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DISSERTATION

Identification and Characterization of the Secretome in Neurofibromatosis Type 1 Tumors

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Abkürzungsverzeichnis

Akt	Protein Kinase B
AUC	Area under the curve (Fläche unter der ROC-Kurve)
CBA	Cytometric Bead Array
cNF	Cutaneous Neurofibroma (Dermale Neurofibrome)
EGFR	Epidermal Growth Factor Receptor (Epidermaler Wachstumsfaktor-Rezeptor)
ELISA	Enzyme-linked Immunosorbent Assay (enzymgekoppelter Immunadsorptionstest)
GTP	Guanosintriphosphat
IFNγ	Interferon gamma
IGFBP1	Insulin-like Growth Factor Binding Protein 1 (Insulin ähnlicher Wachstumsfaktor Bindungsprotein 1)
IL-6	Interleukin 6
MIA	Melanoma inhibitory activity (Melanom inhibierende Aktivität)
MiR-21	Micro RNA 21
MPNST	Malignant peripheral nerve sheath tumors Maligne periphere Nervenscheidentumore
mTOR	Mammalian Target of Rapamycin (Ziel des Rapamycins im Säugetier)
NF1	Neurofibromatose Typ 1
Nf1prx1	Mausmodell der NF1-Röhrenknochendysplasie
PCR	Polymerase chain reaction (Polymerase Kettenreaktion)
PIK3CA	p110α Protein
PI3K	Phosphoinositid-3-Kinase
PNF	Plexiforme Neurofibrome
PTEN	Phosphatase und Tensin homolog
RANTES	Regulated on Activation, normal T cell expressed and secreted
Ras	Rat sarcoma
RasGAP	Ras GTPase
ROC	Receiver Operator Characteristic (Grenzwertoptimierungskurve)
qRT-PCR	Real-Time-quantitative-PCR (quantitative Echtzeit-PCR)
SOX9	SRY (sex determining region Y)-box 9
TNFα	Tumor necrosis factor alpha (Tumornekrosefaktor)

1. Zusammenfassung

Neurofibromatose Typ 1 (NF1) ist mit einer Inzidenz von 1:2500 (1) die häufigste monogenetische Erkrankung und mit benignen und malignen Tumoren (Neurofibrome) des Nervensystems assoziiert. Die bei etwa 50% der Patienten mit NF1 auftretenden diffus, infiltrativ wachsenden plexiformen Neurofibrome (PNF) können durch Raumforderungen schwere Komplikationen verursachen und zu malignen peripheren Nervenscheidentumoren (MPNST) transformieren. Die Wahrscheinlichkeit einer malignen Transformation korreliert mit der PNF-Belastung der NF1-Patienten. Derzeit können PNF und MPNST nur durch eine klinische Untersuchung identifiziert werden. Die Diagnose interner PNF erfolgt über bildgebende Verfahren des ganzen Körpers. Eine MPNST Diagnose ist zur Zeit erst möglich, wenn Symptome auftreten und eine erfolgreiche Behandlung nicht mehr möglich ist. MPNST sind die Hauptursache für die geringere Lebenserwartung von NF1-Patienten. Ein Früherkennungsprogramm für NF1, das sowohl eine frühere Diagnose der Tumorbelastung durch PNF oder der malignen Entartung anzeigt, als auch eine prädiktive Aussage über den generellen Krankheitsverlauf treffen kann, ist derzeit nicht verfügbar.

Unsere Arbeitsgruppe hat sich deshalb der Identifizierung von Serummarkern als prognostische Indikatoren gewidmet. Diese sollenmögliche Risikogruppen für die Entwicklung von MPNST erkennen und maligne Tumore sicher diagnostizieren. Statt nur auf einen einzelnen Biomarker, setzten wir auf ein breites Markerprofil.

Unsere Daten zeigten in Seren von NF1-Patienten deutlich höhere *Melanoma inhibitory activity* (MIA)-Konzentrationen als in gesunden Kontrollen. Innerhalb der NF1-Patientengruppe wiesen Personen mit PNF oder besonders vielen dermalen Neurofibromen (cNF) erhöhte MIA-Serumkonzentrationen auf. Zur Identifikation weiterer Serumproteine führten wir eine Screening-Studie durch. Hierfür wurden neben Proteinen, die in PNF und MPNST überexprimiert und potentiell sezerniert werden auch solche Proteine einbezogen, die das Tumorwachstum bei NF1 systemisch beeinflussen könnten. Frühere Untersuchungen zeigten, dass die NF1-haploinsuffiziente systemische Umgebung bei NF1-Patienten das Tumorwachstum fördert, insbesondere auch durch Zellen des angeborenen Immunsystems wie Mastzellen und Monozyten (2). Aus verfügbaren Daten- und Literaturbanken sowie Vordaten der eigenen Arbeitsgruppe wurde eine Liste mit Kandidatenproteinen (n=115) erstellt und an einer NF1-Patienten Kohorte (n=104) mittels eines antikörperbasierten Microarrays simultan getestet. Es

gelang uns mit dem *Insulin-like Growth Factor Binding Protein 1* (IGFBP1) und *Regulated on Activation, normal T cell expressed and secreted* (RANTES) zwei Proteine zu identifizieren, die spezifisch veränderte Konzentrationen in NF1-Patienten mit MPNST aufwiesen. Des Weiteren konnten wir mit *Interleukin 6* (IL-6), *Interferon gamma* (IFNγ), *Epidermal Growth Factor Receptor* (EGFR) und *Tumor Necrosis Factor alpha* (TNFα) vier potentielle NF1-Marker ermitteln, die im Vergleich zur Kontrollgruppe signifikante Unterschiede im Serum der NF1-Kohorte aufwiesen.
Zuletzt befassten wir uns mit der Auswirkung einer Veränderung des Tumorsuppressorgens PTEN in MPNST und Neurofibromen. PTEN ist wesentlich an der Modulierung von Zytokinsignalwegen in MPNST-Zellen beteiligt und ein potentielles therapeutisches Target. Dabei konnten wir verstärkte PTEN-Promotor Methylierungen in MPNST nachweisen, die eine Unterückung der Expression und damit einhergehend erhöhte Tumorigenität der Zellen bewirken kann.
Zusammenfassend konnten wir ein potentielles Markerprofil zur früheren Diagnose von NF1 identifizieren, das sogar eine Differenzierung von MPNST erlaubt. Unsere Erkenntnisse wurden unter dem Aktenzeichen 10 2012 020 496.5 als „*Biomarker zur Diagnostik und Behandlung von Neurofibromatose Typ 1“* zum Patent eingereicht.

2. Abstract

Neurofibromatosis type 1 (NF1) is the most common monogenetic disorder with an incidence of 1:2500 (1) and is associated with benign and malignant tumors (neurofibromas) of the nervous system. Plexiform neurofibromas (PNF) are a specific form of NF1 tumors with diffuse and infiltrative growth. They lead to severe growth-associated complications and can transform into malignant peripheral nerve sheath tumors (MPNST). The incidence of MPNST is associated with the individual PNF burden of NF1 patients. At present, only a fraction of NF1 patients with PNF can be identified by physical examination. Although advanced imaging technology monitors internal PNF burden, MPNST cannot be identified. MPNST are usually only detected when symptoms arise and hence too late for successful therapy, making this tumor the main reason for the reduced life span of NF1 patients. However, early presymptomatic NF1 diagnosis is currently unavailable, making it difficult to assess overall PNF tumor burden and therapeutic efficacy or follow-up tumor development.

Therefore our working group focussed on the identification of a surrogate serum marker, which should serve as a prognostic indicator for NF1. A surrogate serum marker may provide the basis for identification of cohorts at increased risk for malignant transformation of PNF, and for diagnosis of MPNST prior to symptomatic detection. Moreover, we established a marker profile to avoid relying on a single serum marker.

Our data showed higher concentrations of Melanoma inhibitory activity (MIA) in NF1 patient sera compared to healthy controls. Within the NF1 patient cohort only affected individuals with PNF or numerous dermal neurofibromas (cNF) show higher MIA serum levels. For investigation of further surrogate serum markers we performed a screening study. We incorporated proteins, which we assumed to be overexpressed or potentially secreted by PNF or MPNST and considered also proteins which influence tumor growth systemically. Previous investigations showed that a systemic NF1-haploinsufficient environment supports tumor growth in NF1 patients mainly mediated by cells of the innate immune system like mast cells and monocytes. (2). Investigation of available public databases, scientific literature and previous data of our research group lead to a comprehensive list of candidate proteins (n=115). Those proteins were simultaneously analysed in NF1 patients (n=104) using an antibody based micro array. We identified two proteins, which showed altered levels in MPNST-affected NF1 patients only: *Insulin-like growth factor-binding protein 1* (IGFBP1) and *Regulated on activation, normal T cell expressed and secreted* (RANTES). In addition, we determined *Interleukin 6* (IL-6), *Interferon gamma* (IFNγ), *Epidermal Growth Factor Receptor* (EGFR) and *Tumor Necrosis Factor alpha* (TNFα) to be potential markers for NF1 exclusively. Furthermore, we determined the impact of tumor suppressor gene *PTEN* alterations in MPNST and neurofibromas. PTEN is a modulator of cytokine response and a potential therapeutic target for MPNST therapy. We detected increased methylation of the *PTEN* promotor in NF1 patients with MPNST, reduced expression and increased tumorigenic potential.

In summary, we identified a potential marker profile for earlier NF1 diagnosis, which even allows a differentiation of MPNST. Our findings have been patented as *Biomarker zur Diagnostik und Behandlung von Neurofibromatose Typ 1“* (reference 10 2012 020 496.5).

Neurofibromatose Typ 1

Diagnostik

State of the art

NF1
- Körperliche Untersuchung
- Molekulargenetik
- Röntgendiagnostik/ MRT

cNF oder PNF (interner Tumorload)
- Körperliche Untersuchung
- Röntgendiagnostik/ MRT

MPNST
- MRT
- Histologie

Biomarker

NF1
- IL-6*, IFNγ*, EGFR*, TNFα*
- MIA**

cNF oder PNF (interner Tumorload)
- MIA**

MPNST
- IGFBP1*, RANTES*
- Pten***

* Publikation 1
** Publikation 2
*** Publikation 3

Abb. 1: Überblick über die drei vorangestellten Publikationen:

Publikation 1: Park SJ, Sawitzki B, Kluwe L, Mautner VF, Holtkamp N, Kurtz A. *Serum biomarkers for neurofibromatosis type 1 and early detection of malignant peripheral nerve-sheath tumors.* BMC Med. 2013 Apr 23;11:109.

Publikation 2: Kolanczyk M, Mautner VF, Kossler N, Nguyen R, Kühnisch J, Zemojtel T, Jamsheer A, Wegener E, Thurisch B, Tinschert S, Holtkamp N, Park SJ, Birch P, Kendler D, Harder A, Mundlos S, Kluwe L. *MIA is a potential biomarker for tumour load in neurofibromatosis type 1.* BMC Med. 2011 Jul 4;9:82.

Publikation 3: Bradtmöller M, Hartmann C, Zietsch J, Jäschke S, Mautner VF, Kurtz A, Park SJ, Baier M, Harder A, Reuss D, von Deimling A, Heppner FL, Holtkamp N. I*mpaired Pten expression in human malignant peripheral nerve sheath tumours.* PLoS One. 2012;7(11):e47595.

3. Einleitung

Neurofibromatose Typ 1 (NF1; ehem. Morbus von Recklinghausen) ist ein autosomal dominantes Tumorsyndrom, das mit einer Inzidenz von 1:2500 auftritt (1). Die NF1 gehört zu den häufigsten tumorprädisponierenden, genetischen Erkrankungen, verursacht durch eine Mutation des Tumor Suppressor Gens *Nf1* (3, 4). Das *Nf1-Gen* codiert für das Neurofibromin (319 kD), ein Ras GTPase aktivierendes Protein (RasGAP), das ein negativer Regulator der Ras-Signaltransduktion ist. Der Verlust beider *NF1*-Genkopien führt zu einer andauernden Aktivierung des Ras-Signaltransduktionsweges und somit zu einer Prädisposition für Tumorwachstum. Die Krankheit zeigt 100% Penetranz mit extrem variabler Expressivität. Das klinische Bild der NF1 ist sehr vielgestaltig. Neben melanogenen Veränderungen der Haut und der Augen (Café-au-lait-Flecken, sommersprossenartige Pigmentierungen, Lisch-Knötchen), Knochenveränderungen (Dysplasien), kognitiven Defiziten und Astrozytomen/ Optikusgliomen (bei Kindern) entwickeln nahezu alle NF1-Patienten gutartige Nervenscheidentumore, sogenannte dermale Neurofibrome (cNF). Etwa 50% der NF1-Betroffenen entwickeln PNF, die jederzeit und in jeder Körperregion entstehen können. Oftmals zunächst ein ästhetisches Problem, verursachen sie aber bei entsprechender Lage und Größe Organkompressionen und führen aufgrund der massiven Entstellungen zu sozialer Stigmatisierung der NF1-Betroffenen (5, 6). Darüber hinaus besteht bei etwa 16-26% der Patienten die Gefahr, dass die PNF zu MPNST entarten (Vergleich: Inzidenz der Gesamtpopulation 0.001%; 7). MPNST sind sehr aggressive Sarkome mit einer mittleren Überlebensrate von 17 Monaten und extrem therapieresistent. Gegenwärtig ist die komplette chirurgische Resektion die einzige Therapie von Neurofibromen und MPNST (8, 9, 10, 11), was jedoch durch die meist erst späte Diagnose und daher raumfassenden, bei MPNST oft invasiven und metastasierenden Tumoren meistens unmöglich ist. Strahlentherapie führt zwar zu einer lokalen Kontrolle des Tumors und verzögertem Auftreten von Rezidiven, hat aber kaum Einfluss auf die Überlebenszeit (10, 12). Weiterhin kann die Exposition gegenüber therapeutischer Bestrahlung einen zusätzlichen wesentlichen Risikofaktor für die Entstehung weiterer MPNST darstellen (7, 13, 14).

Eine frühere Diagnose, sowohl der Tumorbelastung durch PNF, als auch der malignen Entartung, sowie eine prädiktive Aussage über den generellen Krankheitsverlauf ist derzeit nicht verfügbar.

4. Methodik

Dies ist eine zusammenfassende Darstellung der angewandten Methoden. Eine ausführliche Beschreibung ist in den drei beiliegenden Publikationen zu finden.

4.1 Publikation 1*: Identifizierung eines potentiellen Biomarkers in NF1*

Die Auswahl der potentiellen Biomarker für NF1 erfolgte durch Recherche wissenschaftlicher Publikationen und Datenbanken. Zur Ermittlung der Proteinlevel wurden maßgefertigte humane Zytokinarrays (Quantibody; Raybiotech Inc., GA, USA) verwendet. In einer Vierfachbestimmung wurden alle Kandidatenproteine simultan detektiert. Im ersten Durchlauf wurden 30 Kandidatenproteine an NF1- (n=60) und Kontrollseren (n=20) analysiert. Im zweiten Durchlauf wurden die aus dem ersten Durchlauf signifikanten Kandidaten mit 26 neuen Kandidatenproteine an weiteren NF1- (n=104) und Kontrollseren (n=41) reanalysiert. Eine Validierung der Zytokinarrayergebnisse erfolgte mittels kommerziellen ELISA (Enzyme-linked Immunosorbent Assay) für IGFBP1 und durchflusszytometrischem CBA (Cytometric Bead Array).

4.2 Publikation 2: *MIA als potentieller Biomarker für Tumorlast in NF1*

Um die Hypothese einer Fehlsteuerung in der Knorpeldifferenzierung von NF1-Knockoutmäusen zu bestätigen, wurde nach dem Zielprodukt der *SOX9*-Transkription, dem MIA in Serum und Tumorgewebe von NF1-Patienten gesucht. Die Bestimmung der MIA-Level im Serum von NF1-Patienten (n=42) und gesunden Probanden (n=22) erfolgte mit Hilfe eines kommerziell erhältlichen ELISA Tests (Roche Diagnostic, Indianapolis, IN, USA). Die NF1-Patientenanamnese kann der Publikation entnommen werden. Der Nachweis des MIA in humanem Tumorgewebe erfolgte immunhistologisch in sechs kutanen und drei plexiformen Neurofibromen und sieben MPNST. Die MIA-Expressionslevel in Nf1Prx1-Knockout- und Wildtypmäusen erfolgte an aus dem Knie entnommenen Knorpelgewebe mittels quantitativer Echtzeit-PCR (qRT-PCR).

4.3 Publikation 3: *Verminderte Pten Expression in MPNST*

Der Nachweis der Pten Expression in MPNST (n=6) und Neurofibromen (n=9) erfolgte immunhistologisch. Die Auswertung erfolgte entsprechend dem prozentualen Anteil der positiven Zellen: ein Anteil von <5% wurde als negativ, ein Anteil von 6-100% als positiv bewertet. Somatische Mutationen in *PTEN* und *PIK3CA* wurden mittels PCR detektiert.

Mittels Pyrosequenzierung bisulfitbehandelter DNA wurde die Pten-Promotoranalyse bestimmt.

5. Ergebnisse

Im Folgenden werden die Ergebnisse der drei vorliegenden Publikationen zusammengefasst:

5.1 Publikation 1: *Serum Biomarker für Neurofibromatose Typ 1 und frühe Detektion Maligner Nervenscheidentumore*

Ziel der vorliegenden Studie war die Identifizierung eines Biomarkers für NF1 assoziierte Nervenscheidentumore. Nach Datenbankrecherche wurden 115 Proteine als mögliche Kandidaten ausgewählt. 79 dieser Kandidaten wurden bereits beschrieben in PNF oder MPNST exprimiert worden zu sein oder kanzerogene bzw. im Fall von 35 Kandidaten immunmodulatorische Eigenschaften zu haben. Aus diesem Kandidatenpool wurden schlussendlich 56 Kandidaten ausgewählt, deren Nachweis mittels eines Antikörper Array erfolgte.

Insgesamt wurden Seren von 104 NF1-Patienten mit unterschiedlichen Tumorarten und 41 Kontrollprobanden in zwei Durchgängen analysiert. Die Analyse ergab, dass sich die Serumkonzentration von NF1-Patienten und der gesunde Probandenkohorte in vier Proteinen signifikant unterschied: IL-6, IFNγ, EGFR und TNFα. Während die Werte für EGFR in der NF1-Patientengruppe signifikant niedriger war, konnte für die anderen drei inflammatorischen Zytokine ein signifikant höherer Level als in der Kontrollgruppe verzeichnet werden.

Die Gruppierung der NF1-Kohorte bzgl. ihrer Tumorart ergab für IGFBP1 und RANTES signifikant erhöhte Serumlevel in Patienten mit MPNST im Vergleich zu denen, die keine MPNST aufwiesen. Unter Berücksichtigung der volumetrischen Daten (n= 87) korrelierten lediglich die Serumlevel von IGFBP1 mit denen der internen Tumorlast.

Das diagnostische Potential der einzelnen Kandidaten ist durch die Ermittlung der Area under Curve (AUC) der jeweiligen Receiver Operating Characteristic-Kurve (ROC Kurve) ermittelt worden. Die Fläche unter der ROC-Kurve (Grenzwertoptimierungskurve) ist das Maß für die Güte eines Test. Hierbei wurde die Spezifität bei einer festgelegten Sensitivität von 90% ermittelt. Für alle sechs potentiellen Kandidaten war die AUC signifikant. Die größte AUC in mitten der NF1-Gruppe besaß das IFNγ (0.90), gefolgt von TNFa (0.88), IL-6 (0.83) und EGFR (0.73). In der Patientenkohorte mit MPNST war die AUC von IGFBP1 (0.77) größer als von RANTES (0.65).

Ein weiterer Ansatz zur Identifizierung eines NF1 Serummarkers zeigte sich in Publikation 2. Es konnte kürzlich gezeigt werden, dass NF1-Tumore für ihr Wachstum *Sox9* benötigen. *Sox9* ist ebenfalls ein wichtiger Regulator der Knorpelentwicklung – dessen Störung ein bei NF1-Patienten ebenfalls symptomatischer Phänotyp darstellt. Wir spekulierten deshalb, dass einige *Sox9*-abhängige Gene aus dem Knorpel, die in *Nf1Prx1*-Knockout Mäusen dereguliert sind, auch als relevante Biomarker für Tumore bei NF1-Patienten dienen könnten. Einer dieser abberant regulierten Faktoren ist MIA.

5.2 Publikation 2: *MIA als potentieller Biomarker für die Tumorlast in Neurofibromatose Typ 1*

Der immunhistologische Nachweis von MIA in NF1 Tumoren erfolgte an kutanen (n=6), plexiformen Neurofibromen (n=3) und MPNST (n=7). In allen drei Tumorarten konnte MIA nachgewiesen werde, wobei die Dichte MIA-positiver Zellen in den MPNST am größten war. Morphologische Unterschiede der MIA-negativen und MIA-positiven Zellen konnten nicht verzeichnet werden.

Um die MIA-Serumlevel zu bestimmen, wurden NF1-Patienten (n=42) und gesunde Probanden (n=22) untersucht. Es wurde gezeigt, dass die MIA-Serumlevel in der NF1-Kohorte mit 15.16 ± 1.26 pg/ mL signifikant höher war als in der Kontrollgruppe mit 4.54 ± 0.40 pg/mL ($p<0.001$, ungepaarter T-Test mit Welch-Korrektur).

Innerhalb der NF1-Kohorte wiesen Patienten mit nur PNF (n=27) eine signifikant höhere MIA-Serumkonzentration auf, als die Patienten ohne pNF (n=15; $p=0.0329$) während im Vergleich der Patienten mit MPNST und ohne MPNST kein signifikanter Unterschied zu verzeichnen war.

Wurden die Patienten nach ihrer Tumorart gruppiert, konnte festgestellt werden, dass höhere MIA-Level in 9 Patienten mit <100 subkutanen Neurofibromen bzw. 7 Patienten mit >100 kutanen Neurofibromen im Vergleich zu den ohne subkutanen bzw. kutanen Neurofibromen. zu verzeichnen waren.

Zur Beurteilung des Zusammenhangs der internen Tumorlast und des MIA-Serumspiegels konnten 30 der 42 NF1-Patienten aufgrund vorhandener MRT-Daten berücksichtigt werden. Dazu wurden die Patienten in vier Gruppen eingeteilt: a) Patienten mit sehr niedriger interner Tumorlast (0-100 mL, n=16), b) mit niedriger Tumorlast (<350 mL, n=5), c) mit hoher Tumorlast (<1000 mL, n=5) und d) sehr hoher interner Tumorlast (>1000 mL, n=4). Die statistische Varianzanalyse (ANOVA mit Bonferroni Korrektur)

ergab signifikante Unterschiede zwischen den Patienten mit sehr niedriger interner Tumorlast und Patienten mit hoher bzw. sehr hoher interner Tumorlast.
Die Betrachtung des Bestimmtheitsmaßes R^2 zur Identifizierung eines linearen Zusammenhangs zwischen Tumorlast und MIA Serumlevel ergab, dass erhöhte MIA-Serumlevel mit einer erhöhten Tumorbelastung einhergingen.

Die dritte Publikation wendete sich der Erfassung molekularer Veränderungen zu, die die Bildung von MPNST beeinflussen. Der Focus lag insbesondere auf MPNST mit fehlendem *Phosphatase and Tensin homolog* (Pten) und dem Einfluss von *Mammalian Target of Rapamycin Inhibitoren* (mTOR) als mögliches Behandlungskonzept.
Vorangegangene Studien haben gezeigt, dass in vielen MPNST eine erhöhte Aktivität des mTOR Signalweges vorliegt. Eine mögliche Erklärung könnte in der Veränderung des *Pten* zu liegen, das ist ein wichtiger Regulator des mTOR Signalweges ist.

5.3 Publikation 3: *Verminderte Pten-Expression in MPNST*

In dieser Studie wurde die Rolle des Proteins Pten in humanen MPNST und benignen Neurofibromen untersucht. Pten ist ein Schüsselregulator des PI3K/Akt/mTOR-Signalweges in humanen MPNST und benignen Neurofibromen.
Die Immunhistologie zeigte, dass die Pten-Expression in MPNST (n=16) signifikant niedriger war als in Neurofibromen (n=16) oder normalem Nervengewebe.
Um die möglichen Mechanismen für die Pten-Herunterregulierung bzw. die mTOR/Akt-Aktivierung in MPNST aufzuklären, wurden weitere Experimente durchgeführt:
die Mutationsnanalyse ergab, dass keine somatischen Mutationen in *PTEN* (n=31) und *PIK3CA* (n=38) vorlagen. Allerdings konnten häufiger *PTEN*-Promotor Methylierungen in primären MPNST (11/26) und MPNST-Zelllinien als in gutartigen Nervenscheidentumoren festgestellt werden. Darüber hinaus wurde ein umgekehrter Zusammenhang zwischen Pten regulierendem MiR-21 und Pten-Proteinlevel in MPNST-Zelllinien beobachtet. Die Untersuchung von *NF1-/-* und *NF+/+* Schwann Zellen und Fibroblasten zeigte, dass die Pten-Expression nicht von NF1 reguliert wird. Um die Bedeutung des Pten-Expressionslevels für die Behandlung mit Rapamycin zu bestimmen, wurden MPNST-Zelllinien (n=5) mit dem mTOR-Inhibitor behandelt. Alle Zelllinien waren ohne signifikante Korrelation zum Pten-Level sensitiv auf Rapamycin. Mit der Kombination des mTOR- Inhibitors Rapamycin und dem Statin Simvastatin konnte ein verstärkter antiproloferativer Effekt beobachtet werden.

6. Diskussion

Derzeit ist die Therapierbarkeit von PNF und MPNST schwierig. Kurativ ist lediglich eine komplette chirurgische Resektion, die aufgrund des meist invasiven Wachstums nicht möglich ist. Die Chemo- oder Radiotherapie führt ebenfalls zu unbefriedigenden Resultaten, da die Exposition mit therapeutischer Bestrahlung ein wesentlicher Risikofaktor für die Entstehung weiterer MPNST ist (7, 13, 14).

Aufgrund der hohen Wahrscheinlichkeit, mit der NF1-Patienten MPNST entwickeln, war das Ziel dieser drei Arbeiten eine frühe Erkennung der Hochrisikogruppe und hierfür einen Serummarker zu identifizieren. Dieser Serummarker sollte eine Abschätzung der Tumorlast zulassen und/ oder prognostische Eigenschaften einer MPNST Entwicklung innehaben, da die interne Tumorlast ein nachgewiesener Risikofaktor für die Entwicklung von MPNST ist.

Im Feld der NF1-Forschung umfasst die in Publikation 1 beschriebene Studie die bisher größte Patientenkohorte zur Identifizierung eines potentiellen Biomarkers.

Die Auswahl der Kandidaten beruhte auf Anhaltspunkten, dass systemische *NF1 +/-* Haploinsuffizienz in NF1-Patienten zu einer Überexpression von Zytokinen führt (15, 16). Daraus folgerten wir, dass der Grad der immunologischen Deregulierung indirekt mit einem erhöhten Risiko für Tumorwachstum und maligner Transformation einhergeht. Unsere Daten lassen ein systemisches proinflammatorisches Profil in NF1-Patienten erkennen, das die o.g. Beobachtung untermauert. Es wurden insgesamt vier mögliche Kandidaten ermittelt, die als diagnostischer Marker für NF1 dienen könnten: IL-6, IFNγ, EGFR, TNFα. Allerdings konnte kein Zusammenhang zwischen der Tumorlast und den proinflammtorischen Zytokinen hergestellt werden wie oftmals in anderen Tumoren beschrieben (17). Die Ursache der erhöhten Zytokinexpression in der NF1-Patientenkohorte gegenüber der Kontrollgruppe lässt sich nicht genau ergründen und kann durch eine erhöhte Mastzellpopulation oder Monozytenaktivität oder generelle Veränderung des Immunstatusses in NF1-Patienten begründet sein (16, 18).

Die Gründe für die verminderten EGFR Serumlevel in NF1-Patienten sind unklar. In NF1-Tiermodellen wurde gezeigt, dass der EGF- Signalweg die Tumorgenese fördert und insbesondere NF1-Schwann Zellen und Fibroblasten empfindlich auf EGF reagieren (19). Deshalb scheint die Bewahrung des EGF-Rezeptors auf der Zelloberfläche in NF1-Patienten das zirkulierende EGFR zu reduzieren und eine Tumorgenese fördernde

Umgebung zu unterstützen. Der konservierte EGF-Rezeptor könnte eine ständige Bereitschaft für die EGF-Signalkaskade bedeuten und somit möglicherweise die Überlebensrate von Zellen erhöhen (20).
Darüber hinaus identifizierten wir zwei Kandidaten, die mit der Gegenwart von malignen peripheren Nervenscheidentumoren einhergehen: IGFBP1, RANTES.
RANTES ist ein inflammatorisches Chemokin, das die chemotaktische Aktivität in T-Zellen und Monozyten vermittelt (21). RANTES wurde bereits im Zusammenhang mit Brustkrebs beschrieben und scheint eine Rolle in der Tumorentwicklung inne zu haben (22).
Besonders zu unterstreichen ist die Beobachtung des IGFBP1, das mit der internen Tumorlast zu korrelieren scheint und somit ein Indikator für eine maligne Transformation vorhandener Tumore sein könnte. Eine Beobachtung, die durch eine vorhergehende MRT-Studie unterstützt wird, die basierend auf volumetrischen Daten, einen Zusammenhang zwischen interner Tumorlast und dem Risiko einer malignen Umwandlung von PNF in MPNST darstellt (23). IGFPB1 reguliert über proteolytische Spaltung die Verfügbarkeit der *Insulin-Like Growth Factors-I* (IGF-I) und IGF-II. Die Regulierung erfolgt über verschiedene Hormone wie Insulin, Glucagon und Cortisol (24, 25).
Ein reziproker Zusammenhang zwischen IGFBP1-Level und Karzinogenese wurde kürzlich gezeigt (26, 27). Die Expression von IGF-1, Wachsumshormonrezeptoren in NF1-Patienten mit PNF und MPNST und die Korrelation zwischen IGF-I-Rezeptorlevel und erhöhtem Mitoseindex von PNF deuten auf eine Abhängigkeit der Tumore auf IGFBP1-regulierende Faktoren hin (28, 29). IGFBP1 könnte den Zugang zu IGF in PNF und MPNST modulieren, allerdings muss dieser Mechanismus weiter untersucht werden. Die erhöhten IGFBP1- und RANTES-Level in NF1-Patienten scheinen das Ergebnis erhöhter Sekretion von den Tumorzellen selbst und/ oder der Immunzellen in Erwiderung auf den neoplastischen Prozess zu sein.

MIA scheint ein weiterer möglicher Biomarker zur Beurteilung der Tumorlast in NF1-Patienten zu sein und war im Fokus der zweiten Publikation. NF1-Tumore scheinen für ihr Wachstum den Hauptregulator für die Knorpeldifferenzierung *SOX9* zu benötigen (30). Es wurde die Hypothese aufgestellt, dass einige der knorpelspezifischen Gene in einem *Nf1Prx1*-Mausmodell fehlgesteuert sind. Diese Hypothese wurde überprüft indem gezielt nach dem Produkt der *SOX9* Transkription in Knorpelgewebe *Nf1*-defizienter Mäuse und Serum bzw. Tumorgewebe von NF1-Patienten gesucht wurde. Vorange-

gangene Studien beobachteten, dass eine erhöhte *Mia* Expression in *Nf1* defizientem Knorpelgewebe von *Nf1prx1*-Mäusen mit der bereits gezeigten erhöhten *SOX9*-Exprimierung in Zellkernen dieser Mutanten einhergeht (31). In *in vitro* kultivierten Chondrozyten wurde nachgewiesen, dass der *MIA* Promotor über *SOX9* reguliert wird (32), deshalb lag es nahe, dass die *MIA* Expression in NF1 Tumoren ebenfalls durch *SOX9* geregelt wird, zumal diesem Transkriptionsfaktor bereits ein wesentliche Bedeutung für das Überleben von MPNST-Zellen bemessen worden ist (33). Unter anderem gehen diese Ergebnisse mit der vorhergehenden Beobachtung einer erhöhten MIA Expression in Gliomen einher (34). Die Beobachtung, dass die MIA Serumlevel in NF1-Patienten mit PNF oder einer sehr großen Anzahl an Neurofibromen erhöht ist, scheint im Zusammenhang mit der internen Tumorlast zu stehen. Durch eine Validierung an einer größeren NF1-Patientenkohorte könnte MIA ein weiterer wertvoller Biomarker für die Tumorlast in NF1-Patienten darstellen.

Mögliche molekulare Veränderungen, die die Bildung von MPNST beeinflussen standen in der dritten Publikation im Mittelpunkt. Das Ziel war die Bewertung der Veränderung des Tumorsuppressorgens Pten in MPNST und Neurofibromen und die mögliche Konsequenz der Behandlung mit mTOR Inhibitoren.

Es wurde gezeigt, dass die Pten-Expression in MPNST signifikant niedriger ist als in Neurofibromen, was darauf hinweisen kann, dass die Herrunterregulierung bei maligner Entartung mitwirkt. Da Pten eine Schlüsselrolle des Akt/mTOR Signalweges innehat, könnte der Pten-Zustand den therapeutischen Erfolg von mTOR-inhibierenden Medikamenten beeinflussen. Dass MPNST-Zellinien *in vivo* und *in vitro* sensitiv auf mTOR-Inhibitoren ansprechen, wurde erst kürzlich gezeigt (35, 36, 37).

Weiterhin wurde gezeigt, dass die PTEN-Methylierung in etwa 50% der Fälle ausschließlich in MPNST, aber nicht in cNF oder PNF vorkommt. Daher wurde vermutet, dass die PTEN-Methylierung ebenfalls mit maligner Entartung einhergeht und diese frühzeitig anzeigen könnte. Dazu könnte der Nachweis zirkulierender Tumorzellen, die eine PTEN-Methylierung aufweisen, ebenfalls als prognostischer Biomarker genutzt werden.

Zusammenfassend lässt sich sagen, dass mehrere potentielle Biomarker für die Tumorlast, sogar für die maligne Transformation in NF1-Patienten identifiziert werden konnten. Das Set an Serummarkern aus der Publikation 1 wurde von der IPAL GmbH auf Patent- und Marktfähigkeit geprüft und seit Oktober 2012 zum Patent angemeldet.

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8. Eidesstattliche Versicherung

„Ich, Su-Jin, Park, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Identification and Characterization of the Secretome in Neurofibromatosis Type 1 Tumors“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.icmje.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o.) und werden von mir verantwortet.

Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit der Betreuerin, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o.) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum ______________________________

Unterschrift

9. Anteilserklärung

Su-Jin Park hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Impact factor 2013: 6,679

Park SJ, Sawitzki B, Kluwe L, Mautner VF, Holtkamp N, Kurtz A, *Serum biomarkers for neurofibromatosis type 1 and early detection of malignant peripheral nerve-sheath tumors*, BMC Med 11:109, 2013
Anteil 85 Prozent, Beitrag im Einzelnen: Durchführung aller experimentellen Versuche: Arrays und Immunologische Assays, Auswertung und Analyse der Daten, Anfertigung des Manuskriptes.

Publikation 2: Impact factor 2011: 6,035

Kolanczyk M, Mautner VF, Kossler N, Nguyen R, Kühnisch J, Zemojtel T, Jamsheer A, Wegener E, Thurisch B, Tinschert S, Holtkamp N, Park SJ, Birch P, Kendler D, Harder A, Mundlos S, Kluwe L, *MIA is a potential biomarker for tumour load in neurofibromatosis type 1*, BMC Med 4;9:82, 2011
Anteil 5 Prozent, Beitrag im Einzelnen: Acquirierung und Archivierung von NF1-Patientenseren

Publikation 3: Impact factor 2012: 4,092

Bradtmöller M, Hartmann C, Zietsch J, Jäschke S, Mautner VF, Kurtz A, Park SJ, Baier M, Harder A, Reuss D, von Deimling A, Heppner FL, Holtkamp N, *Impaired Pten expression in human malignant peripheral nerve sheath tumours*, PLoS One 7(11):e47595, 2012
Anteil 10 Prozent, Beitrag im Einzelnen: Durchführung einiger Experimente, Zellproliferationsassays

Unterschrift, Datum und Stempel der betreuenden Hochschullehrerin

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Unterschrift des Doktoranden/der Doktorandin

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10. Druckexemplare der ausgewählten Publikationen

Publikation 1:

Park SJ, Sawitzki B, Kluwe L, Mautner VF, Holtkamp N, Kurtz A, *Serum biomarkers for neurofibromatosis type 1 and early detection of malignant peripheral nerve-sheath tumors*, BMC Med 11:109, 2013

Publikation 2:

Kolanczyk M, Mautner VF, Kossler N, Nguyen R, Kühnisch J, Zemojtel T, Jamsheer A, Wegener E, Thurisch B, Tinschert S, Holtkamp N, Park SJ, Birch P, Kendler D, Harder A, Mundlos S, Kluwe L, *MIA is a potential biomarker for tumour load in neurofibromatosis type 1*, BMC Med 4;9:82, 2011

Publikation 3:

Bradtmöller M, Hartmann C, Zietsch J, Jäschke S, Mautner VF, Kurtz A, Park SJ, Baier M, Harder A, Reuss D, von Deimling A, Heppner FL, Holtkamp N, *Impaired Pten expression in human malignant peripheral nerve sheath tumours*, PLoS One 7(11):e47595, 2012

Park et al. BMC Medicine 2013, 11:109
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BMC Medicine

RESEARCH ARTICLE Open Access

Serum biomarkers for neurofibromatosis type 1 and early detection of malignant peripheral nerve-sheath tumors

Su-Jin Park[1], Birgit Sawitzki[2], Lan Kluwe[3], Victor F Mautner[3†], Nikola Holtkamp[1†] and Andreas Kurtz[1,4*†]

Abstract

Background: Neurofibromatosis type 1 (NF1) is a hereditary tumor syndrome characterized by the development of benign nerve-sheath tumors, which transform to malignant peripheral nerve-sheath tumors (MPNST) in about 8 to 13% of patients with NF1. MPNST are invasive sarcomas with extremely poor prognosis, and their development may correlate with internal tumor load of patients with NF1. Because early identification of patients with NF1 at risk for developing MPNST should improve their clinical outcome, the aim of this study was to identify serum biomarkers for tumor progression in NF1, and to analyze their correlation with tumor type and internal tumor load.

Methods: We selected candidate biomarkers for NF1 by manually mining published data sources, and conducted a systematic screen of 56 candidate serum biomarkers using customized antibody arrays. Serum from 104 patients with NF1 with and without MPNST, and from 41 healthy control subjects, was analyzed. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test, followed by Bonferroni correction.

Results: Our analysis identified four markers (epidermal growth factor receptor, interferon-γ, interleukin-6, and tumor necrosis factor-α) for which significantly different serum concentrations were seen in patients with NF1 compared with healthy controls. Two markers (insulin-like growth factor binding protein 1 (IGFBP1) and regulated upon activation, normal T-cell expressed and secreted (RANTES)) showed significantly higher concentrations in patients with NF1 and MPNST compared with patients with NF1 without MPNST. A correlation with internal tumor load was found for IGFBP1.

Conclusion: Our study identified two serum markers with potential for early detection of patients with NF1 at risk for developing MPNST, and four markers that could distinguish between patients with NF1 and healthy subjects. Such markers may be useful as diagnostic tools to support the diagnosis of NF1 and for timely identification of MPNST. Moreover, the data suggest that there is a systemic increase in inflammatory cytokines independently of tumor load in patients with NF1.

Keywords: Neurofibromatosis type 1, Serum biomarker, Antibody array, Cytokines, Malignant peripheral nerve-sheath tumor

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Background

Neurofibromatosis type 1 (NF1) is an autosomal dominant tumor syndrome, with an estimated incidence at birth of 1 in 2500 [1] and complete penetrance. NF1 is caused by mutations in the *NF1* gene [2,3], coding for the tumor-suppressor protein neurofibromin, which acts as a Ras-negative regulator via its Ras-GTPase activating protein (GAP) domain. Monoallelic and biallelic loss of *NF1* leads to increased Ras activity in affected cells.

Among the defining features of NF1 is the development of benign peripheral nerve-sheath tumors, which can arise at virtually any site in the body. Whereas cutaneous neurofibromas (CNF) are mostly visible and palpable, subcutaneous neurofibromas, internal plexiform neurofibromas (PNF) and malignant peripheral nerve-sheath tumors (MPNST) are difficult to detect, quantify, or monitor [4].

MPNST are the major cause for the reduced life span of patients with NF1, and they will lead to death if not detected early and treated in time. The primary forms of treatment are selective resection of benign PNF, and radical surgical resection of MPNST [5-8]. However, the invasive growth pattern of MPNST frequently prohibits complete tumor removal, especially when diagnosed late in their development. Moreover, although chemotherapy and radiotherapy may delay recurrence, they have little effect on long-term survival [7,9].

The lifetime risk of MPNST for patients with NF1 patients has been estimated to be about 8 to 13% and thus is more than 1000 times higher for these patients than for the general population. Moreover, many patients with NF1 develop MPNST at the unusually young age of around 30 years [10,11], compared with the median age of diagnosis of 62 years in the general population [12]. Because MPNST develop by malignant progression of pre-existing PNF, the risk to develop an MPNST increases to almost 50% in patients with NF1 and PNF [12,13].

It is possible to detect dermal and superficial neurofibromas directly by optical or ultrasonography methods [14], whereas PNF and MPNST are often diagnosed only after clinical symptoms occur. Systematic analysis of the internal tumor load of patients with NF1 by whole-body magnetic resonance imaging (MRI) suggests an association between the risk for MPNST development and internal PNF tumor load [15]. However, these imaging techniques are not applicable as a routine screening tool. The search for surrogate biomarkers for timely identification of patients at risk for malignant transformation has mostly been based on the assumption that overexpression of proteins in PNF and MPNST subsequently leads to increased systemic concentrations [16-19]. Among such factors, serum levels for midkine and for stem cell factor were found to be significantly increased in a cohort of 39 patients with NF1, although no correlation with tumor load or MPNST was found [20]. Recently, we identified melanoma-inhibitory activity (MIA; also known as cartilage-derived retinoic acid-sensitive protein (CD-RAP)) as a marker for the internal tumor load in a cohort of 42 patients with NF1 [21]. MIA was shown previously to be a biomarker for malignant neuroectotermal tumors [22]. In another study, 92 genes encoding putative secreted proteins in neurofibromas and MPNST were analyzed for their potential as serum markers [23]. Of these, only adrenomedullin (ADM) was confirmed as differentially expressed and increased in the serum of patients with NF1, and serum concentrations were found to be even higher in a small sample of patients with MPNST (n = 5).

Tumorigenesis in NF1 is strongly influenced by the haploinsufficient *NF1+/−* systemic environment, which may also promote invasion of PNF and MPNST by *NF1+/−* monocytes and mast cells [24-30]. Therefore, we included immunomodulating cytokines in the present screen for serum biomarkers, in addition to factors secreted by tumor cells in PNF and MPNST. Of the 56 candidate proteins analyzed, we identified four proteins with significantly altered serum concentrations in patients with NF1 compared with non-NF1 control subjects, but independently of tumor load. Two proteins were significantly increased in patients with MPNST, and one of these also correlated with internal tumor load.

Methods

Ethics approval

The study was approved by the internal review board (Ethics Committee of the Ärztekammer Hamburg number OB-089/04) in compliance with the Declaration of Helsinki, and informed consent was obtained before sample collection.

Patients and serum collection

Serum samples from patients with NF1 were obtained from the Department of Maxillofacial Surgery (University Hospital Eppendorf, Hamburg, Germany). All patients with NF1 were clinically diagnosed according to published guidelines and criteria [31]. Serum samples from healthy control subjects were obtained from the Institute of Medical Immunology (Charité - Universitätsmedizin Berlin) from anonymized leftover diagnostic samples. For detailed information on the patient cohorts, see Additional file 1. Venous blood (1 to 10 ml) was collected, then separated by centrifugation within 2 hours of collection, and serum samples were immediately frozen in aliquots and stored at −80°C until use. Fresh aliquots were used for each analysis.

Candidate marker selection

Selection of candidate markers was based on a manual literature search of publications and publicly available

databases describing 1) protein levels in serum, plasma, or cell supernatants from patients with nervous system or epithelial tumors or from cell lines, or 2) differential gene expression between the normal peripheral nervous system, neurofibroma, and MPNST. and 3) immunomodulatory cytokines (see Additional file 2). The list of identified candidate factors was further reduced by selecting factors with known functional roles in tumorigenesis such as growth promotion, migration and metastasis, angiogenesis, and immune modulation, based on information from the Gene Ontology and GeneCards databases [32,33]. The final selection of candidate factors was based on the availability of suitable screening platforms. Of the 115 initially identified potential serum proteins, a list of 56 candidate factors was compiled for screening of serum samples based on the availability of antibodies for customized array analysis (Figure 1, see Additional file 2).

Serum screening

Customized human cytokine arrays (Quantibody; RayBiotech Inc., GA, USA) were used to determine serum protein concentrations. Analyses were performed in accordance with the manufacturer's instructions. Imaging was performed using the accompanying software (Quantibody Array Testing Software; RayBiotech Inc.). Potential marker proteins were initially identified by screening of 30 candidate proteins using 60 NF1 sera (n = 27, n = 13, and n = 20, respectively, for patients with NF1 with PNF, with MPNST, and without tumors) and 20 control sera. Secondary screening was performed on the five proteins that showed significant differences in the pre-screening round (platelet-derived growth factor (PDGF)-BB, insulin-like growth factor binding protein (IGFBP)1, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-4, TNF-related apoptotic ligand (TRAIL)-R2), together with another set

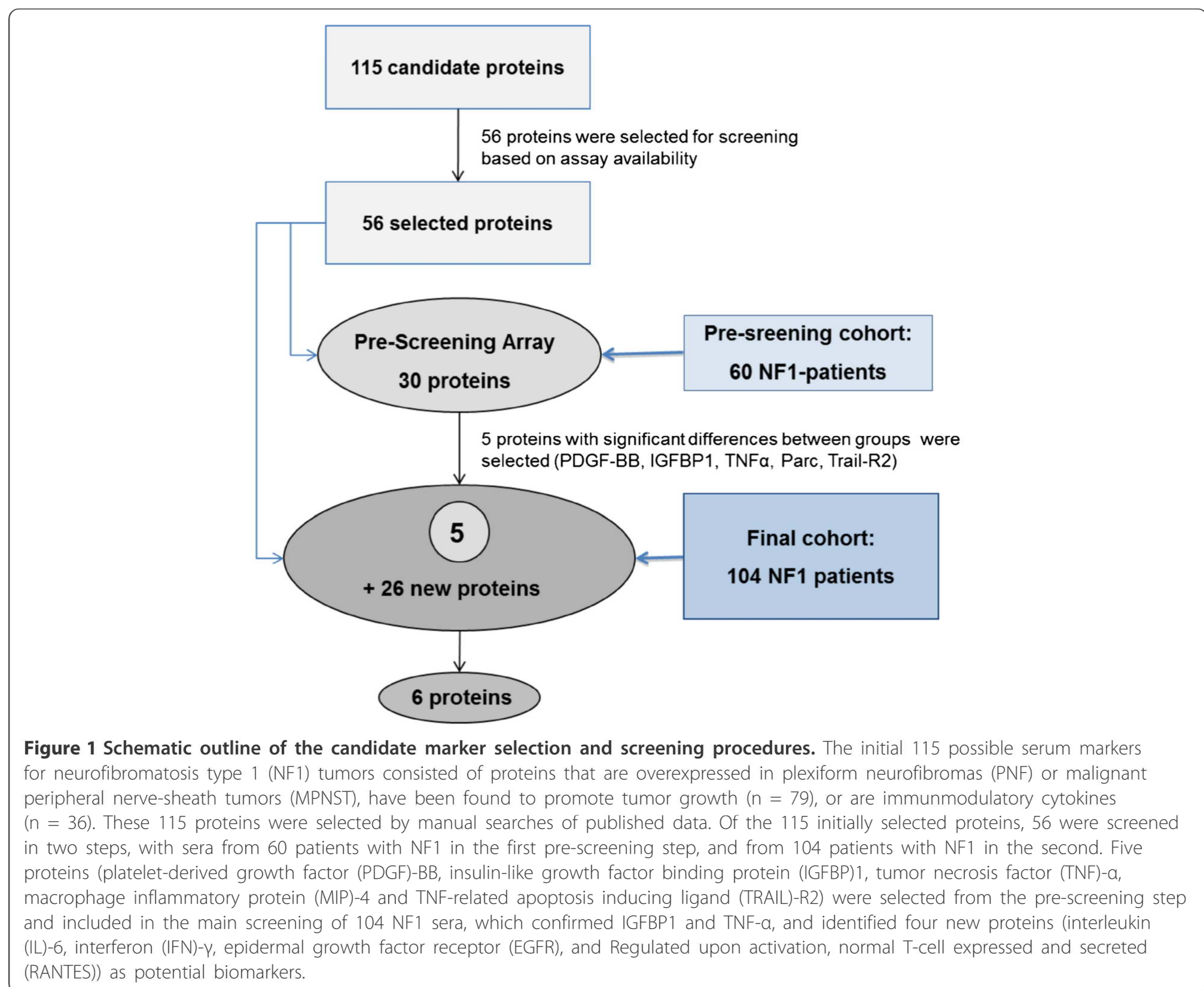

Figure 1 Schematic outline of the candidate marker selection and screening procedures. The initial 115 possible serum markers for neurofibromatosis type 1 (NF1) tumors consisted of proteins that are overexpressed in plexiform neurofibromas (PNF) or malignant peripheral nerve-sheath tumors (MPNST), have been found to promote tumor growth (n = 79), or are immunmodulatory cytokines (n = 36). These 115 proteins were selected by manual searches of published data. Of the 115 initially selected proteins, 56 were screened in two steps, with sera from 60 patients with NF1 in the first pre-screening step, and from 104 patients with NF1 in the second. Five proteins (platelet-derived growth factor (PDGF)-BB, insulin-like growth factor binding protein (IGFBP)1, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-4 and TNF-related apoptosis inducing ligand (TRAIL)-R2) were selected from the pre-screening step and included in the main screening of 104 NF1 sera, which confirmed IGFBP1 and TNF-α, and identified four new proteins (interleukin (IL)-6, interferon (IFN)-γ, epidermal growth factor receptor (EGFR), and Regulated upon activation, normal T-cell expressed and secreted (RANTES)) as potential biomarkers.

Table 1 Characteristics of patient cohorts recruited for the study

	Controls	NF1 patients w/o PNF w/o MPNST	NF1 patients with PNF w/o MPNST	NF1 patients with MPNST
n	41	35	39	30
mean age in years	47 (range 24–66)	32 (range 14–48)	34 (range 15–63)	34 (range 16–62)
♀/♂	22/19	22/13	17/22	17/13
whole body MRI		25/35	33/39	30/30

of 26 candidate proteins (see Additional file 2). In the second round, 104 NF1 sera and 41 control sera were screened. Altogether, 56 candidate proteins were screened, and 104 NF1 and 41 control sera were used. The candidate proteins were simultaneously scanned by multiplex detection in quadruplicate spots per array. Hence, all sera were analyzed in at least quadruplicates. A flowchart of the screening procedure is provided (Figure 1). Serum factors with significantly different levels between groups (with the exception of epidermal growth factor receptor (EGFR)) were verified in a limited subset of NF1 (n ≥ 11) and control (n ≥ 5) serum samples using ELISA for IGFBP1 (Abcam, Cambridge, UK), and cytometric bead array (CBA) (BD Bioscience, Heidelberg, Germany) for RANTES (regulated upon activation, normal T-cell expressed and secreted), interferon (IFN)-γ, interleukin (IL)-6 and TNF-α. The analyses were performed in accordance with the manufacturers' instructions. Capture beads were analyzed on a flow cytometer (FACSCalibur, BD Biosciences, Heidelberg, Germany), and flow-cytometry data were evaluated with FCAP Array analysis software (Soft Flow Inc., MN, USA) (see Additional file 3).

Statistical analysis

Serum levels of the candidate markers in the NF1 patient group and control group were analyzed with respect to median levels and interquartile ranges. To verify all data for normal distribution, the Kolmogorov-Smirnov test was used. Stratified patient groups were compared using the Mann–Whitney *U*-test for continuous non-parametric variables. For assessing the discriminatory power of individual markers, the receiver operating characteristic (ROC) curve and area under the curve (AUC) were calculated. For significance testing, the non-parametric Mann–Whitney *U*-test followed by Bonferroni correction was used. Two-tailed tests were used for all analyses. $P<0.05$ was considered significant. Statistical analysis

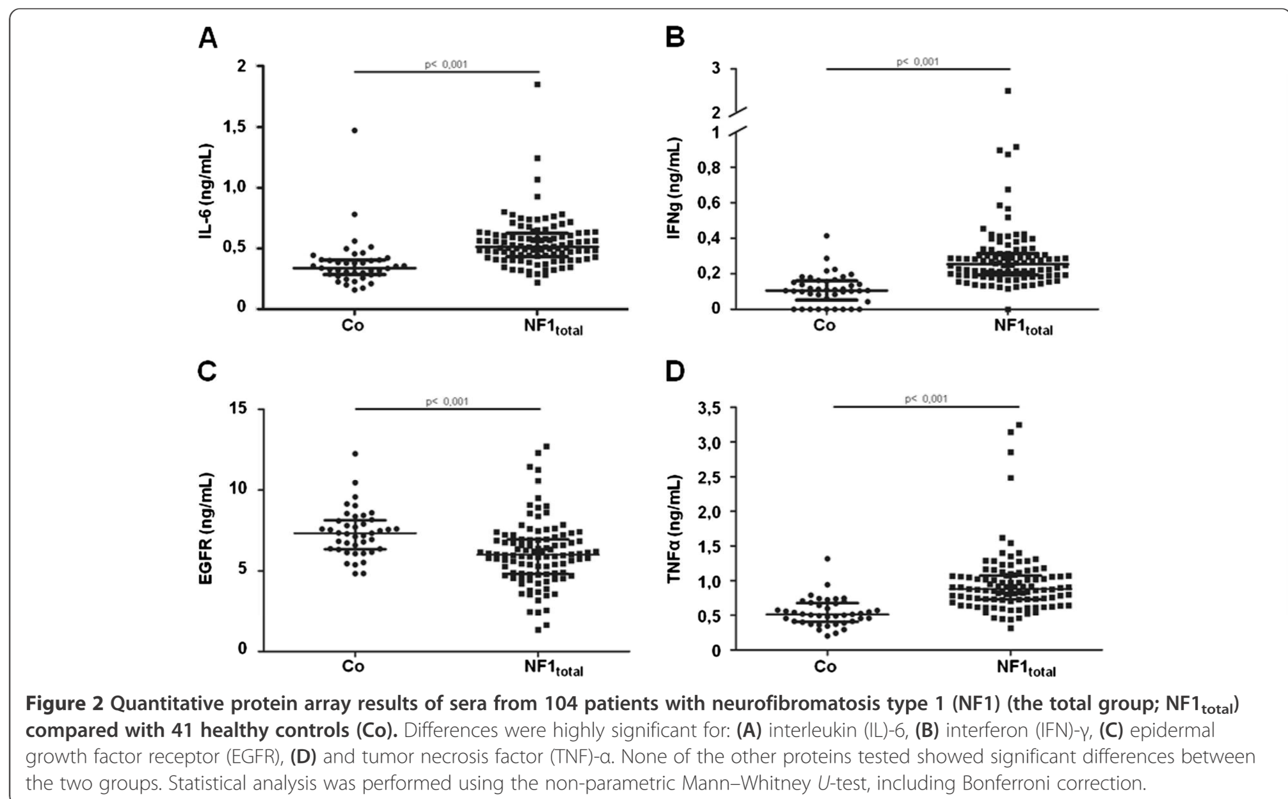

Figure 2 Quantitative protein array results of sera from 104 patients with neurofibromatosis type 1 (NF1) (the total group; $NF1_{total}$) compared with 41 healthy controls (Co). Differences were highly significant for: **(A)** interleukin (IL)-6, **(B)** interferon (IFN)-γ, **(C)** epidermal growth factor receptor (EGFR), **(D)** and tumor necrosis factor (TNF)-α. None of the other proteins tested showed significant differences between the two groups. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test, including Bonferroni correction.

was performed using SPSS version 18 software (SPSS, Inc., IL, USA) and GraphPad Prism software (version 5.0 GraphPad Software Inc., CA, USA).

Results and discussion

In the present study, we used antibody arrays to identify serum biomarkers for NF1 in general and for NF1-associated nerve-sheath tumors in particular. Manual data mining identified 115 proteins as potential serum markers for NF1. Of these 115 proteins, 79 are expressed in PNF or MPNST, or have been described as tumorigenic serum factors. The other 36 proteins are immunomodulatory cytokines. These proteins were selected because of evidence that systemic *NF1* haploinsufficiency in patients with NF1 may result in overexpression of cytokines [34,35] (see Additional file 2). We reasoned that the degree of immunological deregulation may indirectly signal increased risk for tumor growth and malignant transformation. The sera of 104 patients with NF1 with different tumor types, and 41 matched control subjects (Table 1; see Additional file 1) were analyzed, and 56 of the 115 initially identified candidate proteins were screened (see Additional file 2). Pre-screening was carried out with 60 sera (comparing controls, NF1 without PNF or MPNST, NF1 with PNF, and NF1 with MPNST), using an array of 30 proteins (see Additional file 1), and this identified 5 proteins with significantly increased levels in serum of patients with NF1. When testing for these 5 proteins was performed in the complete cohort of 104 patients, only 2 proteins (IGFBP1 and TNF-α) were confirmed to be significantly different in NF1 sera. We also screened for another 26 proteins in the complete cohort and found significant differences for 6 proteins (Figure 1).

Serum concentrations of all six candidate markers were independent of age and sex in the tested population (mean age was 46 and 32 years for the healthy controls and the NF1 group, respectively). This is important, as circulating levels of the inflammatory cytokines TNF-α and IL-6 may increase with age [36,37].

Significant differences in serum concentration were found between patients with NF1 and healthy subjects for four proteins (Figure 2). The serum concentration of EGFR was significantly lower and the serum

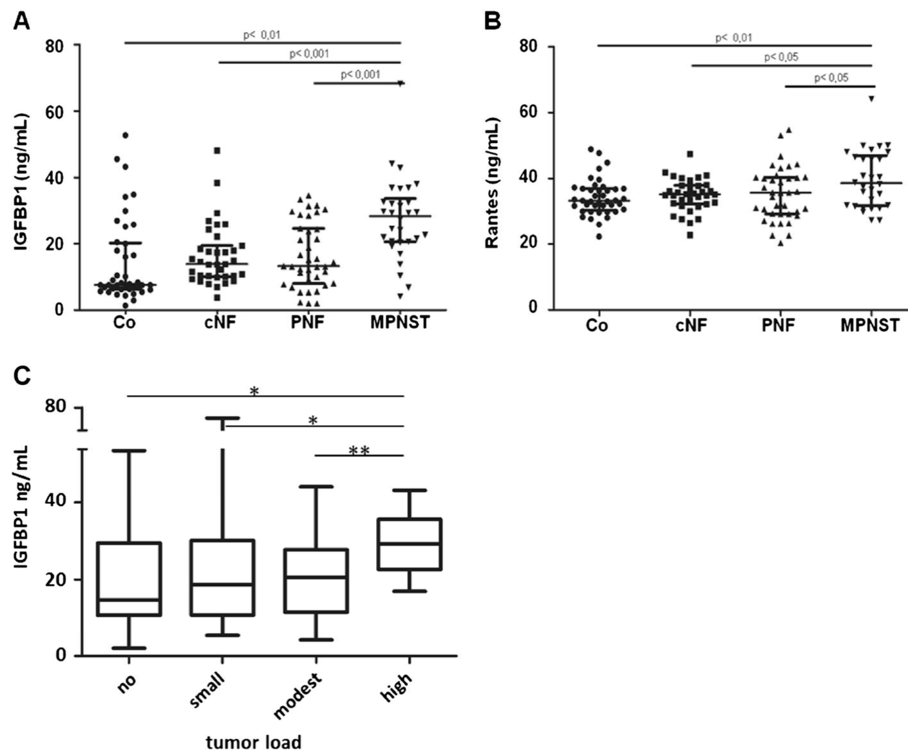

Figure 3 Quantitative protein array results of sera from 41 healthy controls and 104 patients with neurofibromatosis type 1 (NF1) subdivided into three groups. These comprised 35 patients with NF1 with no plexiform neurofibromas (PNF) and no malignant peripheral nerve-sheath tumors (MPNST) (cutaneous neurofibromas; cNF), 39 patients with NF1 with PNF and no MPNST, and 30 patients with NF1 with MPNST. **(A)** Insulin-like growth factor binding protein (IGFBP)1 and **(B)** Regulated upon activation, normal T-cell expressed and secreted (RANTES). **(C)** IGFBP1 serum concentrations in patients with NF1 with different internal tumor loads as measured by MRI-based volumetry (0 cm^3 = no load, 1 to 99 cm^3 = low load; 100 to 500 cm^3 = medium load; >500 cm^3 = high load). Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test, including Bonferroni correction (**P*≤0.05; ***P*≤0.01; ****P*≤0.001).

Table 2 Overview on serum marker features at 90% sensitivity

	NF1 marker				MPNST marker	
	IFN-γ	EGFR	IL-6	TNF-α	IGFBP1	RANTES
Sensitivity:	90,4	90,4	90,4	90,4	90,0	90,0
Specificity:	70,7	14,6	51,2	68,3	50,0	25,7
NPV	88,0	60,3	84,2	87,7	79,3	72,0
PPV	75,5	51,4	64,9	74,0	65,9	58,1
cut off (ng/ml):	0,15	8,57	0,34	0,59	13,77	30,72

The prevalence for NF1 markers was set at 0.5, while the prevalence for MPNST markers was set at 10%. The risk of NF1 patients to develop an MPNST is 8-13%.

concentrations of the inflammatory cytokines IFN-γ, TNF-α and IL-6 were significantly higher in patients with NF1 compared with healthy subjects.

Further stratification of the NF1 cohort into three clinical groups (patients with NF1 with 1) CNF only, 2) with PNF and 3) with MPNST) (Table 1) identified two more proteins, IGFBP1 and RANTES, for which there were significant differences between patients with NF1 with MPNST and those without MPNST. Of note, no difference was detected between the control group and patients with NF1 without MPNST (n = 74) for these two proteins (Figure 3A,B).

A previous study using volumetric analysis of whole-body MRI data for patients with NF1 indicated a correlation between internal tumor load and risk for malignant transformation of PNF into MPNST [4]. Therefore, we attempted to correlate the serum concentration of the six identified serum biomarkers with internal tumor load for the 87 patients with NF1 for which these data were available (see Additional file 1). Importantly, the serum concentrations of IGFBP1, but not of any of the other five markers, correlated with internal tumor load (Figure 3C).

This finding is in line with the correlation between IGFBP1 serum levels and presence of MPNST (Figure 3A), and further identifies IGFBP1 as a potential risk marker for malignant transformation. The data also suggest that increased cytokine levels in patients with NF1 are independent of tumor load. Rather, these results imply that systemic NF1 haploinsufficiency triggers a permanent and systemic inflammatory status in patients with NF1, which is reflected by a significant increase in IFN-γ, TNF-α and IL-6 [34].

Protein array data were confirmed in a small subgroup by CBA and ELISA (see Additional file 3) for IFN-γ, TNF-α, IL-6, IGFBP1 and RANTES. We did not reassess the level of EGFR because of its comparably lower AUC.

The diagnostic potential of the factors we identified was determined by computing the AUC of the individual ROC curves. Specificity was determined at a sensitivity of 90% (Table 2; Figure 4). For all six candidates the

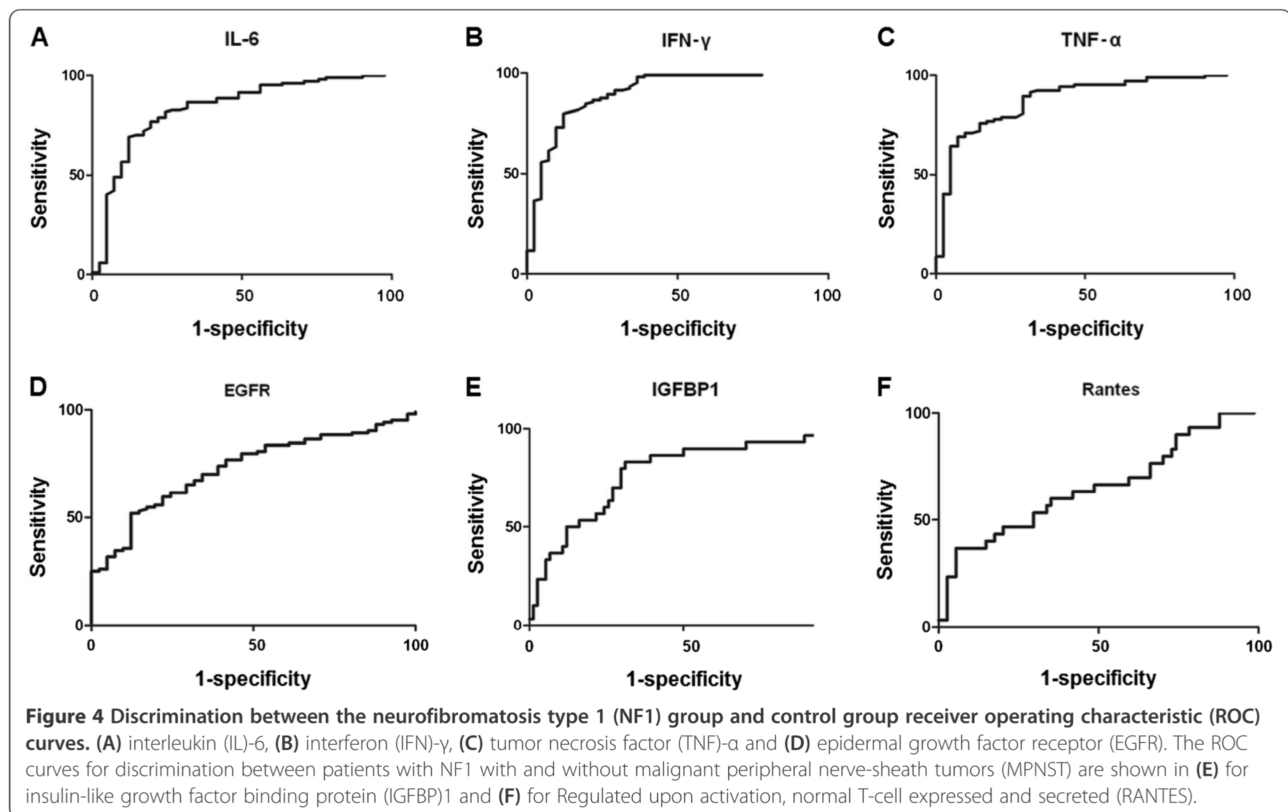

Figure 4 Discrimination between the neurofibromatosis type 1 (NF1) group and control group receiver operating characteristic (ROC) curves. **(A)** interleukin (IL)-6, **(B)** interferon (IFN)-γ, **(C)** tumor necrosis factor (TNF)-α and **(D)** epidermal growth factor receptor (EGFR). The ROC curves for discrimination between patients with NF1 with and without malignant peripheral nerve-sheath tumors (MPNST) are shown in **(E)** for insulin-like growth factor binding protein (IGFBP)1 and **(F)** for Regulated upon activation, normal T-cell expressed and secreted (RANTES).

AUC was significant (P<0.05). The largest AUC for the NF1 markers was found for IFN-γ (0.90), followed by TNF-α (0.88), IL-6 (0.83) and EGFR (0.73). The increased levels of pro-inflammatory cytokines did not depend on tumor load, as often found for patients with other tumors [38]. Rather, our data showed an increased systemic pro-inflammatory state in patients with NF1 compared with non-NF1 controls, supporting our assumption that increased cytokine levels in NF1 are caused by the NF1+/− environment. Whether this is due to an increase in mast cells and monocyte activity, or to other generalized changes in the immune status of these patients, remains unclear [35,39].

In patients with MPNST, the AUC of IGFBP1 (0.77) was larger than that of RANTES (0.65) (Figure 4). RANTES is an inflammatory chemokine known to mediate chemotactic activity in immune cells such as T cells and monocytes [40]. RANTES was also shown to be expressed by breast carcinomas [41], and correlated with a more advanced stage of disease, suggesting a role for cancer progression. Increased serum levels of RANTES and IGFBP1 may be the result of increased secretion by the tumor cells themselves, or by immune cells in response to the neoplastic process, or by both mechanisms.

IGFBP1 binds IGF-I and IGF-II, and prolongs their half-life. Plasma levels of IGFBP1 are regulated by hormones outside of the growth-hormone axis, including insulin, glucagon, and cortisol [42,43]. An inverse correlation has previously been indicated between IGFBP1 levels and carcinogenesis [44,45]. The expression of IGF-I and growth-hormone receptors in PNF and MPNST in patients with NF1, and the correlation between IGF-I receptor levels and the increased mitosis index of PNFs, suggest sensitivity of these tumors to IGFBP1-regulated factors [10,46]. Taken together, IGFBP1 may modulate IGF access to PNF and MPNST, although this mechanism still needs to be elucidated.

The reasons for the reduced circulating EGFR levels that we detected in patients with NF1 are unclear. A possible functional explanation may be enhanced survival of cells that retain their EGFR on the cell surface, providing readiness for EGF signaling [47]. Similarly, EGF signaling has been shown to enhance tumorigenesis in NF1 animal models, and NF1-derived Schwann cells and fibroblasts are highly sensitive to EGF [48]. Hence, it seems that retaining the EGFR on the cell surface leads to reduced circulation of soluble EGFR, and provides an environment that promotes tumorigenesis, as seen in patients with NF1.

Recently, two studies identified MIA and ADM as potential NF1 tumor markers in cohorts of 42 and 32 patients, respectively [21,23]. There was also a trend towards correlation between ADM and MPNST, although the MPNST group was too small to show significance (n = 5). MIA concentration was particularly high in patients with NF1 with either PNF or large numbers of neurofibromas, and correlated with internal tumor burden. Both of these factors seem to be related to tumor burden in NF1, although induction as a result of changed systemic environment due to haploinsufficiency cannot be excluded. It would be intriguing to investigate further what role, if any, a systemic inflammatory environment may play in the early stages of tumorigenesis in patients with NF1.

Conclusions

Our study encompasses the largest cohort of patients with NF1 (n = 104) screened to date for potential serum markers in this rare genetic cancer syndrome. We identified four potential biomarkers, which may assist in the diagnosis of NF1, and two further markers (IGFBP1 and RANTES) that correlate with the presence of MPNST. Intriguingly, IGFBP1 also seems to correlate with internal tumor burden, and thus may indicate increased risk for malignant transformation in patients with NF1. Furthermore, our data reveal a systemic pro-inflammatory profile in patients with NF1, which is probably caused by *NF1* haploinsufficiency. Serum biomarkers that could aid in the early detection of malignant progression would be extremely helpful because therapeutic interventions could be initiated before further spread of the tumor or development of metastasis takes place. Both the current and previous data are very promising for further validation of the data in even larger cohorts. It would be intriguing to further investigate what, if any, role a systemic inflammatory environment may play in the early stages of tumorigenesis in patients with NF1. Multicenter studies in larger cohorts will be necessary to validate the identified markers, and to elucidate a possible role of inflammatory cytokines in tumorigenesis.

Additional files

Additional file 1: List and detailed information of patient and control cohorts used in the study. Abbreviations: nd, not done.

Additional file 2: List of candidate markers selected by manual curation of published data and text. The proteins used in the screenings are shown in bold and italic [49-63].

Additional file 3: Reassessment of protein serum markers interferon (IFN)-γ, interleukin (IL)-6, tumor necrosis factor (TNF)-α, insulin-like growth factor binding protein (IGFBP) and Regulated upon activation, normal T-cell expressed and secreted (RANTES) by cytometric bead array (CBA) and ELISA (arbitrary serum concentration units). Between 11 and 15 randomly selected sera from the different NF1 groups (for IFN-γ, IL-6, TNF-α: all NF1 vs. control; for IGFBP and RANTES: NF1 with no PNF or MPNST, NF1 with only PNF- and NF1 with MPNST) and 5 control sera were tested as indicated (ND, not determined). Where available, associated protein array data are shown. Statistical analysis is shown for CBA/ELISA data (*t*-test).

Abbreviations

ADM: Adrenomedullin; AUC: Area under the curve; CBA: Cytometric bead array; CNF: Cutaneous neurofibromas; EGFR: Epidermal growth factor receptor; ELISA: enzyme-linked immunosorbent assay (ELISA); IFN-γ: Interferon-γ; IGF-1: Insulin-like growth factors; IGFBP1: Insulin-like growth factor binding protein 1; IL-6: Interleukin 6; MIA: Melanoma-inhibitory activity/cd-rap; MIP-4: Macrophage inflammatory protein-4; MK: Midkine; MPNST: Malignant peripheral nerve-sheath tumors; MRI: Magnetic resonance imaging; MRT: Magnetic resonance tomography; NF1: Neurofibromatosis type 1; PDGF-BB: Platelet-derived growth factor-BB; PNF: Plexiform neurofibromas; RANTES: Regulated upon activation, normal T-cell expressed and secreted; ROC: Receiver operating characteristic; TNF-α: Tumor necrosis factor-α; TRAIL-R2: TNF-related apoptosis inducing ligand-R2.

Competing interests

The authors have no competing interests to declare.

Authors' contributions

SP performed array and immunoassay experiments, and analyzed the data. BS advised on the choice of candidate markers, and supervised analytical experiments and analysis. VM collected and provided clinical data and specimens, and advised on experimental design. LK collected and selected serum samples for analysis, and contributed to data acquisition of data; NH coordinated the study, evaluated and interpreted the data, and drafted the manuscript. AK conceived the hypothesis, evaluated data, and critically revised the manuscript. All authors approved the final version of the manuscript.

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RESEARCH ARTICLE Open Access

MIA is a potential biomarker for tumour load in neurofibromatosis type 1

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Abstract

Background: Neurofibromatosis type 1 (NF1) is a frequent genetic disease characterized by multiple benign tumours with increased risk for malignancy. There is currently no biomarker for tumour load in NF1 patients.

Methods: *In situ* hybridization and quantitative real-time polymerase reaction were applied to investigate expression of cartilage-specific genes in mice bearing conditional inactivation of NF1 in the developing limbs. These mice do not develop tumours but recapitulate aspects of NF1 bone dysplasia, including deregulation of cartilage differentiation. It has been recently shown that NF1 tumours require for their growth the master regulator of cartilage differentiation SOX9. We thus hypothesized that some of the cartilage-specific genes deregulated in an Nf1Prx1 mouse model might prove to be relevant biomarkers of NF1 tumours. We tested this hypothesis by analyzing expression of the SOX9 target gene product melanoma-inhibitory activity/cd-rap (MIA) in tumour and serum samples of NF1 patients.

Results: Increased expression of *Mia* was found in *Nf1*-deficient cartilage in mice. In humans, MIA was expressed in all NF1-related tumours and its serum levels were significantly higher in NF1 patients than in healthy controls. Among NF1 patients, MIA serum levels were significantly higher in those with plexiform neurofibromas and in those with large number of cutaneous (> 1,000) or subcutaneous (> 100) neurofibromas than in patients without such tumours. Most notably, MIA serum levels correlated significantly with internal tumour burden.

Conclusions: MIA is a potential serum biomarker of tumour load in NF1 patients which could be useful in following the disease course and monitoring the efficacy of therapies.

Background

Neurofibromatosis type 1 (NF1) is a genetic disorder resulting from mutations in the *NF1* tumour suppressor gene. Susceptibility to neoplastic transformation is the main feature of the disease [1]. The most frequent tumours in NF1 are dermal neurofibromas, which can be found in more than 90% of adult patients [2]. Approximately 50% of NF1 patients develop plexiform neurofibromas (pNFs), which can undergo malignant transformation into malignant peripheral nerve sheath tumours (MPNSTs) [3-6]. MPNSTs are highly malignant tumours with a poor prognosis. The lifetime risk of developing MPNSTs in the NF1 patient is 8% to 13% [7].

Major challenges in clinical practice are to determine tumour burden and to monitor the disease course. While cutaneous neurofibromas are visible on physical examination, the diagnosis of pNFs, especially internal ones, depends on magnetic resonance imaging (MRI), which is costly and laborious. Furthermore, early diagnosis is crucial for complete resection of MPNSTs, which is up to now the only curative treatment [8]. A biomarker for assessment of tumour burden and detection of malignant transformation would therefore be of interest.

Previously, we and others have shown that loss of *Nf1* gene function during murine embryogenesis causes defects of bone and cartilage development [9,10]. One of the observed molecular changes in *Nf1*-deficient

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embryonic cartilage was an upregulation and persistently nuclear localization of the transcription factor SOX9. Interestingly, SOX9 was also recently found to be expressed in NF1-related tumours, where it supports cellular survival [11]. As a master regulator of cartilage differentiation, SOX9 regulates expression of various downstream target genes, including collagen type 2a1, collagen type 11a2, aggrecan and melanoma-inhibitory activity (*MIA*). The last, *MIA*, is also known as cartilage-derived retinoic acid sensitive protein (*cd-rap*) and was originally isolated as a secretory factor from supernatants of melanoma cell cultures [12]. MIA serum level was found to correlate with melanoma spreading [13] and was proposed as a biomarker for monitoring the course of disease and the efficacy of therapies [14]. Various other tumours, predominantly those of neuroectodermal, glial origin, also express *MIA* [15]. Recombinant MIA inhibits melanoma cell growth and cell attachment *in vitro* [16]. Subsequent studies revealed that MIA interacts with extracellular matrix components, laminin and fibronectin, as well as with cellular matrix receptors integrin $\alpha5$, integrin $\alpha4$ [17] and cadherin 7 [18].

In the present study, we examined expression of *MIA* in *Nf1*-deficient mouse cartilage, in human cutaneous and plexiform neurofibromas and MPNSTs, and in sera of NF1 patients with these tumours. MIA in the serum of healthy probands was examined as a control.

Methods

Mouse breeding and tissue processing

The mice were continuously back-crossed to wild-type C57BL/6J to minimize the variation of genetic background. The female Nf1flox mice were crossed to male Nf1flox heterozygous Prx1-Cre-positive males and the offspring genotyped as previously described [9]. Embryos and postnatal tissue samples were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated through an ethanol/xylol series, and embedded in paraffin blocks. Six-micrometer sections were cut and processed for haematoxylin and eosin/Alcian blue staining and *in situ* hybridization.

Patients and samples

The study was conducted with a cohort of 42 NF1 patients and 22 healthy individuals. The diagnosis of NF1 was made using National Institutes of Health criteria. The study protocol was approved by the local institutional review board, and all patients gave their informed consent. Cutaneous and subcutaneous tumours were counted or estimated in case the number was larger than 100. Plexiform neurofibromas, including internal ones, were detected by means of whole-body MRI in 30 of the 42 patients. Because of the limited resolution of whole-body MRI, lesions smaller than 3 cm in the longest diameter, which is often the case for spinal tumours, were not included. Tumour sizes were calculated using a semi-automated volumetric method, and the total internal tumour load was obtained subsequently, including PNs (Plexiform Neurofibromas), spinal tumours and internal nodule neurofibromas, but excluding cutaneous and subcutaneous tumours [19]. An age effect for cutaneous, subcutaneous and internal tumours was examined using a nonparametric Spearman's rank-correlation test.

All serum samples were prepared using a standardized protocol in the laboratory of the Department of Maxillofacial Surgery at the University Medical Center Hamburg-Eppendorf. Whole blood of each patient was kept at room temperature for 30 minutes before being spun down at 4,500 rpm for 10 minutes using a benchtop centrifuge. The supernatant was stored at -80°C in 100-μL aliquots.

In situ hybridization

In situ hybridization was performed on paraffin sections according to standard protocol [9]. Images were collected using a DMR HC microscope (Leica, Wetzlar, Germany) equipped with an AxioCam HRc camera (Zeiss, Jena, Germany) and evaluated using AxioVision 4.1 software (Zeiss, Jena, Germany).

Immunohistochemical detection of MIA

Sections of six cutaneous and three plexiform neurofibromas, as well as seven MPNSTs, from a total of sixteen NF1 patients were stained with monoclonal anti-human MIA antibody (R&D Systems, McKinley Place NE, Minneapolis) diluted at 1:40. Sections were boiled in citrate buffer (pH 6.1) for antigen retrieval. The streptavidin-biotin method was performed using an automated staining system TechMate (Dako, Hamburg, Germany) with an implemented counterstaining. Negative controls were carried out with normal serum without the primary antibody or with antibody preincubated in access (25 ng/μl) of recombinant human MIA (Peprotech GmbH, Hamburg, Germany). Stained sections were analyzed using the BX51 microscope (Olimpus, Hamburg, Germany) and analySIS 5.0 software (Soft imaging system GmbH, Münster, Germany).

Quantitative real-time polymerase chain reaction

RNA was isolated from the knee cartilage of two wild-type and two Nf1Prx1 mice using peqGOLD TriFast (PeqLab Biotechnologie GmbH, Erlangen, Germany) according to the supplied protocol. cDNA was synthesized from 1 μg of total RNA with MuLV Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA). TaqMan Universal PCR Master Mix was then performed on an ABI PRISM 7900 Cycler (Applied Biosystems) using the SYBR Green method (Invitrogen, Darmstad, Germany) according to the manufacturer's instructions. The expression level of *Mia*

was determined in Nf1Prx1 and wild-type tissues and was equilibrated against expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The following primers were used: mGAPDH: 5' GGGAAGCCCATCACCAT CTT 3', 5' CGGCCTCACCCCATTTG 3'; mMIA: 5' GGAGGACCTGACTCTGAAACC 3'; 5' ACTGCAGG-GATAGCGGTAG 3'.

Mia elisa

The MIA ELISA kit was purchased from (Roche Diagnostics, Indianapolis, IN, USA) and the measurements were conducted in duplicate according to the supplied protocol. Internal negative and positive quality controls were provided in each enzyme-linked immunosorbent assay (ELISA) kit and were run in triplicate in each assay.

Results

Mia expression is elevated in *Nf1*-deficient murine cartilage

In situ hybridization revealed expression of *Mia* in the cartilage of the E14.5 to E15.5 mouse embryo (Figure 1). Expression domains of *Sox9, Col2a* and *Mia* overlapped and, in the E14.5 embryo sections, demarcated cartilage anlagen of the future bones (Figure 1A). The expression of *Mia* was found to be more intensive in the *NF1*-deficient cartilage of the Nf1Prx1 mice (Figure 1B). Similar results were obtained with mouse embryos bearing cartilage-specific inactivation of *Nf1* (data not shown). We next quantified *Mia* expression by performing quantitative real-time polymerase chain reaction (qRT-PCR). Absolute quantification was conducted on the RNA isolated from knee cartilage of two mutant and two control mice at P4. *Mia* transcript levels in *Nf1*-deficient tissue were compared to the wild-type tissue and normalized to *GAPDH* expression. qRT-PCR revealed a more than twofold increase of *Mia* expression in Nf1Prx1-deficient cartilage.

MIA is expressed in NF1-associated tumours

MIA was immunohistochemically detected on the paraffin sections of six cutaneous and three plexiform neurofibromas and in seven MPNSTs from NF1 patients. The typical pattern of MIA staining was a mixture of positive and negative nuclei side-by-side (Figure 2). The proportion of MIA-positive cells varied between 50% and 90%. The most intense staining was obtained in MPNSTs, which, however, represents the high density of nuclei in this type of tumour. No morphological difference was observed between MIA-positive and MIA-negative cells. On the basis of the degenerative nuclear atypia of Schwann cells, we deduced that MIA was both positive and negative in Schwann cell nuclei. MIA-positive cells were more often seen in areas of spindle-shaped cells arranged in fascicles.

Serum concentration of MIA in NF1 patients correlates with tumour load

MIA serum level was determined in the 42 NF1 patients and in 22 healthy individuals. The patients' ages ranged from 14 to 72 years (mean age, 36 years). The control group's ages ranged between 19 and 67 years (mean age, 40 years). An age effect was seen in the NF1 patients for the number of cutaneous tumours ($P = 0.023$), but not for subcutaneous tumours ($P = 0.842$) or internal tumours ($P = 0.449$). Additionally, linear regression analysis revealed an association between total internal

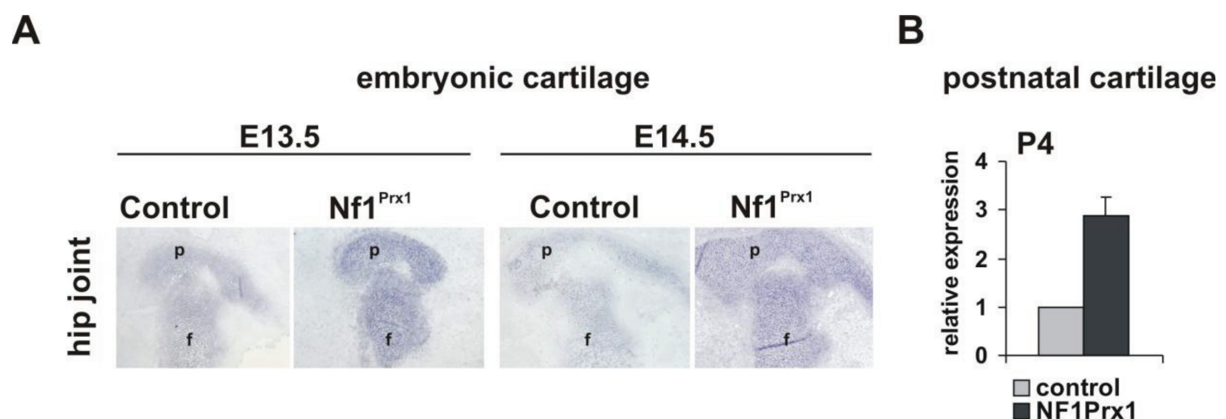
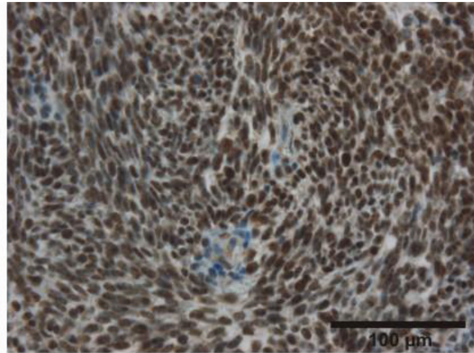
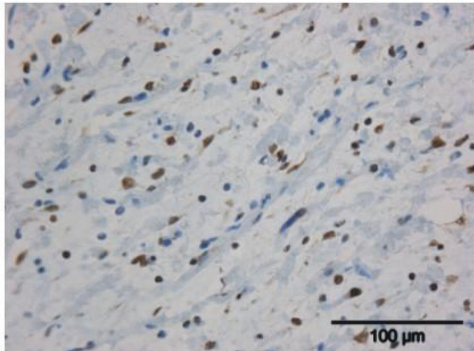


Figure 1 Elevated expression of *cd-rap/Mia* in the *Nf1*-deficient cartilage. **(A)** *In situ* hybridization of the melanoma-inhibitory activity/cd-rap (*mia*)-specific riboprobe on the transverse sections of E14.5 Nf1Prx1 embryos. Intensity of staining reflects abundance of *Mia* transcript. **(B)** Quantitative real-time polymerase chain reaction (qRT-PCR) of *Mia* transcript in the postnatal day 4 knee joints. Data represent means (± SD) of duplicate absolute quantifications for each probe. Transcript of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as control.

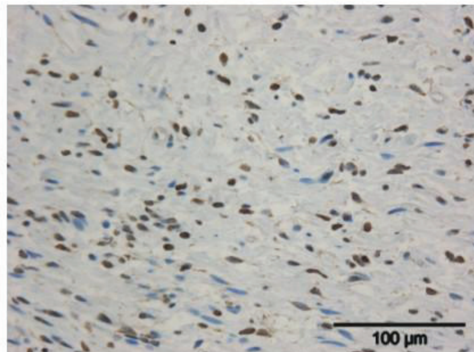
melanoma



cutaneous
neurofibroma



plexiform
neurofibroma



MPNST

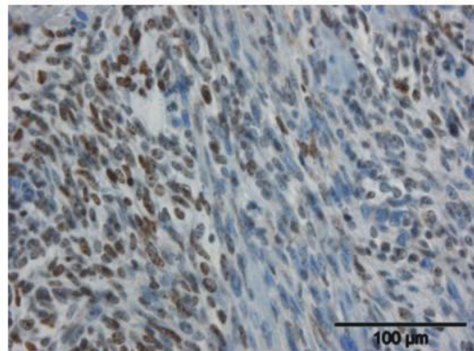


Figure 2 ***MIA*** **is expressed in NF1 tumors**. Immunohistochemical detection of MIA on paraffin sections of NF1-associated tumors. Melanoma samples were used as positive controls. MIA is expressed in each type of the analysed NF1 tumors. Malignant peripheral nerve sheath tumours (MPNSTs) have higher cellular density, yielding more MIA-positive cells per visual field.

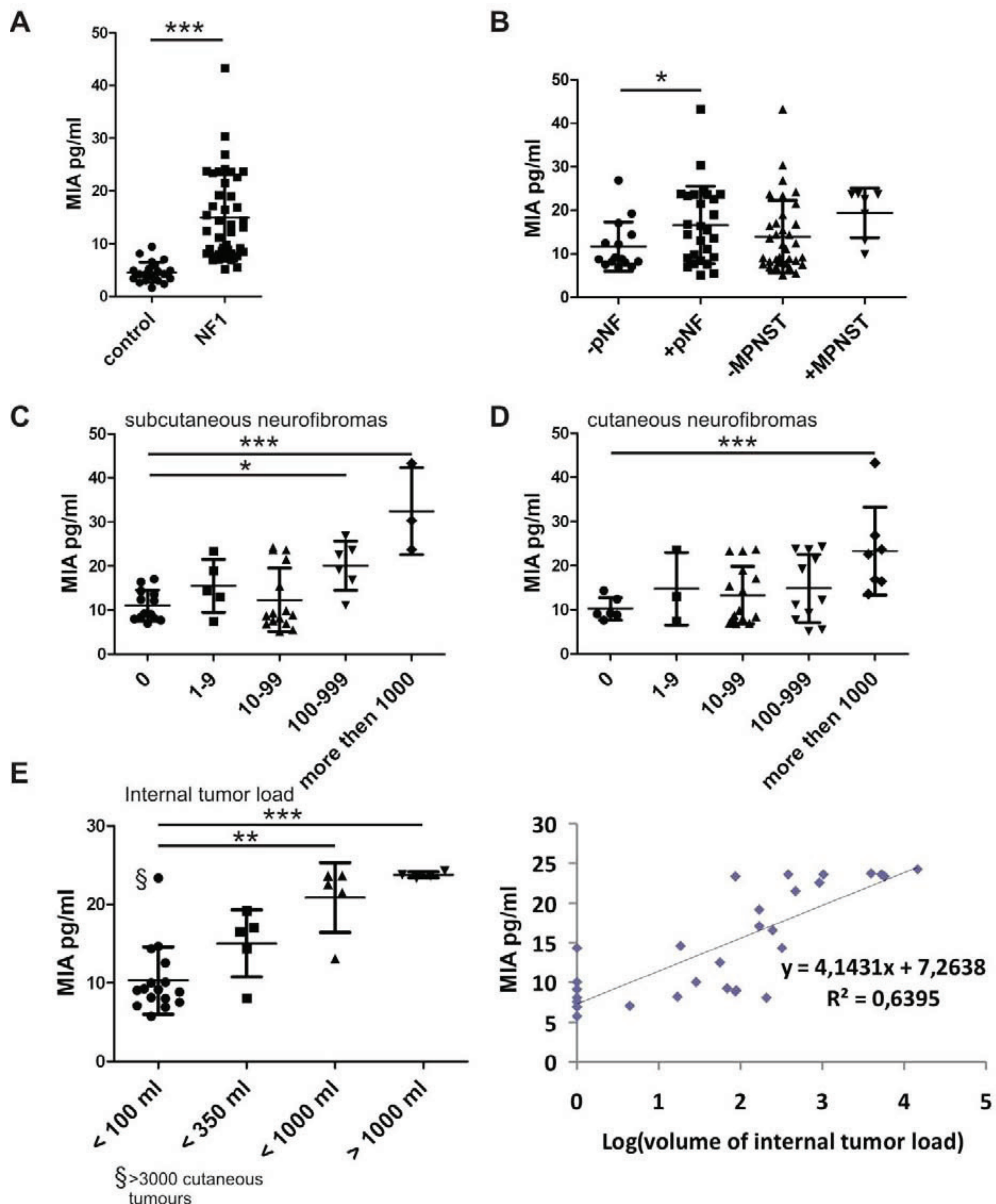


Figure 3 MIA is elevated in serum from NF1 patients and reflects the internal tumor load. **(A)** MIA serum levels in 42 NF1 patients and 22 healthy controls. **(B)** The 42 NF1 patients divided into subgroups according to the absence (-) or presence (+) of pNFs or MPNSTs. **(C, D)** The 42 NF1 patients were further divided with accordingt to cutaneous and subcutaneous tumors load. **(E)** In 30 of the 42 NF1 patients, internal tumor load was determined by whole-body magnetic resonance imaging (MRI). The 30 patients were arbitrarily divided into four groups according to the total tumor load: 0 to 100 mL (n = 16), < 350 mL (n = 5), < 1,000 mL (n = 5) and > 1,000 mL (n = 4). Differences between groups were evaluated using an unpaired t-test **(A and B)** or one-way analysis of variance (ANOVA) with a *post hoc* t-test including the Bonferroni correction **(C to E)**. **$P < 0.01$. ***$P < 0.001$. The linear regression analysis revealed a positive correlation between the logarithm of internal tumor load and MIA serum concentration.

tumour load and the number of subcutaneous tumours (*P* value 8.19E-17 for the F test), but not between internal tumour load and the number of cutaneous tumours.

MIA serum concentration was independent of age and sex (data not shown), but was significantly higher in NF1 patients than in healthy controls: 15.16 ± 1.26 pg/mL versus 4.54 ± 0.40 pg/mL ($P < 0.001$, unpaired *t*-test with Welch's correction) (Figure 3A). Among the 42 NF1 patients, the 27 patients with pNFs had significantly higher MIA serum concentration than the 15 patients without those tumours ($P = 0.032$) (Figure 3B). However, no significant difference in MIA serum level was found between the 7 and 35 patients with and without MPNSTs, respectively (Figure 3B). MIA serum level was also significantly higher in the nine and seven patients with > 100 subcutaneous neurofibromas and > 1,000 cutaneous neurofibromas, respectively, than in those without such tumours (Figures 3C and 3D). Internal tumour load was determined for 30 of the 42 NF1 patients on the basis of whole-body MRI. The patients were divided into four groups: very low internal tumour loads (0 to 100 mL; $n = 16$), low internal tumour loads (< 350 mL; $n = 5$), moderate internal tumour loads (< 1,000 mL; $n = 5$) and high internal tumour loads (> 1,000 mL; $n = 4$) (Figure 3E, left). One-way analysis of variance with the Bonferroni multiple comparison test revealed significant differences between MIA serum levels in patients with very low internal tumour loads and groups with high and very high internal tumour loads (**$P < 0.01$, ***$P < 0.001$). Also, linear regression analysis revealed an association between the total internal tumour load and MIA serum level (*P* value of 1.95E-7 for the F-test). The line that best predicts MIA level from values of logarithm of internal tumor load volume was identified by regression analysis: $R^2 = \sim 0.64$ (Figure 3E, right). These data indicate that elevated MIA serum level may be indicative of an increased internal tumour burden. Since we observed an association between total internal tumour load and the number of subcutaneous tumours, a study involving a larger cohort size is necessary to reveal the relative contributions of internal, subcutaneous and possibly also cutaneous tumours to elevated MIA levels.

Discussion

In this study, we found increased *Mia* expression in *Nf1*-deficient cartilage of Nf1Prx1 mice where SOX9 expression and nuclear localization were previously shown [9]. *MIA* promoter was previously shown to be regulated by SOX9 in a dose-dependent manner in cultured chondrocytes [20]. It thus appears likely that *MIA* expression in NF1 tumours is also regulated by SOX9, as this transcription factor was reported to be required for the survival of MPNST cells [11]. Our finding of MIA expression in various NF1-related tumours is consistent with the findings of previous reports that MIA is expressed in glial tumours [15].

The major finding of the present study is that MIA serum levels correlate with the internal tumour load in NF1 patients. Provided that this correlation can be confirmed in a larger cohort of NF1 patients, MIA would be a valuable biomarker for the internal tumour load.

In malignant melanoma cells, MIA was shown to bind integrin $\alpha 5$ and reduce ERK activity [17]. MIA/cadherin-7 interactions were shown to regulate cell-cell adhesion of malignant melanoma cells, influencing their migration [18]. It was also reported that MIA augmented transforming growth factor-β-mediated chondrogenic differentiation of human mesenchymal cells *in vitro* [21] and inhibited articular cartilage mineralization *in vivo* [22]. It will be interesting to examine whether any of these effects of MIA play a role in NF1-related tumorigenesis and skeletal dysplasia. While more studies are needed to understand the contribution of MIA to NF1 pathology, the presented correlation of MIA serum level with the internal tumour load suggests that it is a promising candidate as a biomarker of the tumour load in NF1.

Conclusions

MIA is a potential biomarker of tumour load in NF1 patients and should be further evaluated for application in monitoring the clinical course and therapy outcomes of patients.

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Authors' contributions

MK formulated the hypothesis, coordinated the study, evaluated data and conceived the manuscript. VM provided clinical data and specimens. NK performed MIA ELISA measurements. RN performed whole-body MRI evaluations. JK provided expertise on the ELISA system handling and data acquisition. TZ performed statistical analysis. AJ helped in establishing the MIA immunohistochemistry protocol. EW performed real-time PCR experiments. BT performed *in situ* hybridization analysis. ST provided NF1 tumour samples and sera. NH provided logistical support and helped in collection of the serum samples. SP helped in collection of the serum samples. PB provided logistical support and helped in collection of the serum samples. DK provided support in obtaining serum probes and critically revised the manuscript. AH performed histological and immunohistological analysis of the surgically removed tumour material. SM critically revised the manuscript. LK coordinated clinical data and specimen acquisition, was involved in the evaluation and interpretation of data, and conceived and critically revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Impaired Pten Expression in Human Malignant Peripheral Nerve Sheath Tumours

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Abstract

Malignant peripheral nerve sheath tumours (MPNST) are aggressive sarcomas that develop in about 10% of patients with the genetic disease neurofibromatosis type 1 (NF1). Molecular alterations contributing to MPNST formation have only partially been resolved. Here we examined the role of Pten, a key regulator of the Pi3k/Akt/mTOR pathway, in human MPNST and benign neurofibromas. Immunohistochemistry showed that Pten expression was significantly lower in MPNST (n = 16) than in neurofibromas (n = 16) and normal nervous tissue. To elucidate potential mechanisms for Pten down-regulation or Akt/mTOR activation in MPNST we performed further experiments. Mutation analysis revealed absence of somatic mutations in *PTEN* (n = 31) and *PIK3CA* (n = 38). However, we found frequent *PTEN* promotor methylation in primary MPNST (11/26) and MPNST cell lines (7/8) but not in benign nerve sheath tumours. *PTEN* methylation was significantly associated with early metastasis. Moreover, we detected an inverse correlation of Pten-regulating miR-21 and Pten protein levels in MPNST cell lines. The examination of *NF1*−/− and *NF1*+/+Schwann cells and fibroblasts showed that Pten expression is not regulated by *NF1*. To determine the significance of Pten status for treatment with the mTOR inhibitor rapamycin we treated 5 MPNST cell lines with rapamycin. All cell lines were sensitive to rapamycin without a significant correlation to Pten levels. When rapamycin was combined with simvastatin a synergistic anti-proliferative effect was achieved. Taken together we show frequent loss/reduction of Pten expression in MPNST and provide evidence for the involvement of multiple Pten regulating mechanisms.

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Introduction

Malignant peripheral nerve sheath tumours (MPNST) are aggressive soft tissue sarcomas that develop with an incidence of 1:100.000. Although rare in the general population MPNST develop frequently in patients with the genetic disorder neurofibromatosis type 1 (NF1), which occurs with an incidence of 1:3500 [1]. The life-time risk of developing MPNST is estimated to be 8–13% [2] for NF1 patients, who account for about 50% of all MPNST. MPNST generally arise from plexiform neurofibromas (pNF) in NF1 patients and constitute the major cause for reduced life expectancy with only 21% of patients surviving longer than 5 years after diagnosis. Loss of the tumour suppressor gene (TSG) *NF1* is only a first step in tumourigenesis. During the course of malignant progression, further alterations are acquired in TSG and oncogenes like *TP53*, *CDKN2A*, *EGFR*, and *PDGFRA* [3,4,5,6,7]. Although sporadic and NF1-associated MPNST share many similarities in their molecular pathogenesis, Ras mutations are linked to sporadic MPNST whereas *PTEN* monosomy segregates with NF1-associated cases [8]. The currently dim treatment options for MPNST patients may be improved by a better knowledge on molecular alterations, which could lead to novel strategies of targeted therapy. Neurofibromin, the *NF1* gene product, is a negative regulator of the Ras oncoprotein. Moreover, it was shown that the Akt/mTOR (mammalian Target of Rapamycin) pathway is activated in *NF1* deficient cells [9]. This pathway is attractive for targeted therapy since different mTOR inhibitors are already approved for clinical application. Recently we found allelic loss of *PTEN* (Phosphatase and tensin homologue deleted from chromosome 10) in 58% MPNST [7]. Pten protein is a major regulator of the Pi3k/Akt/mTOR pathway. Loss or down-regulation of Pten expression leads to the activation of this pathway and thus promotes malignant progression. *PTEN* is the second most frequently altered TSG and inactivated in a variety of tumour entities including glioblastoma, prostate cancer and melanoma. Pten has lipid phosphatase activity and dephosphorylates phosphatidylinositol-(3,4,5)-triphosphate

(PIP3) to phosphatidylinositol-(4,5)-bisphosphate (PIP2). Thereby it antagonizes the activity of the phosphatidylinositol-3-kinase (Pi3k) which converts PIP2 to PIP3. Via this mechanism Pten controls the Akt/mTor pathway, which promotes multiple functions, including cell growth and survival, proliferation, apoptosis, invasion, migration and angiogenesis.

Recently, a transgenic mouse model provided evidence for an important role of Pten in development of benign and malignant nerve sheath tumours [10]. The authors demonstrated that in addition to a constitutively active K-Ras mutant a reduced *Pten* dosage was necessary for tumour formation. Deletion of both *Pten* alleles was observed in malignant but not in benign nerve sheath tumours. This study points towards a crucial role of Pten in nerve sheath tumour formation, however, the employed mouse model does not reflect the genetic nature of NF1 patients and the question why mice haploinsufficient for *Pten* and *Nf1* completely lacked tumour development remains unsolved.

Here we determined the frequency of Pten alterations in human MPNST and neurofibromas and examined underlying mechanisms.

Materials and Methods

Tumour Tissue, DNA and RNA Extraction

Paraffin embedded and frozen tumour and nerve samples were collected in the following German hospitals: University Hospital Eppendorf (Hamburg), Otto-von-Guericke-University (Magdeburg), Robert-Rössle-Hospital (Berlin), and Charité – Universitätsmedizin Berlin. Following initial diagnosis in local neuropathologies, all tumour samples were reviewed by the same experienced pathologist (AvD). Tumour sections were examined histologically prior to extraction of nucleic acids and proteins. DNA and RNA from frozen tumours (6 MPNST and 9 neurofibromas), all cell lines and cell cultures were extracted with Trizol reagent (Invitrogen, Karlsruhe, Germany). RNA integrity was analysed with a Bioanalyzer from Agilent (Böblingen, Germany). Samples with an RNA integrity number (RIN)$<$7 were excluded. RIN of cell lines was $>$9. DNA extraction from paraffin embedded material was carried out according to the QIAamp DNA Mini Kit protocol (Qiagen, Hilden, Germany). The investigations were carried out with the informed consent of the patients.

Immunohistochemistry and Scoring

Immunohistochemistry on paraffin embedded slices was performed with the BenchmarkTM system from Ventana (Strasbourg, France). Pten antibody (A2B1, dilution 1:80) was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Visualization was performed with diaminobenzidine. Negative controls without primary antibodies were carried out. Scoring was performed according to the percentage of positive cells: $<$5% was classified as negative (−), 6–100% was classified as positive. 6–30% of positive cells were scored with +, 31–60% with ++, $>$60% with +++. A blinded repeated test produced similar results.

Immunofluorescence double staining was performed manually. Antigen retrieval was achieved by heating. Pten (A2B1, dilution 1:80) S100 and neurofilament from DakoCytomation GmbH (Hamburg, Germany), dilution 1:1000, antibodies were used. For visualization we utilized 1:100 dilutions of Cy3- and Alexa Fluor 488-conjugated antibodies. Nuclei were counterstained with DAPI. Normal skin tissue served as positive control. Slices were photographed with the confocal laser mikroscope LSM5 Exciter from Zeiss (Jena, Germany).

PTEN and *PIK3CA* Mutation Analysis

The nine coding exons of *PTEN* were sequenced bidirectionally with nine primer pairs labelled either with M13-forward and –reverse sequences conferring PCR products between 160 and 364 bp. PCR was performed in a volume of 15 µl with 20 ng of DNA, GoTaq DNA Polymerase (Promega, Mannheim, Germany) applying 35 cycles. Primer sequences are available on request. For sequencing the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Darmstadt, Germany) was utilized. For *PIK3CA* analysis a set of five primer pairs was employed, covering exon 1, 9 and 20. The primer sets [11], PCR and single strand confirmation polymorphism (SSCP) have been described before [12]. For enhanced sensitivity all PCR products were separated on 8% and 14% acrylamide gels.

PTEN Promotor Methylation Analysis

Promotor methylation was done as previously described [13] and performed by Varionostic GmbH (Ulm, Germany). Briefly, DNA was bisulfite-treated followed by quantitative positional methylation analysis (pyrosequencing). The methylation analysis was restricted to CpG island 3 which contains 5 CpG sites. A methylation of$<$8% of DNA was regarded as unmethylated. The assay does not detect methylation of the *PTEN* pseudogene *PTENP1*.

Expression Analysis by Real Time RT-PCR

Reverse transcription and DNA digestion of 1 µg RNA was achieved with the Quantitect reverse transcription kit (Qiagen, Hilden, Germany). Subsequent PCR reactions were performed with Taqman Mastermix FAST and gene specific MGB probes. PCRs were performed in triplicates in a volume of 20 µl containing cDNA equivalents of 10 ng RNA. PCR reagents, probes and the 7900 HT fast real time PCR system were from Applied Biosystems (Darmstadt, Germany). Data were accepted as valid if the standard deviation of Ct values (threshold cycles) of triplicate reactions was $<$0.5 cycles. *ACTB* and *RPS3* were employed as endogenous controls.

Expression of miR-21 and miR-217 was analysed with the Taqman microRNA reverse transcription kit and subsequent amplification with Taqman Universal PCR master mix and Taqman microRNA assays (all from Applied Biosystems). RNU44 was used as endogenous control.

Western Blots

Subconfluent cell cultures were lysed in ice cold lysis buffer (1% Triton X100, 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA) containing protease and phosphatase inhibitor cocktail. For lysates of solid tumours 1–3 g of tissue was homogenized in lysis buffer. After heat denaturation approximately 40 µg per sample was loaded on to 12% acrylamide gels. For comparability of different gels cell line T265 was run on every gel as an internal standard. After blotting membranes were stained with Ponceau S to verify protein transfer. Membranes were blocked and incubated overnight at 4°C with primary antibodies. The p-p70S6 kinase antibody (detects also the p-p85S6 isoform, dilution 1:2000 (R&D Systems, Wiesbaden, Germany) and Pten antibodies (either A2B1 from Santa Cruz Biotechnology or Pten antibody from Zytomed, both with1:200 dilutions) were used. The β-actin antibody (AC-15, dilution 1:6.000) was from Sigma (Munich, Germany). After washing membranes were incubated for 1 h with second horseradish conjugated antibodies. Visualization was performed with ECL or advanced ECL (Amersham Biosciences, Freiburg, Germany).

Cell Culture Assays

All cell lines were maintained in DMEM Glutamax-I (1000 mg/L glucose) with 10% FCS and 5 µg/mL gentamycin from Invitrogen (Karlsruhe, Germany). MPNST cell lines S462, S520, 1507.2, S805 were established and provided by V. F. Mautner, University Hospital Eppendorf, Germany [14]. MPNST cell line STS26T was provided by G. H. De Vries (Hines VA Hospital, Illinois, USA) [15]. MPNST cell line ST88-14 was provided by J. DeClue (NIH, Bethesda, USA) [16]. NFS-1, low passage culture 31002 and dermal fibroblasts have been described elsewhere [7,17]. Mouse embryonal fibroblasts from *Nf1* wildtype and *Nf1−/−* mice were prepared and genotyped by D. Kaufmann (Institute of Human Genetics, Ulm, Germany) [18]. With the exception of MPNST cells 31002 and STS26T, which were obtained from sporadic MPNST, all other MPNST lines were from NF1 associated MPNST. Neurofibroma derived Schwann cells were cultured on laminin coated dishes and cultured in DMEM supplemented with 10% FCS (Invitrogen), 2.5 µg/ml insulin (Sigma, München, Germany), 0.5 mM IBMX (Serva, Heidelberg, Germany), and 10 nM heregulin (Peprotech, Hamburg, Germany). 0.5 µM forskolin from Sigma was used for normal Schwann cells (Sciencell, Carlsbad, CA) but not for neurofibroma derived *NF1−/−* Schwann cells since these cells are selectively expanded when forskolin is omitted [19]. For comparative analysis Schwann cells were set to the same medium conditions (DMEM 0.5% FCS) for at least 72 h before harvest. When proliferation assays were performed cell lines were maintained in DMEM containing 5% serum. 3×103 cells were seeded in 300 µL medium into 24 well plates and allowed to adhere over night. Drugs were added in 100 µL to obtain the indicated concentrations. Negative controls contained vehicle only. Cell proliferation was evaluated on day 4 post-treatment with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Mannheim, Germany). The experiments were performed in quadruplicate and repeated at least three times. Rapamycin and simvastatin were purchased from Calbiochem (Darmstadt, Germany). Fractional product concept was used to determine whether drug combinations yielded additive or synergistic effects.

Determination of Phospho mTOR/total mTOR and VEGF

Phosphorylated mTOR and total mTOR were simultaneously determined with the MSD 96-well Multi-spot Phospho (Ser2448)/ total mTOR assay from Meso Scale discovery (Gaithersburg, MD). VEGF concentration was measured in with the Multi-array 96-well plate from Meso Scale discovery. Optimal protein concentration was between 10–20 µg in 25 µl as determined by serial dilution. The assay was performed according to the manufacturer's recommendation.

Statistical Methods

SPSS version 12.0 was used for statistical analysis. Survival rates were determined using the Kaplan–Meier method and the log rank test. Association of parameters was assessed with Pearson correlation and Fisher exact test. A p-value of <0.05 was considered as significant.

Results

Pten Expression in Nerve Sheath Tumours and Nervous Tissue

Pten immunohistochemistry shows a significantly ($p = 0.045$, Pearson correlation) reduced Pten expression in MPNST ($n = 16$, 3 sporadic and 13 NF1-associated MPNST) as compared to neurofibromas ($n = 16$) (Figure 1a–c). Median proportion of Pten positive cells was 30% in neurofibromas and 5% in MPNST. In line with these data, three tumours with transition from pNF to MPNST showed lower Pten expression in the malignant part. An example is shown in Figure 1b, with 5% Pten positive cells in the MPNST versus 70% positive cells in the pNF part. Figure 1c shows Pten expression pattern in the pNF with typical spindle shaped cell morphology. Pten staining was visible in the cytoplasm and in the nucleus. Given that nuclear Pten has been associated with more differentiated, resting cells we compared its subcellular distribution in our nerve sheath tumour samples. The ratio of nuclear Pten was higher in neurofibromas as compared to MPNST (mean of 50% versus 30% as shown in Figure S4).

Western blot analysis shows reduced Pten expression particularly in 2 of 4 MPNST (Figure 1d). However, in tumour lysates utilized for Western blot, non-tumourous cell types like endothelial cells contribute to Pten signals. Production of vascular endothelial growth factor (VEGF) increases upon activation of the Akt/ mTOR pathway. Since phosphorylated proteins were difficult to detect in lysates from primary tumours, we determined VEGF levels and found stronger expression in MPNST than in neurofibromas (Figure 1d).

In order to compare Pten expression levels of healthy nerve tissue with nerve sheath tumours we examined three nerves without diagnostic findings (Figure 2). Because all 3 samples were highly positive we aimed to find out the exact cell type expressing Pten in nervous tissue. Immunoflourescence double staining revealed expression of Pten in neurofilament-positive axons as well as in S100-positive Schwann cells (Figure 2b & c). The cross section in Figure 2b shows nerve fibers with a central axon, double positive for neurofilament and Pten, ensheathed by Pten positive Schwann cells.

Next, we determined Pten expression in 9 MPNST cell lines, 4 neurofibroma derived Schwann cell cultures and dermal fibroblasts by western blot (Figure 3a & b). We observed that MPNST cell lines expressed Pten at lower levels than Schwann cell cultures or dermal fibroblasts. The only cell line with undetectable Pten expression was NFS-1.

Pten is not Regulated by Neurofibromin

Less Pten positive cells were detected in MPNST when compared to neurofibroma (Figure 1a). This finding parallels the situation of neurofibromin positive cells in these tumours. To determine if Pten might possibly be regulated by neurofibromin we examined murine embryonal fibroblasts (MEFs) and human Schwann cells with *NF1+/+*and *NF1−/−* status for Pten expression. Pten protein expression was similar in *NF1+/+*and *NF1−/−* negative cells, however, p-p70S6 kinase, an indicator for mTOR activity, was elevated in case of *NF1* deficiency (Figure 3c). The antibody also recognizes the p85 isoform of the S6 kinase. This isoform is, however, not activated by Akt/mTOR.

In case of MEFs we tested the impact of different culture conditions and also determined activation of the Ras/MAPK pathway. An increased level of p-p70S6 kinase and of p-MAPK was detected in *Nf1−/−* MEFs when they were kept in DMEM without serum or in PBS as shown in Figure S1. In a second round of experiments we determined p-mTOR in MEFs kept in PBS for different times. Usage of the Mesoscale system was more sensitive and allowed better resolution than western blot. We could confirm stronger basal mTOR activity in *Nf1−/−* cells (Figure S1b).

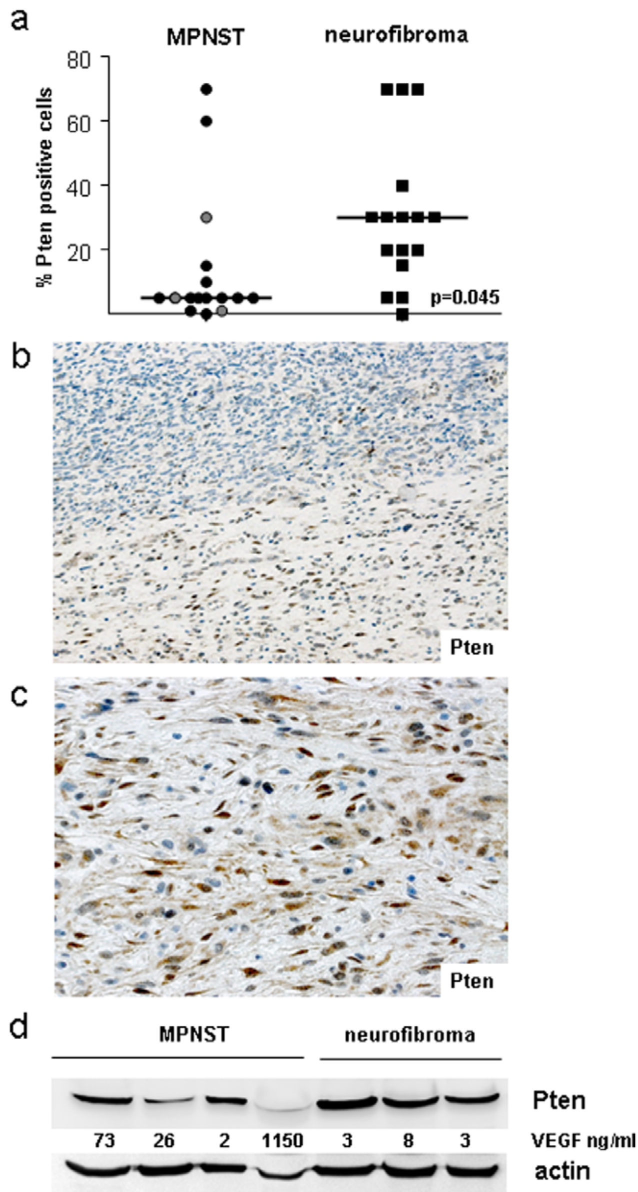

Figure 1. Pten expression in MPNST and neurofibroma. a) Proportion of Pten-positive tumour cells as determined by immunohistochemistry. Each dot represents one tumour. Sporadic MPNST are depicted in grey b) MPNST 29250 with a transition to pNF. Note stronger Pten expression in the pNF (lower part). c) Higher magnification of pNF. Original magnification 200x and 400x. d) Western blot of MPNST and neurofibromas. VEGF concentrations in tumour lysates are indicated.
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Mechanisms of *PTEN* Regulation

Since NF1 status had no influence on Pten expression, we proceeded with further experiments to elucidate mechanisms responsible for Pten downregulation in MPNST.

To analyse an involvement of *PTEN* mutations we examined all *PTEN* coding exons in 24 solid MPNST and seven MPNST cell lines. In addition, we screened for *PIK3CA* sequence alterations, since somatic mutations in this gene can also lead to Akt/mTOR pathway activation. Activating mutations of the p110alpha subunit of Pi3k, encoded by the *PIK3CA* gene, have been identified in a broad spectrum of tumours, e.g. breast cancer and glioblastomas.

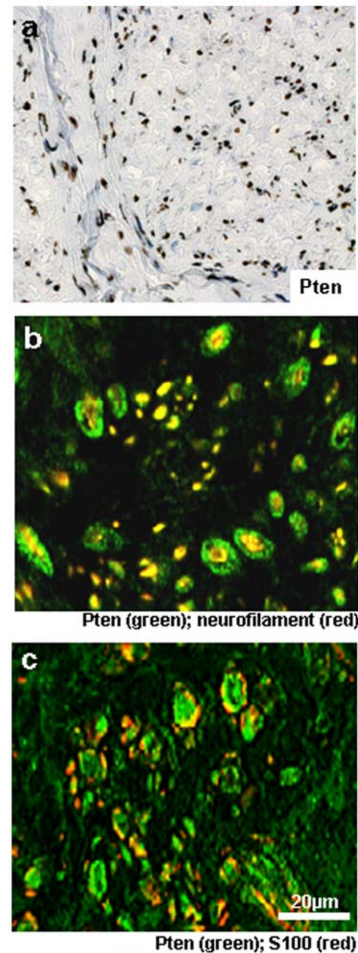

Figure 2. Pten expression in nerve tissue (a-c) and Schwann cell cultures (d). a) Standard DAB staining. b) Double staining for Pten (green) and neurofilament (red). c) Double staining for Pten (green) and S100 (red). Original magnification 400x.
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We analysed exon 1, 9 and 20, which have been identified as hot spot regions for mutations [20] in 38 MPNST. Somatic mutations were neither detected in *PTEN* nor in *PIK3CA*.

Next, we determined *PTEN* promotor methylation, a common mechanism for Pten down-regulation. A total of 73 samples (55 solid tumours and 18 cell cultures) was analysed by quantitative positional methylation analysis. We focused on methylation of CpG island 3, which contains 5 CpG sides [13]. Comparison of 29 benign nerve sheath tumours (5 neurinomas, 24 neurofibromas) and 26 solid MPNST (3 sporadic and 23 NF1-associated MPNST) revealed a significantly higher methylation frequency in MPNST ($p = 0.001$, Pearson correlation). With the exception of 3 neurofibromas, which contained 10%, 23% and 30% methylated DNA, all other benign tumour samples were unmethylated. Five MPNST had DNA methylation levels >50%, two between 30–50% and four between 8–29%. Standard deviation between individual CpG sides was generally small (<10%). Clinical data were available for 21 MPNST patients including 3 sporadic cases without metastasis. The tumours were grouped in two categories: methylation <29% ($n = 14$) and methylation ≥29% ($n = 7$). We detected a significant association (log rank $p = 0.015$) of *PTEN* methylation and appearance of metastasis (Figure S3). Pten immunohistochemistry of the primary MPNST was available for

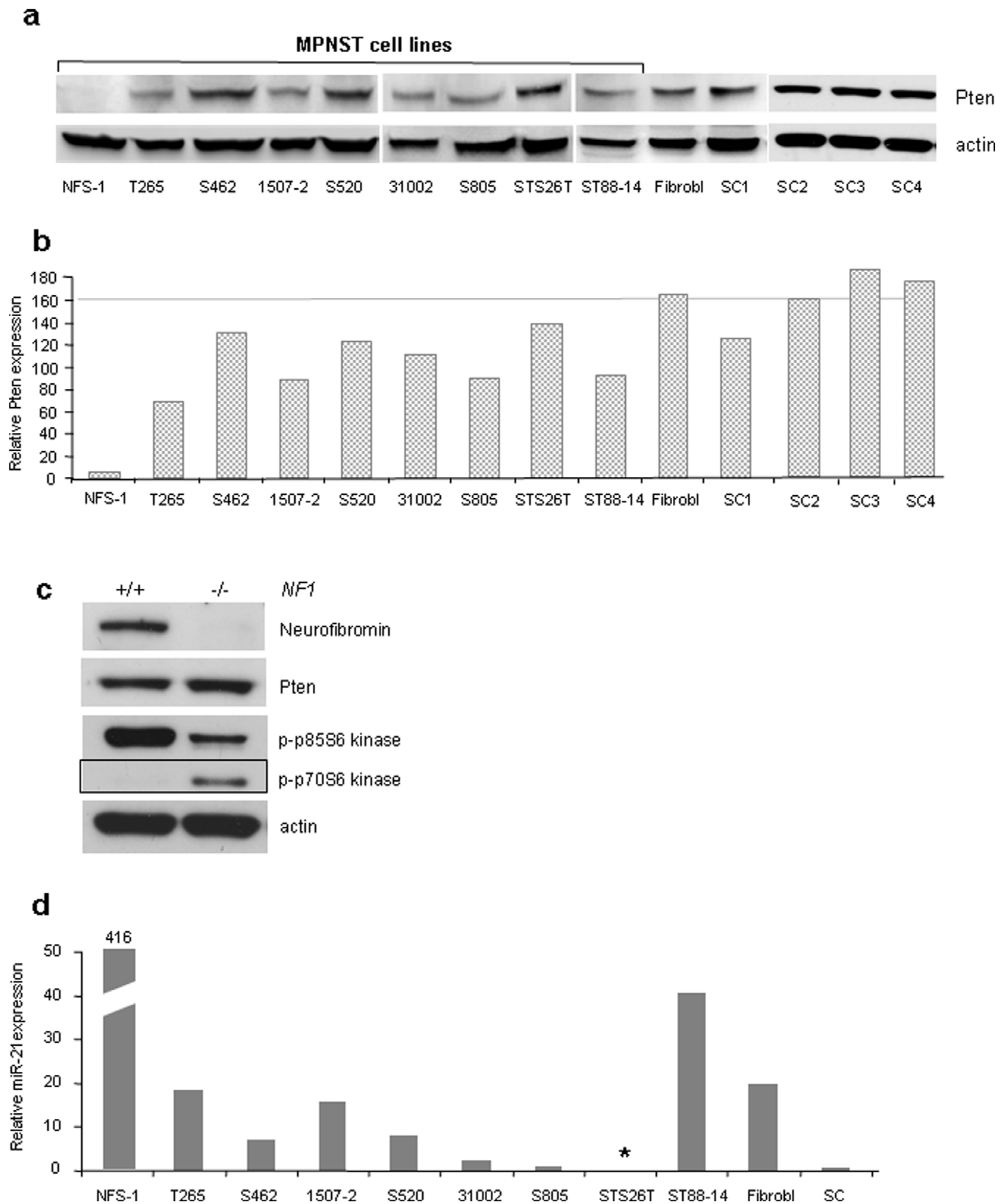

Figure 3. Western blot analysis of Pten and p-p70S6 kinase (isoform p-p70 of the S6 kinase, indicates mTOR activation) and examination of miR-21 expression by real time PCR a) Pten analysis of MPNST cell lines, dermal fibroblasts and neurofibroma derived Schwann cell cultures (SC1-4). The grey line indicates mean Pten expression of 4 Schwann cell cultures. b) Quantification of Pten expression (normalized with β-actin). c) Expression of Pten and p-p70S6 kinase in *NF1* positive (+/+) and *NF1* negative (−/−) Schwann cells. d) Relative expression of miR-21 as determined by real time PCR. * Endogeneous control RNU44 was not detectable. This cell line was thus omitted from analysis. SC = neurofibroma derived Schwann cells.
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only 14 tumours with clinical data and revealed no significant correlation with metastasis. All MPNST that were analysed with more than one method are listed in Table S1.

Moreover, we analysed 18 cell cultures comprising 9 neurofibroma cultures from NF1 patients, one Schwann cell culture of nervus suralis (non-NF1 patient) and 8 MPNST cell lines. DNA samples from cultures of benign tissues were unmethylated. In contrast, 7 out of 8 MPNST cell lines contained methylated DNA (median 71%, range 12–90%).

RNA of high quality was available from 6 solid MPNST, 9 neurofibromas and several cell cultures (9 MPNST cell lines; *NF1−/−* Schwann cells and dermal fibroblasts). The samples were examined by real-time PCR with a *PTEN* specific minor groove–binding (MGB) probe. *PTEN* transcript expression did not correlate well with *PTEN* promotor methylation and Pten protein levels. Thus, we hypothesized that post transcriptional mechanisms may be involved in Pten regulation. Because microRNAs (miR) are well known regulators of Pten we determined expression of miR-21 and miR-217. miR-21 showed an inverse correlation ($p = 0.015$; Pearson correlation) with Pten protein levels in MPNST cell lines (Figure 3d). miR-217 was not detectable in neurofibroma derived Schwann cells and the majority of MPNST cell lines (data not shown).

Pten Expression and Sensitivity to Rapamycin

Next, we wanted to know whether the Pten expression status of MPNST cell lines would correlate with their sensitivity to the mTOR inhibitor rapamycin. Using 5 MPNST cell lines and dermal fibroblasts we observed a dose dependant inhibition of proliferation (Figure 4a). The IC50 of rapamycin was around 10 nM for most MPNST cell lines. Highest sensitivity to rapamycin was observed in Pten negative NFS-1 cells (<10 nM) and lowest sensitivity was present in dermal fibroblasts (IC50>100 nM). The correlation of Pten expression and sensitivity to rapamycin was not significant (Figure 4b; $p = 0.06$; Pearson correlation).

Synergistic Effect of Rapamycin and Simvastatin

Because statins affect the activity of Ras superfamily members and have been shown to mediate a growth inhibitory effect on MPNST cells we evaluated whether the combination of rapamycin and simvastatin would be synergistic. To find a simvastatin concentration that mediates just a mild anti-proliferative effect as single drug, we tested different concentrations of 0.1 µM, 1.0 µM, and 10 µM. The IC50 of simvastatin on MPNST cell lines ranged between 0.1–1.0 µM. However, a concentration of 10 µM simvastatin was necessary to inhibit fibroblast growth by 50%. Combined treatment approaches with low drug doses were performed (0.1 µM simvastatin and 1.0 nM rapamycin). The results show a synergistic effect of this drug combination in 4 of 5 cell lines (Figure S2a). Because we previously observed an inhibitory effect of the multi-tyrosine kinase inhibitor sunitinib on MPNST cell lines [17] we wanted to analyse sunitinib in combination with rapamycin and simvastatin. A synergistic effect was, however, not achieved when we combined sunitinib with either simvastatin or rapamycin (Figure S2b & c). These combinations actually led to an antagonistic effect in about half of the cell lines.

Discussion

The aim of this study was to evaluate Pten alterations in human MPNST and neurofibroma and possible consequences for treatment with mTOR inhibitors. We demonstrate that Pten expression in MPNST is significantly lower than in neurofibroma indicating that its down-regulation could contribute to malignant progression.

Because Pten is a key regulator of the Akt/mTOR pathway, the Pten status may impact the therapeutic success of mTOR inhibiting drugs. Previously it was shown that MPNST cell lines are sensitive to mTOR inhibitors in vitro and in vivo [9,21,22]. The IC50 of the tested mTOR antagonists (rapamycin and everolimus) was around 10 nM. However, beside NF1 status, the cell lines used in these studies were not examined for other molecular alterations that might cause activation of the Akt/mTOR pathway. We hypothesized that loss of Pten expression could contribute to activation of this pathway and that patients with Pten-deficient tumours might particularly profit from treatment with mTOR inhibitors. In the 5 MPNST cell lines tested no significant correlation between Pten levels and rapamycin sensitivity was detected.

In contrast to solid MPNST, which generally harbour only few Pten positive cells (Fig. 1a) most MPNST cell lines maintain Pten expression although on a lower level compared to Schwann cells (Fig. 3a and b). A possible explanation for this finding is senescence induction by complete loss of *PTEN* expression [24]. Thus, partial Pten expression may have a selective advantage over a complete loss at least in the absence of further mutations able to prevent senescence induction [24].

An impact of Pten status on sensitivity to mTOR inhibitors has been observed in *PTEN*-deficient tumour cells and *Pten*-deficient MEFs, which were both more sensitive to mTOR inhibitors than corresponding Pten positive cells [23]. Another study demonstrated that rapamycin enhanced the sensitivity of Pten-deficient glioblastoma cells to treatment with the EGFR inhibitor erlotinib [25]. Moreover, the rapamycin derivate everolimus radiosensitized Pten-deficient prostate cancer cells more than wildtype cells [26]. Our data could not support a clear correlation of Pten expression levels and sensitivity to rapamycin. However, since MPNST are not homogeneous tumours and may harbour different genetic alterations that might influence the Akt/mTOR pathway further studies are needed to clarify the impact of Pten status for therapeutic strategies.

Combination of Drugs

Although mTOR inhibitors show good effects in vitro, in vivo studies demonstrated their limitation as a single agent. A major problem of targeted therapy is the capability of cancer cells to use alternate pathways for survival and proliferation thereby escaping from specific inhibitors. This problem may be circumvented by either targeting the driving mutation (if there is one) or by drug combinations. Here, we tested different combinations of drugs (Figure S2) and found that the combination of rapamycin and simvastatin yielded best results. Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor, is commonly applied as cholesterol-lowering drug. Anti-tumour effects of statins have been described [27,28,29]. The anti-proliferative and pro-apoptotic effects of statins are mediated by multiple mechanisms including blockage of posttranslational modification (prenylation) of Ras superfamily members. Statins have already been shown to improve NF1 symptoms like learning deficits [30], bone healing [31] and to inhibit proliferation of an MPNST cell line [32]. However, simvastatin's exact mode of action on MPNST cell lines needs further analysis in future studies.

We and others found only a weak additive effect when mTOR inhibitors were combined with receptor tyrosine kinase (RTK) inhibitors (e.g. sunitinib and erlotinib) [21]. Thus, inhibition of

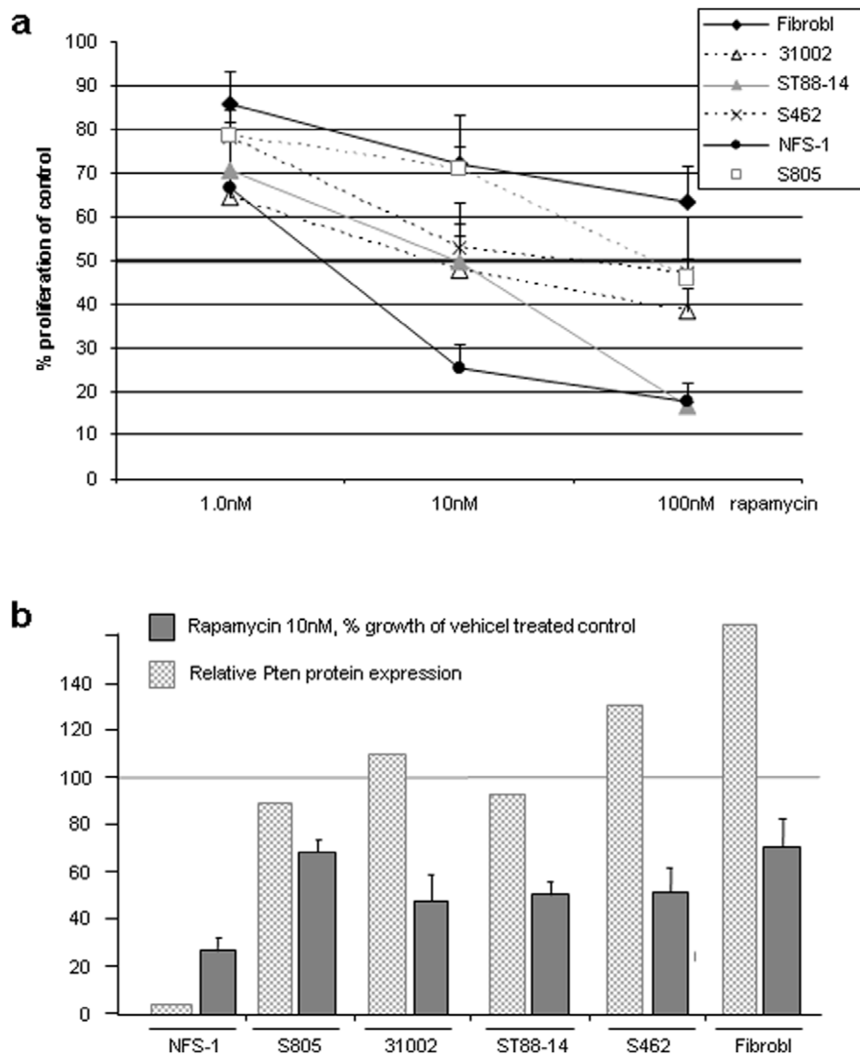

Figure 4. Effect of rapamycin on MPNST cell lines and fibroblasts. a) Dose dependent inhibition of proliferation after 4 days of treatment. b) Pten protein levels correlate with rapamycin sensitivity.
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signalling molecules downstream of RTKs might be more effective because i) multiple RTKs signal via the same downstream cascades and ii) these pathways may be constitutively activated due to mutations in more than one molecule.

Significance of Pten in Neural Tissue

Pten is, like neurofibromin, most strongly expressed in the central and peripheral nervous system (PNS). However, the exact cell types expressing Pten in the PNS were not assessed. Here we show that Pten is strongly expressed in axons and Schwann cells. Subtle reduction in Pten expression could predispose to tumourigenesis in a tissue-specific manner. Neoplasms most commonly observed in NF1 patients are nerve sheath tumours, which originate from Schwann cell precursors. It is tempting to speculate that cell types with strong basal Pten expression are particularly in danger of undergoing malignant transformation upon Pten loss or down-regulation. Pten deficiency is frequently observed in primary glioblastomas but not in astrocytomas grade I-III, underlining its role in malignant progression [33]. Since the mTOR pathway is already activated in NF1-associated tumours due to a lack of *NF1*, it is likely that further activation of the Akt/mTOR pathway due to Pten loss promotes tumour progression in this setting. In animal studies, it was shown that Pten loss altered brain development [34,35,36] and caused nerve sheath tumour formation [10,37].

Pten Regulation

Regulation of Pten expression is complex and may be caused by multiple mechanisms including allelic loss, point mutations, epigenetic silencing, miRs, protein stability (interaction with other proteins) and ubiquitination [24]. Previously, we found reduced *PTEN* gene dosage in more than half of MPNST [7]. Here, we screened for *PTEN* mutations, determined promotor methylation, and the expression of Pten-regulating miRs. Finally, we correlated these data with Pten transcription and protein levels. We can exclude, that *PTEN* mutations play a major role in its regulation or function. Nevertheless, our previous finding of frequent mono-allelic loss in MPNST is likely to account for decreased Pten expression. *PTEN* haploinsufficiency or even subtle Pten down-regulation by 20% has been shown to promote tumour development or progression [38,39]. It was therefore proposed that loss of Pten may be regarded as a continuum rather than a stepwise process [24].

A gene dosage effect in tumour development is also known from patients with PTEN harmatoma tumour syndrome, which is caused by a mono-allelic *PTEN* germ line mutation. Tumours from these patients do not always carry *PTEN* mutations in the second allele [40].

Moreover, *PTEN* promotor methylation correlated significantly with development of metastasis. However, since clinical data was only available for 21 MPNST and metastasis development is influenced by many different factors our finding needs to be confirmed in a larger set of well characterized patients.

A correlation between promotor methylation and transcription was less clear. A possible explanation may be the small set of tumours for which both, DNA and RNA, was available. Moreover, the effect may be masked by other Pten-regulating mechanisms. Since Pten protein and transcript levels did not correlate well in all tumours additional posttranscriptional mechanisms may play a role in Pten regulation. miRs interfere with transcript translation and have been reported to play an important role in Pten regulation [41]. We observed an inverse correlation between miR-21 and Pten protein levels (Fig. 3b & d). miR-21 is among the most commonly and dramatically upregulated miRs in many cancers and is a well known Pten regulator [41,42]. Thus, our data point to a possible role of miR-21 in the regulation of Pten regulation in nerve sheath tumours. However, this issue needs further evaluation and should include larger panels of primary tumours. Taken together, our data suggest that a combination of multiple mechanisms leads to altered Pten expression in nerve sheath tumours.

In summary, accumulating evidence supports the assumption of Pten being an important player in MPNST development. Whether determination of Pten expression status in MPNST might assist in refining therapy needs further evaluation in clinical studies. However, we suggest that approaches with mTOR inhibitors in combination with other agents (e.g. conventional chemotherapy or statins) could improve therapeutic success.

Supporting Information

Figure S1 Lack of *Nf1* does not alter Pten expression but activates the Ras/MAPK and mTOR pathway. a) MEFs of the indicated genotype were kept under different conditions and analysed by western blot. The boxes mark culture conditions, where differences between the two genotypes are most pronounced. b) The assay was repeated with slightly different culture conditions. The significance of *Nf1* status for mTOR activation was determined as the ration between p-mTOR to total mTOR. All samples were tested in duplicates.
(TIF)

Figure S2 Anti-proliferative effect of different drugs on MPNST cell lines and fibroblasts. a) simvastatin, rapamycin and the combination of both drugs b) rapamycin, sunitinib and the combination of both drugs c) simvastatin, sunitinib and the combination of both drugs
(TIF)

Figure S3 Kaplan-Meier curve shows metastasis free time in patients with promotor methylation$<29\%$ and $\geq 29\%$.
(TIF)

Figure S4 Nuclear Pten localization in MPNST and neurofibroma was determined by immunohistochemistry. Each dot represents one tumour. The difference of nuclear Pten in MPNST and neurofibroma was not significant ($p = 0.1$, unpaired t-test).
(TIF)

Table S1 Molecular analysis and clinical data of MPNST patients.
(DOCX)

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Author Contributions

Conceived and designed the experiments: NH CH FH M. Bradtmöller AD. Performed the experiments: M. Bradtmöller CH JZ SJ SP DR NH. Analyzed the data: NH AK VM AD M. Bradtmöller. Contributed reagents/materials/analysis tools: VM AD CH DR AH. Wrote the paper: NH M. Baier.

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11. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

12. Publikationsliste

Park SJ, Sawitzki B, Kluwe L, Mautner VF, Holtkamp N, Kurtz A. *Serum biomarkers for neurofibromatosis type 1 and early detection of malignant peripheral nerve-sheath tumors.* BMC Med. 2013 Apr 23;11:109.

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