Archives of Oral Biology 93 (2018) 133–140

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

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

Yu Isobe[a], Katsu Takahashi[a,*], Honoka Kiso[a], Kazumasa Nakao[a], Masayuki Ikeno[a], Noriaki Koyama[a], Manabu Sugai[b], Akira Shimizu[c], Hironori Haga[d], Kazuhisa Bessho[a]

[a] Department of Oral and Maxillofacial Surgery, Graduate School of Medicine, Kyoto University, Japan
[b] Department of Molecular Genetics, Division of Medicine, Faculty of Medical Sciences, University of Fukui, Japan
[c] Department of Experimental Therapeutics, Institute for Advancement of Clinical and Translational Science, Kyoto University Hospital, Japan
[d] Department of Diagnostic Pathology, Graduate School of Medicine, Kyoto University, Japan

ARTICLE INFO

Keywords:
Fibrous dysplasia
Maxillofacial region
DNA sequencing

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, Gsα. 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, Gsα. 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 Gsα 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

* Corresponding author at: Department of Oral and Maxillofacial Surgery, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail address: takahask@kuhp.kyoto-u.ac.jp (K. Takahashi).

https://doi.org/10.1016/j.archoralbio.2018.05.018
Received 9 December 2017; Received in revised form 29 April 2018; Accepted 23 May 2018

or histidine (Idowu et al., 2007). Somatic mutations in Gsα 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 nevoid 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% CO_2 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 μm 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