Tumorigenesis and Neoplastic Progression

Recurrent Rearrangement of the *PHF1* Gene in Ossifying Fibromyxoid Tumors

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Ossifying fibromyxoid tumor (OFMT) is a soft tissue tumor of unknown lineage. Although most cases are histologically and clinically benign, some show malignant morphological features and local recurrences are not uncommon; a few may even metastasize. In the present study, cytogenetic analysis identified different structural rearrangements of chromosome band 6p21 in tumor cells from three cases of OFMT, including one with typical, one with atypical, and one with malignant morphological features. Mapping of the 6p21 breakpoint by fluorescence *in situ* hybridization (FISH) indicated that the *PHF1* gene was rearranged in all three cases. Further FISH, 5'-rapid amplification of cDNA ends, and RT-PCR analyses disclosed an *EP400/PHF1* fusion transcript in one of the cases. Interphase FISH on tumor sections from 13 additional cases of OFMT showed rearrangement of the *PHF1* locus in four of four typical, two of three atypical, and one of six malignant lesions. Thus, the *PHF1* gene, previously shown to be the 3'-partner of fusion genes in endometrial stromal tumors, is also recurrently involved in the pathogenesis of OFMTs, irrespective of whether they are diagnosed as typical, atypical, or malignant lesions. The *PHF1* protein interacts with the polycomb-repressive complex 2

(PRC2), which, in turn, regulates the expression of a variety of developmental genes. Thus, the results indicate that deregulation of PRC2 target genes is crucial for OFMT development. (Am J Pathol 2012; 181:1069–1077; <http://dx.doi.org/10.1016/j.ajpath.2012.05.030>)

Ossifying fibromyxoid tumor (OFMT) of soft parts is an uncommon tumor of uncertain cellular origin that predominantly occurs in the extremities of adults.¹ Clinically, the tumor often presents as a small painless s.c. mass displaying a partial peripheral ring of calcification on radiography. Microscopically, there are often nests and cords of round-ovoid cells in a myxoid stroma surrounded by an incomplete layer of ossified tissue. However, up to 20% of the tumors lack apparent ossification and are termed nonossifying OFMT.^{2,3} Atypical and malignant examples of OFMT were reported in 1995,⁴ but there has been some subsequent controversy regarding the aggressiveness of OFMT. Folpe and Weiss³ reviewed 70 cases, which they subdivided into typical, atypical, and malignant OFMT on the basis of nuclear grade, cellularity, and mitotic rate. Local recurrences and metastases were seen in all three subtypes, but more often among the malignant lesions (60% recurred locally, and 60% metastasized) than among the typical (12% and 4%, respectively) and atypical (13% and 6%, respectively) lesions. Miettinen et al,⁵ reviewing 104 cases, also noted a correlation between mitotic rate and local recurrence, but questioned the existence of malignant, metastasizing OFMT; according to them, such lesions represented misdiagnoses. A recent study by Graham et al,⁶ using immunohistochemistry (IHC) and gene expression profiling, showed that cases classified as typical show extensive overlap with those classified as malignant.

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Table 1. Clinicopathologic Features of 17 Patients with OFMT

Patient no.	Age (years)/sex	Primary site (size)*	Diagnosis	Relapse time (years)	FISH for <i>PHF1</i> [†]	FISH for <i>SMARCB1</i> [‡]
1	59/M	Shoulder (NA) [§]	Typical	LR: 7, 10, 12, 13, 19 MT: 19 (leg) [§]	Split [¶]	ND
2	47/F	Temple (1.5)	Typical		Split	2 Copies
3	50/M	Foot (4.5)	Typical		Split	2 Copies
4	41/F	Back (5)	Typical		Del 5'	2 Copies
5	76/F	Shoulder (NA)	Typical		Del 5'	2 Copies
6	71/M	Thigh (10)	Atypical		Split [¶]	ND
7	47/F	Leg (2)	Atypical		Failure	Failure
8	53/M	Hip (NA)	Atypical	LR: 6 [§]	Split	2 Copies
9	71/F	Popliteal fossa (3)	Atypical		Del 5'	2 Copies
10	59/M	Neck (NA)	Atypical	MT: 17 (rib), 19 (clavicle) [§]	NR	2 Copies
11	63/M	Toe (3)	Malignant	LR: 2, 4, 6, [§] 7	Split [¶]	ND
12	29/F	Pelvis (7)	Malignant	MT: 3, 5 [§] (peritoneal)	Split	1 Copy
13	42/M	Neck (6)	Malignant		NR	1 Copy?
14	43/M	Paralaryngeal (5)	Malignant		NR	1 Copy
15	82/M	Foot (5.5)	Malignant		NR	1 Copy
16	70/F	Right leg (5)	Malignant	MT: 2 (left leg) [§]	NR	1 Copy
17	70/F	Abdominal wall (4)	Malignant		NR	Failure

Open spaces indicate absence of relapse.

*Largest diameter (in centimeters).

[†]Del 5', loss of the signal mapping the 5' of *PHF1* in $\geq 25\%$ of the nuclei; Split, split signals for the probes mapping 5' and 3' of *PHF1* in $\geq 25\%$ of the nuclei.

[‡]Two copies, two copies of 22q in $\geq 60\%$ of the cells; 1 copy, one copy of 22q in $\geq 60\%$ of the cells; 1 copy?, one copy of 22q in 30% of the cells.

[§]Material reviewed and analyzed in the instance of multiple lesions.

[¶]Results from metaphase FISH. All other cases were analyzed by interphase FISH.

F, female; M, male; LR, local recurrence; MT, metastasis; NA, not available; ND, not done; NR, not rearranged.

The genetic information on OFMT is limited. Graham et al⁶ identified loss of chromosome 22 in five of seven cases analyzed by interphase fluorescence *in situ* hybridization (FISH). Among the three cases with abnormal karyotype published, no recurrent translocation has been found.^{7–9} In the present study, we analyzed three cases by chromosome banding analysis. Because all of them displayed structural rearrangements with a breakpoint in 6p21, a feature found also in one of the previously published cases,⁸ we considered it worthwhile to map the breakpoints by FISH. When finding that all 6p21 rearrangements potentially disrupted the *PHF1* gene (encoding the PHD finger protein 1), previously shown to be the 3'-partner in fusion genes in endometrial stromal cell tumors (ESTs),¹⁰ further analyses using 5'-rapid amplification of cDNA ends (5'-RACE) PCR and RT-PCR were per-

formed to disclose potential fusion gene transcripts. Finally, we analyzed the status of the *PHF1* gene and chromosome 22 in tumor sections from additional OFMTs, representing typical, atypical, and malignant lesions.

Materials and Methods

Patients

The study included 17 patients with OFMT (9 men and 8 women; age range, 29 to 82 years; Table 1). From three of them (patients 1, 6, and 11), fresh and frozen tumor biopsy specimens were available for cytogenetic and molecular genetic analyses (Table 2). From the remaining 14 patients,

Table 2. Cytogenetic, Molecular Cytogenetic, and Molecular Genetic Data on Three Patients with OFMT

Patient no.	Karyotype*	SNP array [†]	FISH [‡]	PCR [§]
1	LR: 19: 46,Y,ins(X;6)(p11;p21p25),t(2;12)(q31;q22); MT: 47,Y,ins(X;6)(p11;p21p25),t(2;12)(q31;q22),+12	ND G: 9q, 12; L: 7q, 8p	ND <i>PHF1</i> pos	ND RACE neg
6	44,XY,6,der(7)inv(7)(p?11q22)ins(7;6)(q?22;p21p25) t(6;12)(p21;q24),der(12)ins(12;6)(q24;p21p1?),-13	L: 6q, 7p, 9q, 12q, 13	<i>EP400</i> pos and <i>PHF1</i> pos	<i>EP400/PHF1</i>
11	45,Y,add(X)(p11),del(1)(p35),der(3)t(3;16)(p12;p11), der(6)t(6;7)(p21;p15),der(7)t(X;7)(p2?;p15)t(X;6)(p11; p21), del(13)(q?21),der(17)t(?13;17)(q2?2;q2?5),20	ND	<i>PHF1</i> pos	RACE neg

For more detailed information on detected imbalances, see Supplemental Table S3 (available at <http://ajp.amjpathol.org>).

*Rearrangements involving the *PHF1* locus in 6p21 are boldfaced. In patient 11, the same karyotype was found in the primary lesion and in three relapses, spanning 4.5 years.

[†]Chromosomes and chromosome arms affected by imbalances, as detected by SNP array analysis.

[‡]Metaphase FISH for *PHF1* and partner genes involved in potential fusions.

[§]Results of RT-PCR and 5'-RACE-PCR analyses.

G, gains; L, losses; LR, local recurrence; MT, metastasis; ND, not done; neg, negative; pos, positive; RACE neg, no fusion transcript detected at 5'-RACE PCR.

tumor sections were retrieved from the consultation files of one of the authors (C.D.M.F.).

Histopathological Review

H&E-stained sections and IHC stains were re-examined. Tumors from five patients were classified as typical, five were classified as atypical, and seven were classified as malignant OFMT (Table 1). Tumors were designated as typical, atypical, or malignant, based on morphological features alone (see *Results*). From five patients, the primary tumor was unavailable for review, with only local recurrences being reviewed for patients 8 and 11 and distant metastases being reviewed for patients 10, 12, and 16. From patient 1, both the primary lesion and the metastasis were analyzed.

IHC Data

IHC was performed at diagnosis at Brigham and Women's Hospital (Boston, MA) on sections (4 μ m thick) of formalin-fixed, paraffin-embedded tissue, using the following antibodies and conditions: epithelial membrane antigen (EMA; Dako, Carpinteria, CA; clone E29; 1:200 dilution, no pretreatment), S100 protein (Dako; polyclonal; 1:3000; no pretreatment), pan-keratin (Dako; clone MNF116; 1:700; 10-minute protease digestion), keratin AE1/AE3 (Dako; AE1+AE3; 1:200; 10-minute protease digestion), glial fibrillary acidic protein (GFAP; Dako; polyclonal; 1:15,000; citrate buffer and pressure cooker), desmin (Sigma, St Louis, MO; clone DE-U-10; 1:5000; citrate buffer and pressure cooker), and INI-1 (BD Biosciences, San Jose, CA; clone 25; 1:200; EDTA and pressure cooker). The Envision Plus detection system (Dako) was used for all antibodies. Appropriate positive and negative controls were used throughout.

Cytogenetic and FISH Analyses

Chromosome Banding Analysis

Cell culturing, harvesting, and G-banding were performed as described, and the karyotypes were written following the recommendations of the International System for Human Cytogenetic Nomenclature.^{11,12}

Metaphase FISH

Metaphase spreads for FISH analysis were available from the same tumors that had been analyzed cytogenetically, except for the local recurrence in patient 1 (Table 2). The bacterial artificial chromosome (BAC) and fosmid probes that were used to delineate the breakpoints in 6p21 and on partner chromosomes were obtained from the BACPAC Resource Center (<http://bacpac.chori.org>, last accessed February 2012; see also Supplemental Table S1 at <http://ajp.amjpathol.org>). The BAC and fosmid clones were selected based on their location in the National Center for Biotechnology Information Map Viewer (<http://www.ncbi.nlm.nih.gov/projects/mapview>, build 36.3, last accessed February 2012) and

the University of California, Santa Cruz, Human Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>, last accessed February 2012). To identify unequivocally tumor cells, whole chromosome painting probes (Applied Spectral Imaging, Migdal Haemek, Israel) were used. Clone preparation, hybridization, and analysis were performed as previously described.¹³

Interphase FISH on Tumor Sections

To search for rearrangements involving the *PHF1* locus in additional tumors, cut sections (4 μ m thick) from paraffin-embedded tumor tissue from an additional 14 patients were retrieved and subjected to interphase FISH, with two BAC probes flanking the gene (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Deletions affecting chromosome 22 were investigated with the LSI TUPLE1/LSI ARSA probe mix (Vysis, Downers Grove, IL), hybridizing to subbands 22q11.2 and 22q13.3. Hybridization and analysis were performed as previously described.¹³

SNP Array Analysis and Data Interpretation

DNA from two OFMTs (patients 1 and 6) was analyzed with single-nucleotide polymorphism (SNP) array to detect global copy number aberrations. The DNA was extracted from fresh frozen tumor biopsy specimens using the DNeasy Tissue Kit, including the optional RNaseH treatment, according to the manufacturer's instructions (Qiagen, Valencia, CA). DNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Saveen & Werner AB, Malmö, Sweden). DNA was hybridized to the Illumina HumanCNV370 Genotyping BeadChips (Illumina, San Diego, CA), following standard protocols supplied by the manufacturer. Data analysis was performed using the BeadStudio software version 3.1 (Illumina), and imbalances were identified through visual inspection.

PCR Analyses

From patients 1, 6, and 11, total RNA was extracted from frozen tumor tissue using the RNeasy lipid tissue kit, according to the manufacturer's instructions (Qiagen). Concentrations were measured as previously described, and the quality was assessed by using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The cDNA synthesis, 5'-RACE PCR, and nested PCR were performed using the SMARTer RACE cDNA Amplification kit, according to the manufacturer's instructions (Clontech, Mountain View, CA). The primers used for 5'-RACE PCR are listed in Supplemental Table S2 (available at <http://ajp.amjpathol.org>). Primers specific for *PHF1*, *EP400*, *FAM188B*, and *AQP1* were designed to search for fusion transcripts using RT-PCR (Table 3). RT-PCR was performed as previously described.¹³ Transcripts were amplified using an initial denaturation for 5 minutes at 95°C; followed by 35 cycles of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C; and a final extension for 10 minutes at 72°C. Amplified fragments were purified from agarose gels and directly sequenced using the Big Dye version 1.1 cycle sequencing kit (Applied Biosystems,

Table 3. Primers Used for 5'-RACE PCR and RT-PCR

Primer name	Primer sequence
AQP1-130F	5'-AGGCCTCTCCAGCTTCTAGG-3'
AQP1-361F	5'-ATTAACCCGCTCGGTCCTT-3'
FAM-117F	5'-GTCAGGGAGTTCTCAGCAG-3'
FAM-396F	5'-GTGCCATCAAGATGCTCAGA-3'
FAM-802F	5'-AAGAAGAGAGCCGGAAGGTC-3'
FAM188B-1277F	5'-GCAGACAGCATCAAAACCAA-3'
FAM188B-1638F	5'-CAGCAGTTCAGTCCAACAGG-3'
FAM188B-1925F	5'-CAACGTTTCAACGATGTGG-3'
EP400-6549F	5'-ACAGTGAGGACGCGATGATG-3'
EP400-6599F	5'-CTGAAGACCCTGCAGGAGAG-3'
PHF1-250R	5'-AAGCTGGGTCCCAAAGTGAGGAGG-3'
PHF1-250R short	5'-AGCTGGGTCCCAAAGTGAGG-3'
PHF1-327R	5'-AGCCCATCAGTCCATCTGGCCAG-3'
PHF1-327R short	5'-AGCCCATCAGTCCATCTGGC-3'
PHF1-426R	5'-GGACCAGACACACCTCCCTA-3'
PHF1-518R	5'-GCGACAGACACAACAGAGGA-3'

Foster City, CA) on an ABI-3100 Avant genetic analyzer (Applied Biosystems). The BLASTN software (<http://www.ncbi.nlm.nih.gov/blast>, last accessed February 2012) was used for the analysis of *PHF1* and *EP400* sequence data.

Results

Histopathological Features

A total of 18 tumors from 17 patients (from patient 1, both the primary lesion and a metastasis were studied) were reviewed by two of the authors (V.Y.J. and C.D.M.F.). All tumors demonstrated at least focal areas of typical OFMT, with reticular and cord-like growth of round-to-ovoid tumor cells characterized by uniformly sized vesicular nuclei embedded in a fibromyxoid background. Most tumors showed a multilobular growth pattern and at least partial encapsulation by a thick fibrous shell, some with characteristic ossification. In general, cytologic atypia ranged from minimal to none, and was observed to be moderate at most (notably in atypical and malignant cases). Tumors showing increased cellularity, at least focal nuclear atypia, more prominent mitotic activity, and bone formation within tumor lobules (whether present in fibrous septa) were classified as malignant; tumors showing only a subset of these features were labeled atypical. **Figure 1**, A–H, shows representative images of typical (patients 2 and 4), atypical (patient 9), and malignant (patient 16) tumors.

IHC staining was performed on one tumor from each patient (from patient 1, only the primary lesion was analyzed). Overall, of 17 tumors, 9 were positive for S100 (2 focal) and 14 were positive for desmin (1 focal) (**Figure 1**, C and D). INI-1 (SMARCB1) staining was positive (ie, normal/retained), albeit sometimes weakly, in 13 of 13 tumors examined (see **Supplemental Figure S1** at <http://ajp.amjpathol.org>). IHC staining results for all cases are summarized in **Supplemental Table S2** (available at <http://ajp.amjpathol.org>).

Five patients were diagnosed as having typical OFMT. Two showed metaplastic bone in the capsule, and three were nonossifying. Tumors were composed of monomor-

phic round-to-ovoid cells, frequently showing trabecular and cord-like growth patterns. Mitotic counts ranged from zero to seven per 10 high-power fields (median count, 1); three tumors showed no mitoses. Both the primary and metastatic lesions from patient 1 showed mild nuclear atypia and focally increased hypercellularity, whereas the other four lesions were entirely bland. S100 protein staining was positive in four tumors and negative in one tumor; desmin staining was positive in four of five tumors. No keratin (0/5), EMA (0/4), or GFAP (0/5) staining was observed. SMARCB1 (INI-1) staining was positive (ie, normal/retained) in three of three tumors.

Five tumors from five patients were classified as atypical OFMT, including a recurrent hip tumor (patient 8) and a metastasis to the clavicle from a neck primary tumor (patient 10). Four tumors were ossifying; in all instances, ossification was present between tumor cells within lobules. One tumor showed no evident ossification. Nuclear atypia ranged from negligible to moderate, with occasional hyperchromatic nuclei. All tumors showed at least focal hypercellularity with overlapping nuclei. Mitotic

Figure 1. Morphological and IHC findings in OFMTs. Typical OFMT (patient 4) showing multilobular growth (A) and ovoid-to-round cells embedded in fibromyxoid stroma (B). Immunoreactivity for desmin (C) and nuclear staining for S100 (D) (patient 2) are observed in a subset of tumors. E: Atypical OFMT (patient 9) showing increased cellularity. F: Mild atypia and hyperchromatic nuclei showing mild atypia (H). Original magnification: $\times 10$ (A, E, and G); $\times 40$ (B–D, F, and H).

counts ranged from 8 to 29 per 10 high-power fields (median count, 10). Three of five tumors were positive for S100 protein. Desmin staining was diffusely positive in three tumors, focal in one tumor, and negative in one tumor. No immunoreactivity was observed for keratin (0/2), GFAP (0/2), and EMA (0/3). SMARCB1 staining was positive in four of four tumors.

Seven patients were diagnosed as having malignant OFMT, including a locally recurrent toe tumor (patient 11), a peritoneal metastasis from a pelvic primary tumor (patient 12), and a metastatic left leg lesion from a right leg primary tumor (patient 16). Four tumors were ossifying and showed ossification within the tumor lobules; three lacked ossification in the sections examined. All tumors showed areas of hypercellularity, with overlapping nuclei and frequently solid growth, in addition to areas with fibromyxoid stroma. Although all tumors demonstrated nuclear atypia and a pleomorphism, the degree ranged from mild to moderate at most. Some tumors showed focal areas of more spindled morphological features. Mitotic counts ranged from 8 to 27 per 10 high-power fields (median count, 16). S100 protein immunostaining was positive in two tumors, focal in one tumor, and negative in four tumors. Desmin staining was present in six of seven tumors. Keratin staining was focal in two of five tumors, and focal GFAP positivity was seen in one of four tumors. Focal EMA staining was seen in three of six tumors. SMARCB1 staining was positive in six of six tumors (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Cytogenetic Findings

Tumors from patients 1, 6, and 11 were characterized cytogenetically after short-term culturing. All had near-diploid karyotypes with different rearrangements of 6p21 (Table 2). From patient 1, both a local recurrence and a metastasis could be analyzed, revealing identical karyotypes, except for the presence of trisomy 12 in the metastasis. Apart from 6p21, no other chromosome band was recurrently involved, but two tumors each had breakpoints in 7p and 12q.

Metaphase FISH Mapping of Breakpoints in 6p21, 12q24, and 7p15

Mapping of Breakpoints in 6p21

The breakpoints in 6p21 in patients 1, 6, and 11 were mapped with a panel of whole chromosome painting, BAC, and fosmid probes (probes delineating breakpoints are listed in Supplemental Table S1 at <http://ajp.amjpathol.org>). In all three patients, FISH using whole chromosome painting probes showed that none of the rearrangements affecting 6p was a standard translocation; in two of them, there was an insertion of 6p into Xp (patient 1) or 7q and 12q (patient 6), and in one (patient 11), 6p was involved in a three-way exchange with chromosomes X and 7. In patient 1, the 6p21 breakpoint in the ins(X;6) was delineated by fosmid W12-1201P10, mapping to the der(X), and BAC RP11-175A4, mapping to the der(6) (Figure 2). The 6p rearrangement was more complex in patient 6;

the der(7) contained material from 6p21-25, but not in a linear manner, and the der(12) carried an inverse insertion of 6p17-21 in band 12q24. The breakpoint in 6p21 was delineated by fosmid W12-1617B20, mapping to the der(12), and BAC RP11-175A4, mapping to the der(7); the sequence recognized by fosmid W12-1201P10, mapping 5' of *PHF1*, was only present on the normal chromosome 6 homologue and, thus, hemizygotously deleted from the rearranged chromosome 6. In patient 11, the 6p21 breakpoint was delineated by BAC CTD-2307H19, which gave split signals on the der(7) and the der(6); the 3' part of the *PHF1* locus mapped to the der(6)t(6;7), on which RP11-175A4 gave a signal (Figure 2A). Bearing in mind the resolution of metaphase FISH, a minimal shared breakpoint region in 6p21 could be mapped to position 33.43 to 33.49 Mb; this region contains three genes (*LYPLA2P1*, *KIFC1*, and *PHF1*).

The breakpoints in distal 6p in the ins(X;6) in patient 1 and the ins(12;6) in patient 6 differed by several megabases, thus excluding a second shared breakpoint in 6p (data not shown).

Mapping of Breakpoints Potentially Involved in Exchanges with 6p21

Considering the transcriptional orientation of *PHF1* (transcribed from telomere to centromere) and the FISH mapping of the 6p21 rearrangements, potential candidates for 5' partners in fusions with the *PHF1* locus should be located in 6p25 in patient 1, 12q24 in patient 6, and 7p15 in patient 11. The breakpoint in 6p25 in patient 1 was delineated by RP3-406P24, which gave split signals on the der(6) and the der(X); the segment covered by this BAC does not contain any known gene. The breakpoint in 12q24 in patient 6 was delineated by RP11-417L19, mapping to the 5' part of *EP400*; cohybridization of RP11-417L19 and W12-2752E10 (covering *PHF1*) revealed a fusion signal in the der(7) (Figure 2B). The breakpoint in 7p15 in patient 11 was delineated by BAC RP11-902N23, which gave split signals on the der(6) and the der(7); this segment contains two genes (*FAM188B* and *AQP1*).

Interphase FISH Analysis of Paraffin-Embedded Tumors

Interphase FISH using BACs flanking the 5' (CTD-3221B10) and 3' (CTD-2592B23) parts of *PHF1* was successfully performed on cut tumor sections from 13 of 14 additional patients (Table 1). Rearrangement of the *PHF1* locus (defined as aberrant signals in at least 25% of the nuclei) was seen in all four typical tumors (two with split signals and two with loss of the signal flanking the 5' part of the gene), in two of three atypical tumors (one with split signals and one with loss of the signal flanking the 5' part of the gene), and in one of six (split signal) malignant tumors (Figure 2C). The same 14 tumors were also analyzed with regard to deletions on chromosome 22 (Table 1). Of 12 successfully analyzed tumors, zero of four typical, zero of three atypical, and four of five malignant lesions showed loss of one copy of chromosome 22 (Fig-

Figure 2. FISH and PCR analyses of ossifying fibromyxoid tumors. **A:** Complex exchange of material between chromosomes X (red), 6 (green), and 7 (blue) in patient 11; the BAC probe covering *PHF1* (yellow) shows split signals on the der(6) and the der(7). **B:** In patient 6, cohybridization of fosmid W12-2752E10 (red; *PHF1*) and BAC RP11-417L19 (green, 5' of *EP400*) showing a fusion signal (yellow) on the der(7). **C:** Interphase FISH with probes (CTD-3221B10, green, 5' of *PHF1* and CTD-2592B23, red, 3' of *PHF1*) flanking the *PHF1* locus in case 8, revealing split signals. **D:** Interphase FISH on a section from the tumor of patient 16, with probes for chromosome bands 22q11 (red) and 22q13 (green), reveals loss of one copy of chromosome 22. **E:** RT-PCR with primers specific for the *EP400* and *PHF1* genes amplifies an approximately 600-bp fragment in patient 6 (lane 2), but not in patient 1 (lane 3). Lane 1, 100-bp ladder; lane 4, blank. **F:** Sequencing of the amplified transcript in patient 6 reveals an in-frame fusion of exon 37 of *EP400* with exon 2 of *PHF1*.

ure 2D). The fifth malignant tumor was potentially positive (30% of the nuclei showed hemizygous loss). One atypical tumor (patient 10) was negative for both *PHF1* and chromosome 22 rearrangements, whereas one malignant tumor (patient 12) was positive for both rearrangements.

SNP Array Results

Both tumors (a morphologically benign lesion from patient 1 and a morphologically atypical lesion from patient 6) analyzed by SNP array showed chromosomal imbalances; no rearrangements affecting the *PHF1* locus in 6p21 or chromosome 22 were seen. In patient 6, the detected imbalances included a deletion of the 3' part of the *EP400* gene (Table 2; see also Supplemental Table S3 at <http://ajp.amjpathol.org>).

PCR Findings

5'-RACE PCR did not result in any fragment in patients 1 and 11, but it amplified an approximately 750-bp fragment in patient 6. Sequence analysis showed that the fragment was a hybrid cDNA product in which nucleotide 6846 of the *EP400* gene (NM_015409), located in chromosome band 12q24, was fused to nucleotide 221 of *PHF1* (NM_024165). Subsequently, RT-PCR with the primer combination EP400-6549F and PHF1-518R amplified an approximately 600-bp cDNA fragment (Figure 2E). Direct sequencing of the fragment disclosed an in-frame *EP400/PHF1* chimeric transcript, in which exon 37 of *EP400* was fused with exon 2 of *PHF1* (Figure 2F).

Repeated RT-PCR analyses with various combinations of forward primers for the *FAM188B* and *AQP1* genes and

reverse primers for *PHF1* did not result in any *FAM188B/PHF1* or *AQP1/PHF1* fusion transcripts in patient 11.

Discussion

In 2003, Folpe and Weiss³ suggested that OFMT might belong to the group of translocation-associated sarcomas. They based their prediction mainly on the disparate lines of differentiation (schwannian and chondroid) displayed by the tumor cells and the lack of progression-related morphological changes in metastases compared with primary tumors.³ In the present study, we have shown that OFMT is recurrently associated with interchromosomal exchanges, resulting in rearrangement of a target gene in 6p21: *PHF1*. In one of the tumors, an in-frame *EP400/PHF1* fusion transcript was found, whereas no fusion transcript was seen in the other two tumors.

Thus, OFMT is the second neoplasm, in addition to EST, in which *PHF1* is involved in fusions with ectopic sequences. ESTs can be further subdivided into benign endometrial stromal nodules and the malignant variants endometrial stromal sarcoma and undifferentiated stromal sarcoma. The first fusion gene to be identified in EST was *JAZF1/SUZ12*, resulting from a t(7;17).¹⁴ Later, also, the *JAZF1/PHF1* and *EPC1/PHF1* chimeras, caused by a t(6;7) and a t(6;10), respectively, were identified.¹⁰ Recently, high-grade ESTs were characterized by a t(10;17), resulting in the *YWHAE/FAM22* fusion gene.¹⁵ Chiang et al¹⁶ ascertained the relative frequencies of the three then known gene fusions (*JAZF1/SUZ12*, *JAZF1/PHF1*, and *EPC1/PHF1*) by using interphase FISH on sections from 81 ESTs. The most common fusion was *JAZF1/SUZ12* (35%), whereas *JAZF1/PHF1* and *EPC1/PHF1* together accounted for 9% of the cases; the two *PHF1* fusions were only identified in the malignant variants. Furthermore, rearrangement of the *PHF1* locus without simultaneous rearrangement of the *JAZF1* or *EPC1* locus was found in an additional 9% of the cases, suggesting one or more alternate fusion partners in half of the cases with *PHF1* involvement.¹⁶ Although OFMT and EST share no morphological or IHC features, they have in common the involvement of *PHF1* in fusions with various 5' partners. The involvement of the same gene in fusion genes in different tumor types is, however, not exclusive for OFMT and EST. For instance, the *ETV6/NTRK3* fusion gene has been described in a variety of neoplasms, including leukemia, breast cancer, infantile fibrosarcoma, and mesoblastic nephroma of the kidney. Thus, the type of fusion and the cellular context in which it occurs are important for the phenotype of the tumor cells.¹⁷

The *PHF1*-associated chromosomal rearrangements are unusually complex in both tumor types. None of the three OFMTs from which metaphase spreads was available had a standard two-way translocation. Furthermore, at interphase FISH on sections from additional tumors, three of seven OFMTs with rearrangement of the *PHF1* locus had loss of sequences upstream of the gene. The cytogenetic data on EST are still limited, but because of the transcriptional orientations of the genes, neither the

JAZF1/PHF1 nor the *EPC1/PHF1* fusion can arise through a simple translocation.¹⁰

The human *PHF1* gene is one of three human homologues of the polycomb-like (*Pcl*) gene in *Drosophila melanogaster*, and is known to interact with polycomb group proteins, important regulators of homeotic (*Hox*) genes in multicellular organisms.^{18,19} Specifically, *PHF1* has interacted with the polycomb-repressive complex 2 (PRC2), which is essential for silencing of *Hox* genes and other genes regulating development.^{18–21} The PRC2 consists of several proteins, including *SUZ12* (the alternative 3'-partner in EST-associated chimeric proteins), *EZH1*, and *EZH2*, with the latter two constituting the enzymatic subunits responsible for the dimethylation or trimethylation of lysine 27 of histone H3.²⁰ *PHF1* also participated in the cellular response to DNA damage, suggesting that it might be involved in the repair of DNA double-stranded breaks.²² It, thus, seems reasonable to assume that the *PHF1* rearrangements in the cell type(s) from which OFMT arises will affect a variety of biological processes.

A subset of tumors, especially those classified as malignant, lacked rearrangement of the *PHF1* locus, indicating that OFMT is genetically heterogeneous. Graham et al⁶ recently suggested that OFMT develops primarily through inactivation of the *SMARCB1* (also known as *INI-1* or *SNF5*) gene in chromosome band 22q11. They based their hypothesis on the frequent (75% of the cases) finding of weak or absent immunostaining for *SMARCB1* in a series of 46 OFMTs, in combination with hemizygous loss of chromosome 22 in 5 of 7 cases at interphase FISH.⁶ *SMARCB1* is a core subunit of all known variants of the SWI/SNF chromatin remodeling complexes, which are multimeric ATP-dependent regulators of gene expression programs.²³ *SMARCB1* has a tumor repressor role (eg, by repressing the hedgehog and retinoblastoma pathways), and functional inactivation of *SMARCB1* plays an important pathogenetic role in several tumor types. The prototypic example is malignant rhabdoid tumor, in which homozygous deletions or various other mutations of *SMARCB1* are seen in close to 100% of the cases.^{23,24} In particular, there is an antagonistic relationship between SWI/SNF complexes and polycomb group proteins,^{23,25} and an attractive hypothesis would be that the underlying pathogenetic mechanism in most OFMTs is increased PRC2 activity, achieved either through deregulation of *PHF1* or by inactivation of *SMARCB1*.

Although we detected frequent loss of one chromosome 22 at interphase FISH analysis of malignant OFMTs, reduced or absent immunostaining for *SMARCB1* was not observed in any case. The reason for the discrepancy between our results and those of Graham et al⁶ is unclear, particularly because the same antibody and comparable techniques were used, albeit we used the antibody in more dilute form. For several reasons, we believe that other target genes on chromosome 22 should be considered. First, we, and Graham et al⁶ consistently, and by using different FISH probes, found signal patterns suggesting loss of the entire chromosome 22. Hence, the *SMARCB1* locus is not specifically targeted. Second, although we cannot exclude inactivating point mutations in

the remaining allele, loss of one copy of *SMARCB1* is not sufficient for functional inactivation.^{23,24} Third, there are several other well-known tumor suppressor genes, such as *NF2*, that map to chromosome 22. Finally, the only malignant OFMT with an abnormal karyotype that has been reported so far⁸ showed a complex karyotype including -22. Thus, loss of one copy of chromosome 22 might be just one of several recurrent chromosomal imbalances in malignant OFMTs.

In one of the three tumors (patient 6) that could be studied in detail, a partner gene to *PHF1* (ie, *EP400*) could be identified. *EP400*, which is also involved in the dynamics of chromatin structure, encodes an evolutionarily conserved ATPase of the SWI/SNF family. The EP400 protein interacts with several other proteins, including helicases and a histone acetyl transferase, mediating exchanges of different variants of histone H2A. In this capacity, it is involved in different cellular processes, such as regulation of gene transcription and DNA double-stranded break repair. Depending on cellular context, EP400 can activate or repress gene transcription through incorporation of the H2A variant, H2A.Z.²⁶ In cases 1 and 11, the absence of a fusion transcript at 5'-RACE PCR and the lack of known coding sequences in the chromosomal segment attached to the 5'-side of *PHF1* in patient 1 suggest that the important outcome of the chromosomal exchanges in OFMT is loss of 5'-regulatory sequences. This impression is reinforced by the extensive variation in potential 5'-partners, and by the fact that the entire coding region is included in all hitherto known *PHF1* chimeras. However, the effects of structural alterations of the PHF1 protein should also be taken into account; the fusions described so far have been in frame, suggesting that chimeric proteins are expressed. Furthermore, the reported 5'-partners are functionally related in that their wild-type proteins all have been implicated in chromatin structure or DNA accessibility.

In summary, the findings presented herein strongly indicate that the pathogenetic basis of OFMT frequently involves rearrangement of the *PHF1* gene, suggesting that epigenetic deregulation of PRC2 target genes is crucial for tumor development. From a practical, clinical point of view, our results suggest that FISH analysis for rearrangement of the *PHF1* locus could serve as an excellent diagnostic tool, especially to identify OFMT cases with typical and atypical morphological characteristics. Suspected atypical and malignant cases could also be analyzed with regard to the status of chromosome 22, hemizygous loss being common in this subset. Furthermore, the results of our study clearly support the view that malignant OFMTs exist. *PHF1* rearrangements were found in all subsets, although they were more common among typical and atypical cases. Of particular interest, case 1, classified as a typical OFMT, had almost identical karyotypes in a local recurrence and a metastasis, showing beyond doubt that typical OFMTs can metastasize. Finally, it might be worthwhile to look for mutations/deletions of tumor suppressor genes mapping to chromosome 22 in atypical and malignant OFMTs that are negative for *PHF1* rearrangements.

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