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Correlation Between the Histological Grade of Chondrosarcoma and the Expression of MMPs, ADAMTSs and TIMPs

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Abstract. *Background: The malignant degree of human chondrosarcoma can be difficult to determine using only histological findings. We, therefore, assessed the expression of matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase (ADAM) with thrombospondin motifs (ADAMTSs) and tissue inhibitor of metalloproteinases (TIMPs) in chondrosarcoma and ascertained the relationships to histological degree of malignancy and prognosis. Materials and Methods: In 28 chondrosarcoma cases, immunostaining was performed using antibodies against MMP 2, 3, 7, 9, 13, ADAMTS 4, 5 and TIMP 1, 2, 3. Results: The chondrosarcoma were classified into groups of 7, 15 and 6 cases based on histologically malignant grade I, II and III, respectively. All target proteins were expressed in chondrosarcoma. Positive correlations ($p<0.05$) existed between immunostaining scores and histological grades for all proteins except MMP 9, with strong correlations ($p<0.01$) for MMPs 2, 3 and 13, both ADAMTSs and all 3 TIMPs. No correlation existed between prognosis and immunostaining scores. Conclusion: These target proteins could, thus, indicate the degree of malignancy in human chondrosarcoma.*

Matrix metalloproteinases (MMPs) are capable of breaking down the extracellular matrix (ECM) and are believed to play important roles in cancer invasion and metastasis (1,2). Working together, MMPs can break down almost all ECM components in the body. In recent years, a disintegrin and metalloproteinases (ADAMs), a family closely related to the MMP family, and secretory ADAM with thrombospondin motifs (ADAMTSs), have been shown to be involved in the decomposition and metabolism of ECM components (3,4). Furthermore, the activities of these enzymes are inhibited by a common factor, the tissue inhibitor of metalloproteinases (TIMPs) (5,6). Human chondrosarcoma is the second most common bone tumour, accounting for 25% of all primary bone tumours. Chondrosarcoma often affects people over 50 years old. Several studies have examined the relationships between the histological grade of chondrosarcoma (7,8) and the expression of MMPs and TIMPs (9-11). However, the relationship between chondrosarcoma and the expression of ADAMs has not been investigated immunohistochemically. Although the mechanisms for articular cartilage destruction are beginning to be clarified, the expression of ADAMs in chondrosarcoma and the mechanisms for invasion and metastasis have not been elucidated.

In the present study, the expression of MMPs 2, 3, 7, 9 and 13, ADAMTSs 4 and 5, and TIMPs 1-3 was examined using antibodies against these proteins in 28 cases of chondrosarcoma to ascertain their expression and assess the relationship between the histological degree of malignancy and immunostaining score. As recurrence markedly affects prognosis in malignant disease, relationships of expression of these proteins to prognosis was investigated in 15 patients without recurrence after wide excision, to ascertain whether these proteins can serve as prognosticators.

Materials and Methods

Materials. The study subjects were 28 cases of chondrosarcoma, who underwent treatment in the Department of Orthopaedic Surgery, Nihon University Itabashi Hospital, Japan, in the period from 1968 to 2001. Five cases of healthy cartilage were used as control. According to the classification system of O'Neal and Ackerman (8), cases were categorised as grade I (n=7), II (n=15), or III (n=6), excluding dedifferentiated chondrosarcoma. Surgical methods comprised: wide excision including ablation (n=15), marginal excision (n=5), intralesional excision (n=3), or open biopsy alone (n=5). Relationships between the expression of the target proteins and the prognosis were assessed in all 15 patients who underwent wide excision, who did not experience recurrence.

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Key Words: Chondrosarcoma, matrix metalloproteinase, tissue inhibitor of metalloproteinase, a disintegrin and metalloproteinase, immunohistochemistry, histological grade.

Table I. *The antibodies and the working dilutions used in this study.*

Antibodies used	Working dilution	Detection methods kit
Anti-human MMP 2 immunoglobulin mouse monoclonal antibody (Code No. F-68, Lot No. 42-5D11; Daiichi Fine Chemical Ltd., Japan)	1: 50	LSAB
Anti-human MMP 3 immunoglobulin mouse monoclonal antibody (Code No. F-66, Lot No. 55-2A4; Daiichi Fine Chemical Ltd., Japan)	1: 100	Envision+
Anti-human MMP 7 immunoglobulin mouse monoclonal antibody) (Code No. F-82, Lot No. 141-7B2; Daiichi Fine Chemical Ltd., Japan)	1: 20	CSA system
Anti-human MMP 9 immunoglobulin mouse monoclonal antibody (Code No. F-69, Lot No. 56-2A4; Daiichi Fine Chemical Ltd., Japan)	1: 100	LSAB
Anti-human MMP 13 immunoglobulin mouse monoclonal antibody (Code No. F-94, Lot No. 181-14G11; Daiichi Fine Chemical Ltd., Japan)	1: 20	CSA system
Anti-human TIMP 1 immunoglobulin mouse monoclonal antibody (Code No. F-26, Lot No. 147-6D11; Daiichi Fine Chemical Ltd., Japan)	1: 10	LSAB
Anti-human TIMP 2 immunoglobulin mouse monoclonal antibody (Code No. F-70, Lot No. 67-4H11; Daiichi Fine Chemical Ltd., Japan)	1: 100	LSAB
Anti-human TIMP 3 immunoglobulin goat polyclonal antibody (Code No. sc-9906, Lot No. I1102; Santa Cruz Biotechnology, Inc., USA)	1: 50	LSAB
Anti-human ADAMTS 4 immunoglobulin goat polyclonal antibody (Code No. sc-16534, Lot No. O062; Santa Cruz Biotechnology, Inc., USA)	1: 100	LSAB
Anti-human ADAMTS 5 immunoglobulin rabbit polyclonal antibody (Code No. RP1ADAMTS-5, Lot No. KA000503; Triple Point Biologics, Inc., USA)	1: 50	Envision+

Methods. Tissue samples were fixed in 10% formalin and embedded in paraffin, and 4-μM sections were prepared using paraffin block without demineralisation. A panel of immunostaining was performed using antibodies to the following proteins: human MMPs 2, 3, 7, 9 and 13; ADAMTSs 4 and 5; and TIMPs 1, 2 and 3. Table I shows the antibodies and the working dilutions used in this study. For detection, horseradish-peroxidase (HRP)-labelled polymer reagent (Envision+; DAKO, Denmark) was used for MMP 3 and ADAMTS 5; labelled streptavidin-biotin method (LSAB; DAKO) for MMPs 2 and 9, ADAMTS 4 and TIMPs 1-3; and catalysed signal amplification method (CSA system; DAKO) for MMPs 7 and 13. The detection methods were selected after preparing positive controls for each antibody and identifying the technique with optimum results (Table I). The specificity of staining was established using negative control slides that were processed as above, except that the primary antibodies were omitted. The procedures for each method are summarised as follows. After deparaffinisation, sections were immersed in methanol solution containing 0.3% H_2O_2 at room temperature for 30 min, to block endogenous peroxidase. Then the sections were washed 3 times in phosphate-buffered saline (PBS) for 5 min each, and adsorption of non-specific proteins was blocked by incubating with the appropriate normal serum at room temperature for 30 min. The primary antibody was then allowed to react at 4˚C for 12 h. The resulting specimens were washed 3 times using PBS for 5 min each. Then, one of the above detection kits (Envision+, LSAB and CSA) was applied based on primary antibody (Table I), according to the manufacturer's procedure. The resulting specimens were washed 3 times using PBS for 5 min each, and were immersed in solution containing 50 mM Tris-HCl, 0.56 mM 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_2O_2 solution. Colour development was performed under microscopy. After the immunostaining was completed, the sections were counter stained lightly by hematoxylin .

Assessments were made under light microscopy at x100 magnification. The ratio of positive tumour cells was determined in 10 fields and classified into 5 grades: 0 points for 0%, 1 point for 1-25%, 2 points for 26-50%, 3 points for 51-75% and 4 points for 76-100%.

To establish relationships between the immunostaining score and histological grade, Pearson's product moment was used, with values of $p<0.05$ considered statistically significant. The prognosis of patients in the group that received wide excision excluding intralesional excision was assessed in terms of continuous disease-free (CDF) ratio (CDF patients/total patients).

Results

Chondrosarcoma clearly expressed MMPs 2, 3, 7, 9 and 13, ADAMTSs 4 and 5, and TIMPs 1, 2 and 3. All proteins were detected in the cytoplasm of chondrosarcoma cells, but not in the ECM (Figure 1A-F). Two patterns of localisation of immunopositive tumour cells were found. One was diffuse distribution of positive cells throughout the tumour mass, while the other was localisation of most immunopositive cells at the periphery of the tumour. No significant correlations existed between tumour cell localisation and histological grade. All cells were stained relatively equally in the former type, whereas marginal tumour cells displayed more intense staining in the latter type.

The average value of immunohistological scores of MMPs, ADAMs and TIMPs according to histological grades are shown in Table II. Regarding all the target proteins, higher histological grade was associated with higher immunostaining

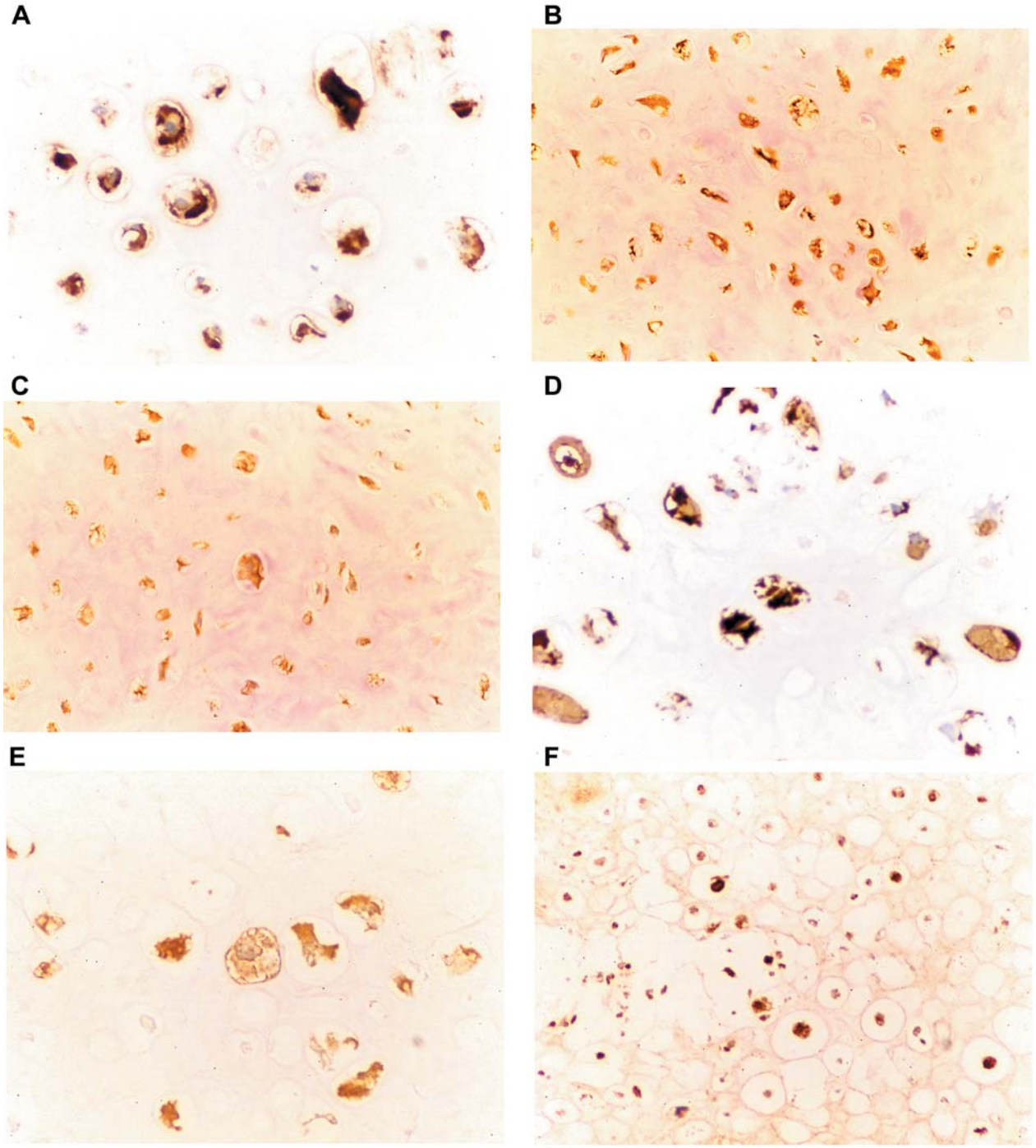

Figure 1 A, B, C, D, E, F. *Immunostaining of MMP 7and 9, TIMP 2 and 3 and ADAMTS 4 and 5, respectively. In chondrosarcoma, all MMPs, TIMPs and ADAMs were expressed. The cytoplasm of chondrosarcoma cells was stained brown. The expression of these compounds was not seen in the ECM.*

score. Regarding the relationships between histological grade and immunostaining scores, significant positive correlations ($p<0.05$) were identified for all target proteins except MMP 9, with strong correlations ($p<0.01$) for MMPs 2, 3 and 13, both ADAMTSs and all 3 TIMPs (Pearson's product moment: MMP 2, 0.593; MMP 3, 0.679; MMP 7, 0.445; MMP 9, 0.305; MMP1 3, 0.539; TIMP 1, 0.587; TIMP 2, 0.528; TIMP 3, 0.521; ADAMTS 4, 0.583; ADAMTS 5, 0.618; $p<0.05$).

Table II. *Immunostaining scores and histological grades. With MMP 2, 3, 7, 9 and 13, ADAMTS 4 and 5 and TIMP 1, 2 and 3, the higher the histological grade, the higher the immunostaining score.*

	N(28)	MMP 2	MMP 3	MMP 7	MMP 9	MMP 13
Grade I	7	0.71	0.57	0.43	0.71	0.14
Grade II	15	2.07	1.13	1.27	1.80	1.27
Grade III	6	2.83	3.33	2.00	1.83	2.17
	N(28)	ADAMTS 4	ADAMTS 5	TIMP 1	TIMP 2	TIMP 3
Grade I	7	0.71	0.29	0.29	1.00	1.00
Grade II	15	1.13	1.00	1.27	2.67	2.45
Grade III	6	2.83	2.33	2.50	2.83	2.83

Table III. *Prognosis (CDF/patient count, total number=15). No significant correlation existed between CDF ratios and immunostaining for MMP 2, 3, 7, 9 and 13, ADAMTS 4 and 5 and TIMP 1, 2 and 3.*

	MMP 2	MMP 3	MMP 7	MMP 9	MMP 13
Score 0	0/0	1/2	1/2	1/3	1/2
Score 1	3/4	3/3	2/3	3/3	3/5
Score 2	2/4	3/4	3/5	2/3	1/1
Score 3	1/2	0/2	1/4	1/4	3/7
Score 4	2/5	1/4	1/1	1/2	0/0
Total	8/15	8/15	8/15	8/15	8/15
	ADAMTS 4	ADAMTS 5	TIMP 1	TIMP 2	TIMP 3
Score 0	1/1	1/2	3/5	0/0	0/0
Score 1	3/4	3/6	1/1	1/1	0/2
Score 2	3/6	2/4	2/4	3/4	2/2
Score 3	0/1	1/1	2/3	2/6	4/6
Score 4	1/3	1/2	0/2	2/4	2/5
Total	8/15	8/15	8/15	8/15	8/15

Expression of all the target proteins appeared to be coordinate , in that increased detection of one was associated with increased signals for all others. Immunostaining of normal cartilage using the above antibodies was performed as a control. In normal cartilage, MMPs 2, 3, 7, 9 and 13 and TIMP1 were not detected, while the mean immunostaining scores for the other proteins were low.

Among the 15 patients who underwent wide excision, the relationships between MMPs, ADAMs and TIMPs and prognosis were investigated, but no significant correlations existed between CDF ratios and immunostaining scores for any targets (Table III)

Discussion

Malignant tumours destroy the surrounding ECM before invading and metastasizing to other tissues (12,13). MMPs are capable of breaking down the ECM and are believed to play important roles in cancer metastasis and invasion (1,2). Thus, research is attempting to ascertain the roles of MMPs in stomach, colon and breast cancers (14,15). For osteochondral tumours, several studies have been conducted (9-11) since Chunbinskaya *et al.* (16) reported the relationship of MMPs and cytokines to chondrosarcoma in 1996. However, the relationships have not been clarified between chondrosarcoma and ADAMs, a family similar to the MMP family. Studies on ADAMs in recent years have clarified that ADAMTSs, which are secretory ADAMs, are involved in decomposition and metabolism of ECM components (3,4).

ADAMTSs 4 and 5 are called aggrecanases, though the nature of these enzymes has long been remained unclear. In 1999, since Arner *et al.* (4) purified and cloned these enzymes, substantial research has been conducted, finding that besides cleaving the aggrecan core protein at Glu^{373}-Ala^{374}, these enzymes also cut at 4 places between globular domains G2 and G3: Glu^{1480}-Gly^{1481}, Glu^{1667}-Gly^{1668}, Glu^{1771}-Ala^{1772} and Glu^{1871}-Leu^{1872} (17,18). The activities of MMP and ADAM are also inhibited by a common group of proteins, the TIMPs (5,6), and the inhibitory action of TIMP 3 on ADAMTSs 4 and 5 is 50-fold higher than that of TIMPs 1 and 2 and 250-fold higher than that of TIMP 4 (5). The mechanisms of articular cartilage destruction are thus beginning to be clarified (19,20). While several studies have examined the expression of MMPs in chondrosarcoma, mechanisms that regulate MMP expression have not been elucidated. To the best of our knowledge, no studies have detailed the relationships between ADAM expression, cancer invasion and metastasis in chondrosarcoma. The present study assessed the presence of MMPs 2, 3, 7, 9 and 13, ADAMTSs 4 and 5, and TIMPs 1, 2 and 3 in chondrosarcoma and ascertained relationships to histological malignancy and prognosis. Immunostaining showed that, in normal cartilage, MMPs 2, 3, 7, 9 and 13 are not detected, while immunohistochemical signals for

ADAMTSs 4 and 5, and TIMPs 1-3 are weak. However, all proteins were widely detected in chondrosarcoma, and positive correlations between histological malignancy and immunostaining scores were identified for all the target proteins except MMP 9. These findings suggest that, when local invasion and metastasis occur in chondrosarcoma, expression increases not only for MMPs and ADAMs, but also for TIMPs. However, in terms of mole ratios, the degree of increase in expression of MMPs and ADAMs that break down the ECM may differ from that of the TIMPs that suppress MMP and ADAM activities. As with rheumatoid synovial fluid (21,22), levels of MMPs and ADAMs are believed to be greater than levels of TIMPs, and the balance between decomposition and suppression systems may be disrupted in chondrosarcoma. Cells weakly immunoreactive for ADAMs and TIMPs are detected in normal cartilage, as these compounds probably function in normal cartilage metabolism. While MMPs were not detected in healthy cartilage in the present study, some of these proteins are thought to be present in normal tissue at very low levels.

Regarding the localisation of immunopositive tumour cells in chondrosarcoma, they are seen inside the ECM, but as with articular chondrocytes, type IV collagen exists around the tumour cells. Chondrosarcoma cells with elevated MMPs and ADAMTSs obviously secrete these enzymes. As MMPs 2 and 9 predominantly break down type IV collagen, MMP 13 breaks down type II collagen and MMPs 3 and 7 and ADAMTSs 4 and 5 break down proteoglycans (aggrecans), the surrounding ECM components are decomposed, and tumour cells are released into the ECM. This explains the two types of chondrosarcoma seen in the present study: immunopositive tumour cells were distributed throughout the tumour mass in one type, but were mostly found in the periphery in the other. Marginal tumour cells then secrete MMPs and ADAMs to break down ECM outside the tumour and, at the same time, directly invade adjacent tissues, resulting in both local invasion and distant metastasis. In events such as pulmonary metastasis, hematogenous metastasis plays an important role. Hematogenous metastasis involves: release of tumour cells from a primary lesion; invasion of connective tissues and vascular basement membranes; stasis and embolism into capillaries in another organ; invasion of vascular basement membranes in the new organ; transfer to parenchyma of the organ; and proliferation in the organ (12,13). MMPs 2 and 9 are believed to break down type IV collagen, which is a component of the basement membrane, and thus may be associated with pulmonary metastasis and prognosis in chondrosarcoma. According to Berend *et al.* (11), MMP-1/TIMP-1 ratios in chondrosarcoma correlate with local recurrence, metastasis and prognosis. Our findings did not show any correlation between the immunostaining scores and prognosis, and this might be attributable to the small subject population or the fact that the compounds used may be poor prognosticators. Further investigations are necessary.

In conclusion, as with digestion of normal cartilaginous tissue, ADAMs are involved in destruction of the ECM in chondrosarcoma. MMPs, ADAMs and TIMPs are detected in chondrosarcoma, and are associated with local invasion and metastasis. Expression of these compounds may indicate chondrosarcoma malignancy. No clear correlation existed between prognosis and immunostaining scores in 15 patients who underwent wide excision.

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