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Original Paper

Chondromyxoid fibroma resembles *in vitro* chondrogenesis, but differs in expression of signalling molecules

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Abstract

Chondromyxoid fibroma is a rare benign cartilaginous bone tumour characterized by morphological features that resemble different steps of chondrogenesis in terms of both cellular morphology, ranging from spindled to rounded cells, and the extracellular matrix formed, which ranges from fibrous to cartilaginous. The presence in chondromyxoid fibroma of signalling molecules that regulate the spatial expression of proteins involved in normal cartilage proliferation and differentiation was investigated in samples from 20 patients and compared with articular chondrocytes from 11 normal donors cultivated in 3D pellet culture. Sections were stained with safranin-O and H&E, and immunohistochemistry was performed for p16, cyclin D1, FGFR3, BCL2, p21, PTHLH, PTHR1 and N-cadherin. Expression patterns were analysed using hierarchical clustering. In chondromyxoid fibroma, specific morphological features correlated with a distinct pattern of expression. Comparison with normal chondrocytes in pellet culture showed a striking morphological resemblance, but with an unmistakably different pattern of expression. N-cadherin, PTHLH, and PTHR1 were expressed to a significantly higher level ($p < 0.01$) in articular chondrocyte pellets but, conversely, there was significantly lower expression of cyclin D1, p16 and BCL2 ($p < 0.05$) in these cells. Morphological similarities reflect common steps in cartilage differentiation, albeit driven by different molecular mechanisms. The proteins we have found to be differentially expressed seem crucial for neoplastic chondrogenesis.

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Introduction

Chondromyxoid fibroma (CMF) is a benign cartilaginous bone tumour with a polymorphous microscopic appearance, as implicated by its name, ranging from a chondroid to a myxoid and even fibrous phenotype [1,2]. It can affect almost every osseous site, but is found more frequently in long (mainly proximal tibia) and flat bones, the iliac bone being the most frequent (~25%) [1,3,4]. The distinct histological features of CMF include lobules of spindle- or stellate-shaped cells with abundant myxoid and chondroid extracellular matrix. Differences in extracellular matrix appearance correspond to variation in proteoglycans and collagen composition, and in the morphology of constituent cells [5]. The cellular areas and the matrix-rich areas, the latter being classified as either myxoid or chondroid, differ in the amount of type I and II collagen and aggrecan. Generally, in cellular areas populated with predominantly spindle-shaped cells, collagen type I is found [5], with no evidence of the presence of collagen type II or aggrecan. Aggrecan production, on the other hand, is evident in the myxoid areas, where the cells display a stellate morphology. Cells possessing rounded morphology and an extracellular matrix morphology and biochemical make-up similar to normal cartilage (presence of aggrecan and collagen type II) characterize the chondroid regions [5].

Morphologically, this spatial difference in cellular morphology and matrix distribution has a striking parallel to the dynamic response of normal articular chondrocytes placed into monolayer culture [6] and subsequently maintained in various high-density culture systems [6,7] (Figure 1). Normal (non-neoplastic) chondrocytes, cultured on standard plastic tissue culture flasks, undergo a well-documented process termed dedifferentiation [6,8]. This transition is characterized by loss of the rounded cell shape to a fibroblast-like morphology and a shift in the proportion of aggrecan (decreasing) and collagen expression (from type II to type I) [9]. It is generally recognized that this process

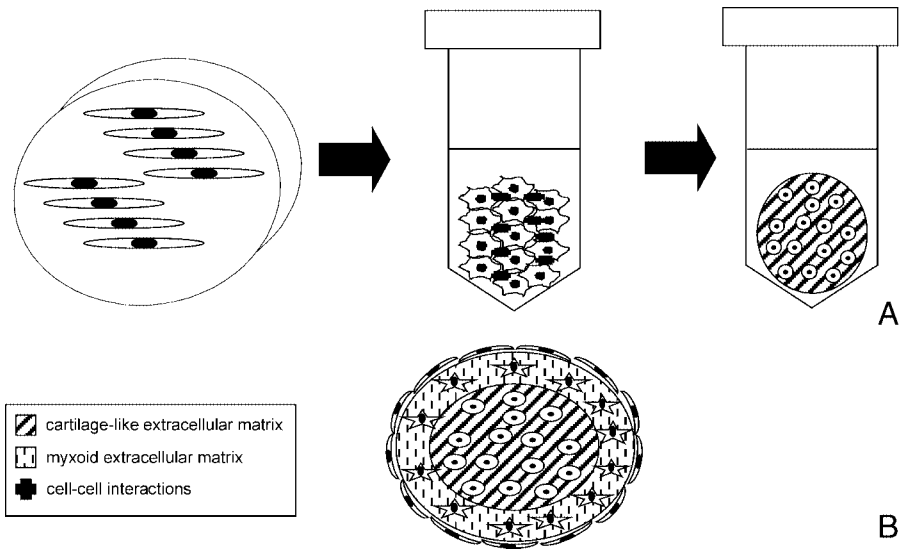

Figure 1. CMF resembles *in vitro* chondrogenesis. (a) Articular chondrocytes grown in flasks acquire a spindle shape (dedifferentiation) but, when cultured in a 3D pellet system, the cells change shape and form several cell–cell and cell–extracellular matrix interactions, finally forming extracellular matrix resembling mature cartilage. (b) Chondromyxoid fibroma resembles *in vitro* chondrogenesis (a). The spindle cells at the periphery of the lobules resemble dedifferentiated chondrocytes morphologically (a). The lobules of myxo-chondroid matrix are similar to the cartilage formed *in vitro* (a)

can be reversed (redifferentiation) under appropriate conditions such as agarose [10], or in other three-dimensional high-density cultures in the presence of differentiation signalling molecules such as TGF-β [9]. During this reverse process, the cells recover their rounded morphology, reflected in a different pattern of organization of the actin filaments. Moreover they revert to expressing collagen type II, aggrecan and other cartilage-specific genes [11,12], while significantly reducing collagen type I production [12].

These processes include cell–cell and cell–extracellular matrix interactions, mainly through integrins and N-cadherin [12], specific extracellular matrix deposition and differentiation toward cartilage formation, and are driven by several signalling molecules.

In particular, a key role is played by parathyroid hormone-related peptide (PTHLH) and fibroblast growth factor (FGF), and cell cycle regulators, in the regulation of cartilage growth in the epiphyseal growth plate [13–15]. Furthermore, these signalling molecules have been shown to be impaired in cartilaginous tumours [16–18].

Based on morphological similarities between cells and extracellular matrix, we hypothesized that the histological features of CMF reflect different steps of *in vitro* chondrogenesis: from dedifferentiated/spindle shape cells to redifferentiated/round chondrocytes with parallel production of either more fibrous or cartilaginous matrix. To test this hypothesis, we performed a comparative study of CMF with cultured articular chondrocytes (dedifferentiated), which were pushed towards redifferentiation through a 3D pellet culture system. We investigated the morphological spectrum of differentiation in combination with the expression pattern using immunohistochemistry for fibroblast growth factor receptor 3 (FGFR3), BCL2, p21, PTHLH, parathyroid hormone-related peptide receptor (PTHR1), cyclin D1, N-cadherin, and p16. This generated spatial information that allowed correlation of the expression profile with the morphological features of cells and extracellular matrix.

Methods

Pathological material

Twenty samples of CMF were selected from 18 primary and two recurrent tumours. The cases were retrieved from the surgical pathology and consultation files of the Leiden University Medical Centre. One primary tumour sample was kindly provided by the Department of Pathology of Ghent University. Formalin-fixed, formic acid (pH 2.1) decalcified and paraffin-embedded archival tumour tissue was available for routine staining and immunohistochemical analysis. All cases were examined following haematoxylin and eosin (H&E) staining to confirm the diagnosis and safranin-O staining to evaluate the amount of sulphated proteoglycans in the extracellular matrix. All specimens were handled according to the ethical guidelines described in the *Code for Proper Secondary Use of Human Tissue in The Netherlands* of the Dutch Federation of Medical Scientific Societies.

Articular chondrocyte pellets (ACP)

As described in more detail elsewhere [19], cells were isolated post mortem within 24 h after death from the knee joints of a total of 11 normal donors who specifically had no clinical history of joint disorders. These donors were selected to be of matching gender and age range. These procedures were performed in

accordance with the ethical guidelines of the Institute of Pathology, University of Bern. To create a three-dimensional environment, 0.5×10^6 cells were centrifuged at 250 *g* for 5 min in 1.5 ml polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) to form a high-density pellet. The cell pellets were maintained in culture for 2 weeks in ITS+ media (Sigma Chemical, St Louis, MO, USA), supplemented with TGF-β1 and dexamethasone, at a final concentration of 10 ng/ml and 39.25 µg/ml respectively [19]. Cell morphology and the production of sulphated proteoglycans in the extracellular matrix were examined in each pellet after 2 weeks using H&E and safranin-O staining, respectively.

Immunohistochemistry

Immunohistochemical analysis was performed on 4 µm sections according to standard laboratory procedures [17,18]. Details of antibodies and antigen retrieval procedures used are listed in Table 2. Briefly, after pre-treatment, sections were incubated overnight with primary antibody, followed by incubation in biotin-labelled rabbit anti-mouse immunoglobulins and subsequent application of biotinylated HRP–streptavidin complex application (DAKO, Glostrup, Denmark). Visualization was carried out in a diaminobenzidine solution (Sigma, St Louis, MO, USA). The slides were counterstained with haematoxylin. Appropriate positive control slides were prepared according to each antibody specificity (Table 1). Moreover, internal positive controls (Table 1) were present in most of the histological slides, allowing evaluation of the antigenic property of the tissue after decalcification. As negative controls, slides were incubated with mouse or rabbit IgG of corresponding (iso-) types and concentration instead of primary specific antibodies.

The specificities of these antibodies have been validated previously and the expression levels were compared to Q-PCR results [16].

Evaluation and criteria used for scoring

The immunostained slides were assessed and scored by three pathologists independently (SR, JVMGB, and PCWH) using the sum of intensity of signal (0 = no expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression) and the number of positive cells (% tumour cells: 0 = 0%; 1 = 1–25%; 2 = 26–50%, 3 = 51–75% 4 = 76–100%) as described previously by us [18] and others [20]. The three authors together, reaching a consensus, revised discrepant scores. The above-mentioned scoring system, emphasizing both staining intensity as well as percentage of cells, has been shown to be highly reproducible in our hands, and has also been used in previous studies on decalcified bone tumour specimens [17,18].

The cellular areas and the matrix-rich areas were evaluated separately if they constituted at least 10% of the surface on the slide. A final weighted score, adapted from Grogan *et al* [21], was calculated as the sum of the scores of single areas multiplied by the relative percentage extension of the area. The mean value of the sum score was reported. Finally, the cellular localization (nuclear, cytoplasmic, and membranous) of immunopositivity was noted.

Statistical analysis

A paired, two-tailed *t*-test was applied in order to evaluate significantly different distributions of final sum score values between matrix-rich and cellular areas in CMF.

An unpaired two-tailed *t*-test, unequal variance, was applied in order to evaluate statistically significant differences in the distribution of CMF's final weighted sum score values versus ACP's final sum score. A value of $p < 0.05$ was considered significant. All statistical analysis, if not otherwise specified, was performed using the SPSS 10 software package. Cluster analysis was carried out using the data of the separate scores for intensity and the number of positive cells. One case of CMF was discarded because of too many absent values. The data were normalized, mean-centred and the average linkage method was applied by means of Cluster and TreeView programs [22]. For similarity, metrics uncentred correlation was used.

Table 1. Details of the antibodies and immunohistochemical protocols used

Antigen	Source	Clone	Staining	Positive control	Internal control	Dilution	Antigen retrieval
cyclin D1	Dako	MC DCS6	Nuclear	Tonsil	Occasional endothelial cells	1:4000	mwo/10 min
p16	Neomarker	MC 16 PO4	Nuclear	Tonsil	Keratinocytes/lymphocytes	1:100	mwo/0 min
FGFR3	Sigma	PC	Cytoplasmic	Umbilical cord	Striated muscle/blood vessel walls/connective tissue/osteoclasts	1:2000	Trypsin
p21	Calbiochem	MC AB1	Nuclear	Colon	None	1:400	mwo/10 min
PTHLH	Oncogene	PC	Cytoplasmic	Skin	None	1:200	Trypsin
PTHR1	Upstate	MC 3d1.1	Cytoplasmic	Skin	Blood vessel walls/osteoblasts	11:100	mwo/10 min
BCL2	DAKO	MC 124	Cytoplasm	Tonsil	Osteoblasts/lymphocytes	1:100	mwo/10 min
N-cadherin	Transduction lab	MC 32	Membranous/cytoplasmic	Heart	Nerves	1:100	mwo/10 min

PC, polyclonal; MC, monoclonal; FGFR3, fibroblast growth factor receptor-3; PTHLH, parathyroid hormone-related peptide; PTHR1, parathyroid hormone-related peptide receptor; mwo, microwave oven.

Results

Morphological and histochemical evaluation

Chondromyxoid fibroma

All the retrieved cases fitted the diagnostic criteria for CMF, being formed by lobules of spindle- or stellate-shaped cells with abundant myxoid and chondroid intercellular matrix [1,2]. In such lobules, a zonal architecture could be recognized. The periphery appeared to be cellular with a low amount of extracellular matrix. Towards the centre of the lobules there was more extracellular matrix with both myxoid and chondroid features, the areas closer to the centre being more similar to hyaline cartilage. The transition between cellular and matrix-rich areas was not well demarcated, with the two areas gradually merging together. This was reflected in a gradual change of cell shape, being slender in the cellular areas, stellate and triangular in the myxoid areas, and round in the cartilage-like areas. Cells were large at the periphery and smaller towards the centre. The location of vessels also followed a zonal architecture, with vessel-rich areas at the cellular periphery of the lobules and absence of vessels in the central cartilage-like areas (Figure 2B).

Articular chondrocyte pellet

The pellet samples showed similar morphology to CMF in terms of both architectural pattern and cell cytology. Rounded cells intermingled with stellate cells were present in most of the pellets, together with abundant extracellular matrix characterized by myxoid and chondroid features. Spindle cells were present mainly in a narrow area at the periphery just beneath the surface (Figure 2E). A striking similarity between the morphological features of spindle and stellate cells of CMF and the articular chondrocytes cultivated in monolayer was evident (Figure 2A, D). In both pellets and CMF lobules safranin-O staining substantiated the morphologically observed pattern. Areas with intense glycosoaminoglycan staining were present at the centre of the lobules of CMF and throughout the ACP, while the peripheral areas showed no positive stain at all (Figure 2C, F). Areas with weak staining showed a myxoid appearance of the extracellular matrix and contained stellate-shaped cells.

Immunohistochemical evaluation

The results are summarized in Table 2.

Chondromyxoid fibroma

In CMF cases the level of immunoreactivity was generally higher in the matrix-rich areas versus the cellular areas, being significantly higher ($p < 0.05$) for p21, cyclin D1, and PTHLH. For N-cadherin significantly higher ($p < 0.001$) expression was observed in the cellular areas (Figure 2G, H, I).

Articular chondrocyte pellet

The absence of staining for BCL2 in all ACPs was noteworthy (Figure 2J). Due to the limited extent of the cellular/peripheral areas (<10% of the surface on the slide), it was not possible to score cellular/peripheral areas versus central/matrix rich areas separately, as for CMF. Hence only a general score was performed. The comparison between CMF and pellets showed differences in expression: significantly higher ($p < 0.05$) expression for p16, cyclin D1 and BCL2 (Figure 2G, J) was found in CMF versus ACP, with BCL2 being completely absent in ACP. Conversely, significantly higher ($p < 0.05$) expression of N-cadherin (Figure 2I, L), PTHLH (Figure 2H, K) and PTHR1 was found in ACP versus CMF. Generally the statistical significance was the same assuming either equal or unequal variance. The different patterns of expression resulted in the two clusters visualized by hierarchical clustering analysis (Figure 3).

Discussion

The morphological spectrum of CMF, in terms of both the type of extracellular matrix produced and the resident neoplastic cells, is broader than what is normally observed in normal mature hyaline cartilage. The

Table 2. Semi-quantitative scoring results specified for cellular or matrix-rich area

Antigen	CMF CA	CMF MA	*t*-test	CMF WS	ACP	*t*-test
cyclin D1	2.6 ± 1.1	3.4 ± 1.1*	<0.01†	3.2 ± 1*	1.9 ± 1.5	0.02†
p16	3.9 ± 1.3	4.1 ± 1.4	0.8	4.1 ± 1.3*	2.2 ± 1.2	<0.01†
FGFR3	6.7 ± 0.5	6.6 ± 0.6	0.3	6.7 ± 0.5	6.4 ± 1.1	0.5
p21	2.8 ± 1.1	3.4 ± 1.1*	0.04†	3.2 ± 1.1	3.2 ± 1.5	0.9
PTHLH	2.3 ± 1	4.3 ± 1.4*	<0.001†	4 ± 1.4	6.8 ± 0.4*	<0.001†
PTHR1	5.7 ± 1.1	5.5 ± 0.9	0.8	5.2 ± 1.6	6.5 ± 0.5*	<0.01†
BCL2	3.1 ± 1.5	3.6 ± 1.5	0.3	3.6 ± 1.5*	0 ± 0.0	<0.001†
N-cadherin	5.3 ± 1.4	4.3 ± 1.3	<0.001†	4.9 ± 1	6.9 ± 0.3*	<0.001†

Values are reported as mean sum score ± standard deviation, * significantly higher mean sum score and † significant *p* values.
CA, cellular areas; MA, matrix-rich areas; WS, weighted score.

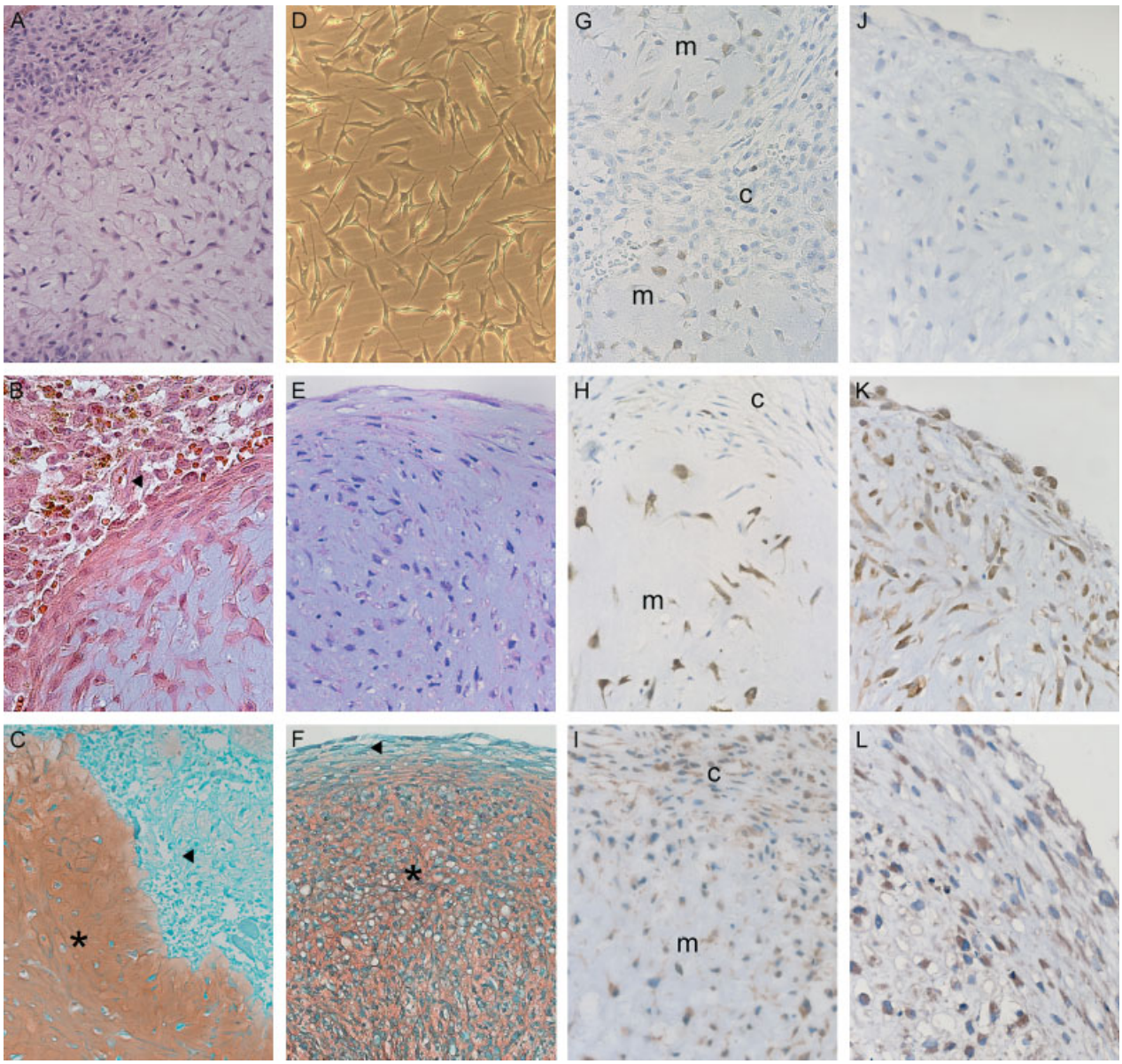

Figure 2. Morphological similarities between CMF and *in vitro* chondrogenesis (from A to F). Left column CMF, right column articular chondrocytes. (A) Spindle and stellate cells in the myxoid areas of CMF (H&E stain 40×, original magnification); (B) architectural organization of CMF lobules: vessels are present at the periphery (arrowhead), where cells are spindle-shaped and there is little interposed extracellular matrix (cellular areas); more to the centre, the cells get rounder and extracellular matrix is more abundant (matrix-rich areas) (H&E stain, 40× original magnification); (C) the architectural organization is substantiated by the safranin-O staining pattern, which is negative in the cellular areas (arrowhead) but positive in the matrix-rich areas (*) (20× original magnification); (D) articular chondrocytes grown in monolayer lose their round shape and become spindle-shaped or stellate (inverted microscope, no stain, 40× original magnification); (E) articular chondrocytes cultivated in 3D pellets are spindle-shaped at the periphery with little extracellular matrix but, more to the centre, the cells get rounder and extracellular matrix is more abundant (H&E stain, 40× original magnification); (F) the extracellular matrix at the periphery of the pellet is negative with the safranin-O stain (arrowhead), whereas at the centre, where the cells are rounder, it is positive (*) (20× original magnification). Expression pattern in CMF and *in vitro* chondrogenesis (from G to L): left column CMF, right column ACP; (G) BCL2 immunostaining is present in CMF and is greater in matrix-rich areas (m) compared with cellular areas (c); (H) PTHLH immunostaining in CMF shows significantly higher expression in matrix-rich (m) versus cellular (c) areas; (I) N-cadherin immunostaining in CMF shows significantly higher expression in cellular (c) than in matrix-rich (m) areas; (J) BCL2 immunostaining is absent in ACP; (K) PTHLH immunostaining in ACP shows significantly higher expression than CMF (40× original magnification); (L) N-cadherin immunostaining in ACP shows significantly higher expression than CMF (40× original magnification)

morphological features observed are suggestive of the recapitulation of *in vitro* chondrogenesis, and this has prompted us to study the phenotype of the neoplastic cells, the specific extracellular matrix present, and their relative profile of expression of different molecules known to be involved in cartilage differentiation. Our study shows that the expression of these regulators of cartilage differentiation and cell cycle regulatory molecules differs significantly in cellular areas versus matrix-rich areas of CMF. Most of the tested proteins were expressed more extensively and intensely in the matrix-rich areas. This could reflect the role of

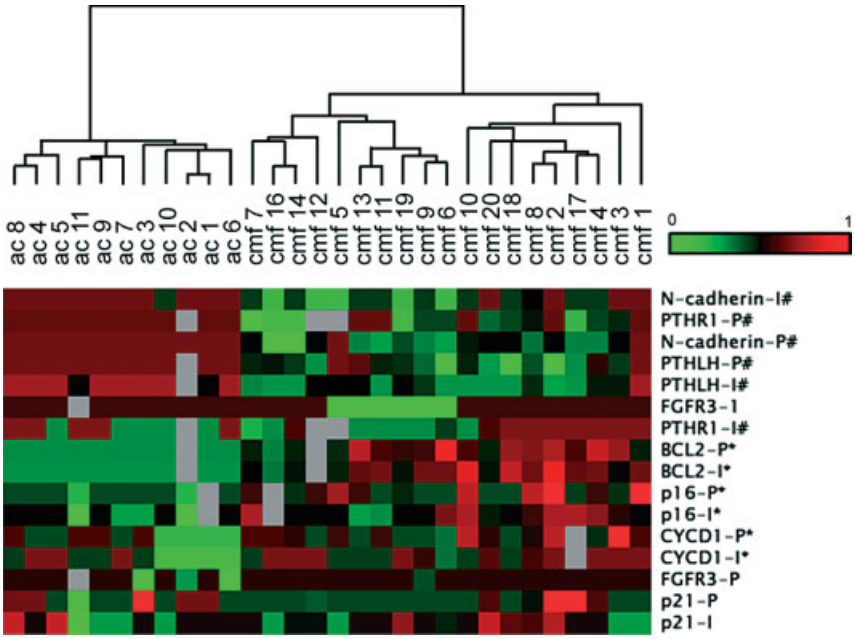

Figure 3. Hierarchical clustering. Two different clusters are evident from the tree in the upper part of the figure. On the left are the articular chondrocytes (ac) and on the right the cases of chondromyxoid fibroma (cmf). In the rest of the figure (heat map), as exemplified by the bar on the right side, the green-coloured blocks represent low values and the red-coloured blocks high values of the proteins studied (I, intensity; P, percentage). The colours in between are intermediate, with black being the mean value; grey blocks represent missing data. #, proteins significantly higher in pellet; *, proteins significantly higher in CMF

these molecules in promoting both the deposition of abundant specific extracellular matrix and the typical cellular phenotype of the resident cells. Conversely, N-cadherin expression was significantly higher in the cellular areas. This pattern strictly resembles the initial mesenchymal condensation in which N-cadherin is present only in undifferentiated precursor chondrocytes and is lost after developing the phenotype of differentiated chondrocytes [12,23]. In this respect the higher expression of N-cadherin in the cellular areas may reflect a role for the homophilic cellular interactions in the commitment of undifferentiated cells towards chondrogenesis, later occurring more towards the centre of the lobule, or conversely in the maintenance of the less differentiated phenotype characteristic of the cellular areas.

We have used an *in vitro* system as a comparative model since we observed a striking morphological resemblance between CMF and cultured articular chondrocytes. In particular, the zonal architecture of CMF lobules strictly resembled that in chondrocyte pellets. A gradient of oxygen and nutrients may be responsible for this architecture since, in both CMF lobules and ACP, these have to diffuse from the periphery towards the centre through the extracellular matrix. The degree of differentiation of cultured chondrocytes has been shown to be inversely proportional to oxygen tension [24]. Consistent with this, a rounder morphology of cells together with intense safranin-O staining, reflecting the phenotype of differentiated chondrocytes, was seen in the central areas of the chondrocyte pellet and in the matrix-rich areas of CMF lobules, where the oxygen tension is expected to be lower. Despite their striking morphological resemblance, CMF and chondrocyte pellets showed different expression patterns (Figure 3). In particular, expression of PTHLH and PTHR1 was significantly higher in the chondrocyte pellet culture system. This could be the result of culture conditions promoting chondrogenesis, since the media used include high levels of TGF-β (10 ng/ml). It is well known that this signalling molecule can induce, specifically in articular chondrocytes, upregulation of PTHLH [25]. However, it is also known that, *in vivo*, PTHLH binding to its receptor PTHR1 leads to the upregulation of BCL2 in proliferative and pre-hypertrophic chondrocytes of the growth plate [26]. Such an effect is not present *in vitro* in the absence of extra doses of PTHLH [26]. In this regard our results with pellets resembled the results of previous experiments [26], since chondrocytes cultured in a high-density system, in the absence of supplemented extra dose of PTHLH, do not express BCL2.

The diffuse positive signal for BCL2 in CMF is noteworthy, especially considering its lower level of PTHLH. This striking difference in BCL2 expression between CMF and normal chondrocytes cultured *in vitro* indicates a different mechanism of signalling/transduction that may be due to the effect of other mediators present *in vivo* and not *in vitro* [26], or the result of differences in cartilage differentiation in neoplasia versus normal cells.

An intriguing result was the different levels of expression found for p16 and cyclin D1. These two molecules counteract one another in regulating cell cycle progression. The cyclin D/cyclin-dependent

kinase (cdk) 4/6 complex phosphorylates Rb (retinoblastoma) proteins, promoting progression of the cell cycle [27]. This action is counterbalanced by the binding of p16 to the complex, which in turn induces an allosteric change in cdk4/6, thereby altering the binding site of D-type cyclins and reducing its affinity for ATP, hence inhibiting cell cycle progression [27]. The presence of both counteracting proteins is in agreement with the clinically benign nature of CMF. In a previous study [28] the expression of p16 in enchondromas and loss of expression in conventional chondrosarcomas was observed, which illustrates the role of this molecule in balancing proliferative activity typical of malignant transformation. The lower level of cyclin D1 in ACPs is of note, considering their higher level of PTHLH and their stimulation by external TGF-β, both known to upregulate cyclin D1 expression [29]. Again this result underlines the difference in signalling/transduction mechanisms between *in vitro* chondrogenesis and CMF, despite their histological similarities. The difference in N-cadherin expression in CMF versus ACP reflects differences in spatial distribution: the expression in CMF is higher in the cellular areas, while in ACP it is largely expressed where cells are embedded in abundant extracellular matrix. Since the ACPs were cultured for only 2 weeks, N-cadherin may still be present at a high level as the condensation phase is perhaps still going through its end stages. FGF signalling is conserved in CMF and ACP, since the expression of FGFR3 and p21 does not differ significantly between them. Both molecules are part of the FGF signalling pathways in which, in chondrocytes, p21 is the downstream molecule of FGFR3 activation, resulting in inhibition of cell cycle progression and indirectly promoting differentiation [13].

Comparison between *in vitro* and *in vivo* conditions is in general problematic. Although the *in vitro* results in the present study have been obtained under the influence of TGF-β1, the possible influence of this condition has been discussed above. Furthermore, recent data showed a diffuse presence of functionally active TGF-β1 in CMF [30].

In conclusion, we have clearly identified and substantiated the morphological similarities between CMF and *in vitro* cell culture chondrogenesis. Similarities include cellular morphology, quality of the extracellular matrix and cytoarchitecture. In our opinion this clearly reflects conservation of the basic process of cartilage formation in this neoplastic condition, further confirmed by the expression of PTHLH as well as FGF signalling molecules. The observed difference in expression of these molecules, between the matrix-rich areas versus the cellular areas of CMF, may reflect their importance in the commitment of neoplastic cells toward cartilage differentiation. The comparisons with ACP showed significantly higher expression of N-cadherin, PTHLH and PTHR1 and, conversely, lower expression of cyclin D1and p16 in ACP versus CMF. The absence of BCL2 expression in ACP is noteworthy. These differences in expression may be crucial in neoplastic chondrogenesis.

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