Activating mutations in the MAP-kinase pathway define non-ossifying fibroma of bone

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Abstract

Non-ossifying fibroma (NOF) is considered the most common benign and self-limiting lesion of the growing skeleton which occasionally results in pathologic fracture. In a sequencing analysis we have identified hotspot *KRAS*, *FGFR1* and *NF1* mutations in 48 of 59 patients (81.4%) with NOF harbouring allelic frequencies ranging from 0.04 to 0.61. Our findings thus define NOF as a genetically-driven neoplasm caused by activated MAP-kinase signalling in the majority of cases. Interestingly, this driving force either diminishes over time or at least is not sufficient to prevent autonomous regression and resolution. Beyond its contribution to a better understanding of the molecular pathogenesis underlying NOF, the data presented here add another benign lesion to the spectrum of *KRAS*- and MAP-kinase signalling-driven tumours.

Keywords: Non-ossifying fibroma, bone tumour, KRAS, FGFR1, NF1, massively parallel sequencing

Introduction

It is estimated that 30-40% of children develop one or more NOF during skeletal growth [1,2]. However, the true incidence of NOF is unknown, and it seems likely that many lesions stay undetected due to lack of symptoms and spontaneous resolution. The radiological features on plain radiographs of NOF are usually so characteristic that if detected as an incidental finding a biopsy is not required. Since most of these lesions cease growing without causing symptoms and follow a course of remodelling and ossification, therapeutic intervention is rarely needed. On the rare occasion when NOF replace at least 50% of the bone width, surgical treatment in the form of curettage may be undertaken to reduce the risk of fracture. Histologically, NOF are composed of spindle cells arranged in a storiform pattern, along with numerous macrophages in the form of siderophages and foam cells. The diagnosis is generally not challenging particularly in the context of the characteristic imaging features. The genetic background of NOF is currently unknown other than when they occur in patients with neurofibromatosis type 1 [3]. NOF can occur multifocally.

Materials and Methods

Patients and samples. Cases of interest were identified by searching the histopathology archives at the Basel Bone Tumour Reference Centre, Switzerland, the Gerhard-Domagk-Institute of Pathology, Münster, Germany, and the Royal National Orthopaedic Hospital, UK. The study was approved by the NREC-approved UCL/UCLH Biobank Ethical Review Committee (reference EC17.14), the Ethical Committee Münster (reference 2018-174fs) and the Ethikkommission beider Basel (reference 274/12) [Supplementary table 1].

Exome sequencing. Whole exome sequencing (WES) was performed using the Illumina HiSeq 2000 platform after constructing short insert ~400bp libraries, preparing flow cells and generating clusters. For WES coding DNA was enriched for using target enrichment by bait capture (Agilent SureSelect v4.0). Summary sequencing statistics for each sample are in the

Supplementary Table 1. Sequencing data have been deposited in the European Nucleotide Archive under the accession number XXXXXX and XXXXX.

Variant detection. FASTQ files were generated by the Illumina Reporter software and the resulting reads mapped onto human genome hs37d5 using the BWA algorithm. A GATK pipeline was used then to call variants [4] once read duplicates were removed and base qualities recalibrated. Variants were assigned probability scores by the internal GATK VQSR algorithm and excluded if they met any of the following criteria: (i) variant presence in a segmental duplication region; (ii) variant present in fewer than three reads; (iii) fewer than 15 reads in total at a genomic position; (iv) variant allele frequency <3% in the sample; and (v) presence of variant in the Exome Aggregation Consortium dataset (released 22.6.2017) at a frequency >2%. Variants identified in constitutional DNA from any of the other local, non-cancer sequencing project at a frequency of 5% (for example, 29 million variants across 284 samples from the Oxford-Illumina WGS500 consortium) were discarded as being more likely due to systematic error in our pipeline than genuine somatic mutations. The consensus mutation classification criteria of the American College Medical Genetics and Genomics and the Association for Molecular Pathology [5] were then applied to determine pathogenic mutations. At the end of this process we identified heterozygous pathogenic mutations in four genes – *KRAS*, *FGFR1*, *NF1* and *PMM2* – of which the *PMM2* mutations were deemed not to be related to the NOF development on the basis that none of our patients carried inactivating bi-allelic or compound heterozygous mutations that predispose to the congenital disorder of glycosylation, type Ia.

Biological validation and Technical Replication. Two custom gene panels consisting of 189 amplicons (Qiagen GeneRead DNAseq V2 panel, hot spot regions of 19 genes) and of 202 amplicons (Qiagen QIAseq targeted DNA panel, complete exonic regions of 4 genes) were used for mutation analysis of *KRAS*, *FGFR1*, *NF1*, and *PMM2*. Target enrichment was carried out according to the manufacturer's instructions: 40ng of genomic DNA was quantified, fragments were size-selected, end-repaired, A-tailed, adapter-ligated, and

sequenced over a lane of a flow cell. Next-generation sequencing was performed on an Illumina MiSeq sequencer using standard chemistry. The Quantitative Multiplex FFPE Reference Standard (Horizon Discovery # HD200) was used as isogenic quality control for performance monitoring and the evaluation of the NGS workflow integrity. Samples with low DNA quality showing low coverages of target genes in NGS data analysis were additionally analyzed with a second GeneRead DNAseq panel consisting of 1028 amplicons assessing the complete exonic regions of 27 genes. Experimental procedures were carried out as described above (targeted regions for all panels are listed in the Supplementary Information). All *KRAS*, *FGFR1*, *NF1*, and *PMM2* mutations were replicated either by NGS-based GeneRead DNAseq gene panel/QIAseq targeted DNA panel sequencing or by Sanger sequencing.

BaseScope™ In Situ Hybridisation. A BaseScope™ Assay v2 (Advanced Cell Diagnostics, Newark, CA, USA) was used to label mutant *KRAS* p.G12D RNA, according to manufacturer's instructions. Briefly, tissue sections were deparaffinized, followed by epitope retrieval (using Target Retrieval Buffer at 98°C 30 min) and protease treatment (2x 30 min at 40°C). Probe hybridization, signal amplification, colorimetric detection, and counterstaining (hematoxylin and ammonia water) were subsequently performed. As a positive control we used a probe recognizing the ubiquitously expressed housekeeping gene *PPIB* in every sample to test RNA preservation. Additionally, the bacterial probe *dapB* was used as a negative control assay. Finally, the specificity of the *KRAS* p.G12D probe has been demonstrated in a previous study [6]. We investigated eight *KRAS* p.G12D mutated and two *KRAS* p.K117N mutated NOF, all of which were processed in a series of three consecutive sections alternatively stained with one of the three described probes.

Immunohistochemistry (IHC). Immunohistochemical staining was performed on ten *KRAS* mutated (four G12A/D/V, three G13D, one Q61L, and two K117N) and five *KRAS* / *FGFR1* / *NF1* wildtype NOF using a phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (monoclonal rabbit, 1:100, #4370, Cell Signaling Technology, Danvers, MA, USA) and a

BenchMark ULTRA Autostainer (VENTANA / Roche, Basel, Switzerland) on 3 µm tissue sections. In brief, the staining procedure included heat-induced epitope retrieval pretreatment using CC1 buffer (95°C; 32 min) followed by incubation with the primary antibody for 16-32 min and signal detection using the OptiView DAB IHC Detection Kit (VENTANA/Roche, Basel, Switzerland) according to the manufacturer's instructions. Positive and negative control stainings using an appropriate IgG subtype (DCS) were included. Stainings were evaluated using a semi-quantitative approach (H-score; ranging from 0-300) determining the percentage of cells at each staining intensity level, using following formula: [1× (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)]. Immunoreactivity was assessed (0, negative; 1+, weak; 2+, moderate; and 3+, strong), defining the staining intensity in the positive control (breast cancer, NST) as strong.

Results

We analysed 61 samples from 59 individuals with histologically confirmed NOF. The patient cohort included 24 females and 35 males (ratio 1:1.5) ranging from 7-22 years of age (median 14 years), fifty-four lesions involved the lower, and seven the upper extremity. All specimens were obtained from patients with pathologic fracture or decreased biomechanical stability of the affected bone and presented with the classical imaging features of NOF (well-demarcated radiolucencies with sclerotic and scalloped borders involving the cortex and medullary cavity of the metaphyseal region).

Our discovery cohort consisted of 19 fresh-frozen (FF) tissue samples from 19 patients that were analysed by exome sequencing. The mean number of sequenced bases per exome was 36 billion, which equates to 203X mean depth-of-coverage and >15X depth for 99.7% of bases within targeted regions [Supplementary Table 2]. Pathogenic mutations were identified only in *KRAS* (9/19), *FGFR1* (4/19) and *NF1* (1/19). We then extended our study to 42 formalin-fixed and paraffin-embedded (FFPE) tissue samples from 40 patients which were analysed by two customised Qiagen gene panels (mean sequencing depth 1550X for the

Qiagen GeneRead DNAseq V2 panel and of 1140X for the Qiagen QIAseq targeted DNA panel).

In total, *KRAS* mutations were detected in 38/59 patients (64.4%, Fig. 1A). The most common alterations were hotspot *KRAS* mutations p.G12A/D/V and p.G13D, accounting for 62.5% of all *KRAS* mutations. Other activating mutations included p.K117N (20%), p.A146P/T/V (10%), p.Q61R/L (5%), and p.A11Q (2.5%). Two *KRAS* mutations at codons 12 and 13 were detected in one NOF, and two NOFs (clavicle and humerus) from a single patient were found to harbour the p.G12D alleles in both lesions. Germline hotspot mutations in *KRAS* were not detected, a finding consistent with the knowledge that they are embryonically lethal. The identified mutations are therefore likely to be somatic (allelic frequencies 0.04 to 0.61, mean 0.19, IQR 0.08; contaminated by non-neoplastic tissue, including intralesional macrophages, 5-25%; detection limit of gene panel and exome sequencing <5% allelic frequency).

To visualize the mutation transcripts in situ, we complemented BaseScope analysis using a specific probe for *KRAS* p.G12D transcripts in eight samples with *KRAS* p.G12D mutations and two samples with *KRAS* p.K117N mutations. All NOFs with *KRAS* p.G12D mutations revealed mutated transcripts in the mononuclear spindle cell population but not in the osteoclast-like giant cells. The amount of signals was lower compared to the positive control and correlates with the rather low allelic frequency found in the mutation analyses (Fig. 2). No specific signals were detected in the negative controls and in the cases with p.K117N mutation (data not shown). Immunohistochemical reactivity against phosphorylated (i.e. activated) p44/42 MAPK (ERK1/2) as a downstream target of *KRAS* signalling in ten mutated and five *KRAS* / *FGFR1* / *NF1* wild-type NOFs showed consistent and significantly stronger positivity in the mutated tumours compared to the wild-type cases (Fig. 3).

FGFR1 mutations were detected in 8/59 patients (13.6%); these included p.N330I (n=7) and p.C381R (n=1). Germline mutations in *FGFR1* have been shown to cause various congenital musculoskeletal phenotypes, including the exceedingly rare osteoglophonic dysplasia characterised by negative regulation of long-bone growth [7]. Since none of the patients

showed clinical features of skeletal deformities other than NOF and the allelic frequencies of mutations were low (mean 0.23, IQR: 25.55), the *FGFR1* mutations discovered were again likely to be somatic. *FGFR1* and *KRAS* mutations were mutually exclusive.

Finally, *NF1* frameshift mutations were identified in NOF from two individuals that had been clinically diagnosed with neurofibromatosis type 1. Polyostotic NOF are characteristic of neurofibromatosis type 1, and Jaffe-Campanacci syndrome, both of which are caused by germline or post-zygotic mosaic mutations in *NF1* in the majority of cases [8]. NF1 negatively regulates the RAS / MAP-kinase signalling pathway and NF1-related disorders belong to the so-called RASopathies, a heterogeneous group of syndromes frequently accompanied by musculoskeletal abnormalities [9]. Specifically, Noonan and cardiofaciocutaneous syndrome, are caused by germline mutations in *KRAS* that cause less pronounced MAP-kinase signalling activation compared to the hotspot mutations detected in our study [10]. In a minority of patients with these syndromes and even less frequently in patients with neurofibromatosis type 1, giant cell granulomas of the jaws which histologically resemble NOF, are known to occur. Syndromic occurrence of both giant cell granulomas of the jaws and NOF in the same individuals is, however, rare and has been described so far only in oculoectodermal and Jaffe-Campanacci syndrome, the former being a rare mosaic RASopathy caused by p.G13D mutations in *KRAS*.. *NF1 mutations were mutually exclusive with FGFR1* and *KRAS* mutations.

Discussion

With the increasing use of DNA sequencing for genotyping neoplasms, mutations that were thought to be restricted to cancer and its precursors are now being increasingly detected in benign tumours and even in lesions considered to represent developmental disorders or hamartomas. *BRAF and NRAS* mutations, for example, commonly occur as early clonal events in melanocytic nevi whereas activating mutations in the *FGFR3* gene, that belong to the most prevalent mutations in bladder cancer, occur in >50% of seborrheic keratoses and epidermal nevi [11-16]. Notably, some of these mutations can be more frequently identified in

benign lesions compared to their malignant counterparts, e.g. *BRAF* V600E mutations are detected in up to 80% of melanocytic nevi, in 60% of dysplastic nevi compared to 40-45% of melanomas [17]. Only recently, somatic activating *KRAS* p.G12D/V mutations have been reported in the endothelial cells of arteriovenous malformations of the brain and *KRAS* p.G12V mutations in endometriosis without cancer [18,19]. Our data in NOF add another benign tumour to the spectrum of *KRAS*- and MAP-kinase signalling-driven neoplasms identifying *bona fide* pathogenic *KRAS* and *FGFR1* mutations that have been reported in a variety of carcinomas, sarcomas and haematological malignancies. Interestingly, *FGFR1* mutations have recently been shown to occur in (sporadic) pheochromocytomas (that can also harbour y *NF1* mutations), and in rare brain tumour subtypes [20-22].

The uniformly benign and self-limiting clinical course of NOF suggests that MAP-kinase signalling activation is either a transient phenomenon or not sufficient to promote ongoing tumour growth over time. Instead the lesions suddenly start to regress and eventually disappear. Fibrous dysplasia (FD), a fibro-osseous lesion of bone caused by mosaic *GNAS* mutations in osteoblastic precursors, shows a remarkably similar fate to that of NOF. Commonly on reaching skeletal maturity, FD lesions stop growing, involute, and mineralise. Eventually *GNAS* mutations can no longer be detected a finding which is explained by increased apoptosis of the FD-mutant cells. It is interesting to speculate that the same mechanism accounts for the disappearance of the mutant cells in NOF [23]. An alternative mechanism is that *KRAS* and *FGFR1* mutations detected at a low frequency arise only in a minor subclonal fraction of the lesional cells and exert an auto- and paracrine tumour-landscaping effect [24]: such a phenomenon is described for the *COL6A3-CSF1* fusion in tenosynovial giant cell tumours, It has also been suggested that *KRAS* mutated cells instigate reciprocal signalling in both mutant and wild-type cells which could theoretically explain the homogeneous phospho-ERK expression observed in NOF [25,26]. However, the young age at which patients present with NOF and the lack of additional driver mutations in exome sequencing renders *KRAS* and *FGFR1* mutations rather unlikely to develop as a (sub-) clonal population.

Unfortunately, in depth functional elucidation of our findings is difficult in a lesion that usually does not require biopsy or resection and therefore is not accessible for a study on mutational allelic frequencies over time or functional studies. Nevertheless, our findings strongly suggest that pathogenic hotspot *KRAS*, *FGFR1* and *NF1* mutations underlie the vast majority of NOF, the most frequent benign tumours of bone.

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Author Contribution

DB, AMF and WH conceived and designed the experiments. DB, AHK, RT, FA, MT, EW, MN, SH, JH, GG, VV, AMF, and WH contributed tumour material and clinical information. MK, JS, AS, BA, and AKra performed DNA extractions and sequencing analyses. MK and JS analyzed sequencing data and interpreted the results. DB, MK, AMF, and WH prepared the manuscript with contributions from all other authors.

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Figure Legends

Figure 1: Pathogenic *KRAS*, *FGFR1* and *NF1* mutations in NOF. A-B: *KRAS* mutations affecting the RAS domain mapped onto the PDB protein structure 4EPV, which was constructed by Sun and colleagues [27]. C: Mutation profile of 61 NOF from 59 patients. Abbreviations: AF: allelic frequency.

Figure 2: (A) H&E stain of a NOF with *KRAS* p.G12D mutation. Representative images of the same tumour using the positive control *PPIB* probe (B) and the *KRAS* p.G12D probe (C). Another tumour with a *KRAS* p.G12D mutation and the mutation specific probe (D). Probe binding is visualized as punctate red dots.

Figure 3: (A) H&E stain of a NOF with *KRAS* p.K117N mutation showing (C) strong expression of p-(Thr202/204)-ERK1/2 in the lesional spindle cell population. (B) Case of a NOF wildtype for *KRAS*, *FGFR1* and *NF1* displaying (D) focal and weak expression of p-(Thr202/204)-ERK1/2 in the spindle cells while intralesional macrophages show non-specific cytoplasmic staining.

Supplementary Information

Supplementary Table 1: Clinical and genetic description of 61 non-ossifying fibromas from 59 patients

Supplementary Table 2: Exome sequencing coverage statistics

Supplementary Information: Panel sequencing basic information