TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Molecular Analysis of Hybrid Neurofibroma/Schwannoma Identifies Common Monosomy 22 and α -T-Catenin/CTNNA3 as a Novel Candidate Tumor Suppressor

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Neurofibromas and schwannomas are benign Schwann cell—derived peripheral nerve sheath tumors arising sporadically and within neurofibromatosis. Multiple tumors are a hallmark of neurofibromatosis type 1 (NF1) and type 2 (NF2) and schwannomatosis. Neurofibromas in NF1 and schwannomas in NF2 or schwannomatosis are defined by distinctive molecular hits. Among these, multiple hybrid neurofibromas/schwannomas may also appear, not yet being defined by a molecular background. We therefore performed molecular analysis of 22 hybrid neurofibromas/schwannomas using array comparative genomic hybridization, immunohistochemistry, quantitative RT-PCR, and functional analyses of cultured Schwann cells. Furthermore, we analyzed *SMARCB1* by fluorescence *in situ* hybridization and multiplex ligation-dependent probe. Monosomy 22 was identified in 44% of tumors of tested patients with hybrid neurofibromas/schwannomas. In addition, in a single case, we detected focal deletion of the α -T-catenin/CTNNA3 gene (10q21.3). To further characterize this candidate, transient knockdown of α -T-catenin in Schwann cells was performed. CTNNA3 depleted cells showed cytoskeletal abnormalities and reduced E-cadherin expression, indicating epithelial-mesenchymal transition-like abnormalities. To conclude, we uncovered loss of chromosome 22 in almost half of all cases with hybrid neurofibromas/schwannomas of patients with multiple peripheral nerve sheath tumors. We tagged α -T-catenin/CTNNA3 as a novel candidate gene. Our functional investigations might indicate involvement of α -T-catenin/CTNNA3 in the biology of peripheral nerve sheath tumors. (*Am J Pathol* 2016, 186: 3285–3296; <http://dx.doi.org/10.1016/j.ajpath.2016.08.019>)

Neurofibromas, schwannomas, and hybrid neurofibromas/schwannomas are benign peripheral nerve sheath and Schwann cell—derived tumors arising sporadically and within tumor syndromes, namely neurofibromatosis type 1 (NF1) and type 2 (NF2) and schwannomatosis.^{1,2} Those three variants differ by genetic background and are associated with risk of malignant transformation in NF1.³ These tumors should be carefully distinguished to improve assignment to the different types of neurofibromatosis.

Although molecular data of neurofibromas and schwannomas are available, no molecular investigation of hybrid neurofibromas/schwannomas has been published, to the best of our knowledge. The latter are defined by intermingled

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areas of both typical neurofibroma- and schwannoma-like areas (Figure 1). Neurofibroma-like areas often ensheath the schwannoma-like ones. Both areas fulfill histopathological criteria of either neurofibroma or schwannoma. Neurofibroma-like areas need to be carefully distinguished from Antoni B regions. Tumors may exhibit a plexiform pattern and often contain larger lymphocytic infiltrates. In our experience, these tumors express SWI/SNF chromatin remodeling complex subunits SMARCB1/INI1, SMARCC2/BAF170, and SMARCA4/BRG in a mosaic pattern (<100% immunopositive nuclei) and there is no evidence of a differential expression in neurofibroma- or

schwannoma-like parts (Supplemental Figure S1). Interestingly, expression of SMARCB1/INI1 and SMARCA4/BRG was significantly higher than expression of SMARCC2/BAF170.⁴ SMARCC2/BAF170 protein expression was low in neurofibromas, peripheral schwannomas, vestibular schwannomas, and malignant peripheral nerve sheath tumors (MPNSTs), but the purpose of reduced BAF170 expression remains unclear because mutations and methylation of *SMARCC2/BAF170* were not identified in those tumors with a low protein expression.⁵ Immunohistochemical expression of SWI/SNF chromatin remodeling complex subunits did not indicate an association to the specific histologic pattern of the two different parts. It is therefore unclear whether the two different parts carry genetic events similar to neurofibromas or schwannomas or if other genetic mechanisms explain their phenotype.

Neurofibromas consist of a mixture of Schwann cells, fibroblasts, perineural cells, and mast cells, and represent a hallmark of NF1, although they may also occur sporadically. In NF1-associated neurofibromas, it has been demonstrated that only Schwann cells harbor two genetic hits (germline and somatic) of the *NF1* tumor suppressor gene (17q11.2). The second/somatic *NF1* hit varies between tumors.⁶ Interestingly, haploinsufficient (*NF1*^{+/-}) microenvironment (eg, mast cells and fibroblasts) is required for initiation of neurofibroma development in mouse models. However, this seems not to be relevant in sporadic tumors.^{7,8} In contrast, schwannomas are nearly completely composed of transformed tumor Schwann cells and show a different molecular setting. Both sporadic and NF2-associated schwannomas typically lose both copies of the *NF2* gene (22q12.2).⁹⁻¹¹ In NF2 patients, the second hit (accompanying the germline hit) comprises small-scale mutations, loss of heterozygosity, and monosomy 22, but only rarely methylation-induced gene inactivation.¹²⁻¹⁵ In particular, loss of 22q is a consistent finding in up to 57% of vestibular schwannoma.^{15,16} Interestingly, schwannomas occur within schwannomatosis, a disease defined by peripheral signs of NF2 and lack of vestibular schwannoma. Schwannomatosis-associated schwannomas exhibit four genetic events: biallelic inactivation of the *NF2* gene (often *NF2* gene mutation plus 22q deletion) and mutations of the SWI/SNF chromatin remodeling subunit *SMARCB1/INI1* (22q11.23) on both alleles. The latter are causative for schwannomatosis in 33% of familial cases.¹⁷⁻¹⁹ Recently, this four-hit model (*NF2*^{-/-}, *SMARCB1/INI1*^{-/-}) has become doubtful because *LZTR1* mutations (22q11.21) have been detected as a predisposing germline mutation event in schwannomatosis patients who are negative for *SMARCB1/INI1* mutations.²⁰⁻²³ Nevertheless, schwannomatosis seems to arise from various genetic abnormalities, as suggested by a novel finding of a *COQ6* mutation and furthermore by evidence of other still unknown involved genes.^{23,24}

We analyzed material of patients experiencing multiple peripheral nerve sheath tumors in the setting of NF2, schwannomatosis, or a still undefined tumor syndrome. We

Figure 1 **A:** Hybrid neurofibromas/schwannomas contain intermingled areas of both typical neurofibroma and typical schwannoma. Grossly, these tumors are encapsulated and are white-gray on cut sections with alternating dark (solid arrow) and pale areas corresponding to the schwannoma-like and neurofibroma-like areas. **D:** Whole mount of the same tumor reflects this pattern in hematoxylin and eosin staining. The neurofibroma-like part is marked with an asterisk (**A**, **D**, and **E**). Representative details of this tumor are given in **B**, **C**, and **E** (dotted arrows show the original site in gross section): a schwannoma-like nodule (20-fold magnification; **B**), a neurofibroma-like part (20-fold magnification; **C**), and a mixed area (10-fold magnification; **E**). Scale bar = 10 mm (**A**).

focused on the subtype of hybrid neurofibroma/schwannoma because nothing is known about their molecular biology in those patients. We aimed to elucidate distinctive molecular mechanisms arising from this particular mixture of intermingled neurofibroma and schwannoma. Using an extensive approach, we searched for novel genetic events in hybrid neurofibroma/schwannoma using fluorescence *in situ* hybridization (FISH), multiplex ligation-dependent probe (MLPA), and array comparative genomic hybridization (aCGH) extended by functional experiments of nontumor Schwann cells as the supposed tumor-initiating cell type.

Materials and Methods

Tumor Specimens and Patients

We analyzed patients with multiple peripheral nerve sheath tumors and focused on the subtype of hybrid neurofibromas/schwannomas. In 6 of 16 patients, a tumor syndrome was diagnosed (eg, NF2 and possible schwannomatosis). Five of 16 patients experienced multiple hybrid neurofibromas/schwannomas. We selected 16 patients with hybrid neurofibromas/schwannomas contributing 31 tumors from our archives. The age of patients with hybrid neurofibromas/schwannomas ranged from 15 to 70 years at time of diagnosis (median, 42.8 years). The size of formalin-fixed, paraffin-embedded (FFPE) tumors was 0.5 to 5 cm in diameter. All but one tumor did not show signs of atypia or malignancy. According to the 2016 World Health Organization classification of tumors of the central nervous system, neurofibromas and schwannomas correspond to World Health Organization grade I. Interestingly, hybrid neurofibromas/schwannomas are not defined by a grade, although World Health Organization grade I could be useful.²⁵ According to the World Health Organization classification of soft tissue tumors, those tumors are not graded.²⁶ One hybrid neurofibroma/schwannoma (number 16) revealed focal transition to a low-grade MPNST. FFPE specimens from lymph node, sciatic nerves, liver, skeletal muscle, kidney, and fetal brain served as controls. In addition, 15 FFPE peripheral schwannomas, 16 FFPE vestibular schwannomas, 15 FFPE neurofibromas, and 12 FFPE MPNSTs were selected for analysis. DNA was isolated according to QIAamp DNA FFPE Tissue Kit protocol (number 56404; Qiagen, Hilden, Germany) from entire tumors as well as separately from neurofibroma- and schwannoma-like areas by microdissection in patients 1, 5, and 9.

Immunohistochemistry, Immunocytochemistry, and FISH

FFPE sections were subjected to immunohistochemistry using a rabbit polyclonal anti-CTNNA3 antibody (1:500; Sigma-Aldrich, St. Louis, MO) and a mouse monoclonal anti-S100 (1:1000; Ventana, Tucson, AZ). Slides were incubated with a biotinylated secondary antibody, streptavidin

peroxidase, and diaminobenzidine using the Dako REAL Detection system for automated immunostaining (Dako REAL Detection system, peroxidase/diaminobenzidine⁺, rabbit/mouse, code K5001; Dako Denmark A/S, Glostrup, Denmark). To perform immunocytochemistry, Schwann cells were cultured on poly-L-lysine-coated chamber slides, transfected with siRNA (Silencer Select Pre-designed siRNA s168107; Ambion, Carlsbad, CA), and incubated with an Alexa Fluor 546-labeled phalloidin antibody (Life Technologies, Carlsbad, CA) to stain F-actin. A confocal microscope (510 meta laser scanning microscope; Zeiss, Oberkochen, Germany) was used for evaluation. Interphase FISH was performed on FFPE material, analyzed by Axioscope, Axioscope-2, and Axio Imager A1 fluorescence microscopes (Zeiss) and documented using an ISIS imaging system (MetaSystems, Althausheim, Germany).²⁷ Triple-color probes were applied, and probe *CEP6* (Abbott Molecular, Des Plaines, IL, labeled in spectrum aqua) served as control.²⁸

Quantitative Real-Time and RT-PCR

Total RNA and DNA were extracted from FFPE samples using the Maxwell 16 LEV Blood Kit (Promega, Madison, WI) and from Schwann cells using the GenElute Mammalian Total RNA Kit (Sigma-Aldrich), according to the manufacturer's protocol. Quantification was performed by NanoDrop ND1000 (peQLab Biotechnologies, Erlangen, Germany). For cDNA synthesis, 0.1 to 0.5 µg/µL RNA was converted using cDNA synthesis kit (High Capacity cDNA Reverse Transcription kit; Applied Biosystems, Waltham, MA). Quantitative RT-PCR (RT-qPCR) was performed with SYBR Green Mastermix (Applied Biosystems). DNA dilution of 0.1 µg/µL was applied for quantitative PCR (qPCR) of *CTNNA3* exon 7, and peripheral blood of healthy donors served as control ($n = 2$). Data were normalized against glyceraldehyde 3-phosphate dehydrogenase (12p13) as internal control and analyzed by $\Delta\Delta CT$ method. Final quantitative data are given as mean values of three independent experiments $\pm$ SD. For primer sequences, see [Tables 1 and 2](#).

Purification of Schwann Cells and Cell Culture

Sciatic nerves were dissected from adult Wistar rats and collected in Hank's balanced salt solution. Nerve segments were incubated at 5% CO₂. Schwann cell medium was removed and replaced by a medium containing 1.25 µL/mL collagenase (Roche Diagnostics GmbH, Mannheim, Germany) and 50 µL/mL dispase II (Roche Diagnostics GmbH) and afterwards incubated. After 24 hours, nerves were triturated and centrifuged, and pellet was resuspended in Schwann cell medium. Cell suspension was plated on flasks (Falcon) coated with 1.875 ng/cm² poly-L-lysine (Sigma, St. Louis, MO) and kept at 5% CO₂. To separate Schwann cells from fibroblasts, we repeatedly used modified cold jet.²⁹ Purity of cultures was assured to be >95% by immunocytochemistry (anti-S100 antibody). Rat Schwann cells were

Table 1 Promoter Methylation Analysis (MSP), Primer Sequences

Gene	Direction	Sequence	T _m (°C)	Product length (bp)
<i>CTNNA3</i> (methylated)	Forward	5'-TGTGTTTGGTTTTTTTTAACGA-3'	55.6	152
	Reverse	5'-ACAATCAATTTCGCTAAACGTT-3'		
<i>CTNNA3</i> (unmethylated)	Forward	5'-GGTAAAGTATGTGTTTGGTTTTTTTTAAT-3'	54.4	161
	Reverse	5'-ACAATCAATTTCACAAACATT-3'		

MSP, methylation-specific PCR; T_m, melting temperature.

cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% penicillin, 1% streptomycin, 2 µmol/L forskolin (Calbiochem, La Jolla, CA), and 10 ng/mL heregulin (R&D Systems, Minneapolis, MN). Normal human Schwann cells (ScienCell; Provitro AG, Berlin, Germany) were cultivated in Schwann cell medium containing 10% fetal calf serum, 1% penicillin, 1% streptomycin, 1 mmol/L sodium pyruvate, 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma), 10 ng/mL heregulin (R&D Systems), 2.5 µg/mL insulin (VWR, Darmstadt, Germany), and 2.5 mg/L fungizone (Invitrogen, Thermo Fisher Scientific, Waltham, MA). To achieve enrichment, mild trypsinization was performed. MPNST cell lines S462, T265, and NSF1 and fibroblast cell line 1.BR.3.GN (European Collection of Cell Cultures, Public Health England, London, UK) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% penicillin, and 1% streptomycin.

Western Blot Analysis

Cells were lysed with radioimmunoprecipitation assay buffer containing a protease inhibitor and transferred to microcentrifuge tubes, incubated on ice, homogenized, and centrifuged. Supernatant containing whole cell protein extract was denatured, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane for electrophoresis. Detection was performed with Luminata Forte Western horseradish peroxidase substrate (Millipore, Darmstadt, Germany). Anti-β-actin polyclonal antibody (Sigma-Aldrich) was applied as control. Image Lab software version 4.1 (BioRad, Hercules, CA) was applied for quantification.

Methylation-Specific PCR

EZ DNA Methylation-Gold Kit (ZYMO Research, Irvine, CA) was applied for analysis of α-T-catenin/*CTNNA3*. DNA amplification was performed using two sets of primers (specific for methylated and unmethylated sequences). We investigated the promoter sequence -111 to 50 (chromosome 10: 69456057-69455927 for methylated and chromosome 10: 69456066-69455927 for unmethylated DNA). For primer sequences, see Table 1.

MLPA and Sanger Sequencing

Four hybrid neurofibromas/schwannomas of three different patients were suitable to allow separate DNA isolation

from neurofibroma- and schwannoma-like areas. MLPA amplification and Sanger sequencing of separate parts were applied to analyze *SMARCB1/INI1*, as described previously.²⁸ For Patient 9, mutation analysis of *NF1* and *NF2* genes was performed by Sanger sequencing and MPLA (P081; P082, P044; MRC Holland, Amsterdam, the Netherlands) on DNA from peripheral blood leukocytes with normal results. Mutation analysis of *SMARCC2/BAF170* of FFPE tumor samples was performed using DNA Clean & Concentrator-25 Kit (Zymo, Freiburg, Germany), according to the manufacturer's protocol, and Sanger sequencing using Big Dye terminator v3.1 (Applied Biosystems). Data were evaluated with DNA star (SeqMan II).

aCGH

aCGH was performed using SurePrint G3 Human CGH Microarray Kit, 4 × 180 K (Agilent, Santa Clara, CA), according to manufacturer's protocols. Between 600 ng and 1 µg of DNA from the patient and 1 µg of sex-matched reference DNA from a pool of healthy donors with a normal karyotype were hybridized. Slides were scanned with the G2565CA Microarray Scanner (Agilent) at 5 µmol/L resolution. Signal

Table 2 Real-Time PCR of Target Genes in Rat Schwann Cells (T_m at 59°C), Primer Sequences

Gene	Direction	Sequence	Product length (bp)
<i>GAPDH</i>	Forward	5'-GTTACCAGGGCTGCCTTCTC-3'	168
	Reverse	5'-GGGTTTCCCGTTGATGAC-3'	
<i>CTNNA3</i>	Forward	5'-ATCCACCCTGCAACAAGAC-3'	142
	Reverse	5'-GAACTCTGGATGCGGATGAT-3'	
<i>CTNNB1</i>	Forward	5'-TGCTGAAGGTGCTGTCTGTC-3'	195
	Reverse	5'-GCTGCACTAGAGTCCCAAGG-3'	
<i>CDH2</i>	Forward	5'-CAGAGAGTCGCCAAATGTCA-3'	163
	Reverse	5'-TTCACAAGTCTCGGCCTCTT-3'	
<i>CDH1</i>	Forward	5'-CAGCGACTGGTTCAGATCAA-3'	228
	Reverse	5'-TATCTCCATGGGATCCTCCA-3'	
<i>SNAI1</i>	Forward	5'-AGCCCAACTATAGCGAGCTG-3'	150
	Reverse	5'-CCAGGAGAGAGTCCCAGATG-3'	
<i>SNAI2</i>	Forward	5'-AACACACACTGGGGAAAAGC-3'	179
	Reverse	5'-AGCAGCCAGACTCCTCATGT-3'	
<i>ZEB1</i>	Forward	5'-GATGACGAATGCGACTCAGA-3'	182
	Reverse	5'-AACTGGGAAAATGCATCTGG-3'	
<i>ZEB2</i>	Forward	5'-CACAGCTCTTCCACCACAGA-3'	242
	Reverse	5'-GGCCCGACATGTAGTCTTGT-3'	

T_m, melting temperature.

Table 3 Molecular Characteristics of Confirmed Hybrid Neurofibromas/Schwannomas Investigated in this Study

Patient	Sex	Tumor	aCGH analysis	α -T-catenin/ <i>CTNNA3</i>			<i>SMARCB1/INI1</i>		
				DNA $\pm$ SD (%)	RNA	Protein	FISH	MLPA	Sequencing
1 (NF2)	M	1a	NA	5 $\pm$ 0.19	(+)	NA	20% A. S.	Normal	Wild type
		1b, plex.	Constitutional loss of 10q21.3, somatic gain of 1q44	46 $\pm$ 18.7	(+)	+++	Normal	Normal	Wild type
2 (NF2)	M	2, plex.	No alteration	59.6 $\pm$ 9.7	(+)	+++	NA	NA	NA
3	M	3	No alteration	69.1 $\pm$ 10.6	(+)	+	NA	NA	NA
4	F	4	No alteration	68.6 $\pm$ 10.2	(+)	++	NA	NA	NA
5	F	5a	NA	69 $\pm$ 6.4	++	++	Normal	Normal	Wild type
		5b	NA	NA			Normal	Normal	Wild type
		5d	Partial monosomy 22: loss 22q11.21-q13.33, loss of 16p13.13, gain of 22q11.1-q11.21	NA		++	NA	NA	NA
6	F	6	Monosomy 22: loss of 22q11.1 - q13.33	76.8 $\pm$ 12.9	++	++	NA	NA	NA
7	M	7	Gain of 3p22.1	90.8 $\pm$ 26.7	(+)	+	NA	NA	NA
8		8	No alteration	120.7 $\pm$ 26.5	(+)	++	NA	NA	NA
9 (TuS)*	M	9a, plex.	Monosomy 22: loss of 22q11.1 - q13.33, gain of 8q22.3	80.4 $\pm$ 19.6	++	++	NA	NA	NA
		9b, plex.	NA	80.2 $\pm$ 1.4	++	+	Normal	Deletion	Wild type
		9c, plex.	NA	131.5 $\pm$ 19.1	++	++	Normal	Normal	Wild type
		9d	NA	102.1 $\pm$ 23.6	++	NA	NA	NA	NA
		9e, plex.	NA	101.4 $\pm$ 9.3	+++	++	NA	NA	NA
		10b, plex.	NA	62.1 $\pm$ 3	(+)	+++	Normal	Normal	Wild type
10 (NF2)	M	10c, plex.	Monosomy 22: loss of 22q11.1 - q13.33	NA	(+)	NA	NA	NA	NA
		10d, plex.	NA				20% A. S.	Normal	Wild type
		11	Partial monosomy 22: 22q11.21 - q13.33, gain of 22q11.1 - q11.21	59 $\pm$ 15.6	(+)	++	NA	NA	NA
12	M	12	Loss of 3q22.3	70.2 $\pm$ 5.2	(+)	++	NA	NA	NA
13	F	13	Monosomy 22: loss of 22q11.1 - q13.33	152.5 $\pm$ 23.3	(+)	+	NA	NA	NA
14		14	Partial monosomy 22: loss of 22q11.21 - q13.33	142.7 $\pm$ 47.3	++	++	NA	NA	NA
		15	No alteration	NA	NA	NA	NA	NA	NA
16 [†]	F	16	Suspicious hyperploidy with gains and losses of whole chromosomes	NA	NA	NA	NA	NA	NA

Only tumors of patients with molecular analyses are shown: tumors were either investigated by aCGH ($n = 16$) or by FISH/MLPA/sequencing for *SMARCB1* alterations ($n = 8$). All losses/gains detected by aCGH are somatic ($\log_2$ ratios), only loss of 10q21.3 displays a presumably constitutional/germline deletion (Table 4). In 7 of 16 tumors, monosomy 22 was detected by aCGH, which results in heterozygous loss of *LZTR1*, *SMARCB1*, and *NF2*. Because a deletion of *SMARCB1* was additionally found in tumor 9b by MLPA/sequencing, altogether 8 of 22 tumors presented loss of *SMARCB1*, and 7 of 22 tumors showed loss of *LZTR1* and *NF2*.

Quantitative PCR data are given as mean values of three individual experiments $\pm$ SD, and relative *CTNNA3* DNA quantity of tumors with marked reduction defined by values below the lower border of the 95% CI are displayed in boldfaced text. Results of quantitative RT-PCR were obtained using the Hs_CTNNA3_1_SG QuantiTect primer assay, generating a short 99-bp mRNA fragment and demonstrating low *CTNNA3* relative mRNA in 56% of hybrid neurofibromas/schwannomas and moderate levels in 39% (means $\pm$ SD, 24.8% $\pm$ 4.9% to 57.7% $\pm$ 10.5%). Protein expression was evaluated by immunohistochemistry (α -T-catenin).

Semiquantitative results of mRNA quantification (real-time PCR) and immunohistochemical protein expression are demonstrated as follows: high, +++; moderate, ++; low, +; very low, (+).

*In this patient with multiple tumors, germline mutations of *NF1*, *NF2*, and *SMARCB1* were excluded.

[†]This tumor histologically shows areas of a low-grade malignant peripheral nerve sheath tumor.

F, female; M, male; aCGH, array comparative genomic hybridization; A.S., additional signals in FISH analysis; deletion, deletion of *SMARCB1* exon 1 to 9 in MLPA; FISH, fluorescence *in situ* hybridization; MLPA, multiplex ligation-dependent probe; NA, not analyzed; NF2, clinically safe diagnosis of neurofibromatosis type 2; plex., plexiform hybrid neurofibroma/schwannoma; TuS, clinically diagnosed as tumor syndrome or probable schwannomatosis.

intensities were measured and evaluated with Feature Extraction software version 10.10.11 and Agilent Genomic Standard Workbench Edition software version 6.5.0.58 (Agilent) applying the Aberration Detection Method-2 algorithm (threshold, 6.0). Aberrations already described as copy number polymorphisms (Database of Genomic Variant, Built GRCh37: February 2009, hg19) were excluded.

Knockdown of α -T-catenin/*CTNNA3* and MTT

siRNAs directed against α -T-catenin/*CTNNA3* (Silencer Select Pre-designed siRNA s168107; Ambion) were used for gene silencing. Transient transfection was performed via the cationic lipid reagent HiPerFect (Qiagen, Venlo, the Netherlands), according to manufacturer's protocol.

Table 4 Chromosomal Gains and Losses Detected by aCGH (SurePrint G3 Human CGH Microarray Kit, 4 × 180 K; Agilent)

No.	Type	Cytoband	Start*	End*	Length* (bp)	Gene
1b	Gain	1q44	Chr1: 246,825,218	Chr1: 246,899,327	74,109	<i>CNST</i> <i>SCCPDH</i>
	Loss	10q21.3	Chr10: 68,956,325	Chr10: 69,098,689	142,364	<i>CTNNA3</i>
5d	Loss	16p13.13	Chr16: 12,093,128	Chr16: 12,261,460	168,332	<i>RUNDC2A</i> <i>SNX29</i>
	Gain	22q11.1-q11.21	Chr22: 17,290,275	Chr22: 21,798,755	4,508,480	Multiple
	Loss	22q11.21-q13.33	Chr22: 21,917,200	Chr22: 51,186,249	29,269,049	Multiple
6	Loss	22q11.1-q13.33	Chr22: 16,133,474	Chr22: 51,186,249	35,052,775	Multiple
7	Gain	3p22.1	Chr3: 41,927,881	Chr3: 42,167,119	239,238	<i>ULK4</i> <i>TRAK1</i>
9	Gain	8q22.3	Chr8: 104,360,779	Chr8: 104,869,423	508,644	<i>CTHRC1</i> <i>SLC25A32</i> <i>DCAF13</i> <i>RIMS2</i>
	Loss	22q11.1-q13.33	Chr22: 16,133,474	Chr22: 19,838,109	3,704,635	Multiple
10	Loss	22q11.1-q13.33	Chr22: 16,133,474	Chr22: 51,170,223	35,036,749	Multiple
11	Gain	22q11.1-q11.21	Chr22: 17,309,519	Chr22: 20,306,819	2,997,300	Multiple
	Loss	22q11.21-q13.33	Chr22: 20,733,427	Chr22: 51,186,249	30,452,822	Multiple
12	Loss	3q22.3	Chr3: 136,358,545	Chr3: 136,429,465	70,920	<i>STAG1</i>
13	Loss	22q11.1-q13.33	Chr22: 16,133,474	Chr22: 50,869,615	34,736,141	Multiple
14	Loss	22q11.21-q13.33	Chr22: 20,733,427	Chr22: 51,186,249	30,452,822	Multiple
16	Gains/losses	Suspicious hyperploidy with gains and losses of whole chromosomes				

*Centromeric and telomeric regions not covered; hence, regions of gains and losses may be larger than indicated. Human reference sequence: Build GRCh37: February 2009, hg19.
aCGH, array comparative genomic hybridization; Chr, chromosome.

Non-specific siRNA (AllStars Negative Control siRNA; Qiagen) was used as control. Total RNA and protein were isolated at 24, 48, 72, 96, and 144 hours after transfection. Primary rat Schwann cells were seeded on a 96-well flat-bottom tissue culture plate with a density of 1 × 104 cells per well for 24 hours, transfected with siRNA and non-specific siRNA, and incubated with MTT solution for 2.5 hours. Absorption was measured with GloMax-multi (Promega).

Results

SMARCB1/INI1 Mutations Were Identified in One of Eight Hybrid Neurofibromas/Schwannomas

To identify possible molecular differences between the different parts of hybrid neurofibromas/schwannomas, we selected eight tumors from four patients (1a, 1b, 5a, 5b, 9b, 9c, 10b, and 10d) for FISH analysis of *SMARCB1/INI1* and investigated schwannoma- and neurofibroma-like parts separately. None of the selected areas harbored gene deletions (Table 3). Furthermore, MLPA and Sanger sequencing for *SMARCB1/INI1* was performed from each of the neurofibroma- and schwannoma-like parts. One of the six tumors contributed by three patients (5a, 5b, 9b, 9c, 10b, and 10d) showed a deletion of exon 1 to 9 of *SMARCB1/INI1* (Table 3).
To summarize, in one of eight hybrid neurofibromas/schwannomas, a deletion of exon 1 to 9 of *SMARCB1/INI1*

was detected. The two different parts being investigated separately did not show different results.

Hybrid Neurofibromas/Schwannomas Frequently Show (Partial) Monosomy 22

To detect genomic gains and losses, aCGH was performed in 16 hybrid neurofibromas/schwannomas (Table 3). In most cases, FFPE tumors used for FISH, MLPA, and sequencing of *SMARCB1/INI1* did not deliver enough material to be included in a further aCGH study; thus, other tumor samples of the same patient with the same diagnosis were investigated by aCGH, as demonstrated in detail in Table 3. The overall number of imbalances was low (Table 4). A (partial) monosomy of chromosome 22 was detected in 44% (7/16) of the patients. Four tumors showed complete monosomy 22, and three cases showed partial losses of chromosome 22 with gains in the centromeric region and heterozygous losses of the almost whole arm of chromosome 22 (22q11.21-q13.33) (Figure 2). There was no evidence for homozygous deleted regions in chromosome 22, notably the genes *LZTR1* (22q11.21), *SMARCB1* and *CABIN* (22q11.23), and *NF2* (22q12.2) were deleted in all cases but did not show additional aberrations. The candidates *COQ6* (14q24.3), *NF1* (17q11.2), and *SMARCA4A* (19p13.2) revealed a balanced chromosomal status. Interestingly, hybrid tumor of Patient 9, who presented multiple hybrid neurofibromas/schwannomas as only definite clinical

Figure 2 Array comparative genomic hybridization (aCGH) analysis performed with the SurePrint G3 Human CGH Microarray Kit, 4 × 180 K (Agilent). A value of zero indicates a balanced chromosome status. **Top panel:** aCGH profiles of the seven hybrid neurofibromas/schwannomas showing partial (dark blue and green) and complete monosomy 22. The **middle panel** exemplarily demonstrates the aCGH profile of chromosome 22 of Case 5 (5d), indicating a partial monosomy 22 with gain of chromosomal region 22q11.1-q11.21. **Bottom panel:** aCGH profile of Case 13, showing a monosomy 22.

sign, showed monosomy 22. Germline *NF1*, *NF2*, and *SMARCB1/INI1* mutations were extensively excluded in this patient.

In summary, monosomy of chromosome 22 was detected in 7 of 16 samples, and evidence for homozygous deleted regions of chromosome 22 was not seen.

A Hybrid Neurofibroma/Schwannoma with Presumable Constitutional Loss of α -T-Catenin/*CTNNA3* instead of Monosomy 22

aCGH of one hybrid neurofibroma/schwannoma identified a deletion of *10q21.3* resulting in loss of 142,364 bp

Figure 3 Array comparative genomic hybridization of chromosome 10 in Case 1b of Patient 1, indicating a heterozygous deletion in chromosomal region 10q21.3 affecting α -T-catenin/*CTNNA3*. The log₂ ratio around -1 (between -0.5 and -1.5) indicates a presumable constitutional loss in *10q21.3*. Somatic heterozygous deletions usually show log₂ ratios between 0 and -1.

(68,956,325 to 69,098,689) containing genetic sequence of catenin (cadherin-associated protein) α 3 (*CTNNA3*) (Figure 3). Log2 ratio around -1 indicated a presumable heterozygous constitutional (germline) deletion because somatic heterozygous deletions usually show log2 ratios lower than 0 and higher than -1 depending on tumor cell content. Because of unavailability of further material of the index patient, mutation was not tested in other than tumor cells. For independent control of the genetic event detected by aCGH, deletion was verified by qPCR based on a short *CTNNA3* fragment covering exon/intron 7.

The mutation affected Patient 1, who did not show monosomy 22 in his tumor. This patient fulfilled diagnostic criteria of NF2 and presented with neurofibromas, schwannomas, hybrid neurofibromas/schwannomas, and meningiomas, and his hybrid tumors showed a normal *SMARCB1*-FISH analysis.

Because deletion of *CTNNA3* is yet undescribed in peripheral nerve sheath tumors, we investigated 20 hybrid neurofibromas/schwannomas from 14 individual patients by qPCR for *CTNNA3* DNA. Means $\pm$ SD relative DNA quantity was $82\% \pm 35\%$ ($n = 20$). To detect those tumors that showed indication of significantly reduced DNA levels, we applied the following approach. We calculated a 95% CI of relative DNA levels for the entire sample. Those tumors that showed *CTNNA3* levels below the lower level of the 95% CI of the entire sample (66%) were defined as being statistically significantly different from the sample (one-sided t -test, $P < 0.05$). By this approach, four tumors of four patients (1a, 2, 10b, and 11) as well as the tumor with *CTNNA3* deletion (tumor 1b) (Supplemental Figure S2A) were identified to show markedly reduced *CTNNA3* levels (Table 3). This was suggestive of *CTNNA3* loss in additional tumors ($n = 3$) of three further patients with hybrid neurofibroma/schwannoma, although we were not able to identify numerical aberrations in region 10q21.3 by reevaluation of aCGH in these samples. This discrepancy could originate from use of different primer sets for qPCR and aCGH because lesions of *CTNNA3* loss would principally include small-scale genetic events. In the presented study, we did not address this issue in more detail because genomic sequencing would have been required.

To address that *CTNNA3* DNA amount may differ between the neurofibroma- and the schwannoma-like part (which was variable between different tumors), we analyzed these two parts separately from four tumors (1a, 1b, 5a, and 9d) by RT-qPCR, but did not detect significant differences: difference between the two parts ($n = 4$) was statistically not significant (means $\pm$ SD relative DNA of neurofibroma-like parts of $60.15\% \pm 15.6\%$ versus means $\pm$ SD relative DNA of schwannoma-like parts of $44.9\% \pm 32.2\%$; paired-test, $P = 0.56$).

In summary, we detected a presumable heterozygous constitutional (germline) deletion of 10q21.3 in one hybrid neurofibroma/schwannoma of a patient with clinically diagnosed NF2. Application of qPCR indicated a reduction of

relative *CTNNA3* DNA quantity in additional hybrid neurofibromas/schwannomas, although the precise extent remains unclear. *CTNNA3* loss may principally include more subtle genetic events (eg, small-scale mutations) not being detected by aCGH.

α -T-Catenin Protein Is Absent in Neurofibroma-Like Parts, Neurofibromas, and MPNSTs

Hybrid neurofibromas/schwannomas are benign Schwann cell–derived peripheral nerve sheath tumors. Therefore, we now examined protein expression of *CTNNA3* in normal Schwann cells, other peripheral nerve sheath tumors, and MPNST cell lines to elucidate expression in normal, benign, and malignant tumor Schwann cells. α -T-catenin was highly expressed in normal primary (rat) Schwann cells (Supplemental Figure S3) and in normal FFPE tissue, such as liver, skeletal muscle, and kidney. Using RT-qPCR, mRNA expression was similarly seen in normal tissues with highest expression in skeletal muscle (Supplemental Figure S2B). Concerning FFPE hybrid neurofibromas/schwannomas ($n = 18$) of 14 individual patients, the following pattern was detected: neurofibroma-like areas were negative in all samples, whereas schwannoma-like areas showed variable α -T-catenin expression (strong in 22%, moderate in 56%, and absent expression in 22% of tumors) (Supplemental Figure S2C). Results of RT-qPCR of extracted *CTNNA3* mRNA of whole tumor samples confirm these data in approximately 50% of samples, taking into account that the portions of neurofibroma- and schwannoma-like areas were not to be respected by RNA analysis (Table 3).

Concerning other Schwann cell–derived tumors ($n = 15$), such as peripheral and vestibular schwannomas, neurofibromas, and MPNSTs, *CTNNA3* mRNA expression was seen in schwannomas (Supplemental Figure S2C) and was not detected in neurofibromas and MPNSTs. Furthermore, no or extremely reduced mRNA expression was found in MPNST cell lines NSF1, T265, and S462 compared to normal cells, confirming data of immunohistochemical protein expression (Supplemental Figure S2D).

Because methylation of α -T-catenin/*CTNNA3* was shown to be monoallelic in tumors, whereas biallelic in normal tissue, we now investigated methylation pattern.³⁰ Methylation was absent only in heart and fibroblasts, but present in hybrid neurofibromas/schwannomas ($n = 20$), different other types of peripheral nerve sheath tumors ($n = 3$), normal human Schwann cells (ScienCell), and FFPE control tissue from lung, kidney, spleen, and leukocytes. In addition, all samples showed an unmethylated band indicating at least partial methylation of cells. Thus, we did not uncover convincing hypermethylation of α -T-catenin/*CTNNA3* or a specific methylation pattern in normal contrasting tumor tissue.

To summarize, loss of *CTNNA3* protein expression seems to be typical in neurofibromas, neurofibroma-like areas of hybrid neurofibromas/schwannomas, and MPNST

being supported by RNA analyses. A role of *CTNNA3* methylation was not elucidated.

CTNNA3 Depletion Induces EMT-Related Gene Activation and Structural Changes of the Perinuclear Actin Cytoskeleton in Schwann Cells

To describe the functional role of α -T-catenin/*CTNNA3*, we established a transient knockdown in primary rat Schwann cells (Supplemental Figure S4) and performed MTT assays for cell viability. Transfected Schwann cells did not show significantly altered viability compared to siRNA-control cells by MTT.

Furthermore, we investigated expression of cadherin- β -catenin complex genes under *CTNNA3* depletion by RT-qPCR: means $\pm$ SD E-cadherin mRNA were markedly reduced to $69.32\% \pm 21.33\%$ at 24 hours and to $51.54\% \pm 3.44\%$ at 48 hours, whereas no significant alterations of β -catenin and N-cadherin mRNA expression were seen (data not shown). Because reduced E-cadherin expression is specific to indicate epithelial-mesenchymal transition (EMT), we analyzed EMT-associated target genes by RT-qPCR and confirmed regulation of *Snail/SNA1*, *Slug/SNAI2*, and *ZEB1* genes in *CTNNA3* depleted Schwann cells (Supplemental Figure S4). Changes of *ZEB2* expression were not obvious. Significance of mRNA expression of these transcription factors was verified by

RT-qPCR, indicating strong expression of these genes in normal Schwann cells.

Finally, we investigated the effect of *CTNNA3* depletion on the cytoskeleton of transfected cells by phalloidin staining to designate the actin cytoskeleton. A granular disaggregation, especially of the nuclear and perinuclear actin cytoskeleton, was demonstrated by confocal microscopy (Supplemental Figure S5).

In summary, depletion of *CTNNA3* in Schwann cells was associated with reduced RNA of E-cadherin and regulation of *Snail/SNA1*, *Slug/SNAI2*, and *ZEB1* genes as well as disaggregation of the actin cytoskeleton.

Discussion

We aimed to elucidate the molecular background of hybrid neurofibromas/schwannomas, which have not yet been investigated so far (Figure 4).² Therefore, we selected 70% of these tumors from >20 patients of our registry who were experiencing a tumor syndrome, including multiple peripheral nerve sheath tumors, and among these contributing 41 hybrid neurofibromas/schwannomas. Both NF2 and schwannomatosis were diagnosed in 19% of patients. Of selected individuals, 31% exhibited multiple hybrid neurofibromas/schwannomas, mostly among other tumors. Remarkably, Patient 9 with a family history of a tumor syndrome exhibited only, but multiple, hybrid neurofibromas/schwannomas. He

Figure 4 Summary of molecular investigations in 16 hybrid neurofibromas/schwannomas. EMT, epithelial-mesenchymal transition.

Figure 5 Tumor distribution in four patients with multiple hybrid tumors is exemplarily shown. Patients were diagnosed to exhibit definite schwannomatosis (**A**, left, and **B**), and one patient shows a family history of hybrid neurofibroma/schwannoma only and was widely excluded to carry a germline mutation of *NF1*, *NF2*, and *SMARCB1* (**A**, right). Patients in **B** had been diagnosed to fulfill criteria of schwannomatosis (not recognizing that hybrid tumors are different from schwannomas). In all patients of our cohort, tumors were principally widespread localized at peripheral nerves, only one tumor was localized at the hypoglossal nerve. Of 41 tumors, 32% were localized at the arms, 17% at the legs, 27% at the trunk, and 15% at the head and neck. A total of 12 (29%) of 41 hybrid tumors showed a plexiform growth pattern.

did not present any clinical signs of *NF1* or *NF2*, and germline molecular analysis did not reveal germline mutations of *NF1*, *NF2*, and *SMARCB1/INI1*. Therefore, the question arises if manifestation of multiple hybrid tumors represents an exceptional (mosaic) form of *NF2* or schwannomatosis or a yet unknown tumor syndrome (Figure 5).

We elucidated the molecular background of hybrid neurofibroma/schwannoma by comprehensive molecular experiments and identified two major alterations. First, monosomy 22 was identified in a considerable number of patients (44%). Second, a novel role of α -T-catenin in those tumors was described. Loss of heterozygosity of *NF2*, including 22q loss, is typical for schwannomas, as reviewed recently by Lassaletta et al.¹⁵ Those researchers demonstrated loss of heterozygosity in 57% of tumors and MLPA alterations other than total loss of chromosome 22 in 13.7% of tumors.¹⁵ Thus, hybrid neurofibroma/schwannoma harbor (partial) monosomy 22, including *LZTR1*, *NF2*, and *SMARCB1*, as they contain schwannoma-like nodules, and encouraging the two- or four-hit hypothesis for schwannoma development in *NF2* or schwannomatosis. We did not perform a detailed mutation analysis to detect small-scale mutations to uncover the complete mutational spectrum because of limited FFPE material. Therefore, it would be interesting to screen for *LZTR1* alterations and small-scale *NF1* or *NF2* mutations.

We herein describe loss of α -T-catenin/*CTNNA3* in one patient and a reduced expression in additional hybrid neurofibromas/schwannomas, indicating a molecular role of α -T-catenin for development of Schwann cell-derived tumors. Ubiquitously expressed α -T-catenin was shown to be involved in urothelial cancer, and single-nucleotide polymorphisms were demonstrated to be linked to asthma development and Alzheimer disease.^{30–32} In our study, α -T-catenin is investigated in nerve sheath tumors for the first time, whereas expression of α -E-cadherin, but not α -N-catenin, was already shown in schwannomas, meningiomas, brain, and peripheral nerves.³³ A role of α -catenin was already demonstrated in breast and colon cancer.^{34–36} α -Catenins directly link β -catenin and actin via a heterodimer formation, which is important for stabilization of adherence junctions of cells to the cytoskeleton.^{37,38} Thus, reduced expression of α -T-catenin in tumor Schwann cells may explain cytoskeletal abnormalities in *CTNNA3* depleted cells because of a failure to link actin. We hypothesize that reduction of α -T-catenin may be an alternative to loss of cellular functions of merlin in *NF2*-associated schwannomas. Herein, *NF2*-deficient tumors present nuclear β -catenin, resulting in activation of WNT, c-myc, and cyclin D1 because of loss of merlin's function to localize β -catenin and N-cadherin near the plasma membrane.³⁹ β - and α -catenin heterodimer formation is moreover known to a preferential binding of E-cadherin. We herein demonstrated

that loss of α -T-catenin in normal Schwann cells is associated with reduction of E-cadherin expression, being a key event of EMT. Markers of EMT include augmented levels of transcription factors that reduce E-cadherin expression. Although Schwann cells are not comparable to epithelial cells, Arima et al⁴⁰ identified increased expression levels of EMT-related genes in NF1-derived Schwann-like sNF96.2 cells and normal human Schwann cells under *NF1* depletion. This observation indicates mechanisms like EMT in neurofibromatosis-associated tumors. In our study, we demonstrate that the EMT program plays a role in Schwann cells being depleted of α -T-catenin and presenting suppressed E-cadherin expression via induction of Snail/SNAI1 and ZEB1. Because α -T-catenin represents a member of the cadherins being important for development of neural crest derivatives, a role of EMT for tumor development of neural crest-derived tumor Schwann cells necessarily should be addressed in further studies. The specific histological and mRNA expression pattern of α -T-catenin/*CTNNA3* being different in the neurofibroma- and schwannoma-like areas of the hybrid tumors may indicate an induction of EMT in the neurofibroma-like, *CTNNA3*-lacking tumor part. This is in accordance with the finding of EMT in NF1-derived Schwann cells published by Arima et al,⁴⁰ underlining that neurofibromas are the typical NF1-derived tumors. Probably, loss of *CTNNA3* in neurofibroma-like tumor areas of hybrid neurofibromas/schwannomas may have a comparable effect on EMT initiation as it has loss of *NF1* in NF1-associated tumors.⁴⁰ Mechanisms of EMT may explain the capacity of neurofibroma-derived Schwann cells to invade into other tissues and to induce collagen deposition.

Finally, one hybrid neurofibroma/schwannoma that histologically showed malignant transition presented with a hyperdiploid karyotype, indicating that transition to MPNST may occur in this entity.

To summarize, we elucidated new molecular events in hybrid neurofibroma/schwannoma, such as monosomy 22 and loss of α -T-catenin. More precise analyses of EMT-like mechanisms in those tumors may be of interest and may uncover additional genetic hits beside chromosome 22 alterations.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.08.019>.

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