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Analysis of the fibroblastic growth factor receptor-RAS/RAF/MEK/ERK-ETS2/brachyury signalling pathway in chordomas

Asem AE Shalaby[1,2,6], Nadege Presneau[1,6], Bernadine D Idowu[2,3], Lisa Thompson[5], Timothy RW Briggs[4], Roberto Tirabosco[3], Timothy C Diss[5] and Adrienne M Flanagan[1,2,3,5]

[1]*UCL Cancer Institute, London, UK;* [2]*UCL Institute of Orthopaedics and Musculoskeletal Science, Stanmore, Middlesex, UK;* [3]*Department of Histopathology, Royal National Orthopaedic Hospital, Stanmore, Middlesex, UK;* [4]*The Bone Tumour Unit, Royal National Orthopaedic Hospital, Stanmore, Middlesex, UK and* [5]*Department of Histopathology, UCLH, London, UK*

Chordomas are rare primary malignant bone tumours that derive from notochord precursor cells and express brachyury, a molecule involved in notochord development. Little is known about the genetic events responsible for driving the growth of this tumour, but it is well established that brachyury is regulated through fibroblastic growth factor receptors (FGFRs) through RAS/RAF/MEK/ERK and ETS2 in ascidian, Xenopus and zebrafish, although little is known about its regulation in mammals. The aim of this study was to attempt to identify the molecular genetic events that are responsible for the pathogenesis of chordomas with particular focus on the FGFR signalling pathway on the basis of the evidence in the ascidian and Xenopus models that the expression of brachyury requires the activation of this pathway. Immunohistochemistry showed that 47 of 50 chordomas (94%) expressed at least one of the FGFRs, and western blotting showed phosphorylation of fibroblast growth factor receptor substrate 2 alpha (FRS2α), an adaptor signalling protein, that links FGFR to the RAS/RAF/MEK/ERK pathway. Screening for mutations in *brachyury* (all coding exons and promoter), *FGFRs 1–4* (previously reported mutations), *KRAS* (codons 12, 13, 51, 61) and *BRAF* (exons 11 and 15) failed to show any genetic alterations in 23 chordomas. Fluorescent *in situ* hybridisation analysis on *FGFR4*, *ETS2* and *brachyury* failed to show either amplification of these genes, although there was minor allelic gain in brachyury in three tumours, or translocation for *ERG* and *ETS2* loci. The key genetic events responsible for the initiation and progression of chordomas remain to be discovered.
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Chordomas are rare tumours accounting for 1–4% of all primary malignant bone tumours and, in the majority of cases, occur in vertebral bones.[1] They are evenly distributed along the axial skeleton[2] and are slow growing and locally destructive, although up to 40% of cases metastasise.[3] Treatment options other than surgery, such as proton/photon-beam radiotherapy when accessible, are of limited benefit, as the tumours are largely resistant to chemotherapy and radiotherapy.[4]

Correspondence: Professor AM Flanagan, MB, FRCPath, PhD, UCL, Institute of Orthopaedics and Musculoskeletal Science, Royal National Orthopaedic Hospital, Stanmore, Middlesex, HA7 4LP, UK.
E-mail: a.flanagan@ucl.ac.uk
[6]These authors contributed equally to this paper.
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Brachyury is a transcription factor that plays an important role in notochord development, as well as in the elongation of the axis and specification of posterior mesodermal elements.[5,6] This molecule is detected only transiently after birth, other than in spermatogonia.[1,7] Brachyury is also expressed in chordomas, but rarely in other neoplasms,[1,8] and this observation is consistent with century-old reports that chordomas exhibit transmitted light and electron microscopic features similar to that of notochord.[9] There is now good evidence that chordomas derive from notochordal precursor cells sited in vertebral bodies.[10,11]

Although little is known about the molecular genetic events involved in chordoma initiation and progression, the regulation of notochord development is well documented, particularly in the ascidian, zebrafish and Xenopus embryos.[12–14] In both zebrafish and Xenopus models, basic fibroblas-

Table 1 Details of antibodies used for immunohistochemistry

Antibody/clone	*Source*	*Antigen retrieval*	*Dilution*	*Incubation time and temperature*	*Method*
Cytokeratin 19 clone b170	Novocastra, Peterborough, UK	Protease 20 min	1:100	30 min at 37°C	Ventana NexES autostainer
Brachyury (H-210): sc-20109	Santa Cruz, CA, USA	Pressure cooker 2 min	1:50	30 min at 37°C	Ventana
FGFR1/Flg (C-15): sc-121	Santa Cruz	Protease 14 min	1:150	30 min at 37°C	Ventana
FGFR2/Bek (C-17): sc-122	Santa Cruz	Protease 14 min	1:100	30 min at 37°C	Ventana
FGFR-3 (C-15): sc-123	Santa Cruz	Protease 14 min	1:100	Overnight at 4°C in a humid chamber	Manual staining
FGFR-4 (C-16): sc-124	Santa Cruz	Protease 14 min	1:50	30 min at 37°C	Ventana

tic growth factor, embryonic fibroblastic growth factor and fibroblastic growth factor receptor (FGFR) 1 signalling are required for brachyury expression.[12,15] In humans, the FGFR family includes four transmembrane receptor tyrosine kinases, and it is through these and their alternatively spliced variants that more than 20 FGF ligands signal and bring about activation of downstream targets through phosphorylation of fibroblast growth factor receptor substrate 2 alpha (FRS2α), an adaptor signalling protein that links FGFR to the RAS/RAF/MEK/ERK and phosphoinositide 3-kinases (PI3K) signalling pathways.[16] FGF regulation of brachyury is mediated through *ETS2*, a gene known to be translocated in patients with acute myeloid leukemia,[17] and ETS2 is also phosphorylated through activation of the RAS/RAF/MEK/ERK pathway.[18] Of particular interest is a report describing a translocation at chromosome 21q22, involving either *ERG* or *ETS2*, in two sacral chordomas.[19] Finally, it is noteworthy that array comparative genomic hybridisation shows that the locus harbouring *FGFR4* shows allelic gain in chordomas.[20]

In this study, having shown that one of the FGFRs is expressed in the majority of chordomas, we have examined the genetic status of a number of molecules in the FGFR-RAS/RAF/MEK/ERK-ETS2/brachyury signalling pathway in 50 chordomas in an attempt to elucidate the molecular genetics of this rare disease.

Materials and methods

This study complies with standards laid down by the Central Office for Research Ethics Committee, UK. The material, 23 snap-frozen and 50 paraffin-embedded tissues, was obtained from the histopathology department of The Royal National Orthopaedic Hospital. The clinical data were retrieved from the clinical notes. The cohort studied consisted of chordomas from 50 patients, 31 men and 19 women, with a median age of 65 years (range 19–84). The tumours were characterised by the typical chordoma morphology and the co-expression of a pan-cytokeratin, cytokeratin 19 and brachyury. The sites of the tumours were sacro-coccygeal ($n=41$), lumbar ($n=6$) and cervical ($n=3$). In a follow-up period ranging from 12 to 120 months, 46% of patients had one local recurrence and 12% had two recurrences. Metastases occurred in 22% of patients involving lung, bone and skin. Five of the tumours had a de-differentiated component but this material was only available from two cases and therefore the de-differentiated component was not analysed in this study. None of the metastatic samples was analysed in this study.

Tissue Microarray

The pathology was reviewed by AMF, AS and RT. A tissue microarray of 50 chordomas was constructed using a manual tissue arrayer (Beecher Instruments Inc, Sun Prairie, WI, USA). At least two representative 0.6 mm cores from each case were taken for the array. An additional 120 cores were used as control material and orientation markers, and included human salivary gland, kidney, thyroid, tonsil, lymph node, placenta, testis, skin, thyroid carcinoma, renal cell carcinomas and breast carcinomas.

Antibodies and Immunohistochemical Analysis

Immunohistochemistry was carried out with the Ventana NexES Autostainer (Ventana Medical Systems, Strasbourg, France) using antibodies, and experimental conditions are detailed in Table 1. Diaminobenzidine (DAB) was used as a chromogen in all reactions. The primary antibody was substituted by an isotype serum that served as a negative control. Each antibody was scored with reference to a positive internal control on the tissue microarray. For FGFR2 and FGFR4, normal kidney served as a positive internal control, whereas epidermis was the positive control for FGFR3 and FGFR1. The scoring system used was as follows: 0—absence of immunoreactivity; 1—'weak', where the intensity of staining was less than that of the positive control; 2—'moderate', where the intensity of staining was as strong as that of the positive control; and 3—'strong', where the intensity of staining was stronger than that of the positive control. In addition, +, ++ and +++ indicated that $<5\%$, between 6 and 49% and more than 50%,

respectively, of the lesional cells were immunoreactive.

Fluorescence *In-Situ* Hybridisation (FISH) Analysis of Brachyury, ETS2, ERG and FGFR4

Fluorescence *in-situ* hybridisation was carried out on the tissue microarray slides after optimisation of the protocol, using probes from the RP-11 BACs library (The Wellcome Trust Sanger Institute, Hinxton, Cambs, UK); RP11-235F13 and RP11830D9 were used for assessing *brachyury* and *ETS2* amplification, respectively. An 'in-house' break-apart probe was prepared for assessing the existence of an *ETS2* or *ERG* rearrangement with BAC clones, RP11-8N7 (centromeric to *ERG* and *ETS2*) and RP11-42C22 (telomeric to *ERG* and *ETS2*) (The Wellcome Trust Sanger Institute). RP11-1008G19 was used to assess *FGFR4* amplification (BAC/PAC resources centre, Oakland, CA, USA). The probes were prepared with reagents from Vysis (Vysis, Abbott Laboratories Inc, Des Plaines, IL, USA). Tissue microarray slides were deparaffinised in xylene and dehydrated in ethanol. The sections were pre-treated using the Paraffin Pre-treatment Reagent Kit II (Vysis): the slides were placed in the pre-treatment solution at 80°C for 50–70 min, and in pepsin solution 0.05% at 37°C for 20 to 25 min. The probes applied to the slides were co-denatured for 5 min at 73°C and hybridised for at least 16 h at 37°C in a Thermo-Brite hybridiser (Iris, Westwood, MA, USA). The slides were washed in formamide-free solutions: 2X Saline-sodium citrate (SCC)/0.3% Igepal (Sigma-Aldrich Company Ltd, Poole, Dorset, UK) at room temperature for 5 min, at 73°C for 2 min and then at room temperature for 1 min. Fifty non-overlapping nuclei, which were clearly identified and contained unequivocal signals, were counted for each case. The ratio between the gene of interest and the centromeric probes was calculated, and amplification was considered if this ratio was more than two. One hundred cells were counted in any case in which the ratio was found to be between two and three. A rearrangement using break-apart probes was considered to have occurred when the distance between the two probes was greater than the size of either of the probes. A case of Ewing sarcoma known to harbour an *EWS-ERG* translocation, confirmed by FISH and quantitative reverse-transcriptase real-time polymerase chain reaction, was used as a positive control for the 'in-house' break-apart rearrangement probe.

DNA Isolation and Mutational Analysis by Denaturing High-Performance Liquid Chromatography (DHPLC) and Direct Sequencing

Genomic DNA was extracted using proteinase K (Qiagen, Crawley, West Sussex, UK) from 23 frozen chordomas. The sampled neoplasms comprised at least 80% tumour. Polymerase chain reaction primers were designed to include the intron/exon boundaries to be analysed in addition to the coding sequence, and are described in Supplementary Table 1. For DHPLC analysis, 50 to 100 ng of genomic DNA was amplified by the following touchdown polymerase chain reaction protocol with annealing temperature reduced by 1°C per cycle from 65 to 56°C, followed by 35 further cycles at 56°C. Each cycle was carried out as follows: 95°C for 45 s, annealing at 56°C for 45 s, extension at 72°C for 90 s, followed by a final extension step at 72°C for 10 min. The polymerase chain reaction was carried out in a total volume of 25 μl containing 2.5 μl of 10X PCR buffer II, 0.2 μM of the dNTP mix, 100 pM of each primer, 0.1 mM of $MgCl_2$ and 1.25 U of optimase enzyme (Transgenomic, Crewe, Cheshire, UK). Before DHPLC analysis, the polymerase chain reaction products were run on an agarose gel to ensure that only the specific product was amplified and that no contaminating bands were present.

A Transgenomic Wave DNA Fragment Analysis System was used to carry out DHPLC analysis (Transgenomic Inc, Omaha, NE, USA). Before DHPLC analysis was performed, the unpurified polymerase chain reaction products were denatured at 95°C for 5 min and cooled to 25°C using a temperature ramp of 1°C/min to produce heteroduplex formations, and 6 μl of the products were loaded onto a preheated DNASep® HT Cartridge column (Transgenomic Inc). DNA was eluted at a flow rate of 0.9 ml/min using a linear acetonitrile gradient that consisted of buffer A (0.1 M triethylammonium acetate; TEAA) and buffer B (0.1 M TEAA, 25% acetonitrile). DHPLC melt software, available from http://insertion.stanford.edu/melt.html, was used to select the optimal temperature to separate hetero-duplexes.

For direct sequencing, 50–100 ng of genomic DNA was amplified by the following touchdown polymerase chain reaction protocol with annealing temperature reduced by 1ºC per cycle from 65 to 56°C followed by 35 further cycles at 56°C as described above. The polymerase chain reaction was carried out in a total volume of 50 μl containing 5 μl of 10X Hotstart buffer I, 0.2 μM of the dNTP mix, 100 pM of each primer and 1 U of Hotstart DNA polymerase (CLP, San Diego, CA, USA). PCR products were purified using a Qiagen purification PCR kit (Qiagen) and sent for DNA sequencing at the Scientific Support Services at the UCL Cancer Institute/Wolfson Institute for Biomedical Research. Sequencing reactions were run using GenomeLab™ DTCS Quick Start chemistry (Beckman Coulter UK Ltd, High Wycombe, Bucks, UK) and were analysed on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

Protein Isolation and Western-Blot Analysis

Ten 10 μm sections were cut from snap-frozen tumours and placed into eppendorf tubes. A tumour

lysate was produced with Radio Immuno Precipitation Assay lysis buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris (pH 8.0)) containing both phosphatase and protease inhibitor cocktails (Sigma-Aldrich). Lysates were incubated on ice for 15 min and then centrifuged at 13 000 r.p.m. at 4°C for 10 min to remove debris. Proteins were quantified with Bio-RAD protein assay base using the Bradford method (BIORAD, Munich, Germany). Thirty micrograms of polypeptides was resolved by SDS-8% polyacrylamide gel electrophoresis (SDS-8% PAGE) and transferred to a poly-vinylidene fluoride (PVDF) Immobilion-P transfer membrane (Millipore Corporation, Bedford, MA, USA) by standard semi-dry electro-transfer methods. The membrane was blocked with PBS, 0.1% Tween 20, 5% BSA for a minimum of 30 min, and probed with the appropriate primary antibody overnight at 4°C. The blots were washed thrice (10 min each) in 1 × PBS with 0.1% Tween 20 (PBS-T) and incubated for 1 h at room temperature with the appropriate secondary horseradish peroxidase-conjugated antibody, followed by further washing, and enhanced using chemiluminescence (ECL) detection (GE Healthcare Ltd, Amersham, Bucks, UK).

The following antibodies were used for western blotting: phospho-FRS2α (Tyr[196]) (dilution 1:1000, Cell signalling Technology, Danvers, MA, USA), phospho-ERK1/2 (dilution 1:1000, overnight incubation at 4°C, Cell Signalling Technology), and re-probed with GAPDH (MAb 6C5, dilution 1:5000, incubation 60 min at room temperature (Advanced Immunochemical Inc, Long Beach, CA, USA) or total ERK1/2 (dilution 1:1000), with overnight incubation at 4°C (Millipore Ltd, Watford, Herts, UK).

Results

Expression of FGFR Protein and Phosphorylated Downstream Molecules in Chordoma

Immunohistochemical analysis for FGFRs on the chordoma tissue microarray showed that 47 of the 50 cases (94%) showed immunoreactivity for at least one of the FGFRs (Table 2, Figure 1). In 39 cases, more than one receptor was detected by immunohistochemistry and, in 17 cases, the tumour cells were immunoreactive for all four receptors: 9 and 13 cases were reactive for two and three markers, respectively. In the majority of cases, more than 50% (+++) of the tumour cells showed immunoreactivity (Table 2). To test whether FGFRs were involved in signalling in chordomas, western blotting was carried out to test for phosphorylation of FRS2α and ERK1/2, as the former is an adaptor signalling protein that links FGFR to the RAS/RAF/MEK/ERK signalling pathway. Western blot analysis showed phosphorylation of FRS2α and ERK1/2 in six chordomas that were also reactive by immunohistochemistry for at least one of the FGFRs (Figure 2). Two chordomas, negative by immunohistochemistry for all four FGFRs, were also negative by western blot analysis for phosphorylation of FRS2α, although these were positive for phospho-ERK1/2 (Figure 2).

Table 2 FGFR 1, 2, 3 and 4 immunoreactivity in chordomas

	FGFR1	*FGFR2*	*FGFR3*	*FGFR4*
3 +++	17	8	13	4
++	0	0	0	1
+	0	1	0	0
2 +++	8	9	10	11
++	1	1	1	2
+	0	0	0	0
1 +++	1	17	15	15
++	0	1	0	2
+	1	3	0	0
0	22	10	11	15
Total number of positive cases	28/50	40/50	39/50	35/50
%	56%	80%	78%	70%

The scoring system employed was as follows: negative immunoreactivity (0). Positive immunoreactivity includes the following subgroups: (1) 'weak', the intensity of the staining is less than the positive control, (2) 'moderate', the intensity of the staining is as strong as the positive control, and (3) 'strong', the intensity of the staining is stronger than the positive control. In addition, +, ++ and +++ indicate that < 5%, between 6 and 49%, and > 50%, respectively, of the lesional cells were immunoreactive.

Screening for *FGFR* Mutations and Amplification

In view of FGFRs being involved in the regulation of brachyury, and the knowledge of mutations in these genes being involved in tumourigenesis, direct sequencing of those exons in which activating germline and somatic mutations have been reported previously was carried out. These included *FGFR1* (exons 3, 6, 11, 12, 14), *FGFR2* (exons 5, 7, 11, 12), *FGFR3* (exons 2, 4, 5, 6, 8, 9, 15) and *FGFR4* (exons 7, 9, 16) (for review see[21] and Supplementary data Table 2). However, sequencing failed to show any mutations in 23 chordomas.

Fluorescence *in-situ* hybridisation was carried out in our cohort of 50 chordomas to determine if there was amplification of *FGFR4,* because an array comparative genomic hybridisation report earlier showed that the locus harbouring *FGFR4* was found to be gained in more than five cases.[20] However, our 50 cases failed to show any amplification of *FGFR4*.

Screening for *Brachyury* and *ETS2* Amplification, and *ETS2* and *ERG* Gene Rearrangement

Brachyury is an embryonic marker expressed in chordomas and is largely specific for this disease. We hypothesised that genetic alterations within this gene or *ETS2*, an upstream molecule of brachyury in the signalling pathway in ascidian and Xenopus models, could account for the development of this tumour. FISH showed that expression of *brachyury*

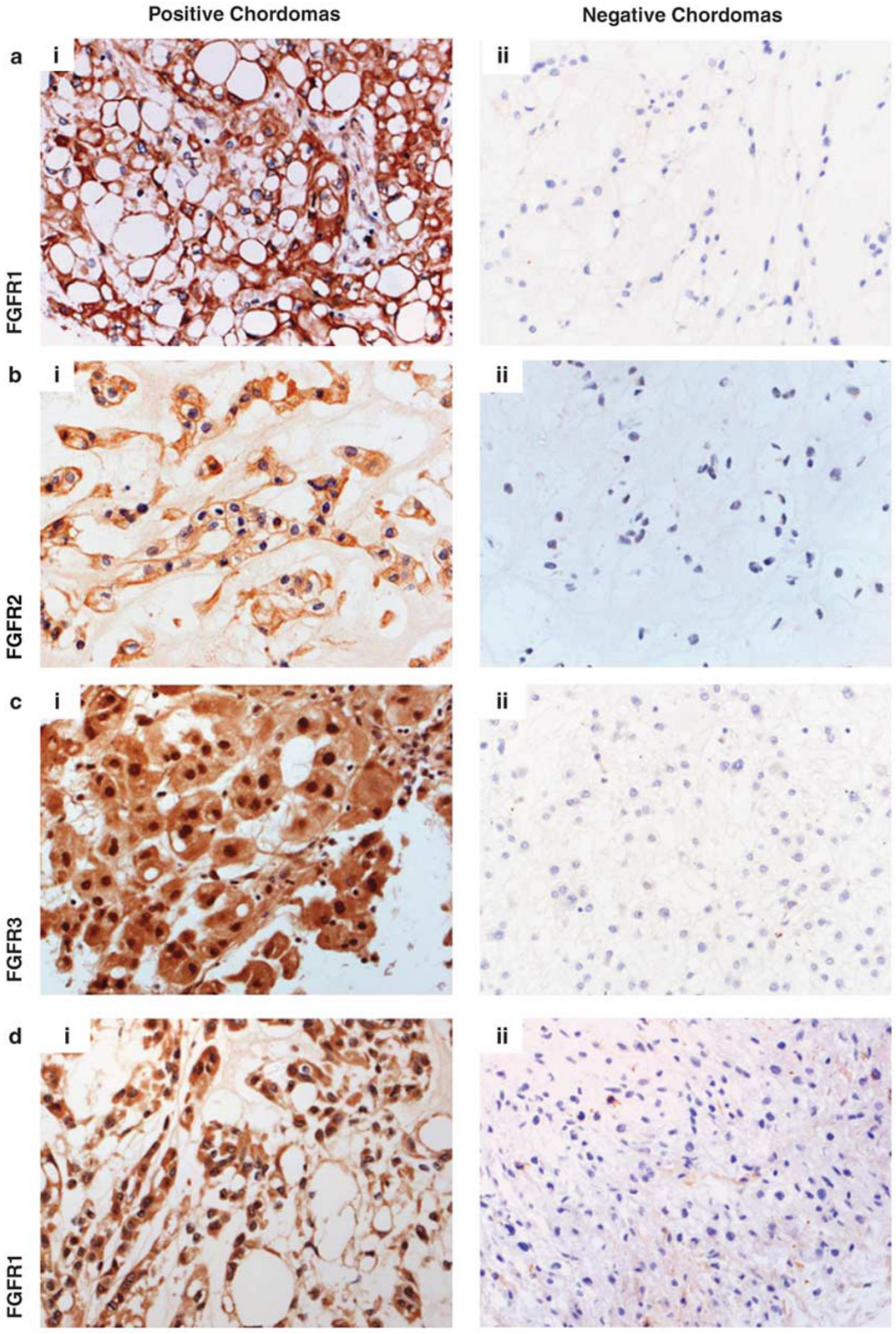

Figure 1 Representative transmitted light photomicrographs of chordomas on tissue microarray. The photomicrographs on the left panel represent positive immunoreactivity and the right panel includes representative chordomas that were not immunoreactive for each of the four FGFRs: FGFR1 (**ai**, **aii**), FGFR2 (**bi**, **bii**), FGFR3 (**ci**, **cii**) and FGFR4 (**di**, **dii**). Magnification $\times 200$.

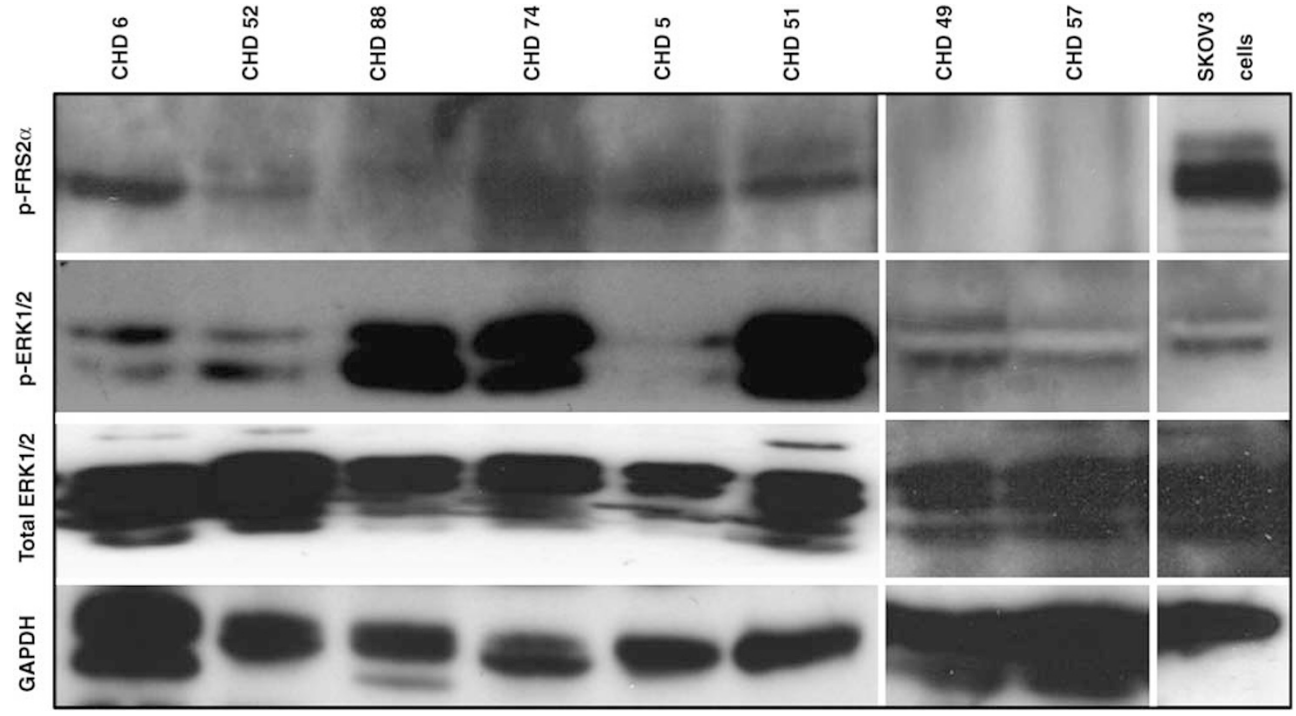

Figure 2 Western blotting analysis for p-FRS2α^{Tyr196} and p-ERK1/2$^{Thr202/Tyr204}$ on eight selected chordomas (CHD). The six chordomas on the left-hand panel were positive by immunohistochemistry for at least one of the FGFRs and showed a band corresponding to p-FRS2α and p-ERK1/2 by western blotting. The middle panel of two chordomas, found to be negative for any FGFR by immunohistochemistry, did not display a band for p-FRS2α. Nonetheless, a band was detected by western blotting for p-ERK1/2 in these two cases. In the right-hand panel, SKOV3 cell line, stimulated with basic FGF, was used as a positive control. The membranes were stripped and re-probed with total ERK1/2 and an anti-GAPDH antibody to ensure an even loading of proteins in each lane.

in chordomas is not accounted for by amplification of either *brachyury* or *ETS2* in 36 and 32 chordomas, respectively. However, in 3 of 39 chordomas, a minor allelic gain showing a ratio of 3:1 for *brachyury* to the centromeric probe was noted (Figure 3).

ETS2 and *ERG* were not rearranged as assessed by an 'in-house generated' break-apart probe in 27 informative cases (data not shown).

Mutation Screening of *Brachyury*, *KRAS* and *BRAF*

Direct DNA sequencing of all coding *brachyury* exons and the promoter region showed that expression of *brachyury* is not accounted for by somatic mutations in 23 cases screened.

As KRAS and BRAF are upstream molecules in the signalling pathway of ETS2 and brachyury, we screened for the common mutations in *KRAS* (codons 12, 13, 51 and 61) and *BRAF* (exons 11 and 15) in 23 chordomas, using DHPLC, but failed to show any abnormalities.

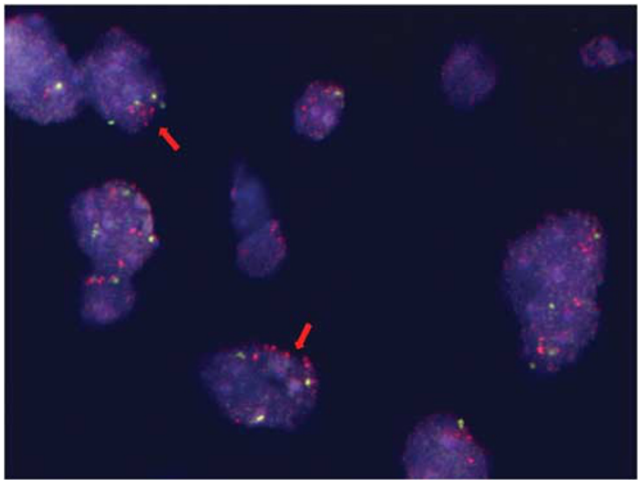

Figure 3 Photomicrograph of interphase fluorescence *in-situ* hybridisation showing an example of minor allelic gain of brachyury. The red signals are for brachyury (red arrows) and the green signals are for chromosome six α satellite probe.

Discussion

The effects of FGFR signalling on tumours is multifold and include increased proliferation, resistance to apoptosis, enhanced motility and invasiveness, increased angiogenesis, metastasis, and resistance to chemo and radiotherapy, all of which can result in tumour progression. Hence, interruption of the FGF signalling system is an attractive therapeutic target, particularly as such therapies can affect tumour cells directly and

tumour angiogenesis.[22–24] Moreover, some agents are currently already in clinical trials.[25]

As brachyury is known to be regulated by FGFR1 and FGFR3 in development,[26,27] we hypothesised that the FGFR signalling pathway is involved in the development of this tumour. We now report for the first time that FGFR1 and FGFR3 are expressed in 56 and 78% of chordomas, respectively. It is noteworthy that over 90% of chordomas express at least one of the four FGFRs. We have also provided evidence that there is activation of the FGFR signalling pathway in chordoma because all six chordomas, which are immunoreactive for one of the FGFRs, are also positive for the phosphorylated forms of both FRS2α and ERK1/2 as shown by western blot analysis. However, two chordomas that are negative for all four FGFRs by immunohistochemistry, and also negative for phosphorylated FRS2α by western blot, are positive for phospho-ERK1/2. This suggests that in the minority of cases, activation of the RAS/RAF/MEK/ERK signalling pathway in chordomas is not dependant on FGFR signalling and is therefore mediated by alternative receptors. As it is known that chordomas also express several other tyrosine kinase receptors including c-MET, PDGFR β and KIT, EGFR, Her2/neu and TrK, which also activate the RAS/RAF/MEK/ERK pathway, these should be considered candidates for the regulation of brachyury.[28–32]

The occurrence of *FGFR* mutations is well documented in the literature, and these are associated in both neoplasia and developmental syndromes. The mutations implicated in the former include those in endometrial, gastric, urothelial and prostatic cancers,[21,33–37] and many of the activating *FGFR2* and *FGFR3* mutations, recently reported in endometrial carcinomas, are identical to germline mutations known to cause syndromes, several of which give rise to skeletal abnormalities including the Apert Syndrome, Beare–Stevenson Syndrome, hypochondroplasia, achondroplasia and the SADDAN syndrome (see Supplementary Table 2 based on Catalogue of Somatic Mutations In Cancer (COSMIC).[35] Apart from FGFR1 being known to be involved in brachyury expression, FGFRs were therefore considered strong candidates for the role of mutation-bearing genes that would be causative in the development/progression of chordomas. Nevertheless, despite the immunoreactivity of 47 chordomas for FGFRs, we have failed to detect mutations in any of the 23 chordomas analysed by direct sequencing in which mutations have been reported earlier. We considered that it was also worth investigating whether *FGFR4* is amplified because Hallor *et al* reported by array comparative genomic hybridisation that there is allelic gain in chordomas on chromosomal band 5q35.2, which harbours *FGFR4*.[20] However, FISH failed to detect an increased copy number of this gene in 50 chordomas. Amplification of the other *FGFR* genes was not studied because there is no evidence in the literature of allelic gain in the loci of these genes in chordomas.

Signalling through FGFRs can activate multiple signal transduction pathways that are known to be implicated in tumourigenesis, and include the phospholipase C γ, phosphatidyl inositol 3-kinase, RAS/RAF/MEK/ERK, and signal transducers and activators of transcription (STAT) pathways.[38–40] However, as analysis of all of these pathways was considered beyond the scope of this work, we focused on the RAS/RAF/MEK/ERK pathway because rearrangement of the locus of *ETS2* has earlier been implicated in pathogenesis of chordoma by karyotyping.[19] *ETS2* is a particularly interesting candidate because this gene lies upstream of brachyury and is known to be involved in its regulation, and it is also phosphorylated through activation of the RAS/RAF/MEK/ERK pathway.[41,42] Furthermore, rearrangements involving *ETS2* have been detected in other neoplasms including acute megakaryocytic leukaemia, acute non-lymphoblastic leukaemia and prostatic cancer.[17,43,44] However, the 'in-house' break-apart BAC probe failed to show a rearrangement in this gene in 23 chordomas. Furthermore, *ETS2* was not amplified as assessed by FISH. Therefore it seems that rearrangement and amplification in this gene is unlikely to represent a common tumourigenic event in chordomas.

We speculated that a genetic event in *brachyury*, which would induce its overexpression, could be involved in the development of chordoma[8,45] despite no *brachyury* mutations being reported in tumours to date, although tumourigenic mutations have been found in related T-box genes in pancreatic and breast cancers.[46,47] In contrast, heterozygous *brachyury* loss in mice results in a short tail and notochordal abnormalities, and in humans, congenital vertebral malformations (hemi vertebra, vertebral bars, supernumerary vertebrae, butterfly and wedge shaped vertebrae), neural tube defects (spina bifida and anencephaly) and sacral agenesis are associated with genetic variations in *brachyury*, the functions of which are variable and not always clear.[48–50] The failure to detect mutations in any of the coding regions, splice junctions and promoter region of this gene in 23 chordomas and the failure to show amplification of *brachyury* in 36 of 39 chordomas imply that the expression of *brachyury* mRNA and protein is likely to be driven by events in upstream molecules. Furthermore, despite the minor allelic gain of *brachyury* in three cases, a finding which corresponds to the array comparative genomic hybridisation data published earlier, we consider that amplification of this gene is also unlikely to account for the driving force in the growth and development of this tumour in view of the low copy number of the gene in the small number of cases that was identified.

We have provided evidence for FGFR-RAS/RAF/MEK/ERK signalling in chordomas and have shown

that some oncogenic events involving *FGFRS, KRAS, BRAF, ETS2* and *brachyury* are rare events in these neoplasms. However, in other studies in which dysregulated FGFR signalling has been implicated in cancer development or progression, there is increased expression of the receptors at a protein and mRNA level in the tumour compared with that in a non-neoplastic precursor,[53] but the difficulty in obtaining notochord for comparison prevents this experiment. Furthermore, the difficulty in carrying out experimental studies to test whether FGFR signalling is abnormal is compounded by the lack of chordoma cell lines, and although there is one, UCH-1,[54] it is extremely slow growing, making it difficult to be used as an experimental model for disease. The possibility remains that there are novel *FGFR* mutations in exons that we have not sequenced and that mutations in FGF ligands exist, as they have been reported in other tumours.[51,52] However, we considered that an analysis of these genes is beyond the scope of this project. Hence, when considering how to take chordoma research forward, it may be more time and cost efficient in the future to carry out genome-wide analysis on a cohort of tumours on a number of different platforms, rather than take a candidate approach as a means of identifying key genetic events in the development of this disease. Generation of cell lines would also be a major contribution to this area of research.

Conflict of interest

The authors declare no conflict of interest.

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Electronic-database information

The URLs for data in this article are as follows: Catalogue of Somatic Mutations In Cancer (COSMIC), http://www.sanger.ac.uk/genetics/CGP/cosmic/.

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