ONCOLOGY

Expression of Matrix Metalloproteinases 1, 2, 9 and Their Tissue Inhibitor-1 in Cartilage-Forming Osteal Tumors

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The expression of MMP-1, -2, -9 and TIMP-1 was studied in 10 benign cartilage-forming osteal tumors (5 osteochondromas and 5 chondromas) and 39 chondrosarcomas (14 central, 4 periosteal, 7 dedifferentiated, and 14 secondary tumors). No expression of MMP and TIMP-1 was detected in benign cartilage-forming osteal tumors. In chondrosarcomas, the expression of MMP-1 was detected in 84.6%, of MMP-2 in 71.8, of MMP-9 in 97.4, and of TIMP-1 in 82.4% cases, the levels of expression of these markers varied from 10 to 60%. The expression of MMP-1 was not associated with patient gender, maximum size and degree of differentiation of the tumor, but was linked with age. The expression of MMP-1 was more often detected in central and dedifferentiated chondrosarcomas; the expression of MMP-1(+) was significantly associated with 3-year relapse-free and 5-year overall survival of the patients. The expression of MMP-1 in the tumor was associated with unfavorable course of the disease. The values of MMP-2 expression in chondrosarcomas did not reflect the main clinical morphological characteristics of the disease and its prognosis. The level of MMP-9 protein expression in chondrosarcomas ≥40% is prognostically unfavorable, while <40% is a favorable factor for 3-year relapse-free survival. The risk of disease relapse within 1 year after the beginning of therapy was maximum in T_3 tumors with expression of MMP-9 protein ≥40%. No relationships between the parameters of TIMP-1 expression in chondrosarcomas and the main clinical morphological characteristics of the disease and its prognosis were detected.

Key Words: *matrix metalloproteinases 1, 2, 9; tissue inhibitor-1 of matrix metalloproteinases; expression; chondrosarcoma*

Invasion into adjacent tissues and metastases to distant organs are among the basic characteristics of malignant tumors [8,10,11]. Destruction of the adjacent basal membrane and extracellular matrix by tumor-associated proteases is an important mechanism of these processes [4,9]. Several protease classes are involved in invasion and metastasizing of tumor cells [6]. A special family is the MMP or matrixine family. These proteases specifically hydrolyze all main proteins of extracellular matrix, primarily collagen [7]. The MMP family consists of more than 20 secreted or cell surface-linked zinc-dependent endopeptidases. The substrates for these endopeptidases are, in addition to the majority of extracellular matrix components, other proteases, chemotaxic molecules, latent forms of

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growth factors, and soluble and membrane-associated proteins binding growth factor [9,12].

Enhanced expression of many MMP in tumors of different genesis was demonstrated. Activation is realized by the paracrine mechanism with participation of growth factors and cytokines secreted by macrophages and lymphocytes infiltrating the tumor and by stromal tumor cells [5,12]. Some MMP are now studied as possible biological markers for prognosis and drug resistance of malignant tumors, for example, of bone tumors [2,4]. The use of natural and synthetic inhibitors of MMP is a possible approach to therapy of tumors low sensitive to the classical antitumor drugs [1].

We compared the expression of MMP-1, -2, and -9 in benign and malignant cartilage-forming osteal tumors and studied the relationship between these values and basic clinical morphological characteristics of the disease and its prognosis.

MATERIALS AND METHODS

The expression of MMP-1, -2, -9, and TIMP-1 was studied by immunohistochemical method in 39 chondrosarcomas: central (n=14), periosteal (n=4), dedifferentiated (n=7), and secondary (n=14). The expression of MMP-1, -2, -9, and TIMP-1 in 10 benign cartilage-forming osteal tumors osteochondroma (n=5) and chondroma (n=5) served as the control.

Immunohistochemical study was carried out on paraffin sections of tumor tissue by the biotin-streptavidin immunoperoxidase method with antibodies to MMP-1, -2, -9, and TIMP-1 (Table 1).

The immunohistochemical reaction with mono- and polyclonal antibodies and reagents was carried out by the same protocol for all the antibodies used in the study.

Sections (4 μ) were deparaffinated and rehydrated by the standard methods. Antigens in cartilage-forming tumors were decamouflaged at 95°C. The slides were removed and cooled in buffer to ambient temperature, washed in washing buffer, and the subsequent steps of the study were carried out according to the common protocol [3]. Activity of endogenous peroxidase was inhibited by application of blocking solution of hydrogen peroxide with subsequent incubation and washing in the buffer. Incubation with the first antibodies was carried out for 30 min with subsequent washing of the sections. Incubation with En Vision visualizing solution was carried out at ambient temperature (30 min) with subsequent washing in washing buffer. The next step was incubation with chromogen solution (5 min, ambient temperature), controlled under the microscope. The preparations were post-stained with hematoxylin.

The cytoplasmatic type of specific staining was evaluated for each antigen. The immunohistochemical reaction was regarded as negative ("-" no reaction) or positive (of moderate ("+") and high ("++") intensity). Slight cellular reaction and reaction of the stroma were neglected. The results of reactions with antigens were expressed in percent (the number of stained cells per 100 analyzed tumor cells in 5 representative visual fields); only medium and high intensity of staining of the tumor cell components was recorded. The expression of the studied markers in bone chondrosarcomas is presented on Figures 1, 2, and 3.

RESULTS

No MMP-1 expression was detected in benign cartilage-forming osteal tumors. In chondrosarcomas, the expression of MMP-1 varied within a wide range: no MMP-1 expression was detected in 6 of 39 tumors, in 4 tumors its level was 10%, in 15 tumors 20%, in six 30%, in six 40%, and in two 60%. No MMP-1 was detected in 4 of 14 (28.6%) secondary chondrosarcomas, in 1 of 14 (7.1%) central chondrosarcomas, and in 1 of 4 (25%) periosteal chondrosarcomas. The expression of MMP-1 was detected in all 7 dedifferentiated chondrosarcomas. Hence, MMP-1 was the most incident in dedifferentiated (100%) and central (92.9%) chondrosarcomas in comparison with other histological variants.

The incidence and levels of MMP-1 expression in chondrosarcomas did not depend on patient's gender. The incidence of MMP-1 in the tumor was 60% for patients aged under 45 years and 93.1% for those aged over 45 years (p=0.028 according to Fisher ex-

TABLE 1. Antibodies Used in the Analysis of Cartilage-Forming Tumors

Antigen	Antibody, clone	Manufacturer	Working dilution
MMP-1	Rabbit pAb	"Diagnostic BioSystems"	1:200
MMP-2	Mouse mAb 17B11	"Novocastra"	1:50
MMP-9	Rabbit pAb	"Dako"	1:50
TIMP-1	Mouse mAb 102D1	"Diagnostic BioSystems"	1:10

act test). It is noteworthy that 85.7% patients with dedifferentiated chondrosarcoma and 100% patients with central chondrosarcoma were aged 45 years and more and hence, the incidence of MMP-1 in the tumor was largely determined by tumor histology, but not patient's age.

No significant relationship between MMP-1 expression in chondrosarcomas and maximum size and degree of differentiation of the tumor was detected. However, the incidence of MMP-1 expression in chondrosarcoma increased significantly with increasing the index of tumor dissemination (T criterion) (p=0.027). In patients with T_1 tumors, the incidence of MMP-1(-) tumors was 50% (3 of 6 cases), in those with T_2 it was 12% (3 of 25 cases), and in those with T_3 it was 0% (0 of 8 cases). MMP-1 expression in chondrosarcomas tended to increase with increasing tumor dissemination: 23.3±3.3% in T_1 tumors, 25.5±2.3% in T_2 tumors, and 31.3±6.7% in T_3 tumors.

Analysis of relapse-free survival in the groups of patients without and with MMP-1 expression in chondrosarcomas revealed significant differences (p=0.048 by Cox's F test). Three-year relapse-free survival of 6 patients with MMP-1(-) chondrosarcomas was 60.0±21.9%. The median of relapse-free survival is not attained in this subgroup, because 4 patients survived 3 years and are living without signs of relapse. In 33 patients with MMP-1(+) chondrosarcomas this value was 28.5±9.4% (relapse-free survival median 11.3 months).

Analysis of 5-year overall survival of patients with chondrosarcoma showed similar results. Six patients without expression of MMP-1 in tumor tissue lived throughout the study (overall 5-year survival 100%, the median not attained over the 5-year period of observation). By contrast, the overall 5-year survival of 33 patients with MMP-1(+) chondrosarcomas was 34.9±11.9% (median 44.7 months; p=0.03 by the Log-Rank test). In 25 of these patients with chondrosarcoma with <40% MMP-1 expression in the tumor, the overall 5-year survival was 37.3±13.7% (median 47.1 months) and in 8 patients with ≥40% MMP-1 expression it was 29.2±24.1% (median 32.8 months).

Hence, the results indicate the prognostic significance of MMP-1 expression in the tumors of patients with chondrosarcoma. The expression of MMP-1 in chondrosarcoma is a prognostically unfavorable factor, particularly if its expression is ≥40%.

On the other hand, multifactorial analysis showed than the expression of MMP-1 in the tumor is less significant for delayed results of treatment of patients with chondrosarcoma than the main clinical morphological factors, such as tumor malignancy (p=0.07), T criterion (p=0.003), and histological variant of chondrosarcoma (p=0.32). Hence, measurement of MMP-1 expression in chondrosarcomas can be used as an additional prognostic factor along with the known clinical morphological prognostic criteria.

In addition, the majority of the analyzed chondrosarcomas with G3-G4 malignancy expressed MMP-1 (17 of 18 cases; 94.4%), and therefore, it was hardly possible to evaluate the relationship between the tumor MMP-1(-) and MMP-1(+) status and disease prognosis. On the other hand, an interesting fact was detected in 21 patients with chondrosarcoma of low and moderate malignancy (G1-G2). Three-year uneventful survival of 5 patients without expression of MMP-1 in the primary tumor was higher (75.0±21.7%) than in 16 patients whose tumors expressed MMP-1 (35.0±18.5%; p=0.16). Hence, the 3-year relapse-free survival in patients with chondrosarcoma of low malignancy and expression of MMP-1 can be 40% lower. These data indicate a relationship between MMP-1 expression in the tumor and prognosis of relapse-free survival in patients with chondrosarcoma.

No expression of MMP-2 in tumor tissue was detected in 10 patients with benign cartilage-forming osteal tumors. Expression of MMP-2 was detected in 28 (71.8%) chondrosarcomas: its level was 10% in 16 tumors, 20% in 9, and 30% in 3 tumors.

The incidence and level of MMP-2 expression in chondrosarcomas were not associated with patients' gender or age, maximal tumor size and its malignancy, and process dissemination criterion T.

The relapse-free and overall survival of chondrosarcoma patients with MMP-2(-) and MMP-2(+) tumors were virtually the same.

Hence, the values of MMP-2 expression in chondrosarcomas did not reflect the main clinical and morphological characteristics of the disease and its prognosis.

No expression of MMP-9 was detected in 10 benign cartilage-forming osteal tumors and in 1 of 39 chondrosarcomas (2.6%). The level of expression was 50-60% in 13 (34.2%) and 10-40% in 25 (65.8%) chondrosarcomas.

No association between MMP-9 expression in chondrosarcomas and patients' gender, maximum size of the tumor, and the histological variant of its structure was detected. However, the level of MMP-9 expression in chondrosarcomas increased significantly with increasing tumor malignancy (p=0.009). In G1 tumors (n=8), the level of MMP-9 expression was 20.6±5.0%, in G2 (n=12) 43.8±6.2%, in G3 (n=8) 50.0±5.7%, and in G4 (n=10) 43.0±4.0%.

Patients with osteal chondrosarcoma were divided into 3 groups by the median of MMP-9 content in the primary tumor: group 1 (N=14) <40%, group 2 (N=14) 40%, and group 3 (N=10) >40%. It is noteworthy that if the primary tumor dissemination corresponded to T_1,

the level of MMP-9 expression was <40% in 83.3% cases (5 of 6 specimens) and in 1 case it was 40%; no tumors with MMP-9 expression >40% were detected. On the other hand, in T_2 tumors, the incidence of MMP-9 expression <40% decreased to 36% (9 of 25 cases), the content of MMP-9 surpassed 40% in only 32% (8 of 25) cases, and the same percentage of tumors expressed MMP-9 at the level of 40%. In the T_3 group, the content of MMP-9 was below 40% in only 1 of 8 (12.5%) cases; tumors with MMP-9 level equal to 40% predominated (5 of 8 cases; 62.5%), while the incidence of MMP-9 expression >40% was comparable to that in T_2 (2 of 8 cases; 25%). Hence, significantly lower incidence of MMP-9 expression <40% in tumors of patients with chondrosarcoma ($p<0.05$) was observed with increasing the T criterion.

Significant ($p=0.007$) differences in shortening of 3-year relapse-free survival in patients with chondrosarcoma were observed with increasing MMP-9 expression in the tumor. In 15 patients with MMP-9 expression below 40%, the 3-year relapse-free survival was 58.3±16.1% (median 34.2 months); in 10 patients with MMP-9 level equal to 40% it was 42.0±17.6% (median 15.4 months); and in 14 patients with MMP-9 content in the tumor above 40% it was 8.4±8.0% (median 8 months).

Overall 5-year survival of patients with chondrosarcoma also decreased with increasing MMP-9 content in the tumor, but the differences were negligible and manifested during the first 3 years of observation. In 15 patients with MMP-9 expression in chondrosarcomas <40%, the 5-year overall survival was 42.9±31.0% (median 52.3 months), in 10 with MMP-9 expression of 40% it was 44.4±18.9% (median 48.5 months), and in 14 patients with MMP-9 content in the tumor >40% it was 41.3±15.7% (median 30.9 months).

Multifactorial analysis of relapse-free survival of chondrosarcoma patients with consideration for the main clinical morphological characteristics and level of MMP-9 protein expression in the tumor showed that T criterion is the most significant prognostic factor for the development of an early relapse in chondrosarcoma patients ($p=0.026$), less so are the MMP-9 content in the tumor ($p=0.06$) and tumor malignancy ($p=0.09$).

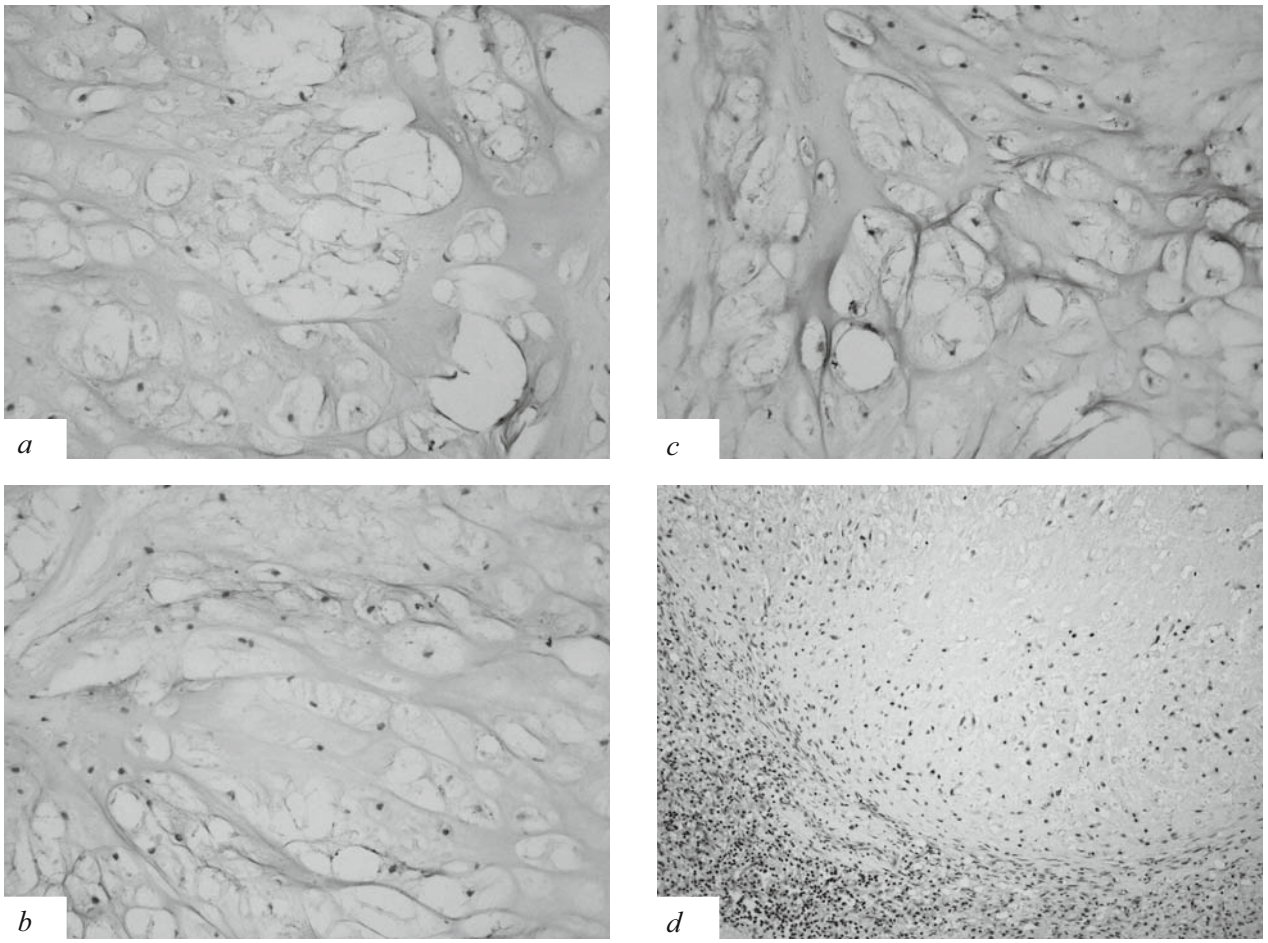

Fig. 1. Patient L. (female), 43 years. Central classical well-differentiated (grade 1) chondrosarcoma of the proximal part of the tibial bone. Negative cytoplasmatic reaction with MMP-1 (*a*), MMP-2 (*b*) in tumor cells, negative immunohistochemical reaction with MMP-9 (*c*) and TIMP-1 (*d*) in tumor cell cytoplasm. Working dilutions of antibodies 1:200 (*a*, *c*), 1:50 (*b*), 1:10 (*d*), ×200.

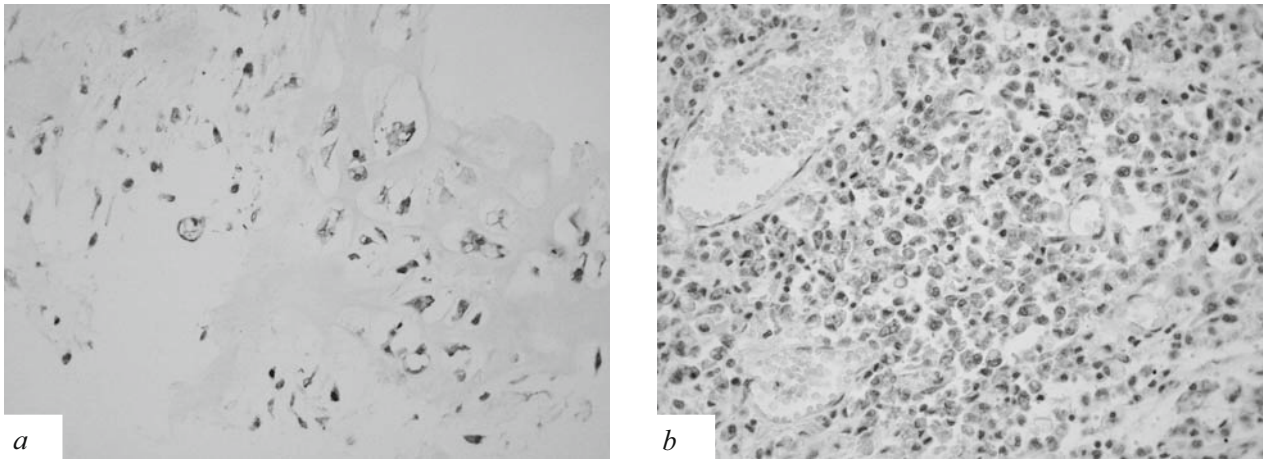

Fig. 2. Patient S., 57 years (*a*). Central moderately differentiated (grade 2) pelvic osteal chondrosarcoma. Positive cytoplasmatic reaction with MMP-1 in tumor cells. Working dilution of antibodies 1:200, ×200. Patient F., 40 years (*b*). Humeral periosteal chondrosarcoma. Positive cytoplasmatic reaction with MMP-2 in tumor cells. Working dilution of antibodies 1:50, ×200.

Significant differences in relapse-free survival of patients with chondrosarcoma in subgroups with MMP-9 content <40% and ≥40% (p=0.02) were detected in clinically unfavorable group of 33 patients (T_2-T_3 tumor process dissemination). Three-year relapse-free survival of 10 chondrosarcoma patients with <40% MMP-9 content in the tumor was 46.9±18.7% and of 23 patients with ≥40% MMP-9 was 17.3±8.9%.

Hence, the content of MMP-9 in tumor tissue reflected the malignancy of chondrosarcoma and dissemination of the tumor process by the T criterion. These data indicate high prognostic significance of the level of MMP-9 expression in chondrosarcomas for prediction of an early relapse. The level of MMP-9 expression ≥40% in chondrosarcomas is prognostically unfavorable, while <40% level is a favorable factor for 3-year relapse-free survival. The risk of relapse development within 1 year from the beginning of therapy was maximum in T_3 tumors with high expression of MMP-9.

In addition, let us note that of 6 patients with chondrosarcoma without MMP-1 expression in the tumor, 5 (83.3%) had also low (<40%) expression of MMP-9. Of 33 patients with osteal chondrosarcoma with MMP-1 expression in the tumor, 13 (39.4%) had high (≥40%) levels of MMP-9 and 10 (30.3%) low levels of MMP-9 (<40%). The differences in 3-year relapse-free survival of patients with unfavorable levels of MMP-1 expression in the tumor between the subgroups with favorable and unfavorable levels of MMP-9 reached 35% (p=0.052). This indicating the need in simultaneous evaluation of the expression of MMP-1 and MMP9 in chondrosarcomas.

The expression of TIMP-1 was studied in 10 benign cartilage-forming osteal tumors and 34 chondrosarcomas. No expression of TIMP-1 was detected in 10 benign tumors and 6 (17.6%) chondrosarcomas. The level of TIMP-1 expression was 10% in 11 chondrosarcomas, 20% in 11, 30% in 2, and 40% in 4 cases.

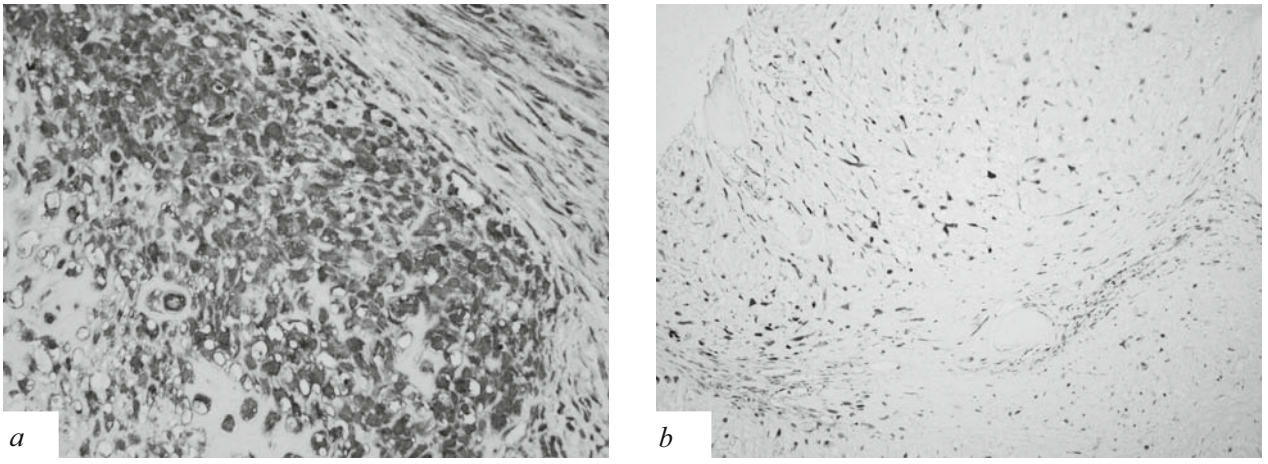

Fig. 3. Patient (female) K., 61 years. Secondary moderately differentiated femoral chondrosarcoma. Positive cytoplasmatic reaction with MMP-9 (*a*) and TIMP-1 (*b*) in tumor cells. Working dilution of antibodies 1:10, ×200.

No appreciable differences in the incidence of TIMP-1 with consideration for patients' gender and age were detected.

A trend to a decrease in TIMP-1 incidence (TIMP-1(-) tumors) with increasing the tumor malignancy was observed (p=0.4). The incidence of TIMP-1(-) tumors in G1 (n=7) was 42.9%, in G2 (n=11) 9.1%, in G3 (n=8) and G4 (n=8) 12.5% each.

The incidence of TIMP-1(-) tumors was higher in secondary (33.3%) than in central (15.4%) chondrosarcomas, TIMP-1(-) tumors were not detected in any other chondrosarcoma variants (p=0.09).

The levels of TIMP-1 expression were not associated with T criterion in chondrosarcoma patients: 16.7% in T_1, 19.1% in T_2, and 14.3% in T_3.

No appreciable differences in relapse-free and overall survival of chondrosarcoma patients with different levels of TIMP-1 expression in the primary tumor were detected.

Hence, no relationships between the presence of TIMP-1 expression in chondrosarcomas and disease prediction and between the level of TIMP-1 expression in chondrosarcoma and the main clinical morphological characteristics of the disease were detected.

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