Diagnostic value of investigating *GNAS* mutations in fibro-osseous lesions: a retrospective study of 91 cases of fibrous dysplasia and 40 other fibro-osseous lesions

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***GNAS* (guanine nucleotide-binding protein/ α -subunit) mutations that induce the activation of G-protein α -subunit participate in the pathogenesis of fibrous dysplasia. The aim of this study was to evaluate the sensitivity and specificity of *GNAS* mutations in fibrous dysplasia and other fibro-osseous lesions, to assess the value of investigating this mutation in the diagnosis of fibro-osseous lesions. We studied 91 cases of fibrous dysplasia. The quality and/or quantity of genomic DNA were suitable for molecular analysis for 51 cases of fibrous dysplasia. *GNAS* mutations were investigated by three techniques: high-resolution melting (exon 8), allele-specific PCR (exons 8 and 9) and/or direct DNA sequencing (exons 8 and 9). Fibrous dysplasia samples were classified blind to the *GNAS* mutation status into six histological subtypes as conventional, fibro-involutive, osteosclerosing, cementifying, osteocartilaginous and with prominent aneurysmal cystic changes. We also studied 14 cases of low-grade osteosarcoma, 21 cases of ossifying fibroma, 3 cases of osteofibrous dysplasia, 1 case of osseous dysplasia of the jawbone and 1 post-traumatic lesion of the ribs. Twenty-three cases of fibrous dysplasia (45%) showed mutations of codon 201 (exon 8, p.R201H or p.R201C). No mutation was found on codon 227 (exon 9). *GNAS* mutations in conventional fibrous dysplasia were detected in the same proportion (47%) as in the other histological subtypes (47%, $P=0.96$), regardless of sex ($P=0.44$), age ($P=0.90$) and location ($P=1$). *GNAS* mutations were not detected in any other fibro-osseous lesions. The *GNAS* mutation was thus specific to fibrous dysplasia in the context of fibro-osseous lesions. The particular mosaicism of mutant and non-mutant cells within the lesion or the existence of other mutations not already described could explain the lack of *GNAS* mutation in cases of fibrous dysplasia. Investigating this mutation may constitute a valuable complementary diagnostic tool, despite its low sensitivity, particularly in unconventional morphologically different subtypes of fibrous dysplasia. *Modern Pathology* (2013) 26, 911–921; doi:10.1038/modpathol.2012.223; published online 1 February 2013**

Keywords: fibrous dysplasia; *GNAS* mutation; low-grade osteosarcoma

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Benign fibro-osseous lesions are a group of polymorphic bone disorders, characterized by the replacement of normal bone by a collagen-rich tissue, comprising fibroblast-like cells and variably abundant immature fibrillar bone. Benign fibro-osseous

lesions include a spectrum of bone lesions, such as fibrous dysplasia, osteofibrous dysplasia and, more specifically, in jawbones, ossifying fibroma and osseous dysplasia.^{1–3} Because of frequent overlapping of clinical, radiological and microscopic features, such lesions can be difficult to distinguish from each other⁴ and also from low-grade osteosarcomas, the malignant counterpart of fibro-osseous lesions of the bone.^{5,6} However, it is important to differentiate between these lesions, because their clinical evolutions are different and involve different treatments.^{7,8} Fibrous dysplasia is the most common benign fibro-osseous lesion. The precise prevalence of fibrous dysplasia is difficult to establish, because the disease is usually asymptomatic and it is thought to represent 5–7% of all benign bone tumors.⁹ Fibrous dysplasia is an intramedullary lesion, histologically characterized by a fibrous connective-tissue stroma containing ovoid or spindle cells with regular oval nuclei, in which trabeculae of woven bone are found, without osteoblastic rimming.¹⁰ The trabeculae of woven bone can be slender and curved, resembling letters of the alphabet, or can form small calcified spherules that mimic cement. These features render the diagnosis of ossifying fibroma difficult in craniofacial locations.¹¹ Clinically, fibrous dysplasia may involve one bone (monostotic disease in approximately 85% of all cases) or several bones (polyostotic disease). In about 5% of all cases, polyostotic fibrous dysplasia may be associated with polyendocrinopathy and jagged *café-au-lait* spots in McCune–Albright syndrome.^{12–14} Fibrous dysplasia can also be associated with soft tissue myxomas in Mazabraud syndrome.^{15–17} This particular disease presentation is explained by somatic mosaicism involving an activating mutation of a signal-transduction G-protein, occurring postzygotically^{18,19} in pluripotent embryonic stem cells.²⁰ Monostotic fibrous dysplasia, polyostotic fibrous dysplasia, McCune–Albright syndrome and soft tissue myxoma coexisting with fibrous dysplasia result from the somatic mutation of the same *GNAS* gene (guanine nucleotide-binding protein/ α -subunit).^{21,22} This gene is located on chromosome 20q13.3, encoding the α -subunit of the heterotrimeric G (*Gsz*) protein complex.^{23–26} Two main mutations have been identified within exon 8 of the *GNAS* gene.²⁷ These mutations are substitutions in codon 201, leading to the replacement of an arginine by a histidine (R201H), or a cysteine (R201C). In rare cases, serine (R201S),²⁸ leucine (R201L)^{29,30} and glycine (R201G) substitutions have been reported.³¹ Exceptional cases of fibrous dysplasia have been linked to mutation in exon 9, leading to the substitution of the glutamine at position 227 by a leucine, an arginine, a lysine or a histidine.³² All mutations lead to constitutive *Gsz* adenylate cyclase activity with overproduction of cyclic adenosine monophosphate (cAMP) in dysplastic cells.³³

Increased levels of intracellular cAMP may induce overproduction of c-fos, one of the two transcription factors forming AP-1, in turn leading to the deregulation of bone protein expression, with increased proliferation of osteogenic cells and inhibition of their maturation.³⁴ Stimulation of osteoclastic resorption upon overproduction of IL-6 and IL-11 has also been observed in dysplastic cells.³⁵ Altogether, this leads to the formation of immature dysplastic bone. *GNAS* mutation has not been described in other types of benign fibro-osseous lesions.^{11,36,37} However, one case of *GNAS*-mutated low-grade osteosarcoma has been reported in the literature³⁸ (one case of R201C *GNAS* mutation in a femoral low-grade osteosarcoma, in a series of five low-grade osteosarcomas mimicking fibrous dysplasia).

There are several methods to detect *GNAS* mutation from paraffin-embedded tissues or frozen samples, ie, Sanger direct sequencing,³⁹ restriction fragment-length polymorphism from PCR,^{11,36,37} an allele-specific probe for PCR quantification,⁴⁰ PCR with mutation-specific restriction enzyme digestion,³² allele-specific PCR,²⁴ coamplification at lower denaturation temperature PCR,²⁶ HRM combined with direct sequencing and, more recently, pyrosequencing.⁴¹ The sensitivity of these techniques is variable and *GNAS* mutations remain unidentified in many fibrous dysplasia lesions, regardless of the molecular method used.^{28,32,39}

The aim of this study was to assess the value of routine detection of *GNAS* mutations to differentiate fibrous dysplasia from other benign fibro-osseous lesions and low-grade osteosarcoma, by (i) evaluating the sensitivity of detection of *GNAS* mutation in fibrous dysplasia, (ii) confirming the specificity of this mutation in fibrous dysplasia and (iii) correlating *GNAS* mutation status with clinical and histopathology findings.

Materials and methods

Selection of Patients and Samples and DNA Extraction

Institutional ethical guidelines were followed for this retrospective study. Between 2000 and 2012, a total of 91 cases of fibrous dysplasia were diagnosed histologically in the University Hospital of Tours (France), including 43 cases of fibrous dysplasia referred by other centers for second diagnosis opinion. Only two patients had a polyostotic form of fibrous dysplasia and the others had monostotic fibrous dysplasia. To select the most representative formalin-fixed and paraffin-embedded tissues, hematoxylin–eosin and saffron slides were reviewed by two pathologists (GdP and FTD) before the molecular studies. Genomic DNA (gDNA) was extracted using the QIAamp DNA formalin-fixed and paraffin-embedded extraction kit (QIAGEN) from 10- μ m sections cut from tissue blocks in 81

cases and from snap-frozen tissue (stored at -80°C) in 18 cases, using the QIAamp DNA Mini Kit (QIAGEN). Tissue blocks and snap-frozen samples were available for eight tumors. Thick sections of formalin-fixed and paraffin-embedded tissue were dewaxed by extraction in 100% xylene and washed with 100% ethanol. The samples were air-dried before DNA extraction. The extracted DNA was quantified using a Spectrophotometer (Cary100Scan, Varian). In 40 cases, the DNA extracted was not suitable for molecular study (all from formalin-fixed and paraffin-embedded tissues), leaving only 51 cases of fibrous dysplasia available for analysis. In parallel, we studied 26 benign fibro-osseous lesions, including 21 cases of ossifying fibroma, 3 cases of osteofibrous dysplasia, 1 case of osseous dysplasia, 1 post-traumatic fibro-osseous lesion of the ribs (all from paraffin-embedded tissue, except for 1 osteofibrous dysplasia from a frozen sample) and 14 low-grade osteosarcomas (12 from formalin-fixed and paraffin-embedded tissue, and 2 from frozen tissues), with suitable DNA for each case.

High-Resolution Melting (HRM) Analysis

HRM screening for *GNAS* mutations was performed on a LightCycler 480 II (Roche) using the LightCycler 480 High Resolution Melting Master Kit (Roche). Each reaction contained $10\ \mu\text{l}$ of $2\times$ Master Mix, $2.4\ \mu\text{l}$ of 25 mM MgCl_2 , $0.4\ \mu\text{l}$ of each $10\ \mu\text{M}$ primer and $2\ \mu\text{l}$ DNA (30 ng), in a total volume of $20\ \mu\text{l}$. The *GNAS* HRM primers were: forward 5'-TCCATTGACCTCAATTTTGTTCAG-3' and reverse 5'-AAGTTGACTTTGTCCACCTGGAAC-3' (Invitrogen). The final product size was 96 bp. The cycling conditions were 95°C for 5 min, followed by 50 cycles at 95°C for 15 s, 68°C for 15 s and 72°C for 20 s. The melting conditions included one cycle at 95°C for 1 min, 40°C for 1 min and 65°C for 2 s, followed by an increase in temperature from 65°C to 95°C at $1^{\circ}\text{C}/\text{s}$. All samples were tested in duplicate. Data were analyzed with the LightCycler 480 SW1.5 software, the normalized melting curves were established for each sample, and samples were compared with controls in a deduced difference plot. Significant deviations from the horizontal line in relation to the curve of the wild-type controls were indicative of sequence changes within the amplicon analyzed. The samples with distinct melting curves compared with the wild-type allele were recorded as positive for a mutation. All mutated samples were confirmed by direct sequencing (20 cases).

Allele-Specific PCR

Allele-specific PCR for *GNAS* R201 (exon 8) and *GNAS* L227 (exon 9) mutations were performed on a LightCycler 480 II (Roche) using the LightCycler 480 SYBR Green I Master kit (Roche). The primers

Table 1 Allele-specific PCR primers used for *GNAS* mutation detection

Primer name	Sequence
GNAS-WT201-F	5'-TCAGGACCTGCTTCGCTGCC-3'
GNAS-R201H-F	5'-CAGGACCTGCTTCGCTGCCA-3'
GNAS-R201C-F	5'-TCAGGACCTGCTTCGCTGCT-3'
GNAS-201-R	5'-AAGTTGACTTTGTCCACCTGGAAC-3'
GNAS-WT227-F	5'-ATGTTTGACGTGGGTGGCCA-3'
GNAS-L227Q-F	5'-ATGTTTGACGTGGGTGGCCT-3'
GNAS-227-R	5'-TGGCCACCACGAAGATGATG-3'

Abbreviation: *GNAS*, guanine nucleotide-binding protein/ α -subunit.

used for the allele-specific PCR are summarized in Table 1.

Each allele-specific PCR reaction contained $10\ \mu\text{l}$ of $2\times$ SYBR Green master mix, $1\ \mu\text{l}$ of each $10\ \mu\text{M}$ primer and 50 ng gDNA in a total volume of $20\ \mu\text{l}$. The cycling conditions were 95°C for 9 min, followed by 45 cycles at 95°C for 10 s, 68°C for 10 s and 72°C for 10 s. The melting conditions included one cycle at 95°C for 5 s, 65°C for 1 min and 97°C for 15 s. All samples were tested in duplicate. Data were analyzed with the LightCycler 480 SW1.5 software. All mutated samples were confirmed by direct sequencing (11 cases).

PCR Amplification and Direct Sequencing Reaction

Samples defined as mutated by HRM or allele-specific PCR were sequenced. PCR was performed using the following primers: forward 5'-TGACTATGTGCCGAGCGA-3' and reverse 5'-CCACGTCAAACA TGCTGGTG-3' (Invitrogen). The reaction contained $2\ \mu\text{l}$ of $10\times$ polymerase buffer, $1.2\ \mu\text{l}$ of 25 mM MgCl_2 , $0.4\ \mu\text{l}$ of 10 mM dNTP, $1\ \mu\text{l}$ of each $10\ \mu\text{M}$ primer, 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 100 ng of gDNA in a total volume of $20\ \mu\text{l}$. The PCR conditions were: 9 min at 94°C , 30 cycles of 30 s at 94°C , 30 s at 48°C and 45 s at 72°C , followed by 7 min at 72°C . PCR products were purified with ExoSap-IT (GE Healthcare) according to the manufacturer's instructions. The sequencing reaction was performed using Big Dye terminator V3.1 cycle sequencing kit (Applied Biosystems), according to the manufacturer's protocol and using the above primers. The sequencing products were ethanol-precipitated before running on a Genetic Analyzer (Applied Biosystems). The sequencing data were analyzed using the Sequencing Analysis software (Applied Biosystems).

Review of Slides

In conjunction with molecular analysis, fibrous dysplasia hematoxylin–eosin and saffron slides were reviewed independently by two pathologists (GdP and FTD) blinded to the *GNAS* mutation status. Each case of fibrous dysplasia was classified

among six histological subtypes: conventional, fibro-involutive, osteosclerosing, cementifying, osteocartilaginous and with prominent aneurysmal cystic changes.

Fibrous Dysplasia Histological Subtypes

The conventional form corresponded to fibrillar immature bony trabeculae distributed in a fibrous paucicellular background. Bony trabeculae showed a typical 'letters of the alphabet' pattern, without osteoblastic rimming (Figure 1a). Fibrous tissue contained small spindle or oval cells with indistinct cytoplasmic contours and with regular ovoid nuclei (Figure 1b). The fibro-involutive form was defined by the abundance of fibrous tissue, low cellular density, and rare and sparse osteogenesis, limited to a few short trabeculae of woven bone (Figure 1c). This form was frequently accompanied by edematous interstitial imbibition responsible for microcystic changes (Figure 1d). The osteosclerosing form was characterized by abundant Pagetoid-like osteogenesis, comprising thick trabeculae of 'normalized' bone, with predominantly mature lamellar architecture and poor cellular fibrous tissue (Figure 1e). This subtype included the liposclerosing myxoid-fibrous tumor, which is a specific anatomic-clinical fibrous dysplasia variant, located at the upper end of the femur (intertrochanteric region). The osteocartilaginous form was defined by conventional fibrous dysplasia containing lobules of hyaline cartilage with peripheral enchondral ossification (Figure 1f). The cartilaginous component was sometimes abundant, in particular in the polyostotic form. The cementifying form corresponded to cellular fibrous tissue with disperse small spherules looking like psammomatoid bodies (cementum-like material calcified to a greater or lesser degree, Figure 1g). The aneurysmal cystic form was defined by the presence of prominent hemorrhagic suffusions that might appear cystic, with osteoclast-like giant cells (Figure 1h). This subtype excluded fractured fibrous dysplasia.

Statistical Analysis

The sensitivity and specificity of the detection of *GNAS* mutations were evaluated in fibrous dysplasia, benign fibro-osseous lesions and low-grade osteosarcoma. Pearson χ^2 and Mann-Whitney's tests were performed to analyze the relationships between the anatomic-clinical data of the two groups (presence or absence of *GNAS* mutations). XLStat

software was used for statistical analysis (Addinsoft, Paris, France). *P*-values less than 0.05 were considered significant.

Results

Clinical and Pathology Findings

Patients with fibrous dysplasia. The aim of the study presented here was to analyze clinical and pathological data from 51 patients with fibrous dysplasia, for whom the quantity/quality of extracted gDNA allowed molecular analysis (Table 2). Ages ranged from 7 to 72 years (mean 33 years). The male-to-female ratio was 0.76 (male 22; female 29). Disease location for 10 patients was craniofacial and for 34 patients it was extra-craniofacial. The most frequent extra-craniofacial site was the femur ($n=16$), followed by the radius ($n=5$), tibia ($n=4$), spine ($n=2$) and pubic bone ($n=2$). Among the craniofacial cases, four were located in the maxilla or the mandible, three in the skull, two in the orbit and one in the frontal sinus. Two patients had a polyostotic form: a 12-year-old girl, with hemimelic disease (patient 13) and a 48-year-old man with diffuse polyostotic disease, causing multiple severe bone deformations (patient 14).

Histologically, 19 patients had a conventional fibrous dysplasia form, and 30 were interpreted as unconventional: 14 fibro-involutive, 8 osteosclerosing (including one liposclerosing myxoid-fibrous tumor), 4 aneurysmal cystic, 3 cementifying and 1 osteocartilaginous fibrous dysplasia (Table 3).

Patients with other benign fibro-osseous lesions (ossifying fibroma, osteofibrous dysplasia, osseous dysplasia, post-traumatic fibro-osseous lesion of the ribs). All these lesions were diagnosed as morphologically typical. Mean age for patients with ossifying fibroma was 27 years (range 1 to 68 years) and the sex ratio was 0.75 (9 females to 12 males). The most frequently observed site was the mandible (8/18 cases), followed by the maxilla (5 cases) and the facial sinus (4 cases). One patient had a juvenile variant form of ossifying fibroma, with recurrence in the eye socket a few months after enucleation of the tumor from the frontal sinus. Three girls aged 10, 15 and 18 years had osteofibrous dysplasia, with an osteolytic lesion located in the upper third of the tibia. The patient with osseous dysplasia was a 39-year-old woman with a jaw lesion, in contact with one tooth. The patient with the post-traumatic fibro-osseous lesion of the ribs was a 51-year-old

Figure 1 Histological subtypes of fibrous dysplasia. (a–b) Conventional form, typical 'letters of the alphabet' immature bony trabeculae and fibrous paucicellular fibroblast-like background; (c) fibro-involutive form, abundant fibrous tissue with slight osteogenesis and edematous interstitial imbibition (d); (e) osteosclerosing form, abundant Pagetoid-like osteogenesis and paucicellular fibrous tissue; (f) osteocartilaginous form, lobules of hyaline cartilage with peripheral enchondral ossification; (g) cementifying form, fibrous cellular tissue with small spherules of cementum-like material; (h) aneurysmal cystic form, hemorrhagic suffusion surrounded by osteoclast-like giant cells.

Table 2 Clinical and pathological findings and GNAS mutation status in patients with fibrous dysplasia

no.	GNAS mutation (exon 8)	Sex	Age (years)	Site	Material for molecular analysis	Molecular detection of GNAS mutations	Histological subtype
1	R201C	F	20	Femur	Paraffin block and frozen sample	High-resolution melting, direct sequencing (exon 8)	Conventional
2	R201C	M	51	Radius	Frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Conventional
3	R201C	F	10	Femur	Paraffin block and frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Fibro-involutive
4	R201C	F	10	Maxilla	Paraffin block and frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Osteosclerosing
5	R201C	F	72	Pubis	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Conventional
6	R201C	F	14	Skull	Paraffin block	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Fibro-involutive
7	R201C	M	44	NA	Paraffin block	Allele-specific PCR, direct sequencing (exon 8)	Fibro-involutive
8	R201C	F	54	Femur	Paraffin block	Direct sequencing (exon 8 and 9)	Osteosclerosing
9	R201C	F	46	Radius	Frozen sample	High-resolution melting, direct sequencing (exon 8)	Fibro-involutive
10	R201C	M	18	Femur	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Cementifying
11	R201C	M	54	Femur	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Fibro-involutive
12	R201H	F	27	Radius	Paraffin block and frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Conventional
13*	R201H	F	12	Femur	Paraffin block and frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Osteochondral
14*	R201H	M	48	Femur	Paraffin block and frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Conventional
15	R201H	M	26	Humerus	Frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Fibro-involutive
16	R201H	M	10	Orbit	Frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Conventional
17	R201H	M	15	Orbit	Frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Aneurysmal cystic
18	R201H	F	57	Femur	Frozen sample	High-resolution melting, direct sequencing (exon 8)	Conventional
19	R201H	F	57	Femur	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Osteosclerosing
20	R201H	M	44	Talus	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Osteosclerosing
21	R201H	F	31	Femur	Paraffin block	Direct sequencing (exon 8 and 9)	Aneurysmal cystic
22	R201H	F	46	Femur	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Conventional
23	R201H	F	12	Maxilla	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Conventional
24	NIM	M	42	Femur	Paraffin block	High-resolution melting (exon 8)	Conventional
25	NIM	F	52	Radius	Paraffin block	High-resolution melting, allele-specific PCR (exon 8)	Conventional
26	NIM	M	28	Femur	Paraffin block	High-resolution melting (exon 8), allele-specific PCR (exon 8 and 9)	Fibro-involutive
27	NIM	M	13	Femur	Paraffin block	High-resolution melting (exon 8), allele-specific PCR (exon 8 and 9)	Fibro-involutive
28	NIM	M	36	Maxilla	Paraffin block and frozen sample	High-resolution melting (exon 8), allele-specific PCR (exon 8 and 9)	Conventional
29	NIM	F	45	Pubis	Paraffin block and frozen sample	High-resolution melting (exon 8), allele-specific PCR (exon 8 and 9)	Conventional
30	NIM	M	12	Rib	Paraffin block	High-resolution melting (exon 8)	Fibro-involutive
31	NIM	F	7	Femur	Paraffin block	High-resolution melting (exon 8)	Osteosclerosing
32	NIM	M	19	Femur	Frozen sample	High-resolution melting, direct sequencing (exon 8), allele-specific PCR (exon 8 and 9)	Aneurysmal cystic
33	NIM	F	32	Metatarsus	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Fibro-involutive
34	NIM	F	16	Radius	Paraffin block	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	Fibro-involutive
35	NIM	F	30	Tibia	Paraffin block	High-resolution melting (exon 8)	Aneurysmal cystic
36	NIM	F	27	Skull	Paraffin block	High-resolution melting (exon 8)	Osteosclerosing
37	NIM	F	19	NA	Frozen sample	High-resolution melting, allele-specific PCR, direct sequencing (exon 8)	NA
38	NIM	F	43	Mandible	Paraffin block	High-resolution melting (exon 8)	Conventional
39	NIM	F	74	Tibia	Frozen sample	High-resolution melting, allele-specific PCR (exon 8)	Fibro-involutive
40	NIM	F	26	NA	Paraffin block	High-resolution melting, direct sequencing (exon 8), allele-specific PCR (exon 8 and 9)	NA
41	NIM	M	64	Spine	Frozen sample	High-resolution melting (exon 8), allele-specific PCR (exon 8 and 9)	Osteosclerosing
42	NIM	F	22	Frontal sinus	Frozen sample	High-resolution melting (exon 8), allele-specific PCR (exon 8 and 9)	Osteosclerosing
43	NIM	F	19	NA	Paraffin block	High-resolution melting, allele-specific PCR (exon 8)	Conventional
44	NIM	F	27	NA	Paraffin block	High-resolution melting (exon 8)	Conventional
45	NIM	M	23	Skull	Paraffin block	High-resolution melting (exon 8)	Conventional
46	NIM	M	43	NA	Paraffin block	High-resolution melting, allele-specific PCR (exon 8)	Fibro-involutive
47	NIM	F	51	NA	Paraffin block	High-resolution melting, allele-specific PCR (exon 8)	Fibro-involutive
48	NIM	M	33	Calcaneus	Paraffin block	Direct sequencing (exon 8 and 9)	Cementifying
49	NIM	M	49	Tibia	Paraffin block	Direct sequencing (exon 8 and 9)	Conventional
50	NIM	M	21	Tibia	Paraffin block	Direct sequencing (exon 8 and 9)	Cementifying
51	NIM	F	39	Spine	Paraffin block	High-resolution melting, direct sequencing (exon 8)	Conventional

Abbreviations: F, female; GNAS, guanine nucleotide-binding protein/ α -subunit; M, male; R201 C, Arg-to-Cys substitution; R201 H, Arg-to-His substitution; NIM, no identified mutation; NA, not applicable; *polyostotic form.

Table 3 Clinical and pathological findings in patients with positive and negative *GNAS* mutations

	Positive fibrous dysplasia <i>GNAS</i> mutations (n = 23)	Negative fibrous dysplasia <i>GNAS</i> mutations (n = 28)	P-values
Sex			
Female	14	14	0.44
Male	9	14	
Age (years, mean $\pm$ s.d.)	34 ($\pm$ 19)	33 ($\pm$ 16)	0.90
Site			
Craniofacial	5	5	1
Extra-craniofacial	17	17	
NA	1	6	
Histological subtypes			
Conventional	9	10	0.96
Unconventional	14	16	
Fibro-involutive	6	8	
Aneurismal cystic	2	2	
Osteosclerosing	4	4	
Osteocartilaginous	1	0	
Cementifying	1	2	
NA	0	2	

Abbreviations: *GNAS*, guanine nucleotide-binding protein/ α -subunit; NA, not applicable.

woman with a small mass on the anterior arch of the 11th rib.

Patients with low-grade osteosarcoma. Mean age of patients with low-grade osteosarcoma was 34 years (range 13–67 years) and the sex ratio was 0.7 (5 male and 7 female). Ten patients had parosteal low-grade osteosarcoma, two had centromedullary low-grade osteosarcoma and two had dedifferentiated low-grade osteosarcoma. The most frequently observed site was the femur (8 out of 12 cases). All cases exhibited *MDM2* amplification or overexpression, assessed by quantitative PCR and immunohistochemistry, respectively.

Molecular Analysis

Patients with fibrous dysplasia. The results of our study are summarized in Tables 2 and 3. The percentage of *GNAS* mutations was 45% (23/51). All mutated cases were codon 201 mutations, with 12 Arg-to-His substitutions (R201H) and 11 Arg-to-Cys substitutions (R201C). No fibrous dysplasia cases were found mutated in exon 9 (eight cases tested by allele-specific PCR and three by direct sequencing). Of the 23 mutated cases, the mutation was found in 11 cases by allele-specific PCR and in 20 cases by HRM, all were confirmed by direct sequencing. Figure 2 shows one case of R201C and one case of R201H *GNAS* mutation. The concordance between HRM and allele-specific PCR was 100% for the 25 cases tested using the two

techniques, including 10 mutated and 15 non-mutated. No interpretation could be obtained for 13 samples, regardless of the techniques used. Non-conclusive results were attributed to the quality or quantity of gDNA extracted. *GNAS* mutation was detected in the same proportion of conventional fibrous dysplasia (47%) as in unconventional histological subtypes (47%, $P=0.96$), regardless of sex ($P=0.44$), age ($P=0.90$) and the location (craniofacial vs other location, $P=1$).

Patients with other benign fibro-osseous lesions (ossifying fibroma, osseous dysplasia, osteofibrous dysplasia, post-traumatic fibro-osseous lesion of the ribs) and low-grade osteosarcoma. *GNAS* mutations were not detected in any ossifying fibroma (12 by HRM and 9 by allele-specific PCR on exon 8) or osseous dysplasia, osteofibrous dysplasia and the post-traumatic fibro-osseous lesion of the ribs (5 cases tested by HRM on exon 8).

GNAS mutations were not detected in any low-grade osteosarcoma (14 by HRM and 13 by allele-specific PCR on exon 8, 13 of whom were tested by both the techniques with a concordance rate of 100%).

Discussion

Fibrous dysplasia is a benign fibro-osseous lesion that can easily be diagnosed when the clinical and radiological presentations are classical, not necessarily requiring morphological study if the lesion is discovered fortuitously and is asymptomatic.^{42,43} Therefore, only 'atypical' lesions are biopsied and can present a diagnostic challenge for pathologists, because of the frequent existence of histological variations, modifying the classical archetype of fibrous dysplasia, especially with the absence of the typical 'alphabetical' woven bone pattern. This can make the differential diagnosis between fibrous dysplasia and other benign fibro-osseous lesions or low-grade osteosarcoma difficult.

Lee *et al*³⁹ recently published a meta-analysis including 155 reported cases of fibrous dysplasia from 8 studies and 48 additional fibrous dysplasia cases from his institution. He assessed the incidence and types of *GNAS* mutations. The overall positive rate of *GNAS* mutations in fibrous dysplasia was 71.9% (146/203). It is of note that the frequency varied according to the molecular method employed. Indeed, the sensitivity of detection of *GNAS* mutations varied from 23.1% (3/13) to 100% (16/16) with PCR–restriction fragment-length polymorphism,^{36,37} 58.3% (28/48) to 88% (8/9) with direct sequencing,^{39,41} 67.1% (51/76) with mutation-specific restriction enzyme digestion,³² 100% (8/8) with protein nucleic acid-mediated PCR,⁴⁴ 100% (9/9) with restriction digestion analysis and direct sequencing,³⁸ and 96% (23/24) with pyrosequencing.⁴¹ The most common *GNAS*

Figure 2 Mutational analysis of genomic DNA isolated from fibrous dysplasia tissue: three different molecular methods of detection of *GNAS* (guanine nucleotide-binding protein/ α -subunit) mutations on exon 8. (a) R201C allele-specific PCR and (b) R201H allele-specific PCR: detection of PCR products in real time after hybridization of the specific primer with the complementary DNA target, ending with the substituted nucleotide; (c) High-resolution melting on exon 8: distinct melting curve of *GNAS* mutations in a patient and a positive control (red curve) compared with the wild-type allele (negative control, blue curve); (d) Direct sequencing on exon 8: CGT>CAT (p.R201H: Arg-to-His substitution *) and CGT>TGT (p.R201C: Arg-to-Cys substitution **). The color reproduction of this figure is available on the *Modern Pathology* journal online.

mutations by far were R201H (66.4%, 95/143) and R201C (30.8%, 44/143) on exon 8, followed by only three cases of Q227L mutation (2.1%, exon 9) and one case of R201S mutation (0.7%, exon 8). In our study, *GNAS* mutations were detected in 45% (23/51) cases of fibrous dysplasia (52% R201H and 48% R201C). Interestingly, we obtained identical frequencies for HRM, allele-specific PCR and direct sequencing. The incidence of the mutation was thus

lower than described in the literature. Regardless of the molecular detection method used, this suggested a low sensitivity of detection of *GNAS* mutations in fibrous dysplasia. Such a result could be explained by the existence of mosaicism, with the coexistence within the lesion of mutant and non-mutant cells in varying amounts.¹⁸ This particular distribution of mutant cells could result from a post-zygotic *GNAS* mutation that generated not only somatic mosaicism

throughout the body (McCune–Albright syndrome, polyostotic fibrous dysplasia),^{45,46} but also somatic mosaicism of normal and mutant osteogenic cells within the lesion.⁴⁷ Mutated cells in the different organs derived from a single clone originating from an ancestral cell mutated during *in utero* development that could contribute to the three embryonic germ layers.^{18,48} On the basis of *in vivo* experiments, Bianco *et al*¹⁹ showed that only a mixture of mutant and non-mutant skeletal stem cells could reproduce fibrous dysplasia tissue when transplanted in immunocompromised mice. These findings suggested that the coexistence of mutant and non-mutant cells was required for the survival of mutant cells and for the development of fibrous dysplasia. However, the critical mutational load necessary to generate fibrous dysplasia tissue remains unknown. The low detection of *GNAS* mutation could also be explained by a ‘normalization’ of fibrous dysplasia cells over time, due to a high level of apoptosis of mutant skeletal stem cells, compared with non-mutant stem cells.⁴⁹ Mutant stem cells and osteogenic cells had an accelerated cell cycle compared with non-mutant cells. This accelerated turnover led to ‘sterilization’ of the fibrous dysplasia lesions in older patients, which could explain why mutated cells may become undetectable.^{49,50} Because of the dynamic nature of this mosaic pattern, the proportion of mutant cells may differ greatly within the lesion, particularly in older lesions. This prompted the use of a highly sensitive molecular technique for the detection of *GNAS* mutations and this could mean that the *GNAS* mutation would be difficult to find in older patients. However, this hypothesis has not been validated by other studies.³⁹ This was also the case in the present study, in which the average age of patients was not significantly different in the ‘mutated’ and ‘not mutated’ groups.

Different strategies have been settled to characterize mutations from clinical DNA samples. Although cost effectiveness is a point to consider, sensitivity must be the major determinant of the choice. Here, all experimental data favored a strategy based on the use of HRM for screening, followed by sequencing for the precise determination of the mutation. The use of allele-specific PCR, which could be less expensive than HRM for a unique mutated allele, needs to be repeated for three alleles. This has a cost, especially for rare clinical material.

With HRM, it was possible to be specific and it was thus proved to be sensitive to detect only known mutations in a defined exon (exon 8), other exons not being routinely studied. Allele-specific PCR appeared even more specific, although involving a single probe designed for each mutation. Restriction fragment-length polymorphism had the same limitations and not all mutations could be detected using this technique. As HRM, direct sequencing provided the greatest number of details of the sequence covering all possible mutations on

the exon examined, but was less sensitive, with a threshold of 20% of mutant cells in the sample.⁴¹ With protein acid-mediated PCR, the specific primer for exon 8 allowed the selective amplification of a low copy number of a mutant allele.⁴⁴ With this molecular technique, Lietman *et al*³⁰ were able to detect one mutant *GNAS* allele out of 1000–5000 cells. However, in their study, gDNA was extracted from peripheral blood, mainly from patients with McCune–Albright syndrome and not from fibrous dysplasia tissue. The most sensitive method described for the detection of *GNAS* mutations in fibrous dysplasia to date is pyrosequencing.⁴¹ Pyrosequencing should therefore be considered in routine diagnostic use, as this technique does not require large amounts of DNA. The main pitfall is the quality of the DNA, which is difficult for bone material. Liang *et al*⁴¹ described a fibrous dysplasia series in which 96% (23/24) of cases of fibrous dysplasia were positive for *GNAS* mutations at codon 201 (19 R201H and 4 R201C). The authors evaluated the sensitivity of pyrosequencing using a premixture of varying ratios of mutant and wild-type control oligonucleotides before the PCR reaction. They evaluated the correlation between the quantitative results and the calculated ratio in the starting materials. Mutant alleles as low as 5% could be detected in the sample (vs 20% with Sanger sequencing), as reported in the literature for the *KRAS* mutation, eg, Ogino *et al*.⁵¹ Pyrosequencing was designed to detect only the two common mutations of codon 201. In our study, we were limited by low DNA extraction yields for 49% (40/81) of formalin-fixed, paraffin-embedded tissues, whereas all frozen samples were of appropriate quality DNA for molecular analyses (18/18). All of formalin-fixed, paraffin-embedded specimens received acidic pretreatment to decalcify bones, which is known to affect DNA and mRNA integrity.³² EDTA decalcification,⁵² in particular if combined with ultrasonic decalcification,⁵³ might increase the quality of the DNA extracted. However, the duration of such a decalcification protocol might make it difficult to implement in routine, apart from implementing in specialized centers.

To confirm the extent and variability of the original material for the outcome of the molecular testing, we next investigated *GNAS* mutations on both formalin-fixed and paraffin-embedded, and frozen samples for eight cases of fibrous dysplasia. In one case, the mutation was found in formalin-fixed and paraffin-embedded material, and not in the frozen sample, two cases did not show mutations on either formalin-fixed and paraffin-embedded or frozen samples, in three cases mutation was found on frozen samples and not on formalin-fixed and paraffin-embedded tissues, and two showed mutations on frozen samples with no analyzable gDNA on formalin-fixed and paraffin-embedded tissues.

What might distinguish our study was that it mostly addressed unconventional fibrous dysplasia

subtypes, which was a challenge to the pathologist in terms of routine activity. Most other published studies have only investigated typical fibrous dysplasia,^{32,37,39,41} not representative of the fibro-osseous lesions examined by pathologists. We looked for *GNAS* mutations in 51 cases of fibrous dysplasia, including 30 unconventional morphologically affected fibrous dysplasia subtypes (Table 3). The same proportions of *GNAS* mutations were found in the conventional fibrous dysplasia (47%) and other subtypes (47%). As controls, we investigated 26 cases of benign fibro-osseous lesions and 14 cases of low-grade osteosarcoma; no cases of *GNAS* mutation were found (exon 8), confirming the specificity of the *GNAS* mutation in fibro-osseous lesions.

In conclusion, we confirmed that *GNAS* mutations were specific to fibrous dysplasia among all fibro-osseous lesions. Regardless of the molecular methods used in this study (HRM analysis, DNA sequencing and allele-specific PCR), a large number of fibrous dysplasia were not associated with a *GNAS* mutation. Those cases of fibrous dysplasia without detectable *GNAS* mutation may be explained by the tumoral mosaicism of fibrous dysplasia, with low rates of mutated cells compared with non-mutated cells, or by the existence of new mutations, which were not sought in our study. We nevertheless concluded that testing for *GNAS* mutations, preferably on cryopreserved material, might, despite the low sensitivity, constitute an important complementary diagnostic tool, particularly with the unconventional, morphologically changed subtypes of fibrous dysplasia. However, the absence of the *GNAS* mutation in a case of fibro-osseous lesions should not rule out the diagnosis of fibrous dysplasia.

Disclosure/conflict of interest

The authors declare no conflicts of interest.

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