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SHORT COMMUNICATION

Expression analysis of genes lying in the NF1 microdeletion interval points to four candidate modifiers for neurofibroma formation

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Abstract The hallmark of neurofibromatosis type 1 (NF1) are multiple dermal neurofibromas. They show high inter- and intrafamilial variability for which the influence of modifying genes is discussed. NF1 patients presenting microdeletions spanning *NF1* and several contiguous genes have an earlier onset and higher number of dermal neurofibromas than classical NF1 patients, pointing to one of the deleted genes as modifier. Expression analysis of 13 genes of the microdeletion region in dermal neurofibromas and other tissues revealed four candidates for the modification of neurofibroma formation: *CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6*.

Keywords Neurofibromatosis type 1 · NF1 modifying genes · NF1 microdeletion syndrome · Neurofibroma

Introduction

Neurofibromatosis type 1 (NF1) [MIM16220] is an autosomal dominant tumor predilection syndrome caused by mutations in the *NF1* gene on chromosome 17q11.2 [1]. Its hallmark symptom is multiple benign dermal neurofibromas. The observed inter- and intrafamilial variability of several NF1 symptoms (e.g., neurofibroma number) has led to the hypothesis that they are modified by other genes [2]. Among the proposed candidates are genes influencing somatic mutation rates [3], genes involved in pathways regulated by neurofibromin, as well as steroid hormones and their receptors [4]. The rare 'NF1 microdeletion syndrome' is of special interest in the search for modifying genes. These patients carry a heterozygous microdeletion of *NF1* and several contiguous genes [5–8] and often have a severe clinical phenotype including mental retardation, congenital heart defects, and an excessive number and early onset of dermal neurofibromas [9]. Fourteen protein-coding and two microRNA genes lie within the most prevalent 1.5 Mb deletion [10–13], which arises from unequal meiotic crossover of two highly homologous low copy repeats flanking *NF1* [14]. It seems likely that the haploinsufficiency of one or more of these genes contributes to the severe phenotype. Candidate genes for mental retardation, congenital heart malformation, and overgrowth have been identified among them [15–17]. None has as yet been associated with dermal neurofibroma formation, thought to be triggered by second hit *NF1* mutations in Schwann cells or their precursors [18] with the subsequent involvement of mast cells and fibroblasts [19, 20]. We hypothesize that interindividual variations in one of the genes of the microdeletion region also account for the variable neurofibroma number in classical NF1 patients. If so, this gene should be expressed in at least one type of neurofibroma precursor cell and presumably also in the neurofibroma itself. We therefore examined the expression of 13 of the 14 protein-coding genes in neurofibromas, peripheral nerve tissue, and cell types involved in neurofibroma formation. The *SUZ12* pseudogene *SUZ12P* was excluded because of its extremely high homology to sequences on chromosome 19. The location and orientation

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of these genes together with the low copy repeats are depicted in Fig. 1.

Materials and methods

Tissues and cells

Nine dermal neurofibromas from seven unrelated NF1 patients and plexiform neurofibroma tissue from four unrelated patients were received. Macroscopically normal peripheral nerve adhering to tumor was obtained in two cases. Two peripheral nerve samples (N. femoralis and N. ulnaris) were received from one NF1 patient. Samples were surgically excised and directly transferred to RNAlater™ (Ambion). Tissue of malignant nerve sheath tumors (MPNST) was obtained from two unrelated NF1 patients and flash frozen in liquid nitrogen. All patients gave informed consent. Primary human fibroblasts derived from the skin of NF1 patients and a healthy control and the NF1-MPNST cell line NFS-1 (kindly provided by Dr. Vincent Riccardi) were cultured in DMEM with 10% FCS. NFS-1, suggested to be of Schwann cell origin [18], shows 26% of the neurofibromin amount of control fibroblasts (J. Griesser, doctoral thesis), indicating an unstable protein. The human mast cell line LAD2 (kindly provided by Dr. Kirshenbaum, NIH, NIAID) was cultured as described [21].

LOH analysis

Two dermal neurofibromas from 4 NF1 patients were subjected to loss of heterozygosity (LOH) analysis as described [22, 23] with markers D17S907 (located within the schlafen family member 12-like gene (*SLFN12L*), 4.16 Mb downstream from *NF1*) and either IVs27TG24.8, D17S1800, or IVs27ac28.4 (located within *NF1*) (kindly provided by Dr. Kehrer-Sawatzki).

Expression analysis

Total RNA was isolated using the RNeasy or the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's protocol and treated with DNase I Amplification Grade (Invitrogen Life Technologies). One microgram thereof was reverse transcribed with the SuperScript™ First-Strand Synthesis system and random hexamers (Invitrogen Life Technologies). cDNA quality was controlled by amplifying part of the housekeeping gene RPII [24]. PCR conditions using 3 µl cDNA as template were 95°C for 2 min, followed by 35–40 cycles of 30 s at 95°C, 30 s at annealing temperature, 30 s at 72°C, followed by 5 min at 72°C. PCR products were separated on 1.5% agarose gels stained with ethidium bromide. Band intensity was measured with Kodak 1D Image Analysis Software and genes displaying less than 10% of the expression measured for the housekeeping gene were defined as not expressed.

The expression of *CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6* was also measured in a LightCycler 1.0 (Roche Applied Science) with the QuantiTect SYBR Green PCR mix (Qiagen) and 2 µl of diluted cDNA according to the manufacturer's protocol. For analysis, the crossing point (C_t) values were determined with the Fit Points method of the Lightcycler 3.5 software and a fluorescence threshold (F_t) of 0.01. The initial template fluorescence (F_0) was calculated as described [25]. Normalization was carried out with the geometric mean of the reference genes *G6PDH* and *TBP* [24]. Primers, annealing temperatures, and product sizes are listed in Table 1.

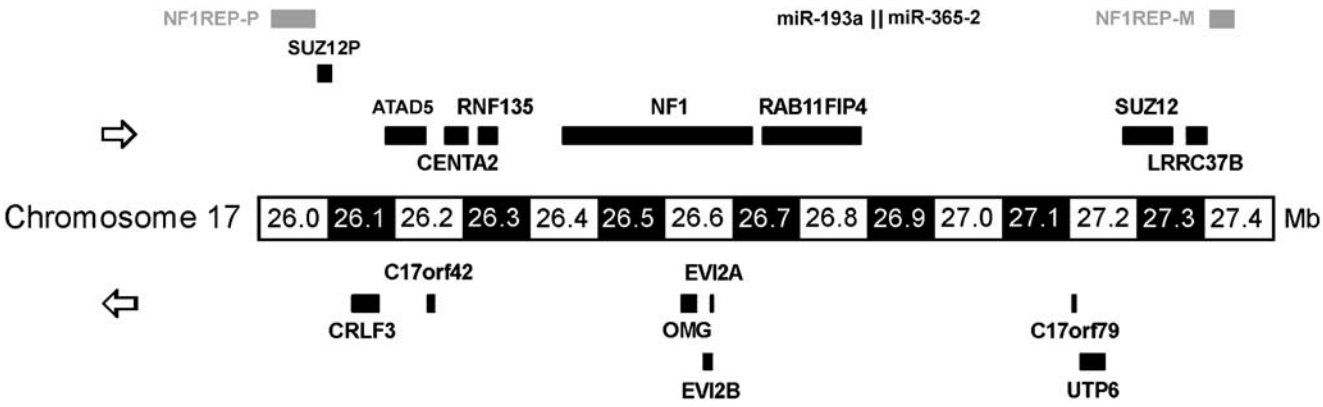

Fig. 1 Location, name, and orientation of our candidate modifying genes on human chromosome 17. The measuring staff shows the physical map coordinated on chromosome 17q11.2 in standard notation from the p-telomere to the q-telomere. Genes depicted above the measuring staff are transcribed in sense; genes below the staff are transcribed in antisense orientation (also indicated by the *arrows*). The *grey boxes* show the low copy repeats (LCR) where the breakpoints of the most common 1.5 Mb microdeletions are located

Table 1 Primers used for the amplification of mRNA of putative NF1 modifying genes

Gene	Primer sequence (5′–3′ end)	Annealing temperature (°C)	Product size (bp)
CRLF3	FW: TGTCCTCAGCCCAGCGTAGTT REV: TCCAGCCTGAGTGACAAGAGTGA	57.8	561
ATAD5	FW: GATCTCATTGCCCGCGCTTTCTCA REV: GGCCAGGACCCCCACCATACTC	65.1	245
C17orf42	FW: GGCGGATCCTCGGGTGTT REV: TGATTTGGTCTTGGCTTATTGTGT	53	320
CENTA2	FW: GGGATATGAAGCCTACGAAGACC REV: CTGGACAGGACACCCCGCAAACT	59.5	159
RNF135	FW: GTCTCACCGCCCACAACCCTATC REV: GCAAGCTTTCCCTCCTCAA	57.8	316
OMG	FW: ATGCTGATGTTGAAGACGAC REV: ATGCCTGTGCCTCTCTGT	65.1	cDNA 146 gDNA 961
EVI2B	FW: CAGATGATGCAGATCTGCCT REV: CTCCTGACACTGGATCTCAA	55.1	278
EVI2A	FW: TATACCCGTCTGTGGGCTAA REV: CTGATTCAGCGGGCCATAGA	55.1	487
RAB11FIP4	FW: CTCGGCTCAAGTCTCAAACAG REV: GGCCGCTCGCAGTCCAG	52.5	cDNA 238 gDNA 796
RAB11FIP4 LC	Qiagen Quantitect Primer Assay		
C17orf79	FW: TCCGCCTCCACTGATACCC REV: GGCCCGCCCACCTACAA	50.2	107
UTP6	FW: TGATCCAGAGAAGAAGAACAC REV: TGAGGCACGCTGGAAAACAC	49.4	101
UTP6 LC	Qiagen Quantitect Primer Assay		
SUZ12	FW: GCCAAAACGAACAAAAGCAAG REV: GACGGAGAGGTAAGCAGGTATCA	55	131
LRRC37B	FW: TTCCTCGCCTCAAGTGGGTTCAAA REV: GCTGCGCTGGGGCTTCTTCCT	60.5	417

cDNA copy DNA, *gDNA* genomic DNA, *LC* LightCycler

Table 2 Expression of candidate modifying genes in different tissues and cultured cells: expression in dermal neurofibromas

Gene	dNF.206	dNF.323	dNF.326	dNF.328	dNF.329I	dNF.329II	dNF.331	dNF.332I	dNF.332II
CRLF3	+	−	−	−	−	+	−	+	+
ATAD5	+	−	−	−	+	+	−	+	+
C17orf42	+	+	−	−	+	+	+	+	+
CENT2A	+	+	+	+	+	+	+	+	+
RNF135	+	+	−	+	+	+	+	+	+
OMG	n.d.	−	−	−	−	−	−	n.d.	−
EVI2B	+	+	−	−	+	+	+	+	+
EVI2A	+	−	−	−	−	+	−	+	+
RAB11FIP4	+	+	+	+	+	+	+	+	+
C17orf79	+	+	+	+	+	+	+	+	+
UTP6	+	+	+	+	+	+	+	+	+
SUZ12	+	−	−	−	−	+	+	+	+
LRRC37B	+	−	−	−	+	+	+	+	+

Expression is represented by [+], no expression is symbolized by [−]. *n.d.* not done, *dNF* dermal neurofibroma. Arabic numerals denote different NF1 patients; roman numerals different tumors (dNF) from the same patient

Table 3 Expression of candidate modifying genes in different tissues and cultured cells: expression in plexiform neurofibroma and nerve tissue

Gene	pNF.330 I	pNF.330 II	pNF.338	dNF.323 nerve	pNF.330 nerve	NF.346 N. fem.	NF.346 N. uln.
CRLF3	−	+	+	+	−	+	+
ATAD5	+	+	+	n.d.	−	+	+
C17orf42	+	+	+	+	+	+	+
CENT2A	+	+	+	n.d.	+	+	+
RNF135	+	+	+	+	+	+	+
OMG	−	−	−	−	−	−	−
EVI2B	+	+	+	+	+	+	+
EVI2A	+	−	−	−	+	+	+
RAB11FIP4	+	+	+	n.d.	+	+	+
C17orf79	+	+	+	n.d.	+	+	+
UTP6	+	+	+	n.d.	+	+	+
SUZ12	+	+	+	+	+	+	+
LRRC37B	+	+	+	n.d.	+	+	+

Expression is represented by [+], no expression is symbolized by [−]. *n.d.* not done, *pNF* plexiform neurofibroma, *dNF nerve* adjacent to dermal neurofibroma, *pNF nerve* adjacent to plexiform neurofibroma, *N. fem. NF1 +/−* nervus femoralis, *N. uln. NF1 +/−* nervus ulnaris. Arabic numerals denote different NF1 patients; roman numerals different parts of one tumor (pNF) from the same patient

Results

LOH analysis

A constitutive *NF1* microdeletion could be excluded for the four NF1 patients tested (NF.326, NF.328, NF.329, and NF.331) as at least one of the two neurofibromas tested showed both *NF1* copies. One neurofibroma of patient NF.326 showed a somatic deletion at both markers (peak reduction by approximately 44%) and was subjected to separate real-time expression analysis together with one NF.326 tumor without LOH. In the remaining three patients, investigation of transcribed polymorphic sites in genes lying within the deletion interval was not informative.

Expression analysis

Four of 13 genes (*CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6*) were found to be expressed in all dermal neurofibromas (Table 2), all plexiform neurofibromas, peripheral nerve tissue (Table 3), and most cultured cells (Table 4). In all plexiform neurofibromas and most nerve tissue samples, six more genes (*ATAD5*, *C17orf42*, *RNF135*, *EVI2B*, *SUZ12*, and *LRRC37B*) were expressed. One gene, *OMG*,

Table 4 Expression of candidate modifying genes in different tissues and cultured cells: expression in cell cultures

Gene	*NF1 +/+* fibros	*NF1 +/−* fibros dNF	*NF1 +/−* fibros dermis	NFS-1 (*NF1 −/−*)	LAD2 (*NF1 +/+*)
CRLF3	−	+	+	+	+
ATAD5	+	+	+	+	+
C17orf42	+	+	+	+	+
CENT2A	+	+	+	+	−
RNF135	+	+	+	+	+
OMG	−	−	−	−	−
EVI2B	+	+	+	+	+
EVI2A	−	+	+	+	+
RAB11FIP4	+	+	+	+	+
C17orf79	+	+	+	+	+
UTP6	+	+	+	+	+
SUZ12	+	+	+	+	+
LRRC37B	−	+	+	+	+

Expression is represented by [+], no expression is symbolized by [−]. *NF1 +/+ fibros* primary control fibroblasts, *NF1 +/− fibros* primary fibroblasts derived from dermal neurofibroma (dNF) or dermis of NF1 patients, *NFS-1* MPNST Schwann cell line, *LAD2* mast cell line.

Table 5 Expression of *CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6* measured by real-time RT-PCR

Sample	*CENTA2*	*RAB11FIP4*	*C17orf79*	*UTP6*
Tissues				
dNF (n=7)	***9.97±7.6***	***8.44±6.62***	1.32±1.57	5.17±4.98
pNF (n=4)	81.06±140.25	5.42[a]±9.1	3.57±0.98	31.59[a]±61.02
MPNST (n=2)	49.1±36.22	1.31±0.32	30.53±15.21	1.23±0.07
Cell cultures				
NF1 +/+ fibros	0.07	0.074	1.65	1.35
NF1 +/− fibros (n=2)	0.034±0.022	0.019±0.0004	1.04±0.06	3.34±1.4
NFS-1	0.08	11.65	3.7	2.97
LAD2	0	5.03	1.74	0.3

Expression is shown relative to the mean of two *NF1* +/− nerves, which was arbitrarily set to 1. Expression in tissues (*dNF* dermal neurofibromas; *pNF* plexiform neurofibroma; *MPNST* malignant nerve sheath tumor) and cultured cells (*NF1 +/+ fibros* primary control fibroblasts; *NF1 +/− fibros* primary fibroblasts derived from NF1 patients; *NFS-1* MPNST cell line; *LAD2* mast cell line). Significant expression changes between peripheral nerve and dermal neurofibromas according to the Mann–Whitney test ($p<0.05$) are highlighted in bold and italic

[a] The mean values are constituted from two values above 1 (upregulation) and two values below 1 (downregulation)

was not expressed in any sample. *CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6* were investigated in detail as candidate modifiers.

Real-time RT-PCR of candidate genes

Real-time RT-PCR of the candidate genes shows an upregulation of *CENTA2* (significant; Mann–Whitney test, p-value 0.04), *RAB11FIP4* (p-value 0.046), and *UTP6* in dermal neurofibromas compared to peripheral nerve tissue (Table 5). No distinct change in regulation was found for *C17orf79* where two samples showed a reduction, four samples were unchanged, and one showed an increase in expression.

Direct comparison of one neurofibroma from NF.326 with LOH (comprising at least *NF1* to *SLFN12L* and thus including *RAB11FIP4*, *C17orf79*, and *UTP6*) to one of the same patient without LOH revealed a reduced upregulation of *RAB11FIP4* (310% to 113%) and a greater downregulation of *UTP6* (45% to 12%) in the tumor with LOH. *CENTA2* and *C17orf79* showed no difference between the two samples (Fig. 2).

In contrast to the dermal neurofibromas, *C17orf79* was upregulated in plexiform neurofibromas and even more so in MPNSTs (Table 5). Great discrepancies were found in some cases between the data from cultured cells and the data from tissue (Table 5). A reduced expression, interesting in view of our hypothesis, was found for *RAB11FIP4* in fibroblasts and *UTP6* in LAD2 cells.

Discussion

We hypothesize that one of the genes lying in the *NF1* microdeletion region not only causes the high number and early onset of neurofibromas in the microdeletion patients but also accounts for the variation of neurofibroma number seen in classical NF1 patients. Accordingly, they would differ in level or activity of the putative modifying protein in cell types involved in neurofibroma formation. As only a difference in an expressed gene can have a modifying effect, we examined the expression of 13 genes of the *NF1* microdeletion region in peripheral nerve tissue, dermal, and plexiform neurofibromas. The majority of cells in a neurofibroma are Schwann cells, with an unknown percentage of *NF1* −/− Schwann cells. The other cell types are underrepresented which is why we also examined representative cell cultures such as primary fibroblasts, a mast cell line and, as representative for Schwann cells, an MPNST cell line.

Only four genes, *CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6*, showed expression in all of the dermal and plexiform neurofibromas investigated as well as in nerve tissue, primary fibroblasts, and the MPNST cell line. They

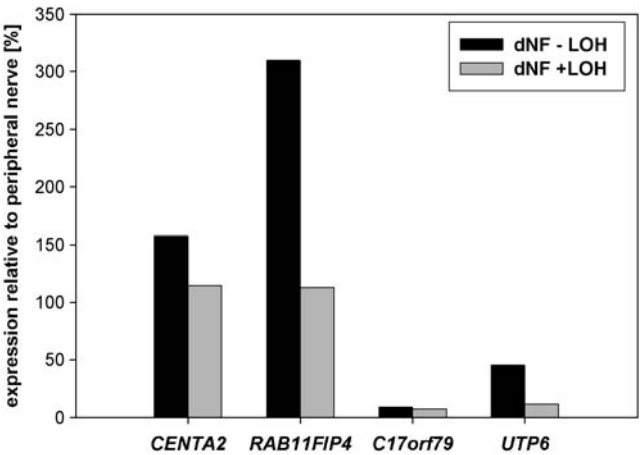

Fig. 2 Expression of *CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6* in dermal neurofibromas from patient NF.326 with and without LOH measured by real-time RT-PCR. Expression is shown relative to the mean of two *NF1* +/− nerves, which was arbitrarily set to 100%. *−LOH* without loss of heterozygosity; *+LOH* with loss of heterozygosity

seem to function in basic cellular processes the disruption of which may promote aberrant growth. *CENTA2* is a GTPase activating protein (GAP) for ARF6 [26], an ADP-ribosylation factor known to regulate tumor cell invasion [27]. The neurally expressed *RAB11FIP4* is involved in the control of membrane trafficking during cytokinesis [28]. *UTP6* influences apoptosome-mediated apoptosis via interaction with Apaf-1 [29]. *C17orf79* (*COPR5*) binds arginine methyltransferase 5 (*PRMT5*) and thereby acts on the promoters of cyclin E1 (*CCNE1*) and the tumor suppressor gene *NM23*. *COPR5* overexpression leads to reduced *CCNE1* levels [30].

Real-time RT-PCR investigation of the candidate gene expression level revealed no clear downregulation in neurofibroma tissue compared to peripheral nerve. Two explanations for these findings are conceivable. First, it is possible that downregulation of a modifying gene occurs only during neurofibroma initiation and it is upregulated thereafter. Second, the level might only be reduced in one neurofibroma cell type but increased in others. Therefore, it seemed reasonable to also investigate the different cell types a dermal neurofibroma is comprised of. However, because of the sometimes strong discrepancies between the *in vivo* and *in vitro* data, these results have to be interpreted with caution. *RAB11FIP4* was found to be reduced in *NF1* +/− fibroblasts, *UTP6* in the mast cell line. Increased expression of both genes was measured in the other cell types. This would support our second explanation mentioned above.

Direct comparison of a neurofibroma with LOH to one without LOH from the same patient showed the expected reduction in the neurofibroma with LOH only in the case of *RAB11FIP4* and *UTP6*. Interestingly, *CENTA2* and *C17orf79* expression did not differ between both tumors. The LOH tumor carries an atypical deletion comprising at least *NF1* to *SLFN12L*. As *CENTA2* is located upstream of *NF1*, it might not be deleted, which would explain the unchanged expression. *C17orf79*, however, is located within the deleted region. In this case, upregulation of the remaining allele in the tumor with LOH is one possible explanation. Another possibility is higher expression of *C17orf79* in one tumor cell type which has a growth advantage in the LOH tumor. Together with the finding of *C17orf79* overexpression in plexiform neurofibroma and MPNST tissue, this points to an interesting function of this gene in tumor tissue.

In summary, *CENTA2*, *RAB11FIP4*, *C17orf79*, and *UTP6* are candidates for modifying NF1 neurofibroma formation. Further studies, especially on protein activity in different NF1 patients, are necessary to elucidate their specific role in neurofibroma formation.

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