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Original Paper

Brachyury, a crucial regulator of notochordal development, is a novel biomarker for chordomas

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Abstract

Chordomas are malignant tumours that occur along the spine and are thought to derive from notochordal remnants. There is significant morphological variability between and within chordomas, with some showing prominent areas of chondroid differentiation. Our microarray data from a broad range of connective tissue neoplasms indicate that, at the transcriptional level, chordomas resemble cartilaginous neoplasms. Here we show that chordomas express many genes known to be involved in cartilage development, but they also uniquely express genes distinguishing them from chondroid neoplasms. The brachyury transcription factor, known to be involved in notochordal development, is only expressed by chordomas. Using a polyclonal antibody, we show that brachyury is expressed in the embryonic notochord and in all 53 chordomas analysed, labelling both chondroid and chordoid areas of these tumours. In contrast, the protein was not detected in over 300 neoplasms, including 163 chondroid tumours. Brachyury was not detected in the nucleus pulposus, arguing against the hypothesis that this tissue derives directly from the notochord. These data provide compelling evidence that chordomas derive from notochord and demonstrate that brachyury is a specific marker for the notochord and notochord-derived tumours.
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Introduction

The notochord is a crucial structure in embryonic development: it is formed by the mesodermal cells as they leave the primitive streak, and provides longitudinal support for the embryo body in addition to producing inductive signals crucial to the formation of ventral neural structures and somites [1]. It is closely related to cartilage and has been considered a primitive form of this tissue [2]. During embryogenesis, most notochordal cells die and are replaced by bone in the vertebral bodies and by the nucleus pulposus in the intervertebral discs [3]. However, whether the nucleus pulposus is formed directly by notochordal cells is unclear [3–5]. Small collections of notochordal cells persist into adult life and are found around the base of the skull in 0.6–2% of autopsies [4,6]. A recent report of 100 autopsies found microscopic notochordal intraosseus remnants in 11% of the clivus and 19% of spinal vertebrae [6]: there is evidence that chordomas arise from these remnants (for review, see [4]).

Chordomas are rare malignant tumours occurring along the spine. In a large study of 325 patients, 48% occurred in the sacrococcygeal region, 38.5% at the skull base and the remainder along the mobile spine [7]. Morphologically, the tumour cells are arranged in lobules and textbook examples have prominent vacuoles conferring the typical physalipherous appearance. However, there is significant morphological variability, both between and within chordomas. Chordomas express cytokeratins, specifically cytokeratins 8, 18 and 19, epithelial membrane antigen (EMA) and HMBE in almost all cases [8–10]; 30–90% also express S100 protein [10–12].

A not infrequent finding in chordomas is the presence of chondroid differentiation [13,14] and, when extensive, the tumours are referred to as chondroid chordomas [15]–a variant about which there has been considerable controversy [13,14,16–19]. Whilst the absence of cytokeratin expression in chondrosarcomas has largely resolved the problem of distinguishing skull-based low-grade/well differentiated chondrosarcomas from chordomas [17], difficulty remains when distinguishing these tumours in needle core biopsies, particularly as cytokeratin expression may

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not be present throughout the chondroid component [18,20,21].

We recently published an overview of the gene expression profile from a spectrum of connective tissue tumours and suggested that the brachyury gene, a T-box transcription factor, was uniquely expressed in chordomas [22]. Brachyury expression is required for the specification of mesodermal identity [23], representing one of the key genes regulating notochord formation. Brachyury is the first molecule identified which specifically links this embryonic structure with this neoplasm [22]. Here we examine in greater depth the expression profile of chordomas and other chondroid neoplasms, discussing their similarities and differences. We also analyse the data with respect to the origin of the nucleus pulposus. Finally, we demonstrate that brachyury expression is pathognomonic for chordomas, both classical and chondroid variants, by screening a large panel of chordomas ($n = 53$), other neoplasms ($n = 323$) and normal tissue ($n = 33$).

Method

This study was approved by the ethics committees of UCL and UCLH NHS Trust, The Royal National Orthopaedic Hospital NHS Trust and Guy's Hospital.

Microarray data analysis

GEM data analysis was carried out using the *R* statistical environment, and programming language and software packages from Bioconductor [24], an open source bioinformatics resource. We used the 'affy' package written to handle Affymetrix data, and specifically the 'rma' algorithm for pre-processing, normalizing, calculation of expression value [25,26]. The 'limma' package was used to select statistically significant differences in expression between tumour groups [27]. Heat maps illustrating levels of expression were created using Cluster and Treeview software [28]. Multidimensional scaling illustrates the similarity of expression between samples, translating their correlation into a two-dimensional (2D) plot. We used the principle coordinates method [29,30].

Tissue retrieval

Cases of chordomas, other tumours and normal tissue were retrieved from the archives of the Royal National Orthopaedic Hospital NHS Trust, University College NHS Trust, the Institute of Neurology, UCL and Guy's and St. Thomas' Hospitals. In tumours where molecular diagnoses can be made using RT-PCR, cases were chosen where this had been performed in-house, as part of the diagnostic service (data not shown).

Chordomas were classified as conventional/classical, chondroid and dedifferentiated variants. Chondroid chordomas were those composed predominantly (at least 60% but generally more) of the phenotype described originally by Heffelfinger, viz. cells arranged singly and embedded in a hyaline-like stroma [15]. Chordomas with focal chondroid areas included those tumours with only minor focal, frequently dispersed, areas (not more than 10% *in toto*) with this phenotype.

Reverse transcription–polymerase chain reaction

RNA was extracted using the TRIzol reagent (Invitrogen, Paisley, UK) followed by purification with RNeasy columns (Qiagen, Sussex, UK) according to the manufacturers' instructions. Tumour RNA was converted to cDNA using the Invitrogen Superscript II RT-PCR kit, according to the manufacturer's instructions, using 200 ng total RNA and OligodT primers (Thermo, Ulm, Germany). PCR was performed using 1 µl cDNA reaction and 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1min, using the PCR Dynazyme kit (Finnzymes, Espoo, Finland). The products were resolved by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide.

The brachyury oligonuclotides were: forward, GCC AGA CTG GAG AGT TGA GG; reverse, CAG GTG GTC CAC TCG GTA CT. The GAPDH oligonucleotides were: forward, GAT CAT CAG CAA TGC CTC CT; reverse, TGT GGT CAT GAG TCC TTC CA

Immunhistochemistry

Thick sections (3 µm) were cut from paraffin-embedded samples, dewaxed in xylene, rehydrated and then pressure-cooked for 2 min in antigen-unmasking solution (Vector Laboratories, Peterborough, UK). The iVIEW™ DAB Detection Kit and NexES immunohistochemistry system (Ventana Medical Systems Inc. Tucson, AR, USA) was used according to the manufacturer's instructions. Briefly, the endogenous peroxidase activity was inhibited and the sections incubated with the primary brachyury antibody (SC-20 109; Santa Cruz, CA, USA) for 30 min, followed by a biotinylated Ig secondary antibody (Ventana Medical Systems Inc.) and a streptavidin–horseradish peroxidase conjugate (Ventana Medical Systems Inc.). Finally, a copper DAB enhancer (Ventana Medical Systems Inc) was applied. Sections were counterstained with Harris's haematoxylin (HD Supplies, UK), dehydrated and mounted with a glass coverslip.

The other antibodies used included S100 (z0311, Dako, Glostrup, Denmark) and MNF116 (M0821, Dako).

Results

We have previously reported that the chordoma gene expression pattern clusters closest to cartilaginous neoplasms, including chondrosarcomas grade 2, chondroblastomas and chondromyxoid fibromas [22]. A closer analysis shows that the gene expression profile of chordomas is most closely related to chondrosarcomas. The relationship between the chondroid tumours and the chordomas can be seen in the multidimensional scaling in Figure 1, in which the relative similarity of expression between the samples is represented in a plot. Chondrosarcoma, chondroblastoma, chondromyxoid fibroma and chordoma were all found to express a range of genes characteristic of hyaline cartilage, including collagen II, aggrecan, cartilage linking protein, fibromodulin and cartilage oligomeric matrix protein, plus the principle chondrogenic transcription factor, SRY-box 9, at far higher levels than other connective tissue neoplasms [31–33] (Table 1). The expression of other genes known to be implicated in cartilage development, including indian hedgehog, parathyroid hormone-related protein, fibroblast growth factor receptor 3, runt-related transcription factor 2, bone morphogenetic proteins and noggin (for review, see [34]) were specifically analysed in chordomas: these were expressed at similar levels to those in cartilaginous neoplasms and other connective tissue tumours.

Although there were similarities between chordomas and cartilaginous neoplasms, there were also important differences. Chordomas were not found to express high levels of the hypertrophic gene collagen X, implicated in cartilage calcification, platelet-derived growth factor alpha, a mitogen for connective tissue cells, or reticulocalbin 3, a putative endoplasmic reticulum protein. In contrast, molecules including cytokeratins 8, 15, 18 and 19, periplakin, CD24 antigen, discoidin domain receptor 1 and the aforementioned brachyury were expressed in chordomas but were not detected in the chondroid neoplasms (Figure 2).

As we have previously shown, the most specific gene in chordomas is brachyury, with expression levels approximately 16-fold greater than in chondroid or other neoplasms (Figure 3). In reality, the level of expression detected in chondroid and other neoplasms is likely to represent background measurements, on the basis that brachyury was only detected by RT-PCR in chordomas and not in other tumours (Figure 4).

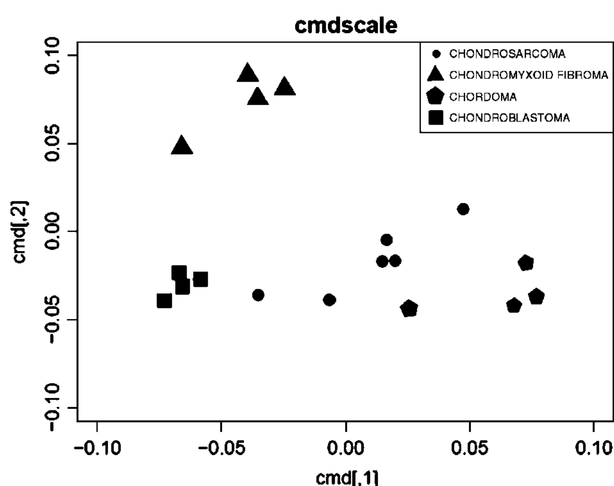

Figure 1. Multidimensional scaling plot showing the relationship between chordomas and chondroid neoplasms. The multidimensional scaling is an optimal 2D representation of the matrix of distances between samples, with the minimum of distortion. In this case the distance is the correlation (actually 1-correlation) between the expression profiles of each sample. See Methods for further details

Brachyury is expressed in the notochord of the embryo and in chordomas

A whole mount of an embryo of 6–8 weeks gestation showed that brachyury expression is restricted to the notochord, where it was co-expressed with

Table 1. Commonly expressed genes in chordomas and chondrosarcomas that are not expressed in a panel of other sarcomas

Gene name	Function
Aggrecan 1	Extracellular matrix protein
Cartilage linking protein 1	Extracellular matrix protein
S100 calcium binding protein A1	Calcium binding
Chondroitin sulphate proteoglycan 4 (melanoma-associated)	Extracellular matrix protein
Proline arginine-rich end leucine-rich repeat protein	Extracellular matrix protein
Chondroitin 6	Extracellular matrix protein
Integrin, α10	Extracellular matrix protein
Fibromodulin	Extracellular matrix protein
Cartilage oligomeric matrix protein (COMP)	Extracellular matrix protein
Collagen II	Extracellular matrix protein
SRY-box 9	Transcription factor

We list a selection of important cartilaginous genes that are highly expressed in both the chondroid neoplasms and chordomas, as opposed to a large panel of other soft tissue sarcoma ($p < 0.05$, see Methods, Data analysis, for statistical details). The other sarcomas include Ewing sarcoma, osteosarcoma, MPNST*, rhabdomyosarcoma, liposarcoma, schwannoma and synovial sarcoma (for complete details, see [22]).
* MPNST, malignant peripheral nerve sheath tumour.

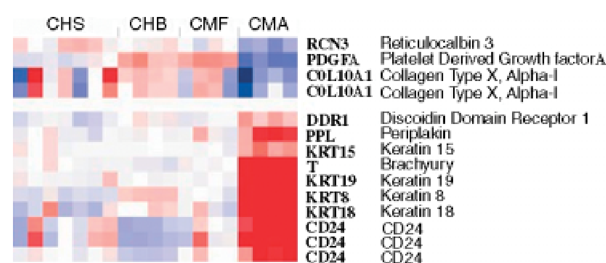

Figure 2. Genes expressed differentially between chordomas and chondroid neoplasms. We illustrate a selection of probe-sets that have a statistically significant difference of expression ($p < 0.01$; see Methods, Data Analysis, for statistical details) between chordoma (CMA) and the chondroid neoplasms — chondrosarcoma (CHS), chondroblastoma (CHB) and chondromyxoid fibroma (CMF). The heat map represents the expression level of genes in colour from low expression (blue), through medium (white) and high expression (red)

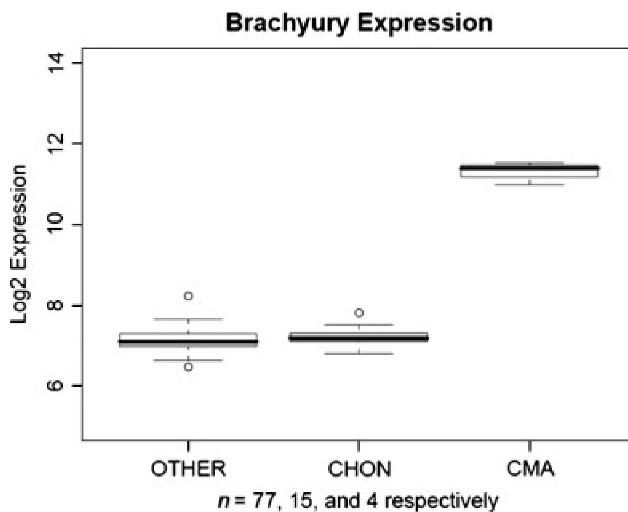

Figure 3. Levels of brachyury expression in chordomas, chondroid neoplasms and other tumours tested. The box and whisker plots show the interquartile range as the box and the 95% confidence range as the whiskers; samples outside this range (outliers) are shown as small circles. The central line is the median. The units of expression in this plot are Log2 expression units calculated using the 'rma' algorithm (see Methods). CMA, chordomas; CHON, chondroid neoplasms; OTHER, all other connective tissue tumours tested

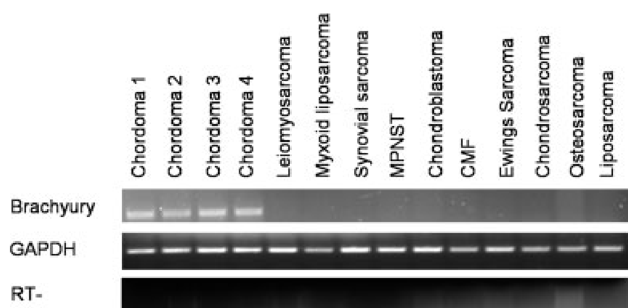

Figure 4. RT-PCR validation of microarray data. Brachyury is only expressed in chordomas and is absent in all the other sarcomas. The *GAPDH* control is expressed in all the tumours. The absence of a product in the reaction lacking reverse transcriptase (RT) indicates that there is no contaminating genomic DNA. MPNST, malignant peripheral nerve sheath tumour; CMF, chondromyxoid fibroma

cytokeratins (Figure 5): being a transcription factor, the staining was restricted to the nuclei. Brachyury expression was present in all but one of the 54 tumours previously classified as chordomas (Table 2). The brachyury antigen was detected in virtually all tumour cells (Figure 5), in both decalcified and non-decalcified material, even in the chondroid areas, indicating that a biopsy would need to be extremely small to result in a misdiagnosis using this antibody. In non-chordoma material, weak diffuse cytoplasmic staining was occasionally detected but this was not interpreted as brachyury expression. Cytokeratin expression was always detected in the cartilaginous areas of chordomas.

As mentioned above, one tumour, sited at the base of the skull, which had previously been diagnosed as a probable chordoma, was found not to express brachyury. On review, this tumour was found not to express cytokeratins, and was reclassified as a chondrosarcoma. The four cases of dedifferentiated chordomas revealed that brachyury was strongly and widely expressed in the areas of conventional chordoma but, apart from focal expression in one case, was not present in the undifferentiated spindle cell component (Figure 5). This loss of brachyury expression coincided with the loss of cytokeratin expression. Five cases of conventional metastatic chordomas were also found to express brachyury strongly in the nuclei throughout this material (image not shown).

Table 2. Summary of chordomas analysed by immunhistochemistry

Chordoma type	Site	Number of cases
Classical chordoma (no chondroid differentiation)	Sacral	23
	Clival	3
Chordomas with focal areas of chondroid differentiation	Sacral	10
	Clival	3
Chondroid chordoma	Clival	6
Dedifferentiated chordoma	Sacral	3
Metastatic chordoma		5
Total		53

Brachyury protein could not be detected in a wide variety of non-neoplastic tissues, including brain, breast, articular and fibrocartilage, squamous and endocervial cervix, endometrium, myometrium, Fallopian tube, ovary, gallbladder, kidney, liver, lung, lymph node, large bowel, sciatic nerve, skin, glandular, transitional and metaplastic squamous epithelium, testis, tonsil, thyroid and salivary gland. It is noteworthy that brachyury protein was not detected in the nucleus pulposus ($n = 7$).

The tumours analysed by immunohistochemistry for brachyury expression ($n = 323$), with a particular emphasis on those neoplasms that are most easily mistaken for a chordoma diagnostically, showed no specific expression of this molecular marker (Table 3).

Discussion

This study strongly supports the hypothesis, and validates our previous work using gene expression microarrays, that chordomas differentiate down a notochordal lineage and may derive from the embryonic notochord. Virchow and Muller first documented the existence of notochordal vestiges around the base of the skull and since then evidence has accumulated linking persistent notochordal remnants with chordomas, i.e. the site of the vestiges corresponds closely with the distribution of chordomas [35], the morphological similarities, by both transmitted and electron light microscopy [36], and the shared immunophenotype (cytokeratin and S100 protein expression [9,37]; for review, see [4]). Although these observations argue

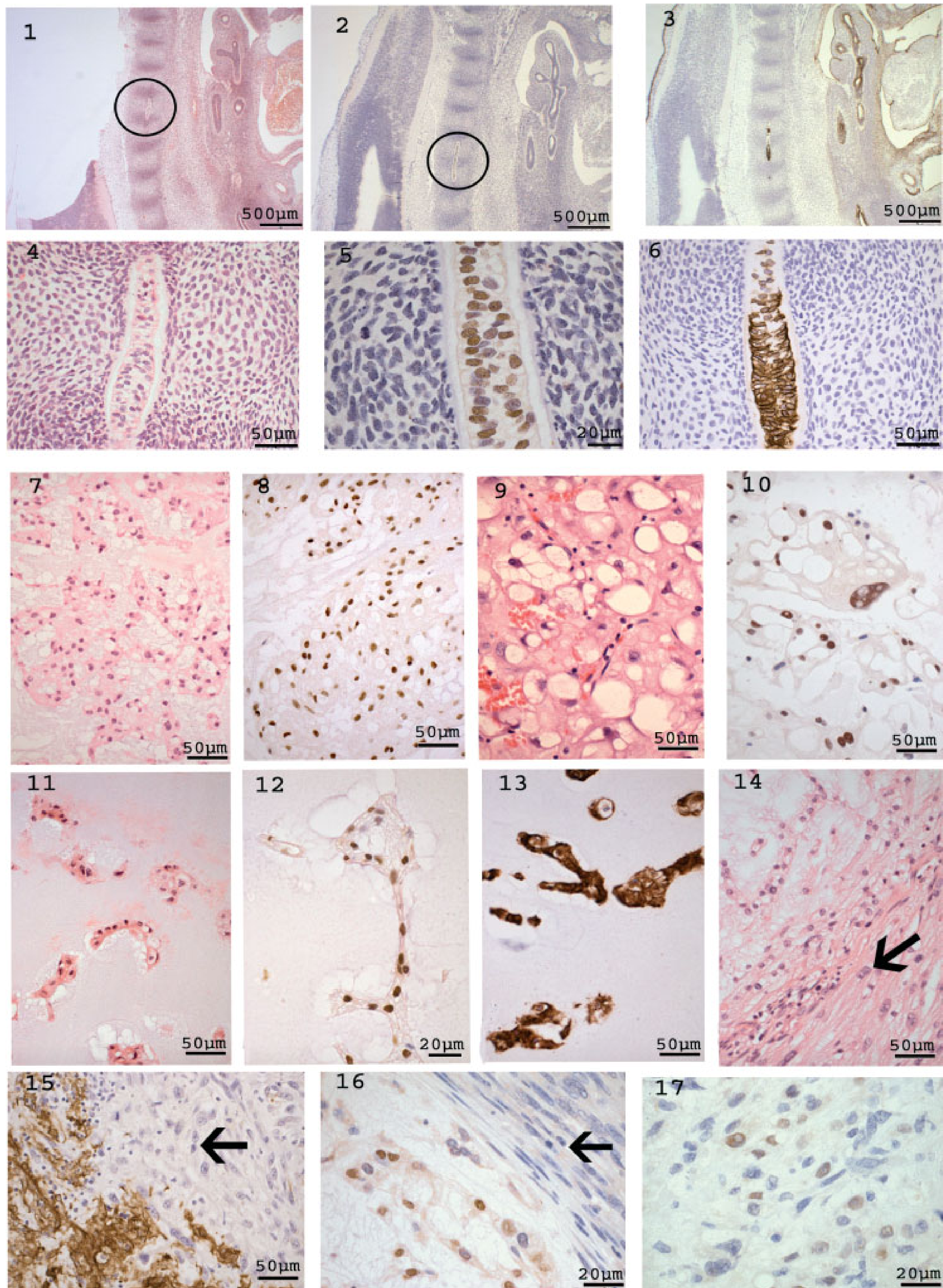

Figure 5. Transmitted light photomicrographs of an embryo [haematoxylin and eosin (H&E), 1 and 4] the notochord showing brachyury (2 and 5) and cytokeratin expression (3 and 6), and chordomas (7–17). Two examples of chordomas (H&E, 7 and 9) with different phenotypes both exhibit strong nuclear brachyury expression in tumour cells (8 and 10). In a clival chondroid chordoma (H&E, 11), the lesional cells express brachyury (12) and cytokeratins (13). In a dedifferentiated chordoma (H&E, 14–17), the dedifferentiated spindle cell component (arrow) does not express cytokeratins (15) but the conventional component expresses cytokeratins and brachyury (15 and16, respectively). Focally, the tumour cells in the dedifferentiated element express brachyury (17)

Table 3. Complete list of all non-chordoma cases assessed for brachyury expression. None of these samples showed brachyury expression by immunohistochemistry

Tumour category	Subcategory	Number of cases analysed
Chondroid neoplasms	Chondroblastoma	14
	Chondromyxoid fibroma	18
	Chondrosarcoma, appendicular: grade 0.5 ($n = 12$), grade 1 ($n = 10$), grade 2 ($n = 22$), grade 3 ($n = 11$)	55
	Chondrosarcoma, base of skull	9
	Chondrosarcoma, clear cell	11
	Chondrosarcoma, dedifferentiated	5
	Chondrosarcoma, extraskeletal myxoid	5
	Chondrosarcoma, mesenchymal (bone)	9
	Chondrosarcoma, myxoid	8
	Enchondroma	12
	Periosteal chondroma	5
	Synovial chondromatosis	12
	Subtotal	163
Other	Adenoma, pituitary	6
	Astrocytoma, gemistocytic	2
	Carcinoma, neuroendocrine	4
	Carcinoma, renal	6
	Carcinoma, squamous cell	2
	Ependymoma	4
	Ependymoma, myxopapillary	4
	Giant cell tumour	2
	Glioblastoma multiforme	5
	Leiomyosarcoma	6
	Lipoma, chondroid	2
	Liposarcoma, myxoid (grade 2)	6
	Malignant melanoma	2
	Mesenchymal phosphaturic neoplasm	2
	Myoepithelial tumour/outside salivary glands, malignant	4
	Myoepithelial tumour/outside salivary glands, benign	7
	Mixed tumour/pleomorphic adenoma of salivary glands	25
	Myxofibrosarcoma	5
	Myxoma, intramuscular	5
	Myxoma, Mazabraud syndrome	5
	Oligodendroglioma	2
	Osteofibrous dysplasia	10
	Osteosarcoma, osteoblastic	3
	Osteosarcoma, chondroblastic	10
	Paraganglioma	1
	Sarcoma, clear cell	8
	Sarcoma, epithelioid	3
	Sarcoma, low-grade fibromyxoid	10
	Sarcoma, Ewing/PNET	6
	Sarcoma, spindle cell	3
	Subtotal	160
Total		323

in favour of chordomas arising from the notochord, much is anecdotal evidence. In contrast, the finding that brachyury, a transcription factor known to be crucial in notochord development, is localized and restricted to chordoma cells at a protein level in all 53 cases of primary chordomas examined, including four recurrent deposits and five metastatic tumours, but not in a wide variety of 323 other neoplasms or in a large number of a diverse spectrum of normal tissues, links chordomas and the notochord together by a specific molecule for the first time.

The gene profiles from chordomas and cartilaginous neoplasms generated from our study supports the similarity, first recognized by Virchow 150 year ago, between these neoplasms. Our data revealed significant overlap between genes expressed in chordomas and those implicated in normal cartilage differentiation, including the expression of SRY-box 9, collagen II, aggrecan, cartilage linking protein, fibromodulin and cartilage oligomeric matrix protein [31,32]: these include genes which we and others have found to be expressed in cartilage tumours [38,39]. This close similarity of gene expression reflects a close evolutionary relationship in which the notochord can be considered a primitive form of cartilage. The substantive evolutionary step from notochord to cartilage is that the

collagen proteins in the notochord are contained within intracellular vacuoles, lending the tissue a gel-like consistency through cell swelling, whereas the collagens produced by chondroid cells are extruded to cross-link and calcify.

Our work also demonstrates differences between cartilage and chordomas. In addition to the identification of brachyury, and keratins which have previously been detected in chordomas [8,9], the microarray analysis identified genes not hitherto recognized as being useful in distinguishing chordomas and cartilaginous neoplasms: these include periplakin, a molecule known to interact with keratins [40]; CD24, which was recently found to be expressed by chordomas [41]; and discoidin domain receptor 1. The latter acts as a receptor for collagen, is regulated by p53, and has been found to be over-expressed in breast carcinoma and osteosarcoma [42–45]. It is therefore an interesting candidate for further investigations. Finally, confirming results of others [20], we found that appreciable levels of collagen X were not detected in chordomas, whereas we detected this gene in cartilaginous tumours. Collagen X is expressed by hypertrophic chondrocytes and is implicated in matrix calcification. The low level is consistent with the virtual absence of calcification in chordomas compared to that seen in chondroid neoplasms [46–48].

Histological review of the group of chordomas in this study confirms previous reports that a significant proportion of chordomas, to a lesser or greater degree, exhibit areas of cartilaginous differentiation [13–15]. Our finding that brachyury and cytokeratins are detected in both the chondroid and chordoid components of chordomas, and in none of the 164 extra-spinal neoplasms showing cartilaginous differentiation, argues that the chondroid component of chordomas arise from the notochord. Nine clival chondroid neoplasms, which were negative for cytokeratin, also failed to express brachyury and therefore were classified as chondrosarcomas. These findings demonstrate that brachyury expression is restricted to the notochord and notochord-derived tumours, and the use of brachyury as a specific biomarker will permit confident discrimination between chordomas and chondrosarcomas. This has important clinical implications, as the treatment of low-grade chondrosarcomas differs to that of chordomas. The former are extremely slow-growing neoplasms with a 85% 10 year survival, and therefore minimizing side-effects is crucial in patients who are likely to live a long time [49]. In contrast, radical surgical cytoreduction with adjuvant radiotherapy in the absence of complete clearance is recommended for chordomas [50].

Parachordomas (originally reported as chordoma periphericum by Dabska [51], are benign tumours occurring in deep soft tissue in extra-axial sites [52,53]. The nature of parachordomas remains unclear and recent reports argue that the lesion may not exist as a separate entity but represents a heterogeneous group of neoplasms, including extraskeletal myxoid chondrosarcomas, myoepithelial neoplasms/mixed tumours and others [54]. It is also unclear whether parachordomas and chordomas can be distinguished on the basis of their immunohistochemical profiles. In addition, there have been a small number of extra-spinal skeletal chordomas described [55,56], and it is proposed that these differ from parachordomas [56]. As we were not convinced that any of the neoplasms in our study fulfilled the criteria described by Dabska [51], we were unable to address the question as to whether parachordomas arise from ectopic notochordal vestiges. Since brachyury is a specific marker for the notochord and notochord-derived tumours (chordomas), analysis of the expression of this molecule should be used to explore the existence of extra-axial skeletal and soft tissue chordomas.

Having identified brachyury as a specific marker for the notochord and notochord-derived tumours, we exploited this finding to address the issue of whether the nucleus pulposus derives from the notochord. The notochord disappears by early childhood [57] and is replaced by the nucleus pulposus of the intervertebral discs [3]. Morphologically, the nucleus pulposus has features similar to cartilage but it is unclear whether it derives from the notochordal population and exhibits a cartilaginous phenotype, or whether the notochord undergoes apoptosis and is replaced by cartilage [3,4]. In our study, as the nucleus pulposus expressed neither cytokeratins, as also shown by others [5], nor brachyury, we found no molecular evidence that this structure develops directly from the notochord. Furthermore, the presence of collagen X in the nucleus pulposus and its ability to calcify favours this structure being developmentally unrelated to the notochord [46–48]. However, it has recently been published that CD24 is expressed by both chordomas and the nucleus pulposus [41], and therefore it could be argued that the absence of brachyury and cytokeratin expression at this site is accounted for by the loss of these molecules as the notochord develops. Hence, we conclude that the relationship of the notochord to the nucleus pulposus remains unclear.

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