumor grade (D), nuclear p53 expression (n-p53) (E), detailed n-p53 expression (F), and n-p53 expression only in complete remission patients (G).

relation between c-p27 expression and poor survival, as observed by Kourea et al.,²⁵ could not be verified in our data. Positive cyclin E1 and CDK4 were found expressed in almost all samples, which is in strong contrast to two earlier reports.^{25,41} This could be due to differences in the immunohistochemistry protocols, for example, use of other dilution levels or reagents.

Sporadic versus NF1-Associated MPNST

Many studies report that the 5-year survival from diagnosis of NF1-associated patients with MPNST is only half of that of sporadic MPNST cases (~30% for NF1 vs. 55% for sporadic).^{1,3,5-8} However, this is not a consistent finding, as others report no significant difference.^{46,47} In the present series, no significant difference

was observed in the survival rate when we compared NF1-associated MPNSTs with sporadic cases; the 5-year survival among the patients in this cohort was about 50% for both groups (Fig. 4A). We can disregard diagnostic uncertainty for the present series, since all cases were reevaluated by reference pathologists, and furthermore, six NF1-associated and eight sporadic cases were analyzed for germline mutations, confirming the clinical status (I. Bottillo et al., unpublished data). The largest difference between the two groups is observed after 36 months, where the disease-specific survival for the NF1-associated and sporadic MPNST patients is 52% (± 10) and 65% (± 8), respectively. However, this difference is not significant ($p = 0.46$). One might speculate that the relatively high survival rate for the NF1-associated patients in our study compared with the previous studies

Table 8. Detailed literature survey of protein expression in malignant peripheral nerve sheath tumors

Antigen	Positive <i>n</i>	Total <i>n</i>	Percentage	Antibody Clone (Supplier)	Reference	Year
p53	28	36	78	DO-7 (DAKO)	30	2007
	14	27	52	NA (DAKO)	28	2003
	10	12	83	DO-7 (DAKO)	48	2002
	3	3	100	DO-1 (Immunotech)	49	2001
	14	49	29	PAb1801 (Oncogene)	27	2001
	5	8	63	PAb1801 (Calbiochem)	50	2001
	11	26	42	DO-7 (Novocastra)	41	2001
	6	12	50	DO-7 (DAKO)	39	2001
	10	35	29	PAb1801 (Oncogene)	25	1999
	6	10	60	NA (Biogenex)	26	1999
	12	15	80	DO-7	51	1998
	3	3	100	DO-7 (Novocastra)	52	1997
	19	28	68	DO-7 (DAKO)	24	1996
	17	26	62	DO-7 (DAKO)	35	1995
	6	6	100	Poly (Nichirei)	53	1994
Total	164	296	55			
Ki67	66	96	69	MIB1 (DAKO)	54	2006
	10	43	23	MIB1 (Marseilles)	27	2001
	4	4	100	MIB1 (Immunotech)	49	2001
	20	34	59	MIB1 (Immunotech)	25	1999
	10	10	100	NA (Amac Inc.)	26	1999
	3	3	100	MIB1 (Immunotech)	52	1997
	23	26	88	MIB1 (Marseilles)	35	1995
Total	136	216	63			
p16 ^{INK4A}	4	15	27	16P07 (NewMarkers)	55	2006
	14	27	52	NA (Santa Cruz)	28	2003
	1	25	4	poly (PharMingen)	41	2001
	1	12	8	JC8 (MGHCC)	22	1999
Total	20	79	25			
n-p27 ^{Kip1}	13	27	48	NA (DAKO)	28	2003
	3	35	9	Ab-2 (Oncogene)	25	1999
Total	16	62	26			
c-p27 ^{Kip1}	9	27	33	NA (DAKO)	28	2003
	19	35	54	Ab-2 (Oncogene)	25	1999
Total	28	62	45			
RB1	10	12	83	Poly (Biogenex)	35	2002
	31	35	89	Clone 3C8 (Bioscience)	25	1999
	27	27	100	PMG3-245 (PharMingen)	41	2001
Total	68	74	92			
p21 ^{Cip1}	35	49	71	EA10 (Oncogene)	27	2001
	16	35	46	Ab-1 (Oncogene)	25	1999
Total	51	84	61			
MDM2	4	26	15	IF2 (Oncogene)	41	2001
	33	49	67	IF2 (Oncogene)	27	2001
Total	37	75	49			
Cyclin D1	10	35	29	Ab-3 (Oncogene)	25	1999
Cyclin E1	14	33	42	Poly (Dr. A. Koff)	25	1999
CDK4	1	27	4	C22 (Santa Cruz)	41	2001

Abbreviations: NA, information not available; MGHCC, Massachusetts General Hospital Cancer Center.

could be correlated to a good monitoring protocol of the NF1-associated patients, allowing for early detection of malignancies.

Summary

We have here presented the most comprehensive in situ protein profiling of sporadic and NF1-associated MPNST so far, which has allowed us to delineate the expression levels of a number of proteins affecting the regulation of the cell cycle in this cancer type.

Notably, tumors with high expression of n-p53, c-p53, or c-p14^{ARF} were correlated with poor survival for both patient groups. Nuclear cyclin D1 was correlated with prolonged survival for the sporadic MPNST patients. Overall, for both groups of MPNST patients, we showed that complete remission was the most decisive parameter to predict disease-specific survival, followed

by nuclear expression of p53 and tumor size. Patients without complete remission, with tumor size larger than 8 cm, and with positive p53 expression had a 60 times greater risk of dying within the first 5 years compared to the remaining patients ($p = 0.000002$). These data suggest that assessment of p53 expression in situ should be performed routinely on these tumors. Patients with curative intent who are in complete remission with a known positive p53 tumor status form a high-risk subgroup for whom adjuvant treatment should be considered.

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