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ORIGINAL PAPER

Neurofibromin specific antibody differentiates malignant peripheral nerve sheath tumors (MPNST) from other spindle cell neoplasms

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Abstract Malignant peripheral nerve sheath tumors (MPNST) derive from the Schwann cell or perineurial cell lineage and occur either sporadically or in association with the tumor syndrome neurofibromatosis type 1 (NF1). MPNST often pose a diagnostic challenge due to their frequent lack of pathognomonic morphological or immunohistochemical features. Mutations in the *NF1* tumor suppressor gene are found in all NF1-associated and many sporadic MPNST. The presence of *NF1* mutation may have the potential to differentiate MPNST from several morphologically similar neoplasms; however, mutation detection is hampered by the size of the gene and the lack of mutational hot spots. Here we describe a newly developed monoclonal antibody binding to the C-terminus of neurofibromin (clone NFC) which was selected for optimal performance in routinely processed formalin-fixed and paraffin-embedded tissue. NFC immunohistochemistry revealed loss of neurofibromin in 22/25 (88 %) of NF1-associated and 26/61 (43 %) of sporadic MPNST. There was a strong association of neurofibromin loss with deletions affecting the *NF1* gene ($P < 0.01$). In a series of 256 soft tissue tumors of different histotypes NFC staining showed loss of neurofibromin in 2/8 myxofibrosarcomas, 2/12 (16 %) pleomorphic liposarcomas, 1/16 (6 %) leiomyosarcomas, and 4/28 (14 %) unclassified undifferentiated pleomorphic sarcomas. However, loss of neurofibromin was not observed in 22 synovial sarcomas, 27 schwannomas, 23 solitary fibrous tumors, 14 low-grade fibromyxoid sarcomas, 50 dedifferentiated liposarcomas, 27 myxoid liposarcomas, 13 angiosarcomas, 9

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extraskeletal myxoid chondrosarcomas, and 7 epitheloid sarcomas. Immunohistochemistry using antibody NFC may substantially facilitate sarcoma research and diagnostics.

Introduction

Malignant peripheral nerve sheath tumors (MPNST) arise either in the peripheral nerve or in extraneural soft tissue cells with Schwann cell or rarely perineurial differentiation [23]. MPNST account for approximately 5 % of all soft tissue sarcomas [6]. The morphology of MPNST can vary greatly and several histological variants with aberrant differentiations add to the diagnostic problem. Cases of well-differentiated MPNST with clear Schwann cell differentiation may be difficult to distinguish from benign tumors like cellular schwannomas. In high-grade MPNST dedifferentiation and loss of typical Schwann cell markers is common. Therefore, the discrimination between MPNST and other sarcomas is challenging. So far, there are no specific immunohistochemical or molecular markers for MPNST [21].

About 50 % of all MPNST occur in patients with the hereditary tumor syndrome neurofibromatosis type 1 (NF1). MPNST is by far the most common malignant tumor in NF1 patients [18]. NF1 patients carry a germline mutation in the *NF1* gene. NF1-associated tumors harbor an additional somatic mutation in the second copy of the *NF1* gene in full accordance with Knudson's two-hit hypothesis for tumor suppressor genes [26]. In addition, *NF1* mutations are also present in a subset of sporadic MPNST [3]. So far, *NF1* gene mutations have been found only in single other adult sarcoma subtypes at a relatively low frequency (11 % of myxofibrosarcomas and 8 % of pleomorphic liposarcomas) [2]. This suggests that loss of the *NF1* tumor suppressor may be sufficiently specific to serve as a marker for MPNST in the differential diagnosis from several other neoplasms. However, there are a broad spectrum of *NF1* gene mutations without any hot spot regions, thus hampering the easy detection of mutations. The vast majority of *NF1* gene mutations lead to protein truncation or even a complete loss of expression. Only about 10 % of mutations comprise missense mutations with production of a full length and possibly stable protein [11].

The *NF1* gene contains 60 exons and 350 kb of genomic DNA rendering a comprehensive mutational analysis very laborious and expensive [8]. Thus, shifting the detection from the DNA to the protein level constitutes an attractive alternative. In addition, non-mutational inactivation of neurofibromin in tumor cells via enhanced proteasomal degradation has been demonstrated, thus further arguing for the usefulness of a reliable detection of the protein [14].

Therefore we aimed to establish an immunohistochemical assay using routinely processed formalin-fixed and paraffin-embedded (FFPE) tissue. To maximize the sensitivity and reproducibility of such an approach we generated a highly specific monoclonal antibody recognizing the C-terminus of neurofibromin in FFPE material.

Materials and methods

Cell culture

Cell lines used for characterization of NFC antibody were cultured as described previously [22]. Murine Schwann cells were isolated from dorsal roots ganglia of new born mice according to published protocols [20].

Samples

Specimens for histology and immunohistochemistry from 342 patients were obtained from the Institute of Pathology, Heidelberg, banked in the archives of the Department of Pathology and administered by the tissue bank of the German National Center for Tumor Diseases, and from the archives of the Institutes of Pathology, University Tübingen, Medizinische Hochschule Hannover, University Medical Center Hamburg-Eppendorf, and University Hospital of Jena. All specimens were diagnosed according to the World Health Organization (WHO) classification in effect at the time of initial diagnosis. All samples were analyzed in an anonymous manner as approved by the local ethics committees at the participating institutions.

Generation of monoclonal neurofibromin antibody NFC

A cDNA fragment encoding for the last 281 amino acids of neurofibromin (transcript variant 1) was cloned into pQCH6 vector. The fragment was expressed in *E. coli* and the fusion protein was purified using a hexahistidine tag. One C57BL6/N and one BALB/c mice were immunized with 20 μg of the fusion protein and boosted on days 12, 16, 20, 28, 96, and 104. Polyethylene glycol fusion of lymph node cells from C57BL6/N with mouse myeloma SP2/O cells was performed on day 105. Immunoreaction was enhanced with Freund's adjuvant. The monoclonal antibody was raised according to the method described by Kohler and Milstein [10].

Screening of clones

All clones were tested in a first screen for immunoreaction with the C-terminal neurofibromin peptide conjugated to ovalbumin by enzyme linked immunosorbent assay (ELISA) [7]. For the second screen, we used immunocytochemistry of FFPE cell lines HEK293 and LN229. Only

one clone (NFC) demonstrated the desired strong and exclusive staining of HEK293 cells. Further characterization was done as described in the "Results" section.

Western blot

Cells were lysed in SDS boiling buffer or RIPA buffer and NuPAGE Sample Reducing Agent (Life Technologies, Darmstadt, Germany) was added. Samples were denatured at 95 °C for 5 min and electrophoretically separated on 4–12 % bis–tris or 3–8 % tris–acetate gels. Proteins were blotted onto nitrocellulose membranes (Life Technologies). After blocking (5 % milk powder, 0.05 % Tween 20 in PBS) at room temperature for 1 h the membranes were incubated overnight at 4 °C with primary antibody in blocking solution. Staining with secondary horseradish peroxidase-conjugated anti-mouse antibody (Cell Signaling Technology, Beverley, MD, USA) for 1 h at room temperature was followed by immunodetection with the Western Blotting Detection System (Medac GmbH, Wedel, Germany).

Immunohistochemistry

Sections cut to 4 µm with a Microm HM 355 S™ microtome (Thermo Fisher Scientific, Waltham, MA, USA) with an electrical cooled object clamp (Cool-Cut™; Thermo Fisher Scientific) were dried at 80 °C for 15 min and stained with anti-neurofibromin antibody (clone NFC) on a Ventana BenchMark ULTRA® immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The Ventana staining procedure included cell conditioning with Ventana (Ventana Medical Systems) cell conditioner 2 (pH 6) for 56 min, pre-primary peroxidase inhibition, incubation with 1:4 diluted NFC hybridoma supernatant at 37 °C for 32 min, incubation with OptiView HQ Universal Linker for 12 min, incubation with OptiView HRP Multimer for 12 min, OptiView Amplification (setting of OptiView Amplifier and OptiView Amplifier Multimer both for 12 min), and incubation with hematoxylin and Blueing reagent for 4 min each.

MLPA

Analyses for multi-exon or whole gene deletions of the *NF1* gene were carried out using the SALSA P081-B2/P082-B2 NF1 MLPA assay (MRC Holland, Amsterdam, the Netherlands) according to manufacture's instructions. SEQPILOT MLPA module (JSI medical systems GMBH, Kippenheim, Germany) was used for data analyses. Normalized peak areas were divided by the average normalized peak areas from four normal controls. A reduction in the peak area values to less than 0.75 was considered an indication of a deletion. Only cases with reduced peak area values in several adjacent probes were considered deleted.

Statistics

Fisher's exact test was used to examine the association of the presence or absence of neurofibromin loss in immunohistochemistry and *NF1* gene deletion in MLPA. *P* values of less than 0.05 were considered significant. *P* values of less than 0.01 were considered highly significant.

Results

Clone NFC is specific for neurofibromin

In total, more than 1,500 clones were investigated for the desired immunoprofile. Only one of these clones (clone NFC) showed highly specific recognition of neurofibromin in ELISA, immunocytochemistry of FFPE cell lines, Western blot, and immunohistochemistry in FFPE tissue. NFC stained paraffin-embedded HEK293 cells (*NF1+/+*) but not LN229 cells (*NF1−/−*) (Fig. 1a, b). In Western blot analyses NFC recognizes a single band slightly above 250 kDa in normal human Schwann cells and sporadic MPNST cell line STS26-T (*NF1+/+*) but not in NF1-associated MPNST cell lines 1507.2 and ST88-14 (*NF1−/−*) consistent with previously published results for neurofibromin expression in these cell lines (Fig. 1c) [22]. The band corresponding to neurofibromin is also visible in immunoblots from murine Schwann cells derived from Nf1(+/flox);Krox20-CRE mice (Nf1+/−) but not in Schwann cells derived from Nf1(flox/flox);Krox20-CRE mice (Nf1−/−) (Fig. 1c). In HeLa cells (*NF1+/+*) transfected with scrambled control siRNA the band is readily detectable, whereas in cells transfected with siRNA targeting neurofibromin the corresponding band is only faintly visible (Fig. 1c). Immunohistochemical NFC staining of an NF1-associated MPNST showed staining of endothelial and infiltrating inflammatory cells only, whereas tumor cells remained completely unstained in accordance with a tumor cell specific loss of neurofibromin (Fig. 2a, b). Altogether these results demonstrate the high specificity of clone NFC for neurofibromin.

Features of NFC immunohistochemistry

NFC staining in different normal FFPE tissues showed strong positivity in central and peripheral nervous tissue, in basal layers of epidermis, glandular tissue, and in various inflammatory cells. Variable staining was seen in connective tissue and skeletal muscle. Of note, endothelial cells were positive in all tissues including tumor tissue with loss of

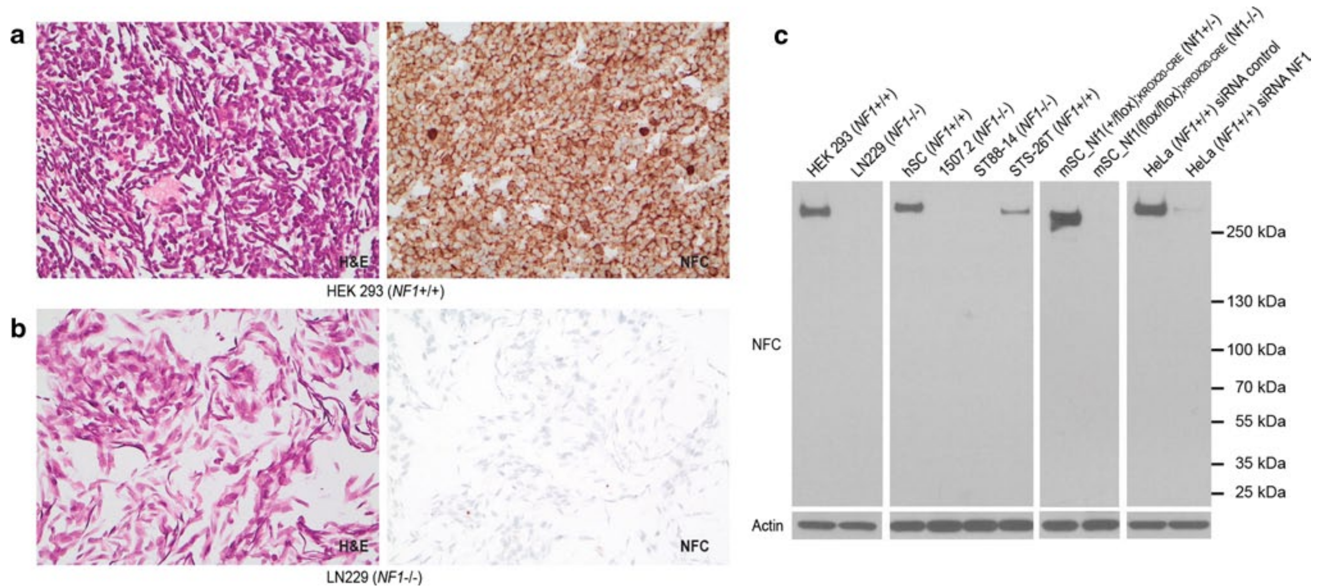

Fig. 1 Mouse monoclonal NFC anti-neurofibromin antibody stains formalin-fixed and paraffin-embedded HEK293 cells (*NF1*+/+) (**a**) but not LN229 cells (*NF1*−/−) (**b**); original magnification ×100. In Western blots NFC produces a strong single band above 250 kDa in human *NF1*+/+ and mouse Nf1+/− cells but not in −/− cells or *NF1*+/+ cells transfected with siRNA targeting neurofibromin (**c**)

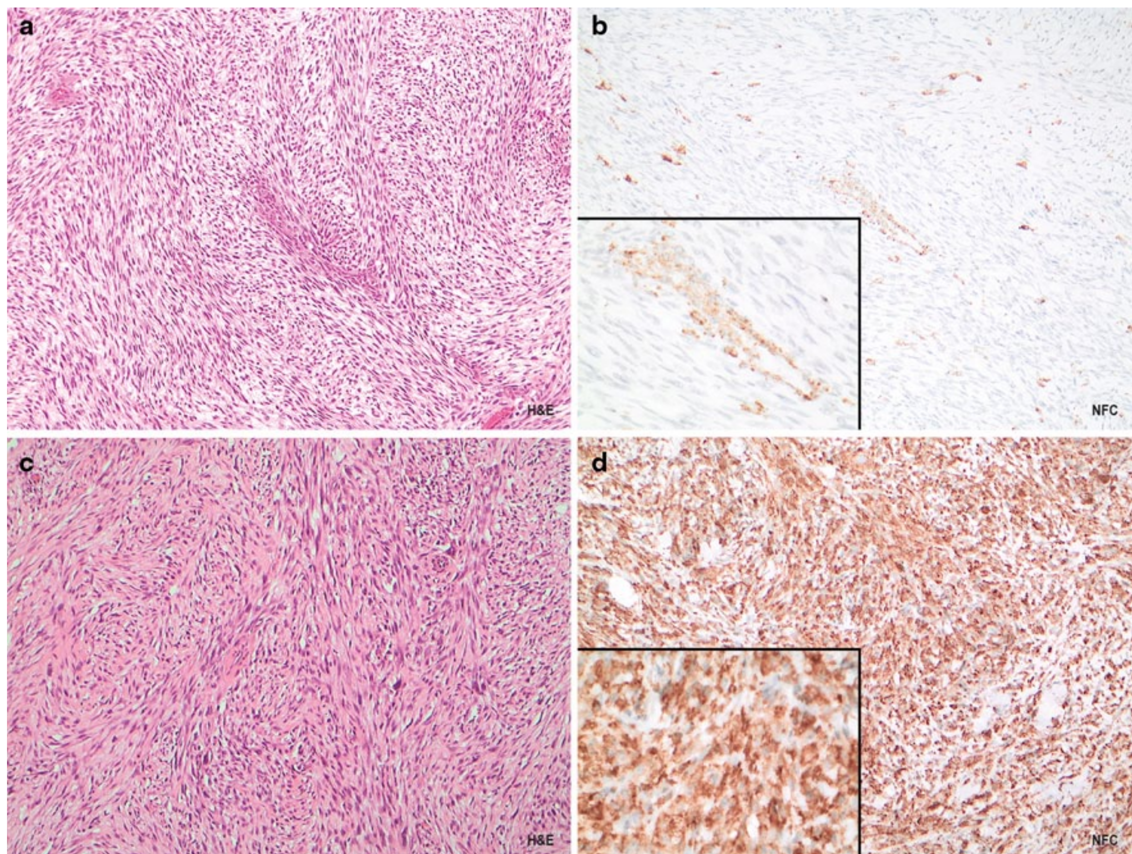

Fig. 2 Immunohistochemistry with clone NFC in NF1-associated MPNST (ID 63340) shows strong staining of non-neoplastic cells like vessels and inflammatory cells, whereas staining of tumor cells is absent (**a**, **b**); original magnification ×100, *inset* ×400. Example of a sporadic MPNST (ID 64352) with retained expression of neurofibromin (**c**, **d**); original magnification ×100, *inset* ×400

neurofibromin in tumor cells. In tumor tissue with retained expression of neurofibromin staining intensity was homogenous (Fig. 2c, d). Necrosis or areas of artificial tissue damage, especially in areas of surgical coagulation, showed complete loss or strong reduction of staining. Of note, staining intensity clearly depended on the age of slides before staining and the fixation time. However, in cases with weak antigenicity, staining intensity increased with prolongation of the pretreatment. This implies that quantitative analysis of neurofibromin in archived material is problematic. However, a qualitative analysis of tumor cell specific loss is possible but indispensably relies on internal positive controls like endothelial and inflammatory cells.

NFC immunohistochemistry in MPNST

In a series of NF1-associated MPNST 22/25 (88 %) showed homogenous tumor cell specific loss of neurofibromin. In a series of MPNST without clinical history of NF1 20/47 (43 %) presented a tumor cell specific loss of neurofibromin. In three of these cases tumor cell specific loss of neurofibromin was heterogeneous with retained neurofibromin expression in some areas of the tumor.

In a second series of sporadic MPNST which derived from the national reference center for soft tissue sarcomas 6/14 (43 %) cases exhibited a loss of neurofibromin, thus confirming the result of the initial series.

Association between NFC immunohistochemistry and *NF1* MLPA

To test whether the staining pattern of tumor cell specific loss of neurofibromin is associated with genetic *NF1* loss, we performed multiplex ligation-dependent probe amplification (MLPA) analyses for detection of *NF1* deletions. The presence of *NF1* gene deletions in NF1-associated MPNST is well established [3, 9, 12, 13, 19, 26]. Indeed, 4/4 NF1-associated MPNST harbored a deletion in the *NF1* gene. Next we analyzed 17 immunopositive and 17 immunonegative sporadic MPNST for *NF1* gene deletion. Thirteen of 17 (76 %) sporadic MPNST with tumor cell specific loss of neurofibromin in immunohistochemistry showed deletions affecting the *NF1* locus, whereas only 3/17 (18 %) immunopositive cases harbored deletions (Table 1; Fig. 3). These results demonstrate a highly significant association between the pattern of tumor cell specific loss of neurofibromin and *NF1* gene deletion in sporadic MPNST ($P < 0.01$; Fisher's exact test).

NFC immunohistochemistry in non-MPNST spindle cell neoplasms

In addition, we stained a series of soft tissue tumors with NFC, many of which may come into consideration in the differential diagnosis of MPNST. Tumor cell specific loss of neurofibromin was present in 0/22 synovial sarcomas, 0/23 solitary fibrous tumors, 2/8 myxofibrosarcomas (25 %), 4/28 (14 %) undifferentiated pleomorphic sarcomas, 1/16 leiomyosarcomas (6 %), 2/12 pleomorphic liposarcomas (16 %), 0/50 dedifferentiated liposarcomas, 0/27 myxoid liposarcomas, 0/7 epitheloid

Table 1 Results of NFC immunohistochemistry and *NF1*-MLPA in 4 NF1-associated and 34 sporadic MPNST

Tumor type	ID	NF1 loss IHC (NFC)	NF1 deletion MLPA
NF1-associated MPNST	61512	Yes	Yes
	63376	Yes	Yes
	63354	Yes	Yes
	63340	Yes	Yes
Sporadic MPNST	64074	Yes	Yes
	61504	Yes	Yes
	61510	Yes	Yes
	63368	Yes	No
	63334	Yes	No
	63348	Yes	Yes
	63364	Yes	No
	63346	Yes	Yes
	63370	Yes	Yes
	63382	Yes	Yes
	63330	Yes	No
	64350	Yes	Yes
	64354	Yes	Yes
	64356	Yes	Yes
	64352	Yes (HET)	Yes
	63360	Yes (HET)	Yes
	64072	Yes (HET)	Yes
	63366	No	No
	63352	No	No
	63380	No	No
	61502	No	No
	61506	No	No
	61508	No	No
	64070	No	Yes
	64800	No	No
	64804	No	Yes
	64806	No	No
	64808	No	No
	64810	No	Yes
	64814	No	No
	64816	No	No
	64818	No	No
	64820	No	No
	64348	No	No

HET heterogenous

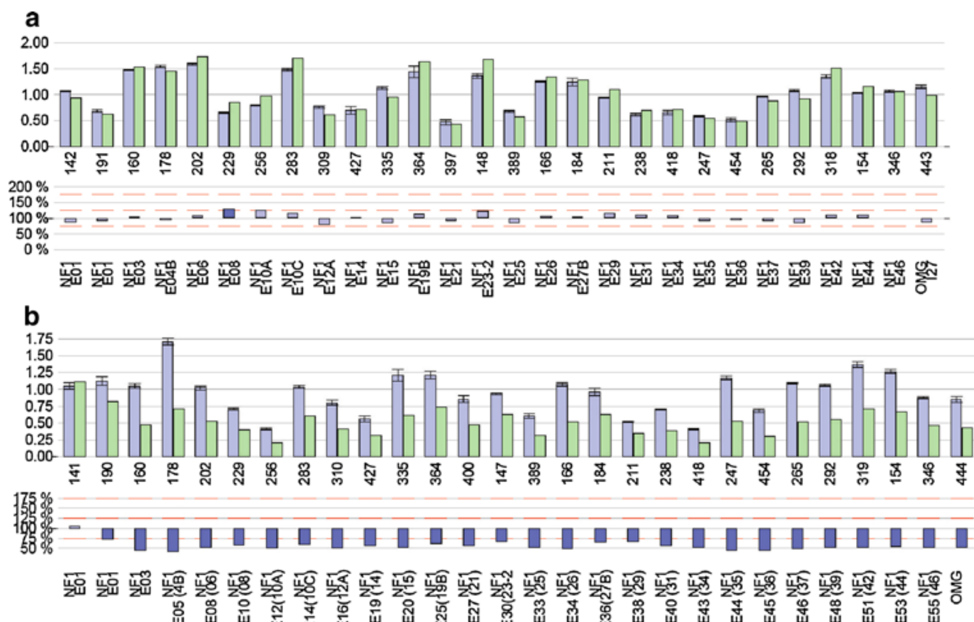

Fig. 3 Representative examples of MLPA results: MLPA analyses of sporadic MPNST without (**a**, ID 61502) and with (**b**, ID 64350) evidence for deletion in the *NF1* locus. Normalized relative peak areas of *NF1*-gene-specific (*gray bars*) and control (*green bars*) probes are shown. A reduction in the peak area values to less than 0.75 (*blue bars*) in adjacent probes indicates a deletion (color figure online)

Table 2 Results of NFC immunohistochemistry in soft tissue tumors

Tumor type	*n*	NF1 loss IHC (NFC)
MPNST		
NF1	25	22 (88 %)
Sporadic	61	26 (43 %)
Synovial sarcoma	22	0
Solitary fibrous tumor	23	0
Myxofibrosarcoma	8	2
Leiomyosarcoma	16	1 (6 %)
Pleomorphic liposarcoma	12	2 (16 %)
Dedifferentiated liposarcoma	50	0
Myxoid liposarcoma	27	0
Schwannoma	27	0
Cellular schwannoma	9	0
Epitheloid sarcoma	7	0
Angiosarcoma	13	0
Low-grade fibromyxoid sarcoma	14	0
Undifferentiated pleomorphic sarcoma	28	4 (14 %)
Extraskeletal myxoid chondrosarcoma	9	0

sarcomas, 0/14 low-grade fibromyxoid sarcomas, 0/27 schwannomas including 9 cellular schwannomas, 0/13 angiosarcomas, and 0/9 extraskeletal myxoid chondrosarcomas (Table 2).

Non-MPNST sarcomas with tumor cell specific loss of neurofibromin were also analyzed with MLPA for *NF1* gene deletion. Results showed *NF1* deletion in 7/9 cases including 1/2 myxofibrosarcomas, 1/1 leiomyosarcoma, 2/2 pleomorphic liposarcomas, and 3/4 undifferentiated pleomorphic sarcomas.

Discussion

Loss of neurofibromin has a marked association with MPNST. NF1-associated MPNST showed loss of neurofibromin in 88 %, which matches well with the expected rate of about 10 % of *NF1* germline mutations predicted to result in stable protein expression and the high prevalence of somatic *NF1* deletions [15, 26]. In NF1-associated MPNST, loss of neurofibromin was always homogenous consistent with loss of *NF1* being an essential early step in NF1-associated tumorigenesis.

Our study confirms the occurrence of *NF1* loss also in a substantial proportion of sporadic MPNST. Whereas biallelic mutation of *NF1* is well established in NF1-associated MPNST, the proof of biallelic inactivation was reported only in 14 % of sporadic MPNST [3]. This contrasts with the complete loss of neurofibromin in 43 % of sporadic MPNST as revealed by NFC staining and suggests that many mutations are not detected even by the combination of different methods or that *NF1* may also be inactivated by epigenetic mechanisms. In three sporadic MPNST, loss of neurofibromin was present only in parts of the tumor tissue, suggesting that in these cases loss of neurofibromin was an event during tumor progression but was not necessary for tumor initiation. In future studies, analyzing NFC expression in rare MPNST variants like epithelioid or perineurial MPNST or additional types of nerve sheath tumors like atypical neurofibromas or epithelioid schwannomas will be of interest [23].

The presence of sporadic MPNST with and without loss of neurofibromin raises several intriguing questions. Do sporadic MPNST without loss of neurofibromin harbor

mutations in other genes in the same pathway resulting in a similar appearance and biological behavior? Of note, *BRAF* and *KRAS* mutations, which would be plausible alternatives for *NF1* loss, occur only rarely in sporadic MPNST [3, 25]. Do sporadic MPNST without *NF1* loss constitute a molecularly distinct entity with morphological overlap or are they actually a mixed group of misdiagnosed other sarcomas? To answer these questions future studies with in-depth molecular and clinical characterization of sporadic MPNST with and without loss of neurofibromin will be necessary.

Immunohistochemical loss of neurofibromin was occasionally observed in myxofibrosarcomas and pleomorphic liposarcomas, two entities in which *NF1* gene mutations were reported previously [2]. In our series an additional case of a poorly differentiated leiomyosarcoma showed tumor cell specific loss of neurofibromin and a deletion in the *NF1* gene. The occurrence of *NF1* mutations in leiomyosarcomas is not well established but there are reports about rare cases of leiomyosarcoma arising in patients with NF1 [1]. Four of 28 (14 %) undifferentiated pleomorphic sarcomas showed loss of neurofibromin accompanied by *NF1* gene deletion in 3 of the 4 cases. This entity is defined by the WHO as soft tissue sarcoma showing no identifiable differentiation. Thus, the neurofibromin-deficient cases of undifferentiated pleomorphic sarcomas might be undifferentiated MPNST, myxofibrosarcoma, or pleomorphic liposarcoma.

Neurofibromin is a GTPase-activating protein for RAS proteins and acts as strong negative regulator of RAS-dependent signaling [27]. Specific therapies for neurofibromin-deficient tumors have not yet been established. However, there is preclinical evidence from Nf1-deficient mouse soft tissue sarcoma models that inhibition of signaling molecules downstream of activated RAS like MEK is a promising therapeutic approach [4, 5, 24]. Therefore, identification of neurofibromin deficiency in tumors can be expected to gain clinical relevance in the future.

Cancer genome analyses have revealed *NF1* mutations in an increasing number of sporadic human malignancies like glioblastoma or ovarian carcinoma [16, 17]. Immunohistochemical detection of neurofibromin deficiency may be useful to screen for additional tumor types harboring *NF1* mutations.

NFC immunohistochemistry is a simple, rapid, and cost-effective method for detecting neurofibromin deficiency in FFPE tissues and thus may be a helpful tool in research and diagnostics.

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