



Direct evidence for the age-dependent demise of *GNAS*-mutated cells in oral fibrous dysplasia

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ABSTRACT

Objective: Fibrous dysplasia (FD) is a benign bone disease characterized by fibro-osseous lesions. FD is caused by somatic mutations in the gene, *guanine nucleotide-binding protein, alpha stimulating activity polypeptide 1 (GNAS)*, which encodes the G protein subunit, Gs α . FD manifests early in life, but the growth of lesions usually ceases in adulthood. FD lesions often exhibit somatic mutation mosaicism. In this study, the relationship between lesion growth and mutation prevalence within a lesion was investigated.

Design: Lesions from five FD patients were characterized by radiographical, histological and immunohistochemical methods. To accurately calculate the prevalence of mutations within lesions, *GNAS* codon 201 in genomic DNA isolated from fresh surgical FD specimens was sequenced.

Results: Uniquely, a lesion in one 46-year-old patient was still growing, enabling simultaneous analysis of both stable-old and active-new FD lesions in the same patient. Immunohistochemical analysis indicated that a newer, proximal lesion was growing while an older, distal lesion was not. The mutation prevalence differed between these lesions; it was low in the old and high in the new lesion. Thus, the frequency of mutated cells had decreased in the older lesion.

Conclusions: This is the first direct evidence for the age-dependent demise of mutated cells in FD, helping to explain why FD lesion growth generally ceases in adulthood.

1. Introduction

Fibrous dysplasia (FD) is characterized by benign fibro-osseous lesions composed of metaplastic fibrous tissue with immature bone remodeling. FD is a sporadic bone disease of genetic origin that can affect single bones (monostotic FD; MFD) or multiple bones (polyostotic FD; PFD). FD occurrence in multiple adjacent craniofacial bones is considered monostotic (craniofacial or facial FD). It can occur as part of McCune–Albright syndrome (Online Mendelian Inheritance in Man Reference Number: 174800). MFD (including craniofacial) affects both genders equally, accounting for 80%–85% of FD cases (Barnes, Eveson, Reichart, & Sidransky, 2005; MacDonald-Jankowski, 2009). FD is caused by somatic mutations in the *guanine nucleotide-binding protein,*

alpha stimulating activity polypeptide 1 (GNAS) gene, which is located on chromosome 20q13.3, and encodes the G protein subunit, Gs α . Mutations in *GNAS* occur post-zygotically to produce a somatic mosaic state and lead to abnormal development of osteogenic mesenchymal tissue (Delaney et al., 2009; Happle, 1986; Schwindinger, Francomano, & Levine, 1992; Shenker, Weinstein, Sweet, & Spiegel, 1994; Weinstein et al., 1991). *GNAS* mutations lead to constitutive Gs α adenylate cyclase activity and overproduction of cyclic adenosine monophosphate in dysplastic cells (Marie, de Pollak, Chanson, & Lomri, 1997). The two most prevalent mutations cause substitution of the arginine 201 (Arg201) residue with either cysteine (Cys201) or histidine (His201) (Alman, Greel, & Wolfe, 1996). A few cases of FD have been linked to substitution of glutamine 227 (Gln227) with leucine, arginine, lysine,

Abbreviations: FD, fibrous dysplasia; *GNAS*, guanine nucleotide-binding protein alpha stimulating activity polypeptide 1; MFD, monostotic fibrous dysplasia; PFD, polyostotic fibrous dysplasia; Arg201, arginine 201; Cys, cysteine; His, histidine; Gln227, glutamine 227; ALP, alkaline phosphatase; CT, computed tomography; BMP2, anti-bone morphogenetic protein 2; PCR, polymerase chain reaction

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or histidine (Idowu et al., 2007). Somatic mutations in Gs α have also been described for MFD (Alman et al., 1996).

FD usually initiates during puberty, and lesion growth tends to stop at the completion of puberty (Riddle & Bui, 2013). However, data on follow-up and clinical outcomes are generally lacking (MacDonald-Jankowski, 2009), as with many other diseases such as cherubism (Sciubba, Fantasia, & Kahn, 2001), hereditary multiple exostoses (Pannier & Legeai-Mallet, 2008), Maffucci syndrome (Goksedef, Baran, Taraci, Gurbuzel, & Cetin, 2012), and keratocystic odontogenic tumors in nevroid basal-cell carcinoma syndrome (McClatchey, Batsakis, Hybels, & Van Wieren, 1975). In light of its association with puberty, sex hormones are thought to contribute to FD (Pensler et al., 1990). However, this assumption is based on clinical observations rather than an adequate explanation of pathological or molecular mechanisms. Recently, by assessing the frequency of mutated colony-forming fibroblasts in patients of different ages, it was suggested that the age-dependent demise of mutated skeletal stem cells was responsible for the normalization of FD (Kuznetsov et al., 2008). However, it is difficult to compare patient groups because of environmental and genetic variation and polyphenism, and the molecular mechanisms underlying this phenomenon are unknown.

The present study focused on craniofacial FD among MFD patients. The mutation prevalence among five FD patients with the same phenotype and genotype was evaluated. One of the five patients had both stable-old and active-new FD lesions, which could be analyzed simultaneously. This unique patient had undergone surgical resection 11 times in 27 years, as previously reported (Sakata et al., 2010). We hypothesized that differences in the growth of this patient's lesions were caused by the age-dependent demise of mutated cells, and we found that the depletion of mutated cells was indeed associated with the normalization of FD lesions.

2. Materials and methods

2.1. Patients

Patient clinical data are summarized in Table 1. We studied five patients diagnosed with FD based on clinical, radiographical, and histological criteria. Conventional radiography was used to determine the poorly-defined margins of lesions. Computed tomography (CT) was also used but margins were less obvious because CT has a poorer spatial resolution than radiography (Fig. 1) (Barnes et al., 2005; MacDonald-Jankowski, 2009, 2015). All patients were Asian. FD characteristics were analyzed, including lesion location, patients' preoperative serum alkaline phosphatase (ALP) activity, treatment, and age at the initial and subsequent operations. This study was performed in accordance with the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Kyoto University, Kyoto, Japan (Approval No. G259). All patients provided written informed consent prior to their participation.

2.2. Image processing

Lesions of the mandible were observed using CT, and were divided into different areas (Figs. 2 and 3). CT images were converted from 16-

bit images to 8-bit images using the image processing software, Photoshop Elements 10 (Adobe Systems, San Jose, CA, USA). The input level was set between 80 and 200 using the command, "level correction." Contrast among bone tissues was determined, and was color-coded red, green and blue. The blue range was designated D', the green range E', and the red range F'.

2.3. Fresh surgical specimens of bone lesions

All FD samples were obtained from the Department of Oral and Maxillofacial Surgery, Kyoto University Hospital, Kyoto, Japan, in accordance with a protocol approved by the Institutional Review Board (the Medical Ethics Committee of Kyoto University). Fresh surgical specimens of bone lesions were obtained from five patients with FD. The material harvested was generally composed of bone spicules and marrow cavity fluid. The bony spicules were minced, and cells were obtained from explant cultures of these tissue pieces.

2.4. Bone marrow stromal cell cultures

Cells from fresh, minced tumor samples were cultured in complete medium consisting of GIBCO® Advanced Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA), 5% fetal bovine serum (HyClone®; Thermo Fisher Scientific, Rockford, IL, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (GIBCO® Penicillin-Streptomycin; Invitrogen). Cells were plated in 6-cm dishes and incubated at 37 °C in a 5% CO₂ atmosphere.

2.5. Tissue specimens

Pathological samples were obtained during surgery at the Department of Pathology, Kyoto University Hospital. The samples were routinely fixed in 10% formalin and embedded in paraffin for histopathological examination and immunohistochemical analysis.

2.6. Immunohistochemistry

Immunohistochemical examination of samples excised from each patient was conducted using anti-ALP and anti-bone morphogenetic protein 2 (BMP2) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Paraffin-embedded sections (5 μ m thick) were subjected to immunostaining with mouse anti-ALP monoclonal antibody (1:100) or goat anti-BMP2 polyclonal antibody (1:250), a biotinylated secondary antibody, and Histofine® Simple Stain MAX PO detection reagent (Nichirei Biosciences, Tokyo, Japan).

2.7. DNA extraction

DNA was extracted from fresh tumor samples and from cultured cells. Total genomic DNA was extracted using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). All procedures were performed according to the manufacturer's instructions.

Table 1
Clinical characteristics of fibrous dysplasia patients.

Patient number	Sex	Site	Preoperative ALP activity (IU/L) (normal values 115-359)	Procedure	Initial operation age(years)	Reoperation age (age)
1	Female	Mandible	326	Debulking	57	–
2	Male	Mandible	175	Debulking	44	–
3	Female	Mandible	179	Debulking	18	–
4	Female	Mandible	250	Debulking	29	–
5	Female	Mandible	313	Debulking	19	24/26/30/33/34/35/37/38/39/41/46

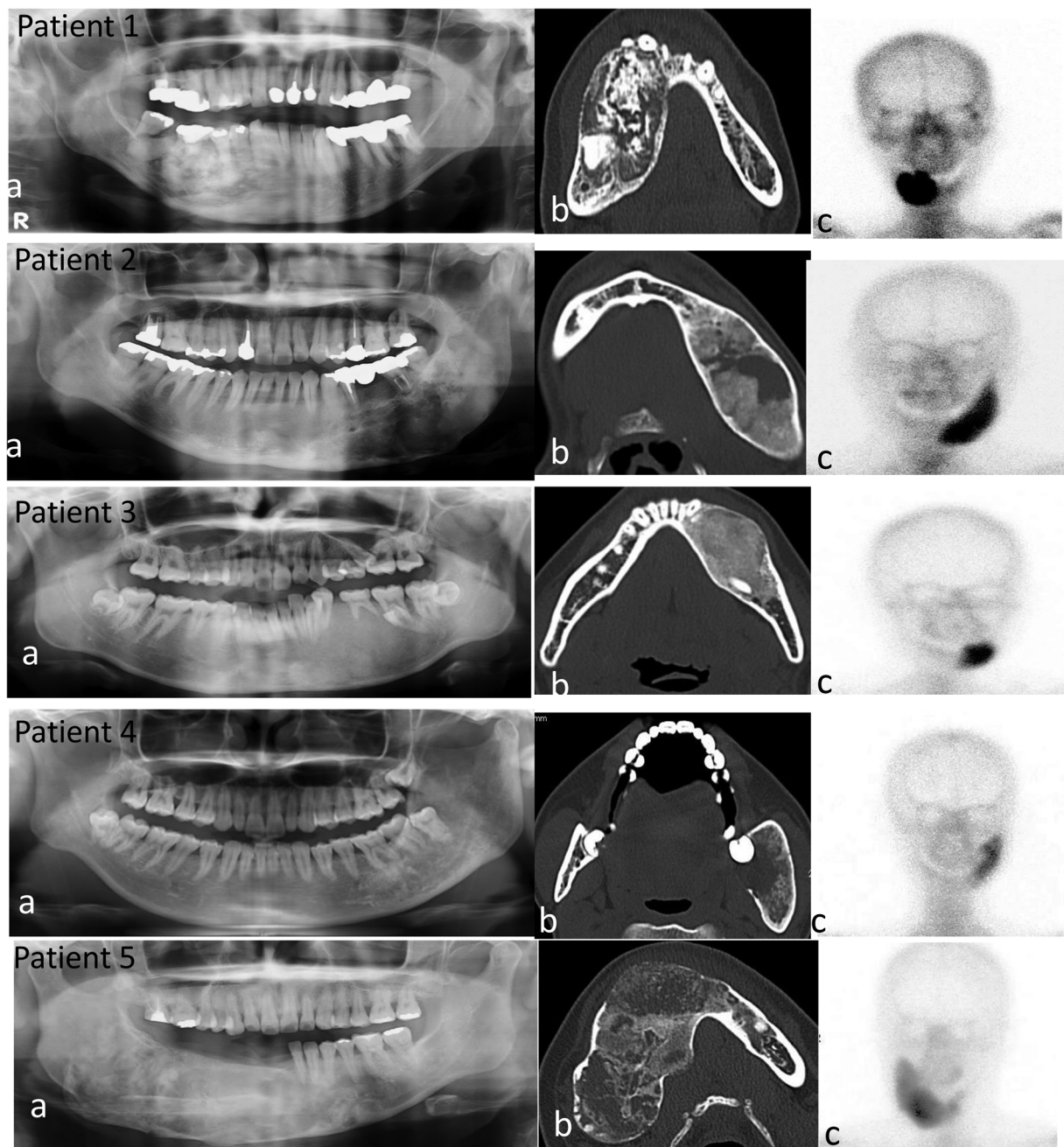


Fig. 1. Patients CT and radiograph. (a) Panoramic X-ray radiographs showing a ground-glass pattern with an unclear border on the right or left side of the mandible. (b) Computed tomography scanning images of the mandible showing marked bone hypertrophy. (c) Bone scintigram of the face showing high-level accumulation in the right or left body of the mandible and part of the right or left submental field.

2.8. Direct sequencing

Mutation analysis was performed by sequencing polymerase chain reaction (PCR)-amplified genomic DNA. DNA, 25 μ L, 2 $\times$ PCR Buffer for KOD FX Neo (Toyobo, Osaka, Japan), 10 μ L deoxynucleotide triphosphate mix (2 mM each), 1 U KOD FX Neo polymerase (Toyobo), and 1.5 μ L each of the forward (5'-TGACTATGTGCCGAGCGA-3') and reverse (5'-CCACGTCAAACATGCTGGTG-3') primers (10 μ M) were subjected to 30 cycles of PCR (predenaturation at 94 $^{\circ}$ C for 2 min, denaturation at 98 $^{\circ}$ C for 10 s, annealing at 64 $^{\circ}$ C for 30 s, and extension at 68 $^{\circ}$ C for 30 s). The PCR products were electrophoresed in 2% agarose gels and visualized by staining with 1 μ g/mL ethidium bromide. After purification, direct sequencing was performed on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye

Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and the same primers for 25 cycles (50 $^{\circ}$ C for 5 s and 60 $^{\circ}$ C for 4 min). The sequencing reaction products were purified from residual BigDye terminators using Centri-Sep Columns (Princeton Separations, Adelphia, NJ, USA).

2.9. Sequencing of subcloned fragments

After purification, isolated DNA was subcloned into the Target Clone Plus vector (Toyobo). Plasmid DNA for transfection was isolated using a Pure Yield[™] Plasmid Miniprep System (Promega, Madison, WI, USA). Sequencing was performed using T3 and T7 vector primers.

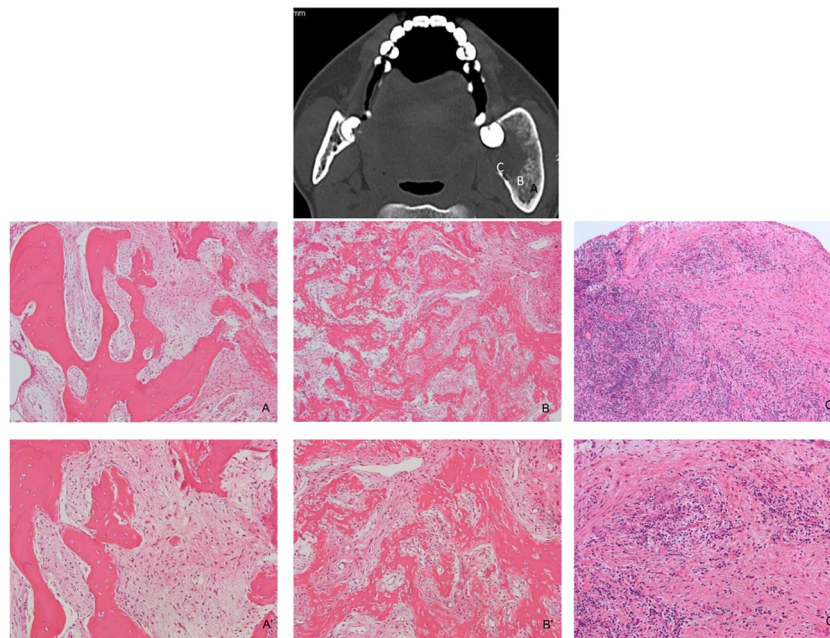


Fig. 2. Microscopic features of fibrous dysplasia in Patient 4. Histopathological examination (hematoxylin and eosin staining). The lesion was composed of irregularly shaped woven bone and fusiform cells with various amounts of collagen. A, B and C: $\times 10$; A', B' and C': $\times 20$.

3. Results

3.1. Patient characteristics

In all five patients, MFD onset was seen in the mandible. Preoperative serum ALP activity ranged from 175 to 326 IU/L, which was normal (the normal values at Kyoto University Hospital are 115–359 IU/L; [Table 1](#)). In panoramic radiographs, a ground-glass pattern was visible in the mandibles, and the borders were unclear. Furthermore, CT images of the mandibles showed severe bony hypertrophy. Bone scintigraphy revealed an intense accumulation of tracer in the mandibles, corresponding with CT findings ([Fig. 1](#)). No malignant transformations were identified. However, Patient 5, a 46-year-old woman, had FD of the mandible requiring repeated surgery with long-term follow-up; she had undergone surgical reduction 11 times in 27 years. The first operation was performed when she was 19 years old.

She had no other significant medical history ([Table 1](#)). Recently, it has been reported that CT can be used to determine the extent, specific dimensions and radiodensity of FD lesions ([Barnes et al., 2005](#); [MacDonald-Jankowski et al., 2004](#)). Based on CT findings and histopathological examination, the FD lesions in Patients 4 and 5 were divided into three parts, 4A–C and 5D–F, respectively ([Figs. 2–4](#)). In Patient 5, lesion 5D was identified on a panoramic X-ray in 1998. In 2006, panoramic X-rays and CT images showed the lesion to have spread from D to E with a proximal enlargement compared with the findings from 1998 ([Fig. 3](#)). Subsequently, in 2014, the lesion was shown to have spread from E to F. CT images of the mandible revealed severe bony hypertrophy with enlargement of the lesion in proximal directions compared with the findings from 2006. The lesion had spread forward over a long interval. This indicated that Patient 5 had FD characteristics that varied from stable-old to active-new.

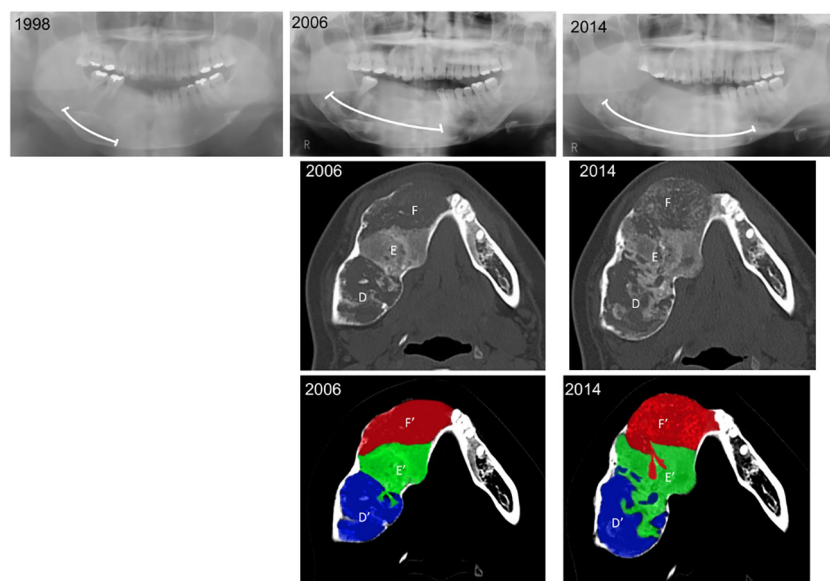


Fig. 3. Panoramic X-ray images and computed tomography images of Patient 5 taken from 1998 to 2014.

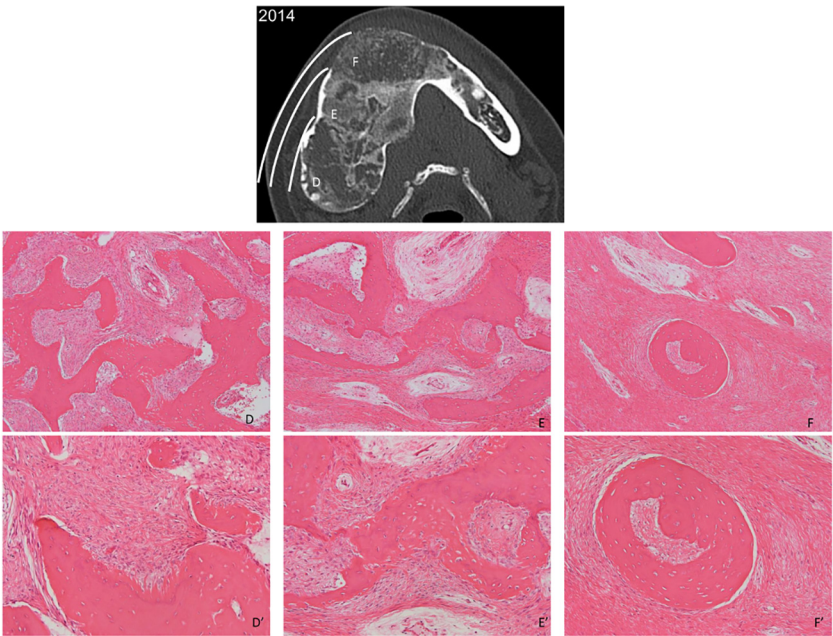


Fig. 4. Microscopic features of fibrous dysplasia in Patient 5. Histopathological examination (hematoxylin and eosin staining). D, E and F: $\times 10$; D', E' and F': $\times 20$.

3.2. Osteogenic activity of lesions reflects their mutation prevalence

Differences in bone growth activity in lesions 5D–F were assessed by performing immunohistochemistry with antibodies against ALP and BMP2. No mitoses or nuclear atypia were seen. The histopathological features of the developing hard tissues varied and consisted of structures such as woven bone and osseous formations. Diffuse ALP and BMP2 expression was observed in fibroblasts, osteoblasts and osteocytes in the reticulum surrounding the bone matrix. In addition, staining was observed in the fibrous matrix of the stroma of bone trabeculae. The immunohistochemical findings for lesions 5D, E and F differed from each other and are summarized in Fig. 5 and Table 2. ALP and BMP2 were expressed at high levels in 5D. However, BMP2 was expressed at low levels in 5F. These results indicate differences in bone growth activity, which confirm that 5D was an old lesion, whereas 5F was a newer lesion.

3.3. Direct sequencing of GNAS mutations in fresh surgical specimens and cultured FD cells

GNAS mutations were verified in fresh surgical specimens and cultured FD cells by direct sequencing. GNAS point mutations in the codon

Table 2

Expression of alkaline phosphatase and bone morphogenetic protein 2 in sections of fibrous dysplasia from Patient 5.

Patient number	Cellular component	Characterization	
		ALP	BMP-2
5-D	Fibroblastic cell	++	+
	Osteoblastic cell	++	+
	Osteocyte	+	+
5-E	Fibroblastic cell	++	+
	Osteoblastic cell	++	+
	Osteocyte	+	–
5-F	Fibroblastic cell	+	+
	Osteoblastic cell	++	–
	Osteocyte	+	–

for Arg201 were not detected in the cultured FD cells from Patients 1, 2 or 3, nor in fresh surgical FD specimens from Patient 3 (Fig. 6). However, it was not possible to distinguish absent from low-frequency mutations in Patients 1, 2 and 3 because of the low sensitivity of direct sequencing. Samples 4A–C from Patient 4 showed a minor adenosine peak in fresh surgical FD specimens and cultured FD cells (Fig. 6). A

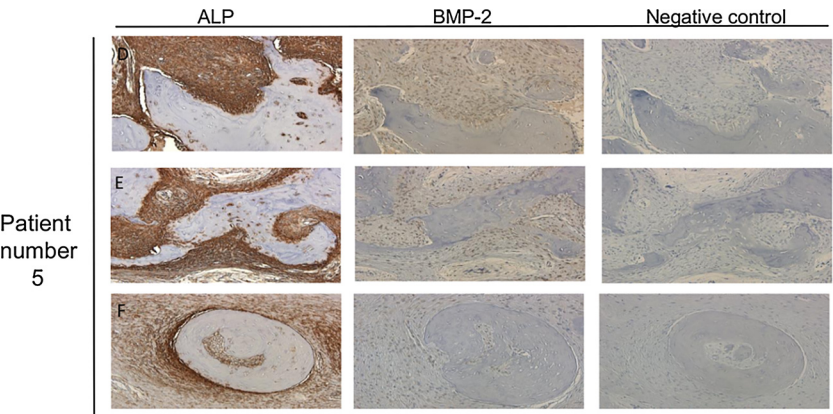


Fig. 5. Immunohistochemical detection of alkaline phosphatase and bone morphogenetic protein 2 in Patient 5 ($\times 20$). BMP2, bone morphogenetic protein 2; ALP, alkaline phosphatase.

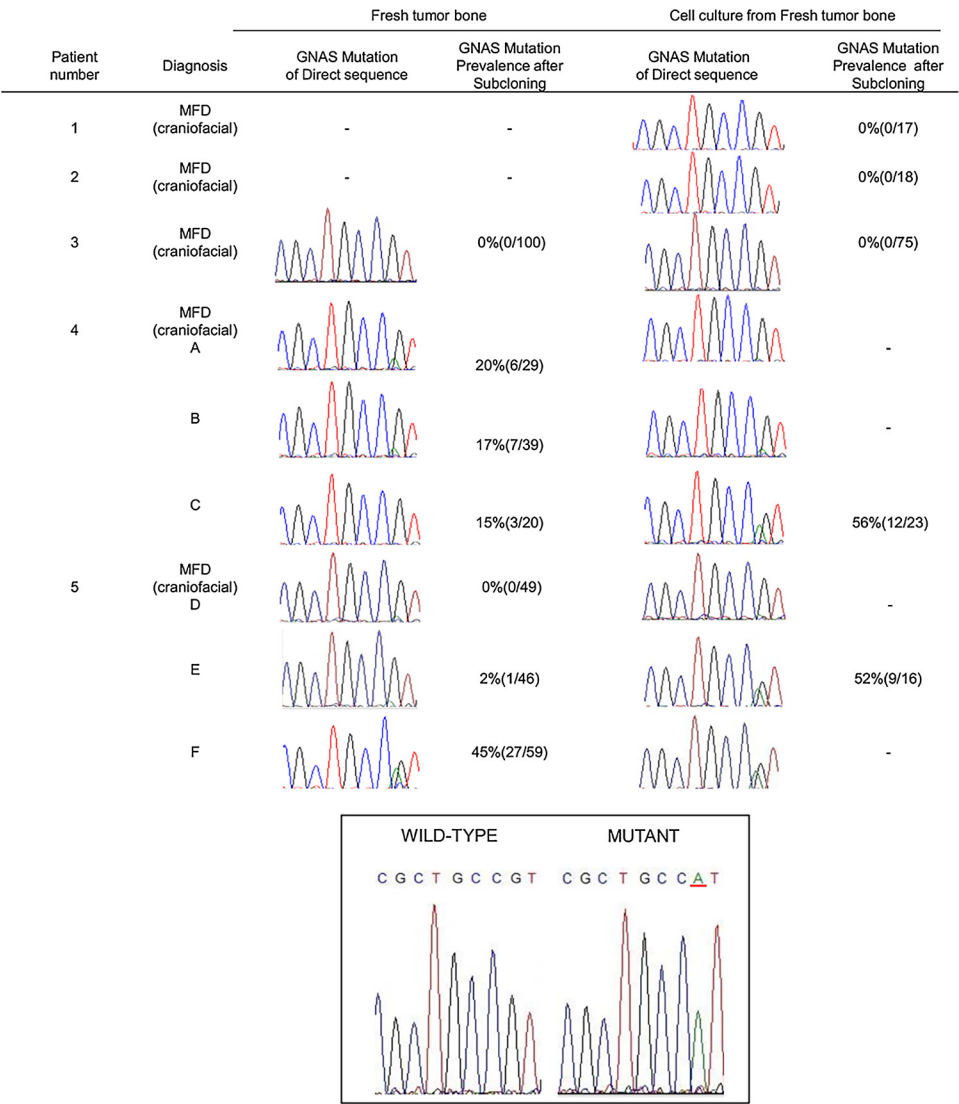


Fig. 6. Sequencing results and percentages indicating the proportion of cultured cells carrying the *GNAS* mutation. Minor adenosine peaks can be seen in green. MFD, monostotic fibrous dysplasia. Bottom panel: mutation type after subcloning. The *GNAS* sense sequences indicate that the second base of codon 201 was mutated from C to A, resulting in a codon change from wild-type arginine (left) to histidine (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

comparatively high-frequency mutation was uncertain for the three different sites of this patient's FD lesion. For Patient 5, the adenosine peak was clearly present in fresh surgical FD specimens and cultured FD cells from samples 5E and F, whereas *GNAS* point mutations were not detected in surgical specimens or cultured cells from 5D (Fig. 6).

Patients 1–5 were respectively aged 57, 44, 18, 29 and 19 years at their first operation (Table 1). No mutation or a comparatively low frequency of mutations was found in two middle-aged and one younger patient (Patients 1–3), but a comparatively high frequency of mutations was found in the other two younger patients (Patients 4, 5). Thus, both low and high frequencies of mutation were found among the younger patients. Furthermore, comparatively low- and high-frequency mutations were detected respectively in the stable-old and active-new FD lesions of Patient 5.

3.4. Prevalence of somatic mutation mosaicism in FD lesions

To calculate the prevalence of somatic mutation mosaicism in FD lesions, sequencing of *GNAS* codon 201 was performed after subcloning genomic PCR products amplified from fresh surgical FD lesions and/or cultured FD cells. For Patients 1 and 2, 17 and 18 clones,

respectively, were isolated from the cultured FD cells. For Patient 3, 100 clones were isolated from the fresh surgical FD specimens and 75 clones from the cultured FD cells. No mutations could be identified in Patients 1–3 after sequencing these clones (Fig. 6). This suggests that the prevalence of somatic mutation mosaicism in the FD lesions of Patients 1, 2 and 3 was less than 5.9%, 5.3% and 1.3%, respectively.

For Patient 4, 29, 39 and 20 clones were isolated from the fresh surgical specimens of samples 4A–C, respectively. Among these clones, we identified six, seven and three mutations respectively. Thus, the prevalence of somatic mutation mosaicism in the FD lesions of samples 4A–C was 20%, 17% and 15%, respectively (Fig. 6). The comparatively high mutation prevalence was similar among the different samples from this patient, whereas CT appearance and histopathological findings were different. This result was consistent with the direct sequencing data.

For Patient 5, 49, 46 and 59 clones were isolated from the fresh surgical specimens of samples 5D–F, respectively. Among these clones, we identified zero, one and 27 mutations, respectively. The prevalence of somatic mutation mosaicism in the FD lesions of samples 5D–F was 0%, 2% and 45%, respectively (Fig. 6). This result was also consistent with that of direct sequencing. Thus, the mutation prevalence in fresh

surgical specimens of this patient differed depending on the site. Distally, lesion 5D was old, with a low mutation prevalence. In contrast, the proximal lesion 5F was newer, with a high mutation prevalence.

To confirm the comparatively high mutation prevalence in Patient 4 and the forward portion of Patient 5's lesion, 23 and 16 clones were isolated from cultured FD cells from samples 4C and 5E, respectively. Twelve and nine mutations were identified among these clones, respectively. The prevalence of somatic mutation mosaicism in samples 4C and 5E was comparatively high at 56% and 52% (Fig. 6), respectively. The mutation prevalence differed between cultured FD cells and fresh surgical specimens from the same patient; it tended to rise in the cultured cells.

4. Discussion

In this study, we hypothesized that differences in lesion growth are caused by the demise of mutated cells. In support of this hypothesis, Patient 5 had FD characteristics that varied from stable-old to active-new. The mutation prevalence differed in this patient, being low in the stable-old lesions and high in the active-new.

Several different PCR-based techniques have been reported for the selective enrichment and analysis of mosaic *GNAS* Arg201 codon mutations (Bianco et al., 2000; Candelieri, Roughley, & Glorieux, 1997; Shenker et al., 1995). These methods allow for the detection of one mutant cell among 200 (Bianco et al., 2000). Stanton et al. (1999) found relatively low mutation prevalence in two out of 11 patients, while Kuznetsov et al. (2008) found relatively low mutation prevalence in seven out of 18 patients. Similar results were obtained in the present study, with a relatively low mutation prevalence in three out of five patients. Furthermore, it has been reported that cells cultured from patients with PFD show a high frequency of *GNAS* mutation, whereas cells from patients with MFD show a low frequency of mutation (Stanton et al., 1999). However, a high frequency of mutation in monostotic craniofacial FD was found in the present study. Kuznetsov et al. (2008) found a low frequency of *GNAS* mutation in cells cultured from older FD patients, whereas those from younger FD patients showed a high frequency of mutation. However, a low frequency of mutation even in younger patients with FD was found in the present study. These previous studies used PCR products amplified from cultured cells. However, cultured cells may not reflect the original mutation prevalence. In the present study, mutation prevalence differed between cultured FD cells and fresh surgical specimens from the same patient. Therefore, to calculate accurate values for the prevalence of somatic mutation mosaicism in FD lesions, clones isolated from genomic PCR products derived from fresh surgical FD specimens were sequenced.

In general, misexpression of BMPs is associated with various bone diseases, and anti-BMP antibodies have been used clinically for the diagnosis of osteogenic lesions (Bessho & Iizuka, 1996). The expression of different BMP subtypes is informative about the bone-forming nature of FD, and BMP2 expression is useful as a clinical index of disease progression and prognosis (Kiso et al., 2015; Sakata et al., 2010). In the present study, Patient 5 had an old lesion (5D) and a newer lesion (5F), as determined by bone growth activity. The mutation prevalence in fresh surgical specimens differed between these lesions. Lesion 5D was old, with a low mutation prevalence (0%, Fig. 5); in contrast, Lesion 5F was newer, with a high mutation prevalence (45%, Fig. 5). Thus, the mutation prevalence differed in the one patient, with the proximal site featuring a higher prevalence. The mutation prevalence was likely lowered in the old lesion because the number of mutant cells was decreased.

The purpose of the present study was to examine patients with the same phenotype and genotype and to show that FD can be normalized by the age-dependent demise of mutated cells. To calculate accurate prevalence values for somatic mutation mosaicism in FD lesions, sequencing of *GNAS* codon 201 was performed after subcloning genomic

PCR products derived from fresh surgical specimens. The mutation prevalence differed within a single patient; it was low in the old and high in the new lesions and, therefore, might directly affect bone growth activity. This report is the first to provide direct evidence for the age-dependent demise of mutated cells. It supports the notion that the demise of mutated cells underlies the cessation of lesion growth in older FD patients. However, to fully demonstrate the age-dependent demise of mutated cells, a more extensive investigation is needed incorporating a statistical analysis of many patients, rather than small case series.

Ethical approval

This study was performed in accordance with the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Kyoto University, Kyoto, Japan (Approval No. G259). All patients provided written informed consent prior to their participation.

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Author contributions

Study design: YI, KT. Performance of experiments: YI, HK, KN, MI, NK. Data collection: YI. Data analysis: YI, HK. Data interpretation: YI, KT, HK, KN, MI, NK, MS, AS, HH, KB. Drafting the manuscript: YI, KT. Revising manuscript content: KT. All authors have read and approved the final article. KT takes responsibility for the integrity of the data analysis.

Declarations of interest

None.

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