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C-erb B-2 STAINING IN PRIMARY SYNOVIAL CHONDROMATOSIS: A COMPARISON WITH OTHER CARTILAGINOUS TUMOURS

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SUMMARY

In this study C-erb B-2 immunostaining has been used to highlight distinct differences between the cartilage found in primary synovial chondromatosis ($n=20$), normal articular cartilage ($n=10$), benign enchondromas ($n=10$), and chondrosarcomas ($n=10$). There was no positive staining in either the normal cartilage or the chondromas, but 15 cases of synovial chondromatosis showed at least some staining, although in the majority of cases fewer than 50 per cent of cells stained positive. There was no correlation between cellularity/pleomorphism and the extent or intensity of staining. Five of the chondrosarcomas were positive, with more than 50 per cent of cells showing positive staining in three of these cases. All positive cases in this series showed a diffuse cytoplasmic staining pattern. Despite these results, there was no Ki-67 positive staining in synovial chondromatosis, which tends to suggest that the demonstrated expression of C-erb B-2 is not related to proliferative activity. The significance of this staining remains undetermined.

KEY WORDS—**synovial chondromatosis; cartilage; immunohistochemistry; C-erb B-2 proto-oncogene; Ki-67**

INTRODUCTION

The proto-oncogene C-erb B-2, located on chromosome 17 (q21), is thought to have a role in normal cell proliferation and development. It produces a 185 kD glycoprotein which is a transmembrane receptor with tyrosine kinase activity[1] and has 78 per cent homology with the epidermal growth factor receptor.[2,3] Oncogenic protein kinases may induce transformation through either inappropriate or excessive protein phosphorylation.

Amplifiction and overexpression of C-erb B-2 has been reported in a wide variety of carcinomas[4] but has only rarely been described in connective tissue tumours.[5,6] Wrba *et al.*[5] found no staining in normal cartilage and benign tumours, with the exception of one osteochondroma, but demonstrated positive staining in the great majority of chondrosarcomas. Primary synovial chondromatosis is thought to be a cartilaginous metaplasia[7] but it may recur locally and although usually benign, rare malignant change has been reported.[8–10] In view of the findings of Wrba *et al.*,[5] it was decided to investigate the C-erb B-2 oncogene expression in the cartilage of primary synovial chondromatosis, compared with normal cartilage, enchondroma, and chondrosarcoma. It was hoped that if a distinct difference in staining could be demonstrated, it might contribute further to our knowledge of the aetiology of this rare condition. The proliferative activity of all cases was also assessed with the Ki-67 antibody, to ascertain whether C-erb B-2 expression could be related to cell proliferation.

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MATERIALS AND METHODS

Twenty cases of primary synovial chondromatosis were identified from files using the criteria of Villacin *et al.*[11] Ten enchondromas, ten chondrosarcomas, and ten cases of normal articular cartilage were also obtained. The latter came from femoral heads removed for avascular necrosis, as this represents one of the few readily available sources. All cases were paraffin-embedded and fresh sections were cut and stained with haematoxylin and eosin. The cases of primary synovial chondromatosis were assessed for cellularity, pleomorphism, and the presence of binucleate forms. The chondrosarcomas were graded using the criteria of Evans *et al.*[12]

Immunohistochemistry

C-erb B-2—Sections 4 μm thick were cut from paraffin wax-embedded tissue blocks. After dewaxing, endogenous peroxidase was blocked with 1·5 per cent H_2O_2 in methanol and sections were then incubated with polyclonal antibody to C-erb B-2 oncoprotein (DAKO) for 30 min at 37°C.

Rabbit anti-human C-erb B-2 was used at a dilution of 1 in 100 in 1 per cent bovine serum albumin (BSA) and was followed by the streptavidin biotin procedure using a DAKO duet kit, colour development with diaminobenzidine and H_2O_2 (Sigma), and counter-staining of nuclei with haematoxylin. PBS washes were used between each step.

Positive controls included a known positive breast carcinoma. Negative controls were included following the same procedure, but with omission of the primary antibody and using an irrelevant rabbit immunoglobulin recommended by the manufacturers (DAKO X903).

Table I—Immunohistochemical staining reaction for C-erb B-2 protein in synovial chondromatosis, normal cartilage, and benign and malignant tumours

	No. of cases	Extent of staining			Intensity of staining		
		0	1	2	0	+	++
Synovial chondromatosis	20	5	11	4	5	6	9
Normal cartilage	10	10	—	—	10	—	—
Enchondroma	10	10	—	—	10	—	—
Chondrosarcoma	10	5	2	3	5	2	3

In all cases, both membrane (M) and cytoplasmic (C) staining was assessed. The intensity and extent of staining were graded using a three-point scale (0=no staining, +=weak staining, and ++=strong staining for intensity; 0=no cell staining, 1=<50 per cent positive cells, and 2=>50 per cent positive cells for extent).

Ki-67—As all cases were from paraffin-embedded, formalin-fixed tissue, an antigen retrieval method similar to that described by Shi *et al.*[13] was used. Sections 4 μm thick were cut and placed on 3-aminopropyltriethoxysilane (APES)-coated slides. After dewaxing, endogenous peroxidase was blocked with 1·5 per cent H_2O_2 in methanol. For antigen retrieval, citrate buffer (pH 6·0) was used in a microwave processing technique. The sections were treated for 20 min (750 W, full power), checking for evaporation at 5 min intervals and topping up as necessary. The sections were then left to stand in the buffer for 15–20 min. For the immunostaining, the polyclonal antibody Ki-67 (DAKO) was used at 1 in 100 dilution, followed by the streptavidin biotin procedure using a DAKO duet kit (DAKO K492), colour development with diaminobenzidine in H_2O_2, and counter-staining with haematoxylin. PBS washes were used between each step.

Normal tonsil was used as a positive control. As all cases had been subjected to varying degrees of decalcification in acid, a series of control experiments was performed to determine the effect of formalin and acid on Ki-67 staining. Slices of normal tonsil were placed in 10 per cent buffered formalin followed by a 5 per cent formic acid for varying times (24–72 h), processed, and embedded. The above procedure for antigen retrieval and Ki-67 staining was then followed and staining intensity and extent were assessed. The optimal microwave oven heating time was also pre-determined by a series of control experiments, using times ranging from 5 to 30 min.

All cases were assessed for Ki-67 staining. Chondrocytes with sharp staining of nuclei (irrespective of intensity) and clear cytoplasm were designated Ki-67 positive. The percentage positivity was determined by counting the number of positive cells amongst 1000 cells from randomly selected areas at × 400 magnification.

RESULTS

Of the 20 cases of synovial chondromatosis, there were 12 male and 8 female, with an average age of 39 years (range 18–50 years). Thirteen cases were in the knee, four in the hip, and one each in the elbow, finger, and foot. Seventeen cases showed high cellularity; in ten cases there was significant nuclear pleomorphism; and binucleated chondrocytes were seen in all cases. There was significant calcification in four cases and marked ossification in two cases.

Of the ten chondrosarcomas, two were grade I, five grade II, two grade III, and one was dedifferentiated.

C-erb B-2 staining

The results are summarized in Table I.

Diffuse cytoplasmic staining was seen in 15 cases of synovial chondromatosis. In 11 cases, fewer than 50 per cent of the cells were stained, but the staining was of strong intensity in five. More than 50 per cent of cells were stained in four cases, all with strong intensity. There was no correlation between cellularity/pleomorphism and either age or site, nor between C-erb B-2 staining extent or intensity and cellularity/pleomorphism ($r=0·2$, $P>0·05$).

There was no positive staining observed in either normal cartilage or enchondroma.

Of the ten chondrosarcomas, five were negative (three grade I and two grade II) and five were positive (two grade II, two grade III, and one de-differentiated). In two cases, fewer than 50 per cent of cells were stained, one with strong intensity. In three cases more than 50 per cent of cells were stained, two with strong intensity.

All positive cases showed a diffuse cytoplasmic staining pattern (Fig. 1), the intensity of which often obscured any membrane staining that may have been present. Sometimes the intensity of staining was variable among cells from the same case. In four cases there was accompanying membrane staining (Fig. 2), but no cases showed only membrane staining.

Control experiments were consistently negative.

Ki-67 staining

Control experiments with normal tonsil showed a slight drop in staining intensity, but no significant reduction in staining extent when tissue was subjected to 24 h treatment in formic acid after 24 h formalin fixation. There was no reduction in staining extent or intensity with acid treatment if tissues were fixed for more than 24 h in formalin. The optimum microwave heating time

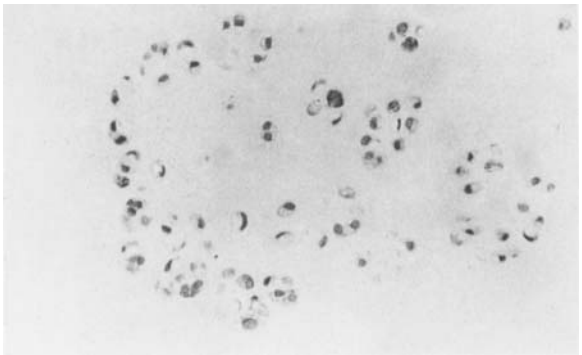

Fig. 1—Diffuse cytoplasmic staining for C-erb B-2 in chondrosarcoma

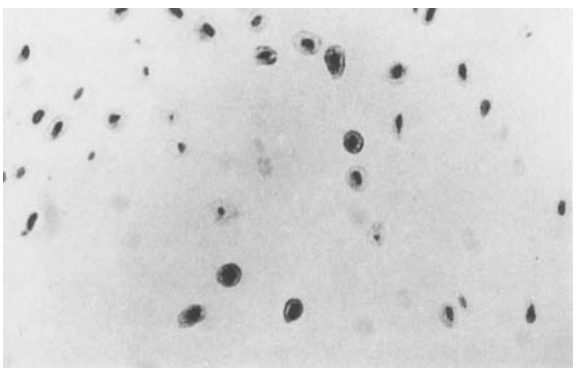

Fig. 2—Diffuse cytoplasmic and membrane staining for C-erb B-2 in synovial chondromatosis

was 20 min. There was a significant increase in staining extent from 5 min (virtually no staining) through to 20 min; heating for longer than 20 min not only resulted in no appreciable increase in staining extent, but also caused loss of tissue from the slides.

There was no identifiable Ki-67 staining in the chondromas, normal articular cartilage, nor in any of the ten cases of synovial chondromatosis. All ten cases of chondrosarcoma stained to a varying extent, ranging from 0·4 to 11 per cent.

DISCUSSION

The cartilage in primary synovial chondromatosis can be extremely cellular, with many binucleated chondrocytes which would be interpreted as malignant if occurring within a bone.[7] The condition is thought to be metaplastic, but the active growth of the cartilage and the presence of binucleated cells suggest a proliferative component.

Seventeen of our 20 cases showed a high degree of cellularity and significant nuclear pleomorphism was present in ten cases, but these features were not related to the age of the patient, the site of the lesion, or clinical recurrence.

Amplification of C-erb B-2 is well recognized in carcinomas[4] but has only rarely been described in connective tissue tumours. Wrba *et al.*[5] investigated C-erb B-2 expression in cartilage and obtained negative results in normal cartilage and 14 of 15 benign tumours, one osteochondroma being positive. Positive staining was seen in 18 of 23 chondrosarcomas. They also reported a low but statistically significant inverse relationship between histological grade and staining intensity. Conversely, George *et al.*[6] found no staining at all in 21 chondrosarcomas.

In this study, we too observed no positive staining in normal cartilage and benign tumours, but positive staining was seen in 15 cases of primary synovial chondromatosis. Fewer than 50 per cent of cells stained positive in the majority of these, but the staining was of strong intensity in nine cases. There was no statistically significant correlation between C-erb B-2 expression and cellularity/pleomorphism ($r=0·2$, $P>0·05$), but the fact that 15 cases of synovial chondromatosis and none of the enchondromas or normal cartilage specimens stained positively is significant (chi-squared, $P<0·001$).

Five out of ten chondrosarcomas showed evidence of C-erb B-2 protein expression and in three of these cases more than 50 per cent of cells stained positive. Unlike Wrba *et al.*,[5] we found no relationship between grade of sarcoma and extent of oncogene expression ($r=0·54$, $P>0·05$).

Most studies with the C-erb B-2 antibody have concentrated on cell membrane staining, but like Wrba *et al.*[5] we found a predominantly diffuse cytoplasmic staining pattern. Cytoplasmic staining has been demonstrated in pancreatic carcinoma[14] and in salivary gland tumours;[15] its significance is undetermined, but its presence warrants documentation.[4] It may possibly represent an intermediate or alternative protein product.[1,16]

Proliferative activity was assessed with a polyclonal Ki-67 antibody as recently recommended by Rose *et al.*,[17] but there was no staining at all in any of the 20 cases of synovial chondromatosis, nor in the enchondromas or normal cartilage. These findings are similar to those described by Apte and Athanasou,[18] who found no Ki-67 staining in the cartilage or synovium in a study of six cases, using fresh tissue, not subjected to the effects of formalin fixation or acid decalcification. As formalin fixation and acid can affect Ki-67 staining, we conducted a series of prior control experiments to guard against false-negative results. Furthermore, like Munakata and Hendricks,[19] we found that the extent of staining increased significantly with increased microwave heating time; on the basis of these experiments, we employed an optimal heating time of 20 min. We are therefore confident that these Ki-67 results are 'true' negatives.

The Ki-67 index for the chondrosarcomas ranged from 0·4 to 11 per cent; these are similar to the results of Scotlandi *et al.*[20] and Vollmer *et al.*,[21] who used fresh tissue.

In view of these Ki-67 results, the cytoplasmic C-erb B-2 staining that we have demonstrated in synovial chondromatosis would not appear to be related to proliferative activity. The enchondromas and normal cartilage were negative for both C-erb B-2 and Ki-67.

In this study we have used C-erb B-2 expression to highlight distinct differences between the cartilage found in primary synovial chondromatosis and both normal

cartilage and benign enchondromas. Significant staining was demonstrated in 75 per cent of cases of chondromatosis and in 50 per cent of chondrosarcomas but no positive staining was seen in any of the other specimens studied. The explanation of the cytoplasmic staining pattern is unclear, but it does not appear to be related to cell proliferation.

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