FOSL1 as a candidate target gene for 11q12 rearrangements in desmoplastic fibroblastoma

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Desmoplastic fibroblastoma (DF) is a benign fibroblastic/myofibroblastic tumor. Cytogenetic analyses have revealed consistent rearrangement of chromosome band 11q12, strongly suggesting that this region harbors a gene of pathogenetic importance. To identify the target gene of the 11q12 rearrangements, we analyzed six cases diagnosed as DF using chromosome banding, fluorescence *in situ* hybridization (FISH), single-nucleotide polymorphism array and gene expression approaches. Different structural rearrangements involving 11q12 were found in five of the six cases. Meta-phase FISH analyses in two of them mapped the 11q12 breakpoints to an ~20-kb region, harboring *FOSL1*. Global gene expression profiling followed by quantitative real-time PCR showed that *FOSL1* was expressed at higher levels in DF with 11q12 rearrangements than in desmoid-type fibromatoses. Furthermore, *FOSL1* was not upregulated in the single case of DF that did not show cytogenetic involvement of 11q12; instead this tumor was found to display a hemizygous loss on 5q, including the *APC* (*adenomatous polyposis coli*) locus, raising the possibility that it actually was a misdiagnosed Gardner fibroma. 5'RACE-PCR in two 11q12-positive DF did not identify any fusion transcripts. Thus, in agreement with the finding at chromosome banding analysis that varying translocation partners are involved in the 11q12 rearrangement, the molecular data suggest that the functional outcome of the 11q12 rearrangements is deregulated expression of *FOSL1*.

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Desmoplastic fibroblastoma (DF, also called collagenous fibroma) is a newly defined neoplastic entity, with <100 cases reported.¹ This rare benign fibroblastic/myofibroblastic tumor was first described by Evans² and occurs predominantly in adult men. Two-thirds of the cases are located subcutaneously, and one-third involves skeletal muscle or deep soft tissue. Common locations are the back, feet and upper extremities. From a morphological point of view, it is paucicellular, composed of uniform stellate fibroblasts, often reactive-appearing, and with myofibroblasts immersed in an abundant dense collagen with variably myxoid stroma.¹ Only five published cases of DF have been subjected to cytogenetic analysis, and an 11q12 breakpoint was detected in all of them. In two of the cases there was a t(2;11)(q31;q12).^{3,4} Two other cases had an add(11)(q12) and a t(11;17)(q12;p11.2), respectively.^{1,5} The fifth case exhibited a complex karyotype including rearrangement of 11q12.³ Thus, although limited,

the cytogenetic information strongly suggests that rearrangement of a gene in 11q12 is recurrent and non-randomly associated with DF. Notably, an identical t(2;11)(q31;q12) was reported in 1998 by Dal Cin *et al*⁶ in a fibroma of tendon sheath, an uncommon, small, benign fibrous nodule that arises near tendinous structures, mostly in the hands of adult males.

To date, no target genes for the 11q12 rearrangements have been identified. Molecular genetic or molecular cytogenetic markers could serve as useful diagnostics tools in the differential diagnosis of DF, which includes histologically similar benign, locally aggressive and low-grade malignant soft tissue tumors, such as nodular fasciitis, fibromatosis, fibroma of tendon sheath, nuchal fibroma, sclerotic fibroma of the skin, Gardner fibroma, neurofibroma, solitary fibrous tumor (SFT) and low-grade fibromyxoid sarcoma (LGFMS).⁷

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In the present study, we investigated a series of six cases diagnosed as DF, including the two cases reported by Sciot *et al*,³ in order to delineate the 11q12 breakpoint and identify putative target genes of the rearrangement. From G-banding analysis, five of the cases showed karyotypes with aberrations involving chromosome band 11q12. We performed fluorescence *in situ* hybridization (FISH) experiments and single-nucleotide polymorphism (SNP) array analysis in order to pinpoint, with higher resolution, the recurrent breakpoint. Global gene expression (GGE) profiling was performed in three of the cases to extract DF-specific expression patterns and hence potential fusion gene candidates. Candidate target genes were then further analyzed by quantitative real-time PCR (qPCR) and/or RACE-PCR.

MATERIALS AND METHODS

Tumors

For the present study all cases classified as DF and for which cytogenetic information and frozen tissue or cells were available, were retrieved from the archives of the Department of Pathology, Leuven Catholic University. Tumor classification was done by an experienced soft tissue pathologist (R. S.), using the criteria for DF in the WHO classification.⁸ Immunostainings were performed as described.³ Data concerning patient sex and age, tumor location, depth and size and karyotype are shown in Table 1.

Cytogenetic Analysis

All cases were analyzed by chromosome banding after short-term culturing according to standard methods. Karyotypes were described according the International System for Human Cytogenetic Nomenclature.⁹

FISH Analysis

FISH was performed in four cases (cases 1 and 4–6). To pinpoint the breakpoint at 11q12, bacterial artificial chromosome (BAC) and fosmid probes spanning the breakpoint region were used (Table 2). DNA extracted from BAC/fosmid

clones was directly labeled by nick-translation with Cy3-, Cy5- or FITC-conjugated dUTP or indirectly labeled with biotin-dUTP and subsequently detected by diethylamino-coumarin-conjugated streptavidin. Whole chromosome paint (WCP) probes (Applied Spectral Imaging) were used to detect chromosomal rearrangements and, when applicable, to discriminate tumor cells from normal cells. Slides were prepared and analyzed as previously described.¹⁰

SNP Array Analysis

Cases 1–3 and 5–6 were analyzed with SNP arrays to detect global copy number aberrations. DNA was extracted from fresh frozen tumor biopsies using the DNeasy Tissue Kit including the optional RNaseH treatment, according to the manufacturer's instructions (Qiagen), and subsequently hybridized onto the Illumina Human Omni-Quad version 1.0 BeadChip (Illumina), containing 1.2 million markers, following standard protocols supplied by the manufacturer. The positions of the SNPs were based on the UCSC

Table 2 FISH results

Probe	Position (Mb) ^a	Case 4	Case 6
RP11-141J21	chr11:64.071–64.241	der(11)	der(11)
RP11-638P2	chr11:65.208–65.385	der(11)	der(11)
RP11-58D3	chr11:65.188–65.388	der(11)	der(11)
RP11-143B16	chr11:65.351–65.517	Split	Split
RP11-692F22	chr11:65.388–65.562	Split	der(5)
G248P8299G1	chr11:65.362–65.402	der(11)	der(11)
G248P88885F8	chr11:65.397–65.441	Split	der(5)
G248P8091E4	chr11:65.438–65.479	der(2)	der(5)
RP11-506O3	chr11:65.641–65.815	der(2)	der(5)
RP11-142G8	chr11:65.876–66.035	der(2)	der(5)

^aBase pair positions are indicated according to the NCBI build 36 (hg18).

Table 1 Clinical and cytogenetic information

Case no.	Karyotype	Sex	Age (years)	Location/depth	Size (cm)	No. of recurrences
1	46,XX,t(10;11)(p15;q12)	F	58	Thigh/deep	3	0
2 ^a	46,XY,t(2;11)(q32;q12),t(9;15)(q34;q13–14)	M	38	Thigh/deep	8	0
3 ^a	46,XY,t(1;6)(q32;q15),-3,?inv(5)(p12q32),add(7)(q32),t(8;22)(q11;p12),t(11;20)(q12;q13),add(15)(q24),add(17)(q24)	M	63	Hand/superficial	3	0
4	46,XY,t(2;4;11)(q34;q32;q12)	M	61	Thigh/superficial	3.5	0
5	46,XX,der(5)del(5)(q12q14)ins(5;?)(q32;?),ins(12;?)(q22;?)	F	20	Preauricular/superficial	2.5	4
6	46,XY,t(5;11)(q21;q12)	M	77	Hand/deep	5	0

^aKaryotypes and clinical data of cases 2 and 3 were reported by Sciot *et al*³ and revised in the present study.

hg18/NCBI Build 36 sequence assembly. Data analysis was performed using the GenomeStudio software 1.6.1 (Illumina), detecting imbalances by visual inspection. Constitutional copy number variations were excluded through comparison with the Database of Genomic Variants (<http://projects.tcag.ca/cgi-bin/variation>).¹¹

GGE Profiling

RNA was extracted from fresh frozen tumor biopsies using the RNeasy Lipid Tissue Kit, according to the manufacturer's instructions (Qiagen). Quality and concentration of the extracted material were measured using a 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop ND-1000 (Thermo Fisher Scientific). RNA from cases 2, 3 and 6 was of sufficient quality, and GGE analysis was performed using Affymetrix Human Gene 1.0 ST Arrays, according to the manufacturer's instructions (Affymetrix). Data from 6 myxofibrosarcomas (MFS), 6 desmoid-type fibromatoses (DFM), 5 SFT, and 17 LGFMS, previously described,¹² were used in the differential analysis. Data were analyzed using Qlucore Omics Explorer v 2.0 (Qlucore AB). For principal component analysis based on Pearson's correlation matrix, data were normalized through the settings mean = 0 and $\sigma = 1$, and variance filtered on the basis of the ratio $\sigma/\sigma_{\max}$.¹² MultiExperiment Viewer (tMeV) v 4.2 was used to analyze mean-centred gene expression data with hierarchical clustering analysis. Pearson's correlation was used as distance method selection, and average linkage clustering as linkage method selection.¹³

Quantitative PCR

To validate the results from the GGE profiling and to further investigate the expression levels of genes located in, or close to, the common breakpoint region, qPCR was performed in cases 1–3 and 5–6 using RNA previously extracted. To detect any differences in the expression level consistent with the hypothesis of breakage within the gene, TaqMan gene expression assays were performed using probes mapping to the 5'- and 3'-ends of each gene. The following TaqMan gene expression assays were used: Hs00973813_g1 (*EFEMP2* 5'), Hs00973820_g1 (*EFEMP2* 3'), Hs01100955_g1 (*FIBP* 5'), Hs01100963_g1 (*FIBP* 3'), Hs04187685_m1 (*FOSL1* 5') and Hs04187686_g1 (*FOSL1* 3'). *ACTB* and *18S* were used as endogenous controls. In order to evaluate the state of mRNA degradation, *ABL1* 5'- (Hs00245443_m1) and 3'-parts (Hs01104721_m1) were also investigated. Total RNA from normal adult fibroblasts (NAF) was used as calibrator. Three cases of DFM, previously analyzed by GGE profiling,¹² were included as controls. qPCR was performed according to the manufacturer's instructions and all reactions were run in triplicate (Applied Biosystems). Calculations were done using the comparative C_T method (ie, $\Delta\Delta C_T$ method),¹⁴ using the SDS software 1.3.1 (Applied Biosystem). Statistical significance analysis of data was performed using the Relative Expression Software Tool (REST 2009).¹⁵

5' RACE-PCR

5' RACE-PCR was performed in cases 2 and 6 to investigate the presence of aberrant transcripts of the candidate gene *FOSL1* (GenBank accession number NM_005438, version NM_005438.3). One microgram of total RNA was analyzed using the SMARTer RACE cDNA Amplification Kit (Clontech), and the FirstChoice RLM-RACE Kit with Platinum Taq DNA Polymerase (5 U/ μ l), according to the manufacturer's protocol (Invitrogen). Primer sequences are presented in Supplementary Table S1.

RESULTS

Histopathology

All cases showed a similar histology, characterized by bland spindle cells embedded in a collagenous stroma (Figure 1a). The tumors were hypocellular and a fascicular arrangement was lacking. There was no nuclear atypia, necrosis or mitotic figures. The collagen was generally fibrillar, but more compact areas were also present. The vessels were inconspicuous. Cleft-like spaces, as typically seen in fibroma of tendon sheath, were not present. The lesions were well circumscribed, except for case 5, which was clearly infiltrative (Figures 1b and c). On immunohistochemistry, some spindle cells expressed alpha-smooth muscle actin. S-100, desmin, CD34 and epithelial membrane antigen were negative. Beta-catenin only stained the cytoplasm of some spindle cells, and nuclear staining was not observed in any of the cases.

Genomic Analyses Support the Importance of 11q12 Rearrangements in DF

Results from G-banding and SNP array analyses are shown in Tables 1 and 3. Five cases (cases 1–4 and 6) showed chromosomal aberrations involving 11q12, in three cases as the sole anomaly. The only exception was case 5, which displayed structural rearrangements of chromosomes 5 and 12, including an interstitial deletion on the long arm of chromosome 5. This deletion was confirmed by SNP array and encompassed a 22-Mb region, including the *APC* (*adenomatous polyposis coli*) locus, in 5q21–23. The 11q rearrangements in cases 1–3 and 6 were all seemingly balanced two-way translocations between 11q12 and different partners: 2q32, 5q21, 10p15 and 20q13. In line with the balanced karyotypes detected in cases 1, 2 and 6 no acquired genomic imbalances were detected by SNP array in these cases. Case 3 displayed an unbalanced karyotype by G-banding and four large deletions affecting chromosome bands 1q42–q44, 3p21–p12, 5q33 and 13q12–q22 were detected by SNP array. Case 4 showed a balanced three-way t(2;4;11)(q34;q32;q12), this case was not analyzed by SNP array.

Metaphase FISH in cases 4 and 6 showed that the BAC probe RP11-143B16 (position chr11:65.351–65.517) was split (Figure 2, Table 2). This probe was not split in case 1, analyzed by interphase FISH, but the presence of a rearrangement

Figure 1 Morphology of the tumors. (a) The tumor (case 1) is well delineated, very rich in collagen and contains haphazardously arranged vessels and spindle cells. The inset highlights the bland nuclei of the tumor cells. (b) The lesion of case 5 infiltrates between the hair follicles (*) and contains cartilage (**). (c) On high-power magnification, the tumor is very similar to case 1.

Table 3 Deletions detected by SNP array analysis in desmoplastic fibroblastoma

Case	Chromosome band	Size (Mb)	Positions (Mb) ^a
3	1q42–q44	17.9	chr1:229.190–247.189
3	3p21–p12	40	chr3:45.569–85.985
3	5q33	3.8	chr5:15.036–15.420
3	13q12–q22	57	chr13:19.149–76.293
5	5q21–q23	21.7	chr5:107.291–129.012

^aBase pair positions are indicated according to the NCBI build 36 (hg18).

leading to dislocation of a segment <10 kb cannot be excluded. In cases 4 and 6, hybridizations with additional BAC and fosmid probes mapped the breakpoints to regions 65.41–65.43 Mb and 65.37–65.43 Mb, respectively (Figure 2, Table 2). In case 5, hybridizations with a WCP probe for chromosomes 11, as well as the BAC probes RP11-143B16 and RP11-692F22, disclosed seemingly normal chromosomes 11 (Figure 2). Loss of the BAC probe RP11-107C15, covering the *APC* locus in 5q22, confirmed the deletion detected by cytogenetic and SNP array analyses (Figure 2).

***FOSL1* Is Highly Expressed in DF**

To identify the genes useful to distinguish DF from histologically similar tumor types, the gene expression profiles of three DF (cases 4–6) were compared to 34 previously described cases of MFS, DFM, LGFMS and SFT.¹² By unsupervised hierarchical clustering analysis including all variables, the DF group formed a distinct cluster most similar to the DFM group (Figure 3a). Because of the similarity between the DF and DFM groups, the expression levels of the genes *EFEMP2*, *FIBP* and *FOSL1*, which were located in, or close to, the common 11q12 breakpoint area (Figure 3b), were compared between these groups only. Scatter plots were obtained without filtering the data. The expression levels of *CTNNB1* and *APC*, known to be mutated in DFM,¹⁶ were also evaluated (Figure 3c). *FOSL1* and *CTNNB1* were the only two genes differentially expressed between the two groups ($P < 0.05$). Using qPCR, both *EFEMP2* and *FIBP* showed similar levels of expression in DF, DFM and NAF (Figures 4a and b). *FOSL1* was expressed at significantly higher levels in all, but one, of the DF samples (case 5, which did not show 11q12 rearrangement), compared with the DFM and NAF samples (Figure 4c; $P < 0.05$). These results were observed using both *18S* and *ACTB* as endogenous controls. In agreement with the similar expression of 5'- and 3'-parts of *FOSL1*, no fusion transcript was detected using 5'RACE.

DISCUSSION

DF is a benign fibrous lesion with limited information on chromosomal changes and no molecular genetic data.

Cytogenetic information strongly suggests that translocations involving chromosome band 11q12 are non-randomly associated with this disease and a molecular marker related to the 11q12 breakpoint could serve as a useful diagnostic tool in the differential diagnosis of DF. In order to identify the target gene of these chromosome rearrangements we investigated six DF, five of which displayed translocations involving 11q12. Cytogenetic and SNP array findings indicated that the rearrangements were balanced, and by FISH the common breakpoint region could be delineated to 20 kb, containing the *FOSL1* gene (previously known as *Fra-1*). *FOSL1* and two additional genes, *FIBP* and *EFEMP2* located close to the common breakpoint region, were considered potential targets on the base of their functions related to fibroblast growth or cancer development. In line with the FISH results, *FOSL1* was the only one showing an altered expression in DF compared with histologically similar tumors. Except for the case not showing rearrangement of 11q12, all DF tumors displayed high expression of *FOSL1*. The expression levels of the first two exons were similar to the last two, suggesting that the whole gene was overexpressed and that there was no break within the gene. Also, repeated attempts using 5' RACE PCR to amplify a fusion product involving *FOSL1* were unsuccessful. As the recurrent breakpoint occurs in 11q12, and there are several different translocation partners, it can be hypothesized that the 11q12 rearrangements cause loss of negative regulatory elements upstream of the *FOSL1* locus. However, loss or exchange of 3'-regulatory sequences cannot be disregarded based on available data. Translocations with similar position effects have been described in leukemias,^{17,18} as well as in soft tissue tumors.¹⁹

FOSL1 belongs to a gene family consisting of four members: *FOS*, *FOSB*, *FOSL1* and *FOSL2*, all encoding leucine zipper proteins. These proteins can contribute to the formation of the transcription factor complex AP-1, dimerizing with proteins of the JUN family.²⁰ AP-1 contributes to different cellular processes, such as proliferation, differentiation and apoptosis, and regulates both basal and stimulus-activated gene expression. Its activation occurs predominantly through the mitogen-activated protein kinase cascade.²¹ *FOSL1* has been reported to have a role in different kinds of cancer such as colorectal, breast, prostate and lung cancer, as well as head and neck squamous cell carcinoma.²⁰ Expression of wild-type *FOSL1* was able to induce tumor development in athymic mice and anchorage-independent *in vitro* growth in rat fibroblasts.²²

Beta-catenin is encoded by the *CTNNB1* gene and binds to the product of the *APC* tumor suppressor gene.²³ Mutations in either *APC* or *CTNNB1* might cause nuclear accumulation of beta-catenin, which interacts with members of the T-cell transcription factor family to activate transcription of target genes, including *FOSL1*.^{24,25} In familial adenomatous polyposis (FAP), constitutional *APC* mutations predispose to colorectal polyps and may lead to DFM. Sporadic DFM is

Figure 2 Fluorescence *in situ* hybridization (FISH) analysis. FISH analysis was used to characterize the breakpoint region at 11q12 in cases 4–6. The bacterial artificial chromosome (BAC) probe RP11-143B16 was split in cases 4 and 6, but not in case 5 (**a–c**). In this case, a whole chromosome paint (WCP) probe for chromosome 11 disclosed seemingly normal chromosomes 11 and, in line with the SNP array findings, the BAC probe RP11-107C15, spanning the *APC* locus, was deleted (**b**). In case 4, the breakpoint region was 65.41–65.43 Mb as the fosmid probe G248P88885F8 was split (**d**). In case 6, the breakpoint region was 65.37–65.43 Mb as the fosmid probes G248P8299G1 and G248P88885F8 were identified on the der(11) and der(5), respectively (**e**). The positions of the BAC and fosmid probes spanning the breakpoint area of 11q12, as well as the genes located in the region, are shown in panel **f**, according to the UCSC Genome Browser on Human Mar. 2006 (NCBI36/hg18) Assembly (<http://genome.ucsc.edu/>).³³

characterized by somatic mutations in either *APC* or *CTNNB1*, resulting in beta-catenin protein stabilization and nuclear accumulation.^{26–28} Furthermore, it has been demonstrated that beta-catenin stabilization in fibroblasts is

sufficient to cause DFM.²⁹ Our results showed that *FOSL1* is weakly expressed in DFM and that the expression levels of *CTNNB1* are higher in DFM than in DF. In agreement with the latter finding, no nuclear expression of beta-catenin was

Figure 3 Global gene expression (GEE) analysis. Unsupervised hierarchical clustering analysis of GGE data was performed using the software tMeV (a). Each box represents a sample and the color indicates the tumor type according to the legend reported to the right. The GGE profile of desmoplastic fibroblastoma (DF) was most similar to desmoid-type fibromatoses (DFM), then to myxofibrosarcoma (MFS) and solitary fibrous tumors (SFT), and least similar to low-grade fibromyxoid sarcoma (LGFMS). Scatter plots of genes located in the breakpoint region in 11q12 (b) and of *CTNNB1* and *APC* (c) show a significant difference in the expression of *CTNNB1* and *FOSL1* between DF (green) and DFM (red). *CTNNB1* and *FOSL1* displayed a lower and higher expression, respectively, in DF compared with DFM ($P < 0.05$). Ordinates represent the expression levels of the analyzed genes as log₂ values.

seen at immunohistochemical analysis of the DF cases. Therefore, in DF, beta-catenin stabilization as such does not seem to be the cause of increased *FOSL1* expression. In addition, *FOSL1* expression was not increased in case 5, which lacked 11q12 rearrangement and carried a large deletion of 5q, including the *APC* locus. The observed differential expression of *FOSL1* among DF, DFM and NAF thus seems to be associated with the 11q12 rearrangement, rather than beta-catenin stabilization.

The importance of being able to distinguish DF from potential mimics is underlined by case 5. On the basis of the genetic results in this case—lack of 11q12 rearrangement and presence of hemizygous loss of the *APC* locus—it is possible that this tumor represents a misdiagnosed Gardner fibroma, which is a rare fibroblastic soft tissue tumor entity occurring in children and young adults. The young age of the patient and the fact that the lesion was obviously infiltrative and recurred several times might support this. However, DF have

also been reported to infiltrate.³⁰ Generally, Gardner fibroma is even more hypocellular than DF, with more coarse collagen, but this was not obvious in our case. In addition, Gardner fibromas are typically positive for CD34 and show nuclear beta-catenin staining in two-thirds of the cases; both markers were negative in case 5.³¹ Gardner fibromas are important to recognize not because of their tendency to recur locally but because they can be harbingers of Gardner syndrome, a variant of FAP. Hence, the diagnosis of a Gardner fibroma calls for extended analysis of the family history and for screening for colorectal polyps. If there are further signs of FAP and a constitutional mutation of the *APC* gene is detected, the patient needs continuous follow-up for colorectal tumors and DFM.³² Bearing the possibility of a misdiagnosis in mind, we contacted the physician who had sent the tumor tissue. After the fourth recurrence the patient has remained disease free. She did not have documented DFM or similar lesions elsewhere. The familial history was negative

Figure 4 Quantitative real-time PCR. Quantitative real-time PCR analysis was performed to investigate expression levels of the 5'- and 3'-parts of the potential target genes, *EFEMP2*, *FOSL1* and *FIBP*, in the 11q12 breakpoint region. The expression levels of *EFEMP2* and *FIBP* were not different between five desmoplastic fibroblastoma (DF), three desmoid-type fibromatoses (DFM) and normal adult fibroblasts (NAF) (a, b). *FOSL1* was expressed at higher levels in DF except in case 5, the only DF without 11q12 rearrangement (c). The individual relative expression levels are $\log_{10}$ values. The expression levels of the 3'- and 5'-parts of each gene is represented by gray and black bars, respectively. Error bars indicate the range.

regarding FAP/Gardner syndrome. A colonoscopy was proposed, but had not been performed. Thus, the possibility exists that a minority of DF develops through other mechanisms, possibly involving loss of the *APC* gene, than deregulation of *FOSL1* through chromosomal rearrangements of 11q12.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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