Expression of hypoxia-inducible factor-1 α and its relationship to tumour angiogenesis and cell proliferation in cartilage tumours

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We investigated the use of hypoxia-inducible factor (HIF) proteins as prognostic markers in chondrosarcoma and the relationship of HIF to the biological characteristics of cartilage tumours. The expression of HIF-1 α , HIF-2 α , proliferating cell nuclear antigen (PCNA) and microvessel density (MVD) were measured immunohistochemically in 29 specimens of cartilage tumour.

There was no HIF-1 α and HIF-2 α staining in any of the nine benign cartilage tumours. In 20 specimens of chondrosarcoma, the rate of HIF-1 α and HIF-2 α expression was 40% and 25%, respectively. The tumour size (≥ 8 cm), histological grade (grade 2 and grade 3) surgical margin (marginal and intralesional) and HIF-1 α expression (positive) correlated significantly with a shorter disease-free survival. There was a significant association between HIF-1 α and the MVD and a strong trend towards a correlation between HIF-1 α and the PCNA index or histological grade.

Our findings suggest that HIF-1 α protein may be a useful objective marker in the assessment of the prognosis in chondrosarcoma, since it plays an important role in tumour angiogenesis and cell proliferation.

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Chondrosarcomas are generally resistant to radio- and chemotherapy which means that surgical wide resection is the only curative treatment. The rates of local recurrence, distant metastases and survival vary from study to study. Consequently, the histological grade of cartilage tumours has traditionally been regarded as the best tool for the assessment of prognosis.¹ However, the histological determination of the grade of the tumour is basically subjective and pathologists do not always agree on the number or appearance of cytological factors in chondrosarcoma cells.² It is often difficult to distinguish between low-grade chondrosarcoma and benign processes such as enchondromas and osteochondromas. Moreover, it is of critical importance to differentiate accurately between low- and intermediate-grade chondrosarcomas because orthopaedic surgeons are beginning to treat low-grade chondrosarcomas in long bones with intralesional procedures and local adjuvants. More elaborate objective methods have been sought to assess prognosis.

It has long been suspected that hypoxia is involved in many pathophysiological processes including neoplasm and inflammation. It is also the prime stimulus for angiogenesis, which is probably one of the key elements involved in

the initiation and development of tumours.³ Hypoxia may play an even more significant role in the evolution of cartilage tumours than in others because of the phenotypic links between cartilage tumour and cartilage which is hypoxic and avascular.⁴

One of the key regulators of the cellular response to hypoxic stimuli is the transcription factor hypoxia-inducible factor (HIF).³ The HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β subunits. The latter is continuously expressed while HIF-1 α accumulates rapidly within the nuclei of the hypoxic cells, primarily because of the prevention of ubiquitination and proteasomal degradation of the protein which usually take place in normoxic cells. The HIF-1 α heterodimer activates the transcription of many genes which code for proteins involved in angiogenesis, glucose metabolism, cell proliferation and invasion. Recently, other members of the family have been described.⁵⁻⁷ HIF-1 α and HIF-2 α are thought to play a significant role in tumour angiogenesis, are widely expressed by various cancers and have been shown to be significant prognostic markers in selected cancers.⁵⁻⁷ Although the expression of HIF-1 α in cartilage tumours has been investigated,^{4,8} the precise correlation of HIF-1 α expression with clinico-

pathological features and the overall impact on prognosis has not been evaluated in patients with chondrosarcoma.

We have examined the immunohistochemical expression of HIF-1 α and HIF-2 α proteins in surgical specimens of chondrosarcoma to evaluate the prognostic importance of this expression. In addition, HIF-1 α expression was also analysed and compared with the microvessel density (MVD) and the proliferating cell nuclear antigen (PCNA) index.

Materials and Methods

We obtained 29 specimens of cartilage tumour from patients treated at our institution between 1986 and 2001. Written informed consent was obtained for participation in the study from all the patients or their guardians.

Slides of each specimen which had been stained with haematoxylin and eosin were reviewed to confirm the diagnosis and histological grade of each tumour using currently-accepted criteria.^{9,10} Blocks containing specific features of chondrosarcoma were selected. An appropriate histological grade was assigned in a blinded fashion by two authors (TK, KA), one of whom was a bone pathologist (KA). In particular, grade-1 chondrosarcoma was distinguished from enchondroma by its growth pattern.¹⁰ Enchondroma is characterised by islands of tumour cartilage surrounded by trabecular bone and/or osteoid, whereas the tumour cartilage of chondrosarcoma infiltrates the marrow spaces and involves the trabecular bone.

There were 20 specimens of chondrosarcoma and nine of benign cartilage neoplasia. According to the histological grade 14 chondrosarcomas were grade 1, three were grade 2, and three grade 3 (which included one dedifferentiated chondrosarcoma and one mesenchymal chondrosarcoma). Of the benign cartilage tumours three were enchondromas, three were osteochondromas and three were chondroblastomas.

Immunohistochemical staining. Each tissue block was cut into sections 5 μ m in thickness, transferred to Matsunami adhesive silan (MAS)-coated glass slides (Matsunami, Osaka, Japan), deparaffinised in xylene, rehydrated in a graded series of decreasing ethanol concentrations and then rinsed in tris-buffered saline Tween-20 (TBST) (50 mM Tris/HCl pH 7.6, containing 0.3 M sodium chloride and 0.1% Tween 20). For the detection of HIF-1 α and HIF-2 α , sections were immersed in Target Retrieval Solution (DakoCytomation Inc., Carpinteria, California), and placed in a hot-water bath for 20 minutes. After antigen retrieval, there followed a cooling-off period of 20 minutes. Sections were immunostained using the Catalysed Signal Amplification system (DakoCytomation Inc.) according to the manufacturer's instructions. The HIF-1 α antibody (ESEE122, diluted 1:1000; Novus Biological Inc., Littleton, Colorado) and HIF-2 α antibody (Ep190b, 1:1000; Novus Biological Inc.) were incubated with the tissue sections overnight at 4°C in a moisture chamber. Target Retrieval Solution (DakoCytomation Inc.) was

used for the detection of cluster of differentiation (CD34), whereas the antigen retrieval procedure was omitted for the detection of PCNA. CD34 antibody (Ready-to use N-series Qbend 10, DakoCytomation Inc.) and PCNA antibody (Ready-to use N-series PC10, DakoCytomation Inc.) were incubated with the tissue sections for 30 minutes at room temperature in a moisture chamber, and then the polymer-peroxidase method was used (EnVision+/HRP, DakoCytomation Inc.). The reaction products were visualised by exposing the sections to 3,3-diaminobenzidine. Nuclei were lightly counterstained for about ten seconds with Gill's formulation #2 haematoxylin. Tissue sections of renal-cell carcinoma with strong nuclear HIF-1 α expression were used as a positive control group. Non-specific reactivity was assessed by omission of the primary antibody and the specificity was confirmed in the tumour section as well as in the control tissue by pre-absorption of the antisera by blocking peptide supplied by the manufacturer.

Immunohistochemical evaluation. Two independent observers (TK, KA) evaluated stained specimens in a blinded fashion. Each score was discussed by all the authors to reach an overall consensus. Cells were considered to be immunoreactive to HIFs when distinct nuclear staining was identified. Cytoplasmic staining, observed occasionally, was ignored because active HIFs are located only in the nucleus. The extent of staining was scored as follows: (-) indicating a negative reaction of the tumour cells; ($\pm$) nuclear staining in < 10% of cells; (+) 10% to 50% of cells; and (++) > 50% of cells. For all analyses, the tumours in which stained tumour cells made up > 10% of the tumour were regarded as positive.

For the evaluation of proliferating tumour cells, we examined at least 500 tumour cells to determine whether their nuclei were positive for PCNA staining. We did not divide the PCNA expression level. Instead we used the percentage of PCNA-positive cells (PCNA index) for all analyses.¹¹ The PCNA index was classified as high if it was $\geq$ 12.6%, a figure representing the median value.

Microvessel counting. Angiogenic activity was assessed by estimating CD34-positive microvessels in the surrounding stroma of invasive tumour nests.¹² In each tumour, hot-spot areas displaying the highest vessel density were identified by scanning tumour sections at low-power magnification ($\times$ 40). The maximum vessel density was determined from these hot-spot areas at fields under high-power field (HPF) magnification ($\times$ 400), and the mean of the counts for the three fields was calculated. Identification of the lumen of a vessel was not necessary for a structure to be defined as a vessel. The MVD was classified as high if it was $\geq$ 12.9/HPF, a figure representing the median value.

The time of follow-up was calculated from the date of the operation. The median follow-up was for 78 months (1 to 192).

Statistical analysis. All survival analyses were evaluated using the Kaplan-Meier method and the log-rank test. The

Fig. 1a

Fig. 1b

Photomicrographs showing the immunohistochemistry of a) hypoxia-inducible factor (HIF)-1 α and b) HIF-2 α in a grade-3 chondrosarcoma of the proximal tibia. Representative chondrosarcoma tissues with (++) staining show dark brown nuclear staining of HIF-1 α and HIF-2 α in a diffuse fashion ($\times 100$).

correlation of HIF-1 α expression with the MVD, PCNA index and histological grade was analysed using Fisher's exact test and the chi-squared test. All analyses were performed by SPSS software (SPSS Inc., Chicago, Illinois) and Statcel (OMS Ltd, Saitama, Japan). A p -value < 0.05 was considered to be significant.

Results

HIF-1 α and HIF-2 α expression in cartilage tumours. There was no HIF-1 α and HIF-2 α staining in any of the nine benign cartilage tumours. In eight (40%) of the 20 specimens of chondrosarcoma, HIF-1 α expression was positive, with five (25%) strongly expressing the protein (++) . In five (25%) specimens HIF-2 α expression was positive, with only two (10%) strongly expressing the protein (++) . All HIF-2 α -positive specimens showed positive HIF-1 α staining. Representative immunostains of HIF-1 α and HIF-2 α are shown in Figure 1.

Survival analysis in chondrosarcomas. None of the patients with chondrosarcoma presented with metastases. The median age at the time of surgery was 42 years (15 to 71). There were eight men and 12 women. Their tumours were sited in the trunk in 12 (9 pelvis, 1 rib, 1 scapula and 1 vertebra) and in the limbs in eight (4 tibia, 3 humerus and 1 femur). The median tumour size, defined as the maximum diameter of the resected tumour was 11.7 cm (4 to 27). A total of 16 patients were treated by wide excision, two by marginal excision, and two by intralesional excision. There was one local recurrence and three patients developed pulmonary metastases. The other 16 patients remained free from disease. The five- and ten-year disease-free survival rates were 81.3% and 63.2%, respectively.

The tumour size (≥ 8 cm), histological grade (grade 2 and grade 3), the surgical margin (marginal and intralesional) and HIF-1 α expression (positive), correlated significantly with a shorter disease-free survival ($p = 0.020, 0.033, 0.017$ and 0.020 , respectively; Table I and Figure 2).

Correlation of HIF-1 α with the MVD, PCNA and histological grade. In order to investigate the effect of HIF-1 α on tumour angiogenesis and cell proliferation, the relationship of HIF-1 α to the MVD, PCNA index and histological grade was analysed in the 20 chondrosarcomas. Of the 29 patients in the study, four (13.8%) showed a high MVD and five (17.2%) had a high PCNA index. Representative immunostains of CD34 and PCNA are shown in Figure 3. There was a significant correlation of HIF-1 α expression with the MVD (Fisher's exact and chi-squared tests, $p = 0.014$ and $p = 0.006$, respectively; Table II). Although neither the PCNA index nor histological grade significantly correlated with HIF-1 α expression, both showed a strong trend towards significance (Fisher's exact test). This trend could achieve significance using the chi-squared test (PCNA, $p = 0.035$; histological grade, $p = 0.035$; Table II). This could be explained by the results of inadequate numbers for statistical power. Two non-conventional chondrosarcomas, one dedifferentiated chondrosarcoma and one mesenchymal chondrosarcoma strongly expressed HIF-1 α and HIF-2 α proteins (++) .

Discussion

In recent years it has been thought that the expression of HIF-1 α and HIF-2 α may be a useful prognostic indicator for a variety of malignancies.⁵⁻⁷ Zhong et al¹³ observed that HIF-1 α expression also occurred in premalignant lesions

Table I. Prognostic variables in chondrosarcoma as determined by univariate analysis of disease-free survival (DFS)

Variable	5-year DFS (%) (n = 20)	95% confidence interval	p-value
Age (yrs)			
< 40	71	38 to 100	0.468
≥ 40	89	68 to 100	
Gender			
Male	70	34 to 100	0.216
Female	89	68 to 100	
Tumour size (cm)			
< 8	100	0 to 100	0.020
≥ 8	66	34 to 98	
Tumour site			
Extremity	100	0 to 100	0.861
Trunk	66	34 to 98	
Histological grade ^{9,10}			
1	93	79 to 100	0.033
2 and 3	50	1 to 99	
Surgical margin			
Wide	84	64 to 100	0.017
Intralesional and marginal	75	33 to 100	
HIF [*] -1α expression			
Negative	91	74 to 100	0.020
Positive	66	25 to 100	

* HIF, hypoxia-inducible factor

such as colonic adenoma, breast ductal carcinoma *in situ* and prostatic intra-epithelial neoplasia, but not in benign tumours, suggesting that HIF-1α expression may take place in the very early stages of carcinogenesis. To date, only two studies have evaluated the expression of HIF-1α in cartilage tumours.^{4,8} They found no staining in benign tumours but there was HIF-1α expression in all grades of chondrosarcoma at similar levels which increased significantly in angiogenic tumours. They could not correlate HIF-1α expression with other clinicopathological features or survivorship. In our study, HIF-1α and HIF-2α were not expressed in any of the benign cartilage tumours, which is consistent with the previous studies. However, in chondrosarcoma, the rate of expression of HIF-1α and HIF-2α was 40% and 25%, respectively, which does not accord with the previous studies. The discrepancy may have been the result of different methods of evaluating the positivity of HIF-1α staining. In the previous studies^{4,8} HIF-1α expression was evaluated within the nucleus and cytoplasm by the staining intensity and number of tumour cells. Our analysis was based only on the nuclear expression since it can be assumed that nuclear HIF-1α protein is the active form.^{13,14} In fact, Nakanishi et al¹⁵ found that despite a significant correlation between HIF-1α protein expression and disease-free survival in transitional cell carcinomas of the upper urinary tract, the expression of HIF-1α mRNA was not associated either with progression or prognosis, proba-

bly because its mRNA expression would not be correlated with the active form of nuclear HIF-1α protein. In addition to previously reported prognostic factors, such as the surgical margin, tumour size and histological grade, the active form of HIF-1 expression may help to predict a group of chondrosarcoma patients with a poor prognosis.

The two most important characteristics of malignant tumours are their unrestrained growth and their capacity to produce metastases. The ability to induce sustained angiogenesis is needed for both. Until now, only two studies have shown that cartilage tumours can possess microvessels in their development and progression, and that their vascularity correlates with the grade of tumour.^{16,17} We have, in addition, demonstrated a significant correlation between HIF-1α expression and a high MVD, suggesting that hypoxia in the microenvironment of the tumour may be involved in angiogenesis in chondrosarcoma.

It is increasingly thought that hypoxia is not the only factor to stimulate HIF-1 activity after the induction of angiogenesis. For example, Feldser et al¹⁸ reported that in the treatment of cultured prostatic carcinoma cells with insulin, insulin-like growth factor (IGF)-1, or IGF-2 induced the expression of HIF-1α protein, which in turn required the expression of IGF-2 mRNA, suggesting the involvement of HIF-1α protein in an autocrine growth factor loop. Zhong et al¹³ also reported that HIF-1α overexpression was associated with aberrant accumulation of

Fig. 2a

Fig. 2b

Fig. 2c

Fig. 2d

Graphs showing Kaplan-Meier event-free survival for chondrosarcoma according to a) tumour size, b) histological grade, c) surgical margin and d) hypoxia-inducible factor (HIF)-1 α expression. The vertical bars indicate the censored patients (no relapse).

p53 oncogene in 75 human cancers, including most cancers of the colon and breast. We used PCNA to explain the relationship between HIF-1 α and the proliferation of chondrosarcoma cells, since PCNA is an assistant factor of DNA synthetase, takes part in DNA biological synthesis and regulates the cell cycle and cell proliferation by tetramer with cyclin, cyclin-dependent kinase (CDK) and p21.¹⁹ Our immunohistochemistry revealed that the expression of HIF-1 α positively correlated with the PCNA index of chondrosarcoma cells, indicating the presence of HIF-1 α in actively proliferating cells, which is in line with other studies.²⁰⁻²²

Surgical treatment with wide resection is the mainstay of treatment for chondrosarcoma. There is no widely

accepted adjuvant therapy and chemo- and radiotherapy have a minimal role. Lin et al⁴ reported that when chondrosarcoma cells were transfected with small interfering RNA targeting HIF-1 α before exposure to hypoxia, HIF-1 α protein and the angiogenic cytokin vascular endothelial growth factor (VEGF) mRNA decreased. They speculated that anti-angiogenic treatment strategies which target the HIF-1 α and VEGF pathways may be worth exploring as experimental therapy for chondrosarcoma. In fact, several HIF inhibitors, including topotecan, 17-N-allylamino-17-demethoxygeldanamycin, 2-methoxyestradiol and small molecules targeting receptor tyrosine kinase-dependent signalling pathways are currently in clinical development for the treatment of

Fig. 3a

Fig. 3b

Photomicrographs showing the immunohistochemistry of a) cluster of differentiation (CD) 34 and b) proliferating cell nuclear antigen (PCNA) in the case shown in Figure 1. Representative chondrosarcoma tissue has CD34-positive microvessels in the interfaces between tumour nests and stroma and diffuse strong positivity for PCNA in the nuclei of the cells ($\times 100$).

Table II. Correlations of hypoxia-inducible factor (HIF)-1 α expression with the microvessel density (MVD), proliferating cell nuclear antigen (PCNA) index and histological grade⁹ in 20 chondrosarcomas according to the number of cases

Variable	HIF-1 α expression		p-value	
	Positive	Negative	Fisher's exact test	chi-squared test
MVD				
Low	4	12	0.014	0.006
High	4	0		
PCNA				
Low	4	11	0.058	0.035
High	4	1		
Histological grade				
1	3	10	0.052	0.035
2 and 3	5	2		

cancers.²³ Our findings are of value for the development of anti-angiogenic chemotherapy for patients with chondrosarcoma.

In conclusion, our study has shown that the active form of HIF-1 α expression is associated with diminished disease-free survival and correlates with the MVD and PCNA index, suggesting that HIF-1 α may play an important role in tumour angiogenesis and cell proliferation in chondrosarcoma. The active form of HIF-1 α expression may be a more objective marker than the histological grade in the assessment of the prognosis of chondrosarcoma.

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