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Immunohistochemical and molecular analysis of p53, MDM2, proliferating cell nuclear antigen and Ki67 in benign and malignant peripheral nerve sheath tumours

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Abstract A series of 26 malignant peripheral nerve sheath tumours (MPNST) and 24 benign peripheral nerve sheath tumours (BPNST) were analysed immunocytochemically for p53 expression and the cell proliferation markers proliferating cell nuclear antigen (PCNA) and Ki67 (with MIB1). In 23/26 MPNST, 5%–65% of the tumour cell nuclei were immunoreactive for Ki67 with MIB1 while none of the 24 BPNST had nuclear staining exceeding 5%. Greater than 50% nuclear PCNA staining was detected in 25/26 MPNST compared with 8/24 BPNST; 17/26 MPNST showed 5–100% nuclear staining for p53 (13/26 > 20%), whereas none of the BPNST had nuclear staining exceeding 1%. The Ki67, PCNA and p53 immunostaining results correlated significantly with benign versus malignant ($P<0.001$, $P<0.001$ and $P<0.005$, respectively) as well as mitotic rate ($P<0.001$, $P<0.05$ and $P<0.05$). Ki67 immunostaining results correlated significantly with PCNA and p53, as did p53 with Ki67 and PCNA ($P<0.001$ in both). Stepwise (logistic regression forward) multivariate analysis of the variable, benign versus malignant, revealed the strongest correlations with PCNA ($P=0.007$) and Ki67 ($P=0.021$). Direct confirmation of the presence of p53 protein was obtained by western blot analysis of 3 MPNST and 5 BPNST. Two MPNST, showing 90% and 30% immunoreactivity, were positive for p53, while one MPNST with 5% immunoreactivity and all 5 BPNST were negative. Southern blot analysis performed on the two MPNST with high p53 protein levels revealed no amplification of the MDM2 gene, suggesting that high p53 levels in MPNST are likely to be due to mutation. The results also indicate that PCNA and Ki67 are potentially useful in distinguishing BPNST from MPNST, particularly in problematic cases of cellular schwannoma versus MPNST. The detection of p53 in a large percentage of cells of a plexiform neurofibroma giving rise to MPNST and Ki67 in 5% and 25% of cells of two similar cases suggests that malignant transformation may be detected in some cases by p53 and proliferation markers prior to overt histological evidence of malignancy.

Key words Peripheral nerve sheath tumours · p53 · MDM2 · Proliferating cell nuclear antigen · Ki67

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Introduction

There have been an enormous number of studies indicating the importance and frequent occurrence of *p53* gene mutations and deletions in malignant transformation. *p53* mutations have been observed in most human malignant epithelial tumours as well as malignant melanomas and gliomas [12, 13, 14, 17, 25, 26]. There are also studies demonstrating *p53* mutations in several bone and soft tissue tumours [2, 10, 23, 24, 31, 37, 40]. More recently, immunocytochemical analysis of p53 protein has been applied to archival material and its detection has often been equated with *p53* mutations. Moreover, the immunocytochemical detection of p53 protein has been suggested to be useful in the distinction between some benign and malignant tumours [9, 20, 28, 32].

With the development of new monoclonal antibodies that work in formalin-fixed tissues, immunocytochemical demonstration of cell proliferation markers such as proliferating cell nuclear antigen (PCNA) and Ki67 are being used to distinguish benign from malignant tumours and in predicting prognosis [5, 15, 16]. The immunodetection of these cell cycle related antigens has been reported to be of diagnostic and prognostic significance in fibroblastic lesions such as fibromatosis and fibrosarcoma [34] and in gastrointestinal stromal tumours [1].

In view of these reports and the demonstration of chromosome 17p deletions and *p53* gene mutations associated with neurofibrosarcomas in neurofibromatosis in (NF)1 patients [30], as well as the particular problems involved in defining criteria for malignancy in peripheral nerve sheath tumours (PNST) [29], we have used immunocytochemical techniques and molecular analysis to

study p53 expression, MDM2 amplification and the cell proliferation markers PCNA and Ki67 in a series of benign and malignant PNST in order to evaluate their potential diagnostic and prognostic value.

Materials and methods

A series of 50 PNST were analysed immunocytochemically for p53 expression and the cell proliferation markers PCNA and Ki67. All cases were obtained from the files of the Department of Pathology, Sahlgren Hospital Göteborgs University Hospital in Göteborg, Sweden. Characteristic examples of malignant PNST (MPNST) and benign PNST (BPNST) were selected. The diagnoses were based on clinical information, review of haematoxylin and eosin and van Gieson-stained tissue sections and previous immunocytochemical staining results. All of the cellular schwannomas and most of the MPNST had also been examined ultrastructurally.

Twenty-six MPNST were selected; 21 of these were classical, developing in a nerve or from a neurofibroma or in patients with a known history of NF1 (8 cases). Five examples of epitheloid malignant schwannoma were selected; 4 of these developed within a nerve and one within a neurofibroma in a patient with NF1. Fourteen of the patients with MPNST were men and 12 were women. Their ages at diagnosis ranged between 16 and 85 years (median 40 yrs). The tumours ranged in size from 3 to 20 cm in largest diameter (median 8 cm). Fourteen were located in the extremities or limb girdles, 3 in the head and neck, 3 in the mediastinum or chest, 3 in the trunk and 2 were intra-abdominal. Histological malignancy grade was based upon cellularity, nuclear atypia, mitotic activity and necrosis. Three tumours were grade 2; 7 were grade 3; 16 were grade 4. Mitotic activity was assessed by counting ten fields using a 20× objective and 10× oculars. The mitotic rate varied between 5 and 50 mitoses per ten high power fields (hpf; median 18 per ten hpf). The extent of necrosis was estimated as the percentage of the surface area of the histological sections, using 10% increments. The percent necrosis ranged from 0% to 80% (median 10%). Of the 26 patients with MPNST, 7 (27%) were alive 2–18 years after diagnosis (median 3 years) without local recurrence or metastases. Two patients died of unrelated causes. Seventeen patients (65%) died of tumour (with local recurrences and/or metastases) 2 months–3 years after diagnosis (median 1 year).

Of the 24 BPNST, there were 8 classical schwannomas, 7 paraspinal cellular schwannomas, 5 cutaneous neurofibromas and 4 plexiform neurofibromas in NF1 patients. Three of the MPNST developed in a plexiform neurofibroma; the histologically benign neurofibroma components were also analysed in these cases but only the MPNST component was used for statistical analysis in this series. The patients with cellular schwannomas were 25–56 years of age; their tumours were 3–20 cm in greatest dimension and their mitotic rates ranged from 0–5 per ten hpf. All patients with cellular schwannomas were alive and well 2–25 years after diagnosis (median 20 years).

All specimens were routinely formalin-fixed and paraffin-embedded. The immunocytochemical techniques, including details of microwave pretreatment, antibodies, dilutions and sources, are summarized in Table 1. Positive controls for p53 included sections from colon and breast carcinomas with known *p53* mutations and p53 immunoreactivity. PCNA and Ki67 positive controls consisted of lymph node germinal centres and skin biopsies (basal epidermal and adnexal cells). The immunostaining results were recorded by selecting those areas that had the greatest number of positively staining cells. The percentage of positive cells was subjectively estimated using 5% increments by three observers.

Statistical analysis was performed by testing correlations between variables using Pitman's test [4], which is a non-parametric method. Two-sided tests were used. In order to elucidate the multivariate correlation between different prognostic variables and the variable, benign versus malignant, a stepwise logistic regression analysis was performed. The other variables, including tumour grade, mitotic rate, percent necrosis and the percentage of nuclei staining for Ki67, PCNA and p53, were all treated as continuous rather than dichotomous variables.

Table 1 Antibodies dilutions and sources used in this study [*PCNA* proliferating cell nuclear antigen, all immunostainings were performed after microwave treatment (three times, 5 min, 750 W) in 10 mM citrate buffer (pH 6). Immunoreactions for p53 were visualized with the Strept ABC/HRP Duet kit (mouse/rabbit) (Dako, code no. K492). Immunoreactions for PCNA and Ki67 were visualized with the Vectastain ABC kit (Burlingame, Calif., USA)]

Antibody	Dilution	Source
p53 (DO-1, Ab-6)	1:10	Oncogene Science, Uniondale, N.Y., USA
p53 (DO-7, code M 7001)	1:25	Dako, Copenhagen, Denmark
PCNA (PC10)	1:200§	Dako
Ki67 (MIB 1)	1:100§	Immunotech Marseille, France

Used for western blot analysis
Incubation time: 2 h at room temperature
§ Incubation time: 18 h at 4° C

For Western blot analysis cell lysates were prepared from fresh frozen material of three MPNST and from five BPNST as previously described [39]. Equal amounts of the lysates were separated electrophoretically on 12% polyacrylamide gels. Proteins were subsequently blotted onto nitrocellulose membranes [41]. Incubation with the p53 monoclonal antibody DO-1 (Oncogene Science, Uniondale, N.Y., USA) was performed for 60 min at a 1:10 dilution followed by incubation with biotinylated goat anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) for 60 min at a 1:1,000 dilution. Antigen-antibody complexes were detected using 4-chloro-1-naphthol and the specificity of the reactions was determined by omission of the primary antibody in a parallel incubation. As a p53 positive control, we used an SV40 transformed cell line derived from a pleomorphic adenoma (TCG 580). TCG 580 expresses high levels of wild type p53 protein (unpublished data).

For Southern blot analysis high molecular weight chromosomal DNA was isolated from two MPNST and five BPNST using standard methods [36]. DNAs were digested with the restriction endonucleases *Taq*I and *Msp*I under conditions recommended by the manufacturer. Digested DNAs were electrophoresed in 0.8% agarose gels in 0.5× TBE buffer (45 mM TRIS, 45 mM boric acid, 1.25 mM ethylenadiaminetetracetic acid and transferred to a Hybond-N+ filter according to the method of Southern. The MDM2 probe was a 600 base pair fragment, corresponding to nucleotides

Fig. 1 A paraspinal cellular schwannoma (**A**) The uniform spindle cells are arranged in poorly defined fascicles (H&E). (**B**) Few tumour cell nuclei (<<1%) are immunoreactive for Ki67. The MIB1 antibody also labels vessel walls. (**C**) The vast majority of the nuclei were immunoreactive for PCNA. There was no p53 immunoreactivity in this case

Fig. 2 Malignant peripheral nerve sheath tumour which involved the right shoulder and nerve plexus of a 51 year old woman. She developed lung metasases and died 2.5 years after the primary tumour was resected. (**A**) Densely packed atypical spindled tumour cells arranged in distinct fascicles. Scattered mitoses are seen (H&E). (**B**) 5–10% of the tumour cell nuclei are immunoreactive for Ki67. (**C**) Almost all of the tumour cell nuclei stain for PCNA. (**D**) In this area all of the tumour cell nuclei stain intensely for p53. The endothelial cells of capillaries and scattered lymphocytes serve as negative internal controls

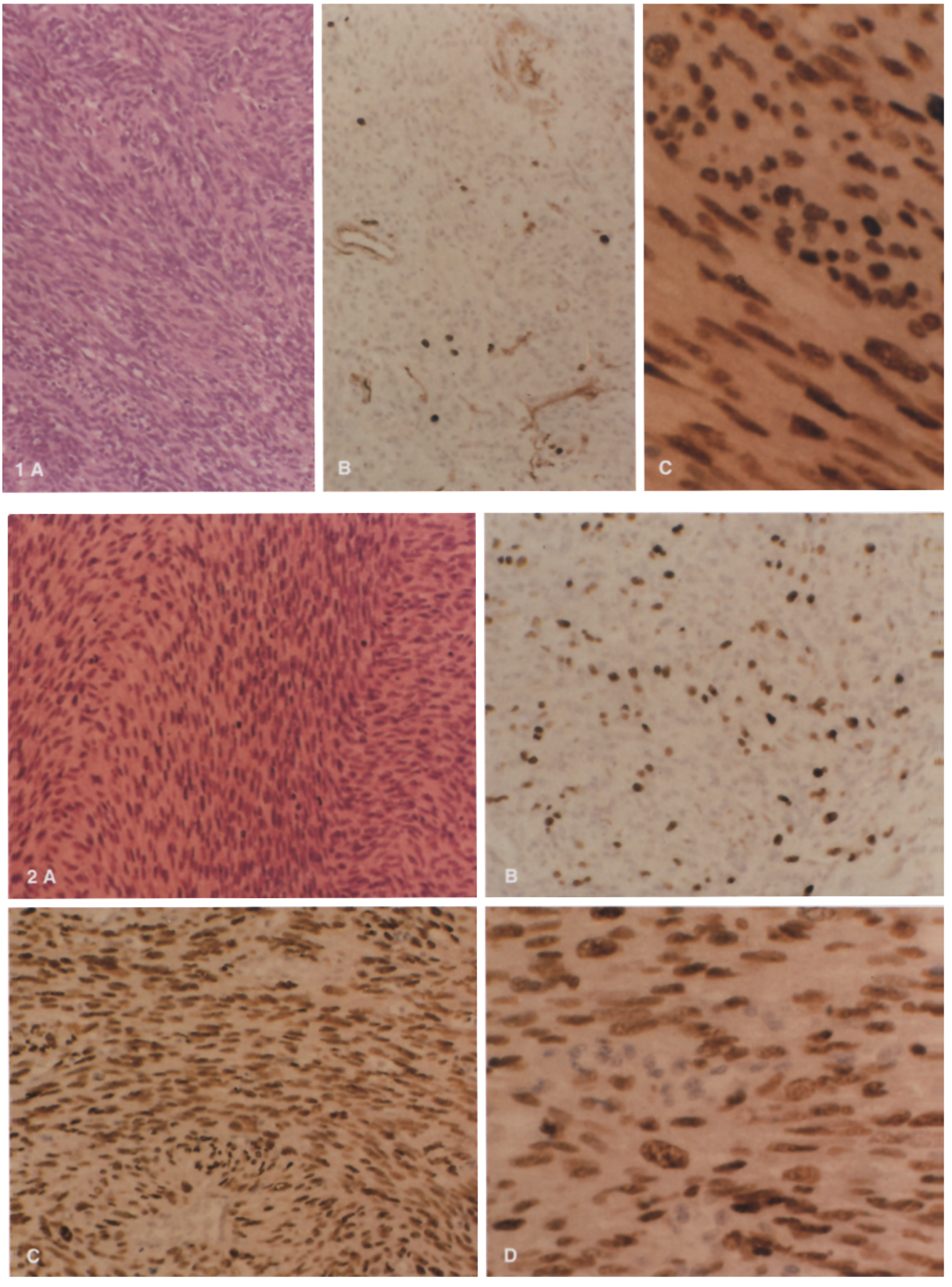
1 A
B
C
2 A
B
C
D

Table 2 Immunostaining results of malignant peripheral nerve sheath tumours (*MPNST*) and benign PNST (BPNST) with Ki67, PCNA and p53

Diagnosis (number)	Ki67	PCNA	p53
MPNST (26)	23/26>5% 13/26>30%	25/26>50% 22/26>75%	26/26>1% 17/26>5% 13/26>20% 9/26>50%
Classical schwannoma (8)	0/8>1%	5/8>50% 0/8>75%	0/8>1%
Cellular schwannoma (7)	0/7>1%	2/7>50% 0/7>75%	0/7>1%
Cutaneous neurofibroma (5)	0/5>1%	0/5>50%	0/5>1%
Plexiform neurofibroma (4)	0/4>1%	1/4>50%	0/4>1%
Plexiform neurofibroma (adjacent to MPNST; 3)	3/3>1% 2/3>5%	0/3>50%	1/3=50% 2/3<1%

53–653 of the human MDM2 cDNA sequence, synthesized by reverse transcriptase-polymerase chain reaction amplification. The probe was labelled with [^{32}P]dCTP by random priming [12] and hybridized to filters as described [38]. The filter was subsequently exposed to Fuji RX films at −80° C with intensifying screens for 1–5 days.

Results

Immunocytochemical staining results for Ki67, PCNA, and p53 are illustrated in Figures 1A–C and 2A–D, and summarized in Table 2. In 23 of the 26 MPNST, between 5% and 65% of the tumour cell nuclei revealed immunoreactivity for Ki67, 13 of which exceeded 30%. None of the 24 BPNST had nuclear staining exceeding 1%. In 4/24 BPNST, the nuclear staining approached 1% (one classical schwannoma, one cellular schwannoma, and two plexiform neurofibromas); in the other cases the nuclear staining was far less than 1%. All but one (25/26) of the MPNST showed nuclear staining for PCNA exceeding 50% (50%–100%); most of them (22/26) exceeded 75%. Eight of the twenty-four BPNST revealed 50% or more nuclear staining (five classical and two cellular schwannomas and one plexiform neurofibroma). None of the BPNST exceeded 75% nuclear staining. In all of the 26 MPNST there was p53 immunoreactivity in 1%–100% of the tumour cell nuclei. In 17/26 cases there was 5%–100% staining, with nine exceeding 50% and 13 exceeding 20%. None of the BPNST exceeded 1% staining. Scattered positive tumour cell nuclei (far less than 1%) were seen in examples of all types of BPNST. In addition, there were histologically benign plexiform neurofibroma areas available for analysis in three of the MPNST. In one of these, the plexiform neurofibroma revealed 50% p53 immunoreactivity whereas the other two contained only scattered positive nuclei (far less than 1%). Two of these three neurofibromas showed higher Ki67 positivity (5% and 25% nuclear staining) than the plexiform neurofibromas that were unassociated with MPNST, while the third case showed 1% positivity. The PCNA percentages in the plexiform neurofibromas associated with MPNST were 1%, 5%, and 15%.

The results of the statistical analysis are detailed in Table 3. All variables except benign versus malignant (including tumour grade, mitotic rate, percent tumour necrosis and percent positively staining nuclei for Ki67, PCNA and p53) were treated as continuous rather than dichotomous variables. The Ki67, PCNA and p53 immunostaining results correlated significantly with benign versus malignant ($P<0.001$, $P<0.001$ and $P<0.005$, respectively) as well as mitotic rate ($P<0.001$, $P<0.005$ and $P<0.005$, respectively). Ki67 immunostaining results correlated significantly with PCNA ($P<0.001$) and p53 ($P<0.001$) immunoreactivity. p53 also correlated with Ki67 ($P<0.001$) and PCNA ($P<0.001$). Ki67, PCNA and p53 did not correlate with the histological grade of malignancy or the extent of tumour necrosis in the MPNST ($P<0.30$). The malignancy grade correlated significantly with mitotic rate ($P<0.001$) but not with necrosis ($P<0.30$).

The step-wise (logistic regression forward) multivariate analysis of the prognostic markers Ki67, PCNA or p53 and the variable, benign versus malignant, revealed the strongest correlation with PCNA ($P=0.007$). When the additional influence of Ki67 and p53 was analysed, Ki67 was the second most significant factor ($P=0.021$) correlating with benign versus malignant. When PCNA and Ki67 were considered simultaneously in the test, the p53 values did not show any additional significant correlation with benign versus malignant ($P<0.103$).

Direct confirmation of the presence of p53 protein in MPNST was obtained by immunoblotting of proteins from three MPNST and five BPNST. The three MPNST showed 90%, 30% and 5% positive cells, respectively by immunocytochemistry while the BPNST were all negative for p53. The two MPNST with 90% and 30% p53 positive cells both showed distinct bands of approximately 53 kDa while one MPNST and the five BPNST lacked the p53 specific band (Fig. 3). The intensity of the bands corresponded well with the immunohistochemical findings in the two p53 positive cases.

Table 3 Results of statistical analysis correlating Ki67, PCNA, p53, benign versus malignant, malignancy grade, mitotic rate and necrosis in 26 MPNST and 24 BPNST (all variables with the exception of benign versus malignant are continuous rather than dichotomous variables)

	Benign versus malignant	Tumour grade (I–IV)	Mitotic rate	Percent tumour necrosis	Percent Ki67 positive nuclei	Percent PCNA positive nuclei	Percent p53 positive nuclei
Benign versus malignant		–	$P<0.001$	–	$P<0.001$	$P<0.001$	$P<0.005$
Tumor Grade (I–IV)	–		$P<0.001$	$P>0.30$	$P>0.30$	$P>0.30$	$P>0.30$
Mitotic rate	–	$P<0.001$		$P>0.30$	$P<0.001$	$P<0.05$	$P>0.30$
Percent tumour necrosis	–	$P>0.30$	$P>0.30$		$P>0.30$	$P>0.30$	$P>0.30$
Percent Ki67 positive nuclei	$P<0.001$	$P>0.30$	$P<0.001$	$P>0.30$		$P<0.001$	$P<0.001$
Percent PCNA positive nuclei	$P<0.001$	$P=0.08$	$P<0.05$	$P>0.30$	$P<0.001$		$P<0.001$
Percent p53 positive nuclei	$P<0.005$	$P=0.18$	$P<0.05$	$P>0.30$	$P<0.001$	$P<0.001$	

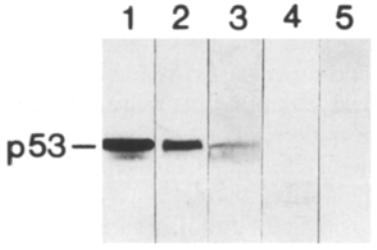

Fig. 3 Immunoblot analysis of p53 from an SV40 transformed control cell line (lane 1), three MPNST showing 90% (lane 2), 30% (lane 3), and 5% (lane 4) p53 positivity by immunohistochemistry, and a classical schwannoma that was negative for p53 by immunohistochemistry (lane 5). The p53 specific band is indicated to the left

To determine whether the MDM2 gene was amplified in the p53 positive MPNST, genomic DNAs from the two MPNST with high p53 protein levels were hybridized to an MDM2 cDNA probe. There were no signs of rearrangements or amplification of MDM2 in the two MPNST (data not shown). We did not find any MDM2 amplification in DNAs from the five BPNST either.

Discussion

Extensive studies of the wild type p53 protein, often called the guardian of the genome, indicate that it induces G_1 arrest, induces apoptosis following DNA damage, inhibits cell growth and preserves genetic stability. Missense and nonsense mutations and more rarely deletions of the tumour-suppressor *p53* gene, mapped to chromosome 17p 13.1, have been identified in a wide variety of human epithelial malignancies, including carcinomas of the colon, breast, prostate, ovary and lung as well as gliomas, malignant melanomas and some bone and soft tissue tumours such as osteosarcoma, rhabdomyosarcoma, malignant fibrous histiocytoma and leiomyosarcoma [2, 10, 12, 13, 14, 17, 23, 24, 25, 26, 31, 37, 40].

There are relatively few DNA sequencing studies in the literature regarding *p53* in soft tissue and bone tumours. *p53* mutations detected by this technique have been identified in osteosarcomas, rhabdomyosarcomas and liposarcomas (2, 24, 31, 40). There are also reports of *p53* mutations in neurofibrosarcoma associated with NF1 [30]. In addition to *p53* mutations, there are also reports indicating that binding of wild-type p53 by various oncoproteins, such as MDM2, may constitute an alternative mechanism for inactivation of p53, particularly in mesenchymal tumours [24, 33]. Thus, MDM2 amplification and overexpression have been described in malignant fibrous histiocytomas and liposarcomas lacking *p53* mutations, as well as in glioblastomas without *p53* gene rearrangements [35]. To test whether such a mechanism might also be operating in MPNST, we analysed DNA available from two of the MPNST with high p53 expression levels. However, no signs of MDM2 rearrangements or amplification were found, suggesting that high p53 levels in these MPNST are likely due to mutation.

Recently immunocytochemistry has been used to study p53 expression in human tumours [18]. Based on the observation that wild-type p53 protein has a very short half-life, in contrast to mutated p53 protein [13], immunodetection of p53 has often been interpreted as the presence of *p53* gene mutation. In fact, there are many reports describing a good correlation between *p53* mutations detected by DNA sequencing and immunoreactivity for p53 using monoclonal antibodies, most of which recognize both wild-type and mutated p53 protein. However, there are also studies demonstrating a lack of correlation between gene rearrangement and immunoreactivity, indicating that immunocytochemical results regarding p53 should be interpreted with caution, especially in terms of DNA rearrangements [3, 43]. To further strengthen our p53 immunocytochemical findings, we also analysed three MPNST and five BPNST by western blot. A p53 specific band of approximately 53 kDa was detected in two MPNST overexpressing p53 by immunocytochemistry. The relative levels of p53 protein detected by western blot correlated very well with the results recorded in the immunohistochemical assay.

In the present study, the p53 immunocytochemical results strongly correlated with malignant histology and biological behaviour. Thus, none of the BPNST, including the cellular schwannomas, revealed any p53 immunoreactivity, except for occasional, scattered stained nuclei (usually less than 1:1,000). In contrast, two-thirds of the MPNST revealed more than 5% nuclear staining and

one-third 50%–100% nuclear staining. There was no correlation between histological grade of malignancy and p53 expression in the MPNST; however, most of the tumours in this study were high grade. Mitotic rate also correlated significantly with p53 expression. These results indicate that p53 immunoreactivity is probably indicative of malignancy in the setting of a PNST. They also suggest that *p53* gene involvement may be common in MPNST unassociated with NF1. An interesting observation was the occurrence of p53 immunoreactivity in the histologically benign-appearing areas of a plexiform neurofibroma within which a MPNST arose, raising the possibility that p53 involvement may occur as an early event in malignant transformation.

The significance of the scattered positive nuclei as seen in the BPNST is difficult to explain, as is the low percentage of positive nuclei found in one-third of the MPNST (1%–5% positivity). We have found that the immunodetection threshold for p53 is significantly lowered by the citrate-buffer and microwave retrieval techniques, and that the results correspond well with those we have obtained in frozen tissue. Scattered p53 immunoreactivity has previously been observed by us and others in a variety of benign tumours as well as in some non-neoplastic cells when using highly sensitive immunotechniques [10]. This finding strongly suggests that such positivity is not a reflection of early clones harbouring *p53* mutations, but rather a reflection of the high sensitivity of the immunomethods used. Recent studies have shown that scattered p53 positive cells in tumours may reflect a situation of high endogenous genetic stress occurring in proliferating tumour cells [22].

Despite the lack of information on *p53* DNA sequencing in PNST (with the exception of occasional cases of neurofibrosarcoma in NF1 patients), the results of our study indicate that p53 immunocytochemistry may be useful in the diagnosis of MPNST and potentially assist in the distinction between cellular schwannoma and MPNST [27, 42]. Detection of p53 has previously been shown to be useful in the distinction between malignant and reactive mesothelial lesions [28], benign and malignant melanocytic lesions [31] and low and high grade gliomas [32].

The difficulties that occur when using mitotic activity in the distinction between benign and malignant neoplasms, especially in PNST, are well known. It is therefore of particular interest that recently developed antibodies capable of detecting cell proliferation associated antigens can be used on routinely fixed and processed tissues [8]. In contrast to S-phase determinations and other techniques previously used, the immunocytochemical application of PCNA and Ki67 antibodies allows estimation of cell proliferation while preserving morphology, allowing assessment of subpopulations in a tissue section.

In the present study, the PCNA and Ki67 (MIB1) immunoreactivity correlated strongly with benign versus malignant as well as p53 expression and mitotic rate. The value of PCNA and Ki67 immunoreactivity in distinguishing between cellular schwannoma and MPNST is of particular interest. High PCNA and Ki67 values, especially if simultaneous p53 expression is found, strongly suggest a MPNST and militate against the diagnosis of cellular schwannoma. Of interest is the observation that histologically benign-appearing areas in two plexiform neurofibromas undergoing malignant transformation revealed a high degree of proliferation marker activity, raising the possibility that this may be yet another indicator of early malignant transformation. We have also found that plexiform MPNST of infancy and childhood [29], a recently described variant of PNST characterized by locally aggressive growth and occasionally leading to the patient's death, showed significantly higher values of PCNA and Ki67 immunoreactivity than benign PNST [19], supporting the contention that this is a malignant PNST rather than a cellular or plexiform schwannoma. Moreover, occasional cases of plexiform MPNST have also revealed p53 immunoreactivity [19], another feature in favour of a malignant rather than a benign lesion. We have also recently found PCNA and Ki67 immunoreactivity to aid in the distinction between pleomorphic xanthoastrocytoma and glioblastoma multiforme [20].

There are few previous reports in the literature on proliferation markers in PNST, especially on their utility in the distinction between benign and malignant tumours. In one recent study of PCNA comparing intracranial or intraspinal cellular schwannoma with classical schwannomas, the cellular variant was reported to show a higher labelling index than the classical schwannoma [11]. A DNA flow cytometric and proliferation marker study of a large series of cellular schwannomas has also been reported recently [7]. However, neither of these two studies made any comparisons with MPNST. Although both PCNA and Ki67 (MIB1) reactivity correlated strongly with benign versus malignant in the present study, the actual values varied strongly, with PCNA staining a much higher percentage of the cell nuclei, especially in the benign lesions. This striking discrepancy between PCNA and Ki67 values is similar to what we have found in several other studies using identical techniques [19, 21]. We and other investigators have found that immunodetection of PCNA, Ki67 and p53 is strongly influenced by the citrate-buffer microwave retrieval method. Therefore, the results obtained in different laboratories using different techniques cannot be compared directly. For example, the actual PCNA and Ki67 values we obtained in cellular schwannomas deviate significantly from those of Casadei et al. [7], who did not use the citrate-buffer microwave retrieval method.

Another problem in assessing proliferation marker immunoreactivity, or for that matter, all types of immunoreactivity, is to determine the weakest level of positive staining to be called positive. Whereas immunostaining for Ki67 using the MIB1 antibody shows clear-cut positive or negative nuclear staining, PCNA immunostaining frequently has a broad spectrum, ranging from strongly positive to moderate, weak, questionably positive and negative. Considering these problems, we chose to inter-

pret all unequivocal staining, whether strong or weak, as positive. This may also explain differences in values when comparing results with other studies. Utilizing the more sensitive microwave technique we have applied in the current study, we have found that the Ki67 antibody (using MIB1) had definite advantages over PCNA. The MIB1 antibody seems to be more selective in identifying the S plus G_2M fraction of cells, while PCNA antibodies seemed to identify all non-resting cells. In addition to the high sensitivity of the technique, the long half-life of the PCNA protein and the existence of non-proliferation related nuclear PCNA populations [6] may explain the very high labelling found in benign tumours.

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