

Analysis of pRb, p16INK4A proteins and proliferating antigens: PCNA, Ki-67 and MCM5 expression in aggressive fibromatosis (desmoid tumor)

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Summary. Aggressive fibromatosis (desmoid tumor) is a mesenchymal lesion originating from fascial, aponeurotic and muscular connective tissue. It rarely becomes histologically malignant. In this study we analyzed the cell cycle regulation proteins: pRb, p16, and proliferating antigens: Ki-67, PCNA, MCM5 with immunohistochemical method in archival material derived from 27 extra-abdominal (E-AD), 18 abdominal (AD) and 5 intra-abdominal (I-AD) cases of desmoid tumor.

None of the examined cases (n=50) of aggressive fibromatosis was pRb-immunonegative. Heterogeneous expression of pRb was observed in 51.85% (14/27) of Group AD cases and in 5.56% (1/18) of Group E-AD cases; positive expression in 48.15% (13/27) of Group AD cases, in 94.44% (17/18) of Group E-AD cases, and in 100% (5/5) of Group I-AD cases. There were no negative cases for p16 staining in any of the examined groups. The number of heterogeneous cases in individual groups was: 33.33% (9/27) in Group AD, 50% (9/18) in Group E-AD and 40% (2/5) in Group I-AD, and positive cases: 66.67% (18/27), 50% (9/18) and 60% (3/5), respectively. Overexpression of PCNA was noted in 98% (49/50) of cases. The positive staining for Ki-67 protein was noted in 25.93% (7/27) in Group AD, in 16.67% (3/18) in Group E-AD and in 60% (3/5) in Group I-AD. None of the examined cases was immunopositive for MCM5 protein.

The noted levels of pRb and p16 expression in desmoid cells reflect their function in cell cycle

regulation. Probably the unsettled cell cycle progression, especially in G1 phase, is not the cause of aggressive fibromatosis pathogenesis.

Key words: Aggressive fibromatosis, Immunohistochemistry, pRb, p16, Proliferating antigens

Introduction

Desmoid-type fibromatoses are clonal fibroblastic proliferations that arise in the deep soft tissues and are characterized by infiltrative growth and a tendency towards local recurrence, as well as by inability to metastasize (Goldblum and Fletcher, 2002). Aggressive fibromatosis is divided into three groups: abdominal, extra-abdominal and intra-abdominal, depending on the tumor location (Weiss and Goldblum, 2001). Desmoid tumor can develop as a sporadic neoplasm (Alman et al., 1997a,b; Fallen et al., 2006), but it can also be related to familial adenomatous polyposis (FAP) (Gebert et al., 1999; Knudsen and Bulow, 2001; Galiatsatos and Foulkes, 2006).

The etiologic factors causing sporadic aggressive fibromatosis have not been well defined. They appear to be associated with trauma, either surgical or physiological, like pregnancy, and with hormones (Reitamo et al., 1986; Bertario et al., 2001; Kempson et al., 2001; Knudsen and Bulow, 2001; Hosalkar et al., 2006; Okuno, 2006). Also, genetic factors might be the cause of desmoid tumor, especially APC mutations (Alman et al., 1997a; Bertario et al., 2001; Li et al., 1998) and β -catenin genes (Shitoh et al., 1999). In many cases of desmoid tumor cytogenetic abnormalities, such

as trisomy of chromosome 8 and 20, were observed (Bridge et al., 1999; Brandal et al., 2003; Mayer et al., 2007). A history of previous abdominal surgery, like colectomy, the position of germline mutation of APC gene and a family history of aggressive fibromatosis are identified as etiologic risk factors in the development of desmoid tumors in FAP (Gebert et al., 1999; Knudsen and Bulow, 2001; Latchford et al., 2006).

Aggressive fibromatosis located in various parts of the body often differ in biological behavior. Moreover, there is a great individual variability in desmoid tumor development dynamics and progression. Wide local resection remains the treatment of choice for most patients with desmoid tumor, but radiotherapy and chemotherapy are also used. Radiotherapy and chemotherapy are also applied as adjuvant therapy for surgical excision (Knudsen and Bulow, 2001; Sturt et al., 2004; Latchford et al., 2006; Hosalkar et al., 2006; Okuno, 2006; Lev et al., 2007). The recurrence rate of aggressive fibromatosis is high and averages from 25 to 65% (Reitamo et al., 1986; Peterschulte et al., 2000; Shields et al., 2001).

Excessive proliferation of cells is often one of the hallmarks of tumorigenesis, which is caused by abnormal cell cycle progression. The cell cycle is a very combined process, precise regulation of which is vital for normal cell activity. Its normal course is regulated by many proteins, which are synthesized or activated at the appropriate moment of the cell cycle. Due to this, the cell cycle cannot be withdrawn to the previous phase. The results of the studies carried out so far concerning cell cycle regulation have shown that disorders of this process, especially of G1/S phase transition, might be the cause of neoplastic transformation (Hirama and Koeffler, 1995; Niklinska et al., 2001; Bartkova et al., 2003; Beasley et al., 2003; Wikman and Kettunen, 2006).

Rb protein (pRb) and p16INK4A (p16) are suppressor proteins which control G1 course and G1/S phase transition (Hirama and Koeffler, 1995; Cordon-Cardo, 1995; Sherr, 1996; Gillett and Barnes, 1998). pRb is a 105-110 kDa (depending on the rate of phosphorylation) nuclear phosphoprotein encoded by retinoblastoma (Rb) gene. pRb binds to the E2F transcription factor, which inhibits cell cycle progression. Its activity is regulated by phosphorylation. When mitotic signal stimulates cell to proliferation, Rb protein is phosphorylated and it releases the E2F transcription factor. Not-bound E2F stimulates expression of the proteins, the presence of which is necessary for G1/S transition (Hirama and Koeffler, 1995; Weinberg, 1995; Dyson, 1998).

p16 is a 15.5 kDa protein encoded by *p16INK4A* (*CDKN2A*) gene. Its main function in regulating the G1 course and G1/S phase transition is executed by competitive binding with the cyclin-dependent kinases (CDKs). p16 inhibits the activity of pRb by binding the CDK4 and CDK6, which phosphorylate Rb protein (Hirama and Koeffler, 1995; Cordon-Cardo, 1995; Sherr,

1996; Clurman and Groudine, 1998; Lundberg and Wienberg, 1999). In many mesenchymal tumors, such as rhabdomyosarcoma (Yoo et al., 2002; Takahashi et al., 2004; Sabah et al., 2006a), gastrointestinal stromal tumor (Schneider-Stock et al., 2003; Schneider-Stock et al., 2005; Sabah et al., 2006b), leiomyosarcoma, liposarcoma (Yoo et al., 2002; Sabah et al., 2006a) altered expression of p16 and pRb is observed.

The antigens commonly used to estimate the proliferating activity of neoplastic cells are PCNA and Ki-67. PCNA, a 36 kDa nuclear acidic protein, is necessary for cellular proliferation as a DNA polymerase σ and ϵ auxiliary protein. Its expression is elevated in the nucleus during the late G1 phase with a maximum expression during the S phase and decline during G2 and M phase. PCNA half life is estimated from 8 to 20 hours (Prelich et al., 1987; Linden et al., 1992; Bozlu et al., 2002).

Ki-67 is thought to be a good proliferating marker, because its concentration in the cell starts to increase in the S phase until G2/M, and after mitosis it decreases rapidly, but it is absent in resting cells (G0). It is composed of two subunits, 345 and 395 kDa. Its estimated half-life is 60-90 min, and that is why Ki-67 can be detected only in cells which are currently in the active cell cycle (Prelich et al., 1987; Bruno and Darzynkiewicz, 1992; Linden et al., 1992; Brown and Gatter, 2002).

The MCM family of proteins (MCM2-MCM7) are relatively new proliferating antigens. They take part in the initiation, but also in elongation of the DNA replication, as the MCM protein complex has the activity of helicase. It places MCM proteins directly in the active cell cycle. The expression level of MCM proteins is constant throughout the cell cycle phases with a peak in late G1. MCM proteins are not present in differentiated and quiescent cells (Kearsey and Labib, 1998; Tye, 1999; Williams et al., 2004; Braun and Breeden, 2007). MCM proteins expression has not been characterized previously in the desmoid tumor.

The aim of the study was to estimate immuno-histochemically the expression of proteins playing the essential role in G1 course and G1/S phase transition: pRb, p16 and to assess the expression of proliferating antigens (PCNA, Ki-67, MCM5) in aggressive fibromatosis cells.

Materials and methods

50 formalin-fixed, paraffin-embedded tissue blocks of aggressive fibromatosis (desmoid) were studied. They included: 27 abdominal (AD group), 18 extra-abdominal (E-AD group) and 5 intra-abdominal (I-AD group) cases. The archival specimens were from the Departments of Pathomorphology in Poland. All the sections were independently examined by two experienced pathologists (AK and JS), using a conference microscope and were histopathologically classified, as recommended by the World Health

Organization Classification of Tumors (Goldblum and Fletcher, 2002) and Weiss and Goldblum (2001).

Immunohistochemical screening

Each tumor section, 4 µm in thickness, was deparaffinized and subjected to antigen retrieval by microwaving in 10 mM of citrate buffer (sodium citrate, pH 6.0) for 15 min. Endogenous peroxidase activity was blocked by incubating the deparaffinized sections in 3% hydrogen peroxide (H₂O₂) for 5 min. Nonspecific antibody binding was reduced by incubation of the sections for 10 min with normal horse serum. The sections were incubated with monoclonal antibodies against pRb, p16, PCNA, Ki-67 and MCM5 (Novocastra, UK) for 1 hour at room temperature. The dilutions and clones of used primary antibodies are demonstrated in Table 1. In the negative control reaction the primary antibody was omitted. This was followed by incubation with the Novocastra Universal Detection Kit. 3,3'-diaminobenzidine (DAB) was used as a chromogen to yield brown reaction products. The sections were counterstained with Mayers hematoxylin, dehydrated and mounted.

For pRb, PCNA, Ki-67 and MCM5 the paraffin embedded sections from tonsil, and for p16 from placenta, were used as positive control. For each case the immunohistochemical reaction for CD34 (Novocastra, UK) was performed (Miettinen et al., 2003).

The immunohistochemical staining of cells was estimated by means of the qualitative method using IMAGEJ v. 1.34 software. The percentage of positive cells per 160-300 aggressive fibromatosis cells, counted with 40 magnification of objective lens, was used to express the results. For proliferating antigens PCNA, Ki-67 and MCM5 the results were graded into three groups: (-) – less than 10% of tumor cells stained positively; (+) – 10-50% of tumor cells stained positively; (++) > 50% of tumor cells stained positively. Lesions scored as (++) were considered as showing high expression (overexpression) of the protein (Nikitakis et al., 2002; Hanly et al., 2006). For pRb and p16 the results were classified as: (-) – less than 10% of tumor cells stained positively; heterogeneous staining - 10-50% of tumor cells stained positively; (+) > 50% of tumor cells stained positively (Yoo et al., 2002; Takahashi et al., 2004;

Sabah et al., 2006a,b). The nuclear staining for pRb (Fig. 1), PCNA (Fig. 2), Ki-67 (Fig. 3) and MCM5 was categorized as positive cases. For p16 nuclear and cytoplasmic staining was detected but only nuclear one was classified as positive (Fig. 4) (Cohen and Geradts, 1997).

Statistical procedure

Basic statistical analyses, such as arithmetic mean, range and standard deviation were performed. Kruskal-Wallis test was used to evaluate versatility of expression of the examined proteins within the analyzed groups. Then, a more thorough analysis was made with Mann-Whitney test (test U), comparing the mean percentage of positive cells for each protein between the investigated groups. The Fisher's exact test was used to compare for contingency tables (four-fold) the frequency of occurrence of the given classes between the tested groups. The results were accepted as statistically significant at $p < 0.05$.

Results

pRb and p16

The average percentage of cells showing nuclear localization of pRb protein was 53.17 (SD=34.54) in abdominal group (AD), 73.87 (SD=61.85) in extra-abdominal group (E-AD) and 73.3 (SD=66.23) in intra-abdominal group (I-AD). The average percentage of positive cells for this protein in E-AD group was statistically significantly higher in comparison to AD group ($p < 0.01$).

None of the examined cases ($n=50$) of desmoid tumor showed negative immunoreactivity of Rb protein.

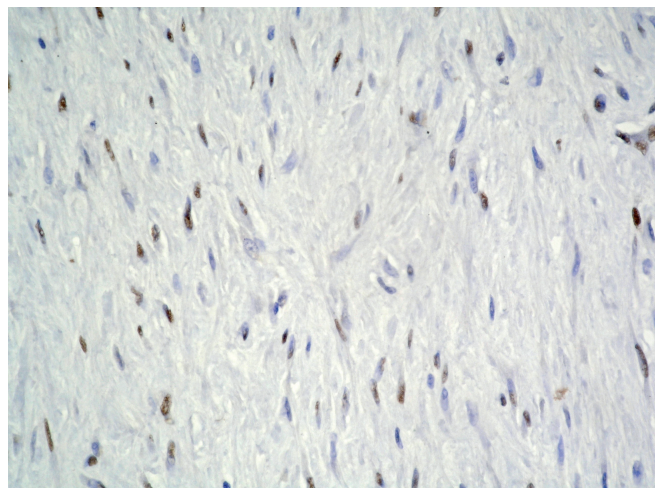


Fig. 1. Immunoreactivity of pRb in desmoid tumor. Nuclei were counterstained with Mayers hematoxylin. x 400

Table 1. Antibodies used in the immunohistochemical analysis of desmoid tumors.

Antigen	Antibody (clone)	Dilution	Source
pRb	13A10	1:40	Novocastra, UK
p16	6H12	1:30	Novocastra, UK
CD34	QBEnd/10	1:50	Novocastra, UK
PCNA	PC10	1:100	Novocastra, UK
Ki-67	MM1	1:120	Novocastra, UK
MCM5	CRCT5.1	1:30	Novocastra, UK

Heterogeneous expression of Rb protein (10-50% of positive cells) was noted for 51.85% (14/27) of cases in group AD and for 5.56% (1/18) of cases in group E-AD. The remaining cases in AD and E-AD groups, that is 48.15% (13/27) and 94.44% (17/18) respectively, demonstrated normal expression of the analyzed protein. All cases of aggressive fibromatosis located intra-abdominally showed normal expression of Rb protein (Table 2). The number of positive cases for pRb expression in E-AD and I-AD groups was statistically significantly higher, compared to AD group ($p < 0.001$).

The percentage of cells positive for p16 was the

highest in AD group (57.14; SD=44.48) followed by the I-AD group (52.76; SD=60.93) and E-AD group (46.32; SD=25.35). The comparison of these data revealed no statistically significant differences between the examined groups of desmoid tumor.

The positive expression of p16 (>50% of positive cells) was observed in 30 out of 50 (60%) cases of aggressive fibromatosis. For neoplasm located abdominally, extra-abdominally and intra-abdominally the positive expression was noted in 66.67% (18/27), 50% (9/18) and 60% (3/5), respectively (Table 2). No statistically significant differences were noted in the

Table 2. Number of positive, heterogeneous and negative cases for pRb and p16 noted in examined groups.

Location	+ n (%)		Heterogeneous staining n (%)		- n (%)	
	pRb	p16	pRb	p16	pRb	p16
Abdominal n=27	13 (48.15)	18 (66.67)	14 (51.85)	9 (33.33)	0 (0.00)	0 (0.00)
Extra-abdominal n=18	17 (94.44)	9 (50.00)	1 (5.56)	9 (50.00)	0 (0.00)	0 (0.00)
Intra-abdominal n=5	5 (100.00)	3 (60.00)	0 (0.00)	2 (40.00)	0 (0.00)	0 (0.00)
Total	35 (70.00)	30 (60.00)	15 (30.00)	20 (40.00)	0 (0.00)	0 (0.00)

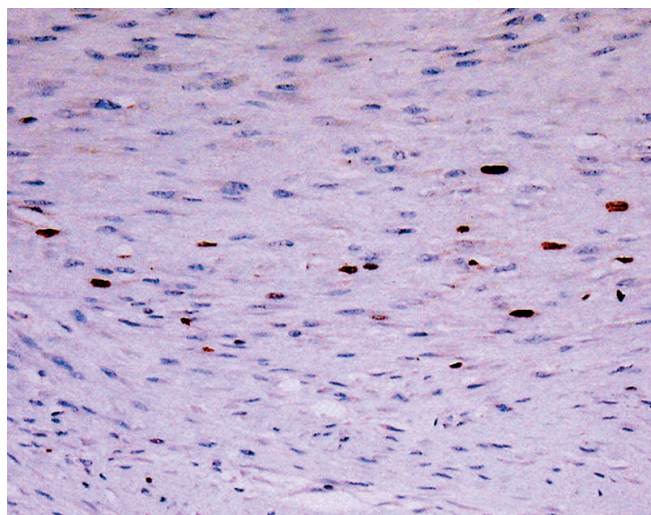


Fig. 3. Immunoreactivity of Ki-67 in desmoid tumor. Nuclei were counterstained with Mayers hematoxylin. x 400

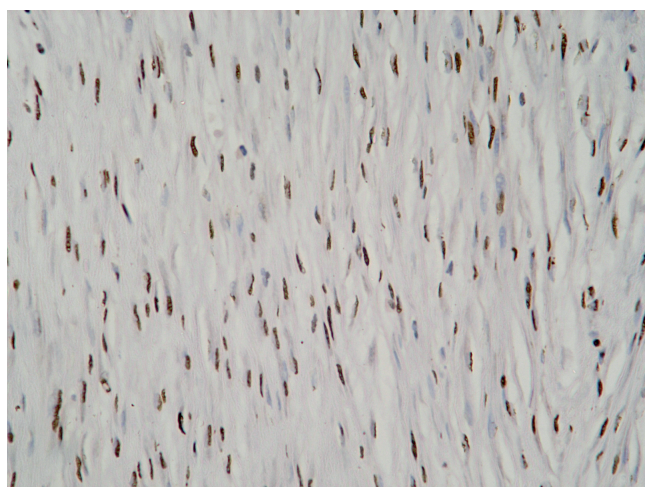


Fig. 2. Immunoreactivity of PCNA in desmoid tumor. Nuclei were counterstained with Mayers hematoxylin. x 400

Table 3. Number of positive and negative cases for PCNA, Ki-67 and MCM5 noted in examined groups.

Location	++ n (%)			+ n (%)			- n (%)		
	PCNA	Ki-67	MCM5	PCNA	Ki-67	MCM5	PCNA	Ki-67	MCM5
Abdominal n=27	26 (96.30)	0 (0.00)	0 (0.00)	1 (3.70)	7 (25.93)	0 (0.00)	0 (0.00)	20 (74.07)	27 (100.00)
Extra-abdominal n=18	18 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	3 (16.67)	0 (0.00)	0 (0.00)	15 (83.33)	18 (100.00)
Intra-abdominal n=5	5 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	3 (60.00)	0 (0.00)	0 (0.00)	2 (40.00)	5 (100.00)
Total	49 (98.00)	0 (0.00)	0 (0.00)	1 (2.00)	13 (26.00)	0 (0.00)	0 (0.00)	37 (74.00)	50 (100.00)

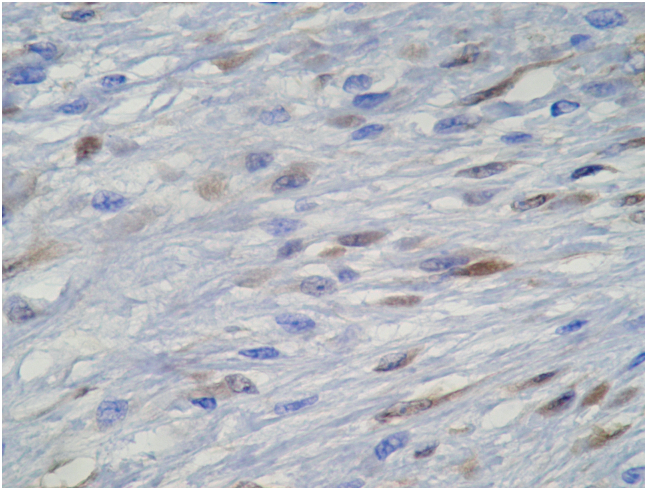


Fig. 4. Immunoreactivity of p16 in desmoid tumor. Nuclei were counterstained with Mayers hematoxylin. x 400

number of positive cases for p16 between those groups.

Proliferating antigens

The mean percentage values of the positive cells for PCNA were 69.51 (SD=55.78) for desmoid located abdominally, 79.26 (SD=69.08) for desmoid located extra-abdominally and 75.48 (SD=52.26) for intra-abdominal location. The average percentage of positive cells for PCNA in E-AD group was statistically significantly higher, when compared to AD group ($p<0.02$).

Overexpression of PCNA was noted in 98% (49/50) of cases with desmoid tumor. In one case of abdominal desmoid tumor the expression did not exceed 50% of positive cells (Table 3). No statistically significant differences were detected in the number of positive cases for PCNA between those groups.

The mean values of the percentage of Ki-67-positive cells in group AD, E-AD and I-AD were 6.92 (SD=2.43), 7.54 (SD=6.65) and 16.66 (SD=0.61), respectively. The values did not differ statistically significantly. Positive staining for Ki-67 protein was noted in 25.93% (7/27) of cases with abdominal desmoid tumor, in 16.67% (3/18) with extra-abdominal tumor and in 60% (3/5) with intra-abdominal desmoid tumor. Overexpression of Ki-67 protein was not observed (Table 3).

Immunohistochemical staining for MCM5 was negative in all cases of aggressive fibromatosis examined in this study. In few cases single cells with nuclear staining for MCM5 were found (Table 3).

Discussion

Numerous studies have proved that disorders in normal transition between cell cycle phases, particularly

of G1/S transition, are the cause of cell neoplastic transformation. Rb protein (pRb) and p16INK4A (p16) play an important role in G1 phase regulation and in the transition from G1 to S phase (Cordon-Cardo, 1995; Hirama and Koeffler, 1995; Gillett and Barnes, 1998; Keenan et al., 2004).

Suppressive protein Rb is one of the so called “guardians” of cell cycle. The highest activity of this protein is observed in G1 cycle phase. In the case of the lack of signal for cell proliferation, unphosphorylated Rb protein binds to the E2F transcription factor, the activity of which is essential for the initiation of the next cell cycle phase (Hirama and Koeffler, 1995; Sherr, 1996; Keenan et al., 2004). Suppressor genes mutations, and thus the loss of proteins from “genome guardians” group, are one of the causes of cell cycle dysregulation and they can lead to the development of neoplasms.

So far, in the available literature only a few studies related to Rb protein expression in aggressive fibromatosis have been published. Müller et al. (1996) investigated 13 cases of desmoid tumor for the presence of pRb and no immunohistochemical staining was observed for this antigen. These authors also pointed to changeable results of staining for Rb of paraffin specimens depending on the time and temperature of antigen exposure in a microwave cooker. Cohen and Geradts (1997) examined 7 cases of aggressive fibromatosis for pRb presence. These authors found in 5 (71.4%) cases of desmoid normal result of staining for Rb protein (uniform nuclear reaction within the whole specimen) and in 2 (28.6%) cases the result deviated from the accepted standard (focal reaction).

Due to the lack of sufficient literature data concerning Rb protein expression in aggressive fibromatosis cells, it has been decided to compare the results obtained in this study with the results obtained for other mesenchymal neoplasms. For instance, Takahashi et al. (2004) investigated pRb expression in 72 cases of rhabdomyosarcoma. pRb demonstrated normal expression (>50% of positive cells) in 26.4% (19/72) of cases, whereas altered expression, both heterogeneous (1-49% of positive cells) and lack of expression, was observed in 53 (73.6%) cases. Despite high differentiation of the obtained results the authors were not able to determine the prognostic significance of altered pRb expression in rhabdomyosarcoma. Sabah et al. (2006b) accepted more rigorous criteria of division of positive heterogeneous and negative cases for pRb expression (respectively: >80%, 20-80%, <20% positive cells). The authors examined a group of 23 gastrointestinal stromal tumors (GIST) of different degree of cell proliferation. Variable expression was noted in 14 cases of GIST: 2 (8.7%) cases evaluated as heterogeneous and 12 (52.2%) as negative. Altered expression of pRb was more frequently observed in tumors of high degree of cell proliferation (10/14, 71.4%) than in tumors of low degree of proliferation (4/9, 44.4%) but these differences were not statistically significant (Sabah et al., 2006b). The same authors in

another study presented the results of pRb expression in a group of 152 mesenchymal neoplasms of different stage of malignancy (Sabah et al., 2006a). The result of staining was heterogeneous only in 9 (5.9%) cases and in 68.8% (94/152) of the cases immunoreactivity was evaluated as negative. The authors did not find any significant correlation between the stage of histological malignancy of the investigated neoplasms and abnormal expression of pRb (Sabah et al., 2006a). In this study no negative pRb expression was detected in the examined desmoid cases. A positive result of immunohistochemical staining was found in 35 (70%) cases in the whole investigated group: 13/27 (48.15%) cases of abdominal aggressive fibromatosis, 17/18 (94.44%) cases of extra-abdominal and 5/5 (100%) cases of intra-abdominal desmoid location. Mean percentage of positive cells in the analyzed groups was: 53.17 (SD=34.54), 73.87 (SD=61.85) and 73.3 (SD=66.23) respectively for the groups AD, E-AD and I-AD. The comparison of mean percentage of positive cells for Rb protein between abdominal and extra-abdominal desmoid location demonstrated statistically significant difference. Mean value of the percentage of positive cells was significantly higher in E-AD group ($p<0.01$). The comparison of mean values of the percentage of positive cells for the remaining groups did not demonstrate statistically significant differences.

The results obtained in this study of investigations concerning pRb expression in aggressive fibromatosis cells are convergent with the data given by other authors. In the majority of desmoid cases examined in this study pRb expression was normal, as in cases described by other authors of benign tumors of mesenchymal origin (Takahashi et al., 2004; Sabah et al., 2006a,b). The level of pRb expression observed in the studies of these authors was lower for malignant neoplasms but the difference was not of statistical significance. On the other hand, the application of immunohistochemical method did not allow estimation of the degree of pRb phosphorylation and, as is known, the level of pRb phosphorylation has an important effect on its activity and cell-cycle regulation, particularly of G1 phase (Hirama and Koeffler, 1995; Sherr, 1996; Keenan et al., 2004).

p16 (p16INK4A), like pRb, is a suppressor protein included in the group of cell cycle "guardian" proteins. p16 plays its suppressor function by competing with cyclin D for CDK4 and CDK6 binding. Via inactivation of CDK4/6, p16 can block the process of pRb phosphorylation in G1 phase and thus stop G1/S transition. The decrease of pRb level in a cell may cause deregulation of the precise mechanism of cell cycle (Cordon-Cardo, 1995; Hirma and Koeffler, 1995; Sherr, 1996; Subramaniam et al., 2006).

In this study no case was found with negative expression of p16 and mean percentage of positive cells for groups AD, E-AD and I-AD was respectively 57.14 (SD=44.48), 46.32 (SD=25.35) and 52.76 (SD=30.93). Heterogeneous pattern of staining was observed in 20

(40%) cases of aggressive fibromatosis from among all the analyzed cases ($n=50$): 9/27 (33.3%) in AD group, 9/18 (50%) in E-AD and 2/5 (40%) in group I-AD. Normal expression was detected in 60% (30/50) of all examined cases: in 18/27 (66.67%) cases of abdominal fibromatosis, 9/18 (50%) cases of extra-abdominal and 3/5 (60%) cases of intra-abdominal fibromatosis.

The study of Cohen and Geradts (1997) presents the results related to p16 immunohistochemical reaction in desmoid cells. In all, that is in 9 (100%) examined cases of deep fibromatosis, normal staining was noted for this protein. The authors accepted the marker of normal staining for p16 a uniform nuclear staining within the cells of the whole histological specimen. Roca et al. (2003) used immunoblotting analysis of aggressive fibromatosis and fibrosarcoma cell extracts to detect the presence or absence of p16 protein. The expression of p16 was found in desmoid cells, but it was completely lost during the transition from the aggressive fibromatosis to fibrosarcoma stage (Roca et al., 2003). So far, in the available literature there is a lack of sufficient data on the expression of p16 in aggressive fibromatosis. Thus, it was not possible to compare the results obtained in this study.

For instance, in another mesenchymal tumor, rhabdomyosarcoma, Takahashi et al. (2004) noted positive immunoreactivity for p16 in 33.3% (24/72) of cases, and in the remaining 66.6% of cases they recognized the immunoreactivity to be altered (the criteria of the expression evaluation were similar to those for pRb). Sabah et al. (2006a) presented the results of studies on the expression of p16 in a group of mesenchymal neoplasms – GISTs demonstrating different degrees of cell proliferation. Altered levels of p16 expression investigated in GISTs of different grade of histological malignancy, i.e. low-, medium- and high-grade was respectively: 59.3%, 62.5% and 72.4%. However, no statistically significant correlation was noted between the degree of intensification of the neoplasm cell proliferation and altered expression of p16. Yoo et al. (2002) accepted similar criteria for the evaluation of p16 expression. They examined 67 cases of soft tissue tumors including: rhabdomyosarcomas, leiomyosarcomas, liposarcomas, malignant fibrous histiocytomas and malignant peripheral nerve sheath tumors. Due to a high number of cases with altered expression of p16 these authors suggested that inactivation of this protein may play a key role in the development of sarcomas.

Sabah et al. (2006b) in their study on 23 mesenchymal tumors (GISTs) found normal immunoreactivity for p16 in all 9/9 (100%) cases of low grade of aggressiveness. At the same time in all 14/14 (100%) cases of aggressive GISTs these authors detected an abnormal level of p16 expression. Thus, the level of p16 expression may serve as a marker of mesenchymal neoplasm aggressiveness, which was also confirmed by other authors (Schneider-Stock et al., 2003, 2005). The results obtained in this study concerning the expression

of p16 are similar to those obtained by Sabah et al. (2006b) in relation to GISTs of low grade of aggressiveness.

Proliferating cell nuclear antigen (PCNA) is a widely recognized and used marker of cell proliferation (Prelich et al., 1987; Linden et al., 1992; Bozlu et al., 2002). In this study no negative expression of this protein was found in the examined cases of aggressive fibromatosis. In the whole investigated group positive (+) expression of PCNA, in the range from 10 to 50% of positive cells, was observed only in 1/50 (2%) desmoid case. In the remaining 49/50 (98%) cases of aggressive fibromatosis overexpression of the examined antigen was detected. Mean percentage of positive cells for desmoid abdominal and extra-abdominal location was respectively 69.51 (SD=55.78) and 79.26 (SD=69.08) and this difference was statistically significant ($p<0.02$). The comparison of the positive cells percentage between groups AD and I-AD and E-AD and I-AD was not of statistical significance ($p<0.01$).

Kouho et al. (1997) analysed 22 desmoid cases immunohistochemically for PCNA. The authors compared PCNA labeling index (calculated as the per cent of positive cells/500 desmoid cells) for 10 cases of primary tumors and 12 recurrent cases. The mean value of the labeling index (LI) for primary and recurrent tumors was respectively 12.6% (range 6.7% - 17.9%) and 14.8% (range 8.7% - 25.1%). In our study the mean value of the percentage of positive cells for PCNA in the whole investigated group ($n=50$) was 73.54 (SD=13.97) (range 33.1 - 93.9). Kouho et al. (2007) did not find a statistically significant correlation between the value of PCNA labeling index and recurrence of desmoid tumor. PCNA labeling index calculated by Saito et al. (2001) for 38 cases of aggressive fibromatosis was 31.8 (range 2.5 - 67.4) and was closer to the results obtained in our study. Serpell et al. (1999), based on the investigations carried out on a group of 24 cases of aggressive fibromatosis found a correlation between the percentage of PCNA-positive cells and the recurrence of desmoid but this correlation was statistically insignificant.

The results presented in our study, concerning PCNA expression were convergent with the results demonstrated by Serpell et al. (1999), where positive result of PCNA expression was found in 83.33% (20/24) of the examined cases. On the other hand, some authors reported about markedly lower PCNA labeling index in desmoid cells (Kouho et al., 1997).

The share of PCNA in the process of DNA repair synthesis, as well as long biological half-time of this protein in a cell, make this antigen possible to be detected also in cells which are not in the active cell cycle, but have entered G0 phase, or in cells in which the cycle was inhibited to repair DNA damage (Prelich et al., 1987; Linden et al., 1992; Zacchetti et al., 2003). The above observations can cause difficulties in the interpretation of the fact of high PCNA expression at simultaneous low division activity of aggressive fibromatosis cells.

Different results were obtained in this study for two other proliferating antigens – Ki-67 and MCM5. Saito et al. (2001) examined 38 cases of aggressive fibromatosis without familial adenomatous polyposis (FAP) for Ki-67 protein. The mean value of labeling index for Ki-67 was 3.0 (range 0 - 28.6). This points to low division activity of desmoid cells.

Hoos et al. (2001) did not find either any positive result of immunohistochemical reaction for Ki-67 in 24 (100%) examined cases of aggressive fibromatosis. Similarly, Kouho et al. (1997) did not find positive cells for Ki-67 or observed only single cells with nuclear reaction for this antigen, which was considered as a negative result in 24 desmoid cases. Leithner et al. (2005) examined a larger group of desmoid cases ($n=80$). They accepted the value of 5% of cells with distinct nuclear reaction as a positive result of staining for Ki-67 antigen. On the basis of this criterion 20 of 80 (25%) examined desmoid cases were positive for Ki-67. Then, Gebert et al. (2007) noted expression of Ki-67 not exceeding 2% of positive cells (negative result) in 36 of 37 (97.3%) cases of aggressive fibromatosis. Only in one case expression of this protein reached the level of 5%.

In our study the mean value of positive cells for Ki-67 in the whole examined group ($n=50$) was 8.12 (SD=12.07) (range 0 - 43.6). Mean values of the percentage of positive cells for groups: AD, E-AD and I-AD were respectively: 6.92 (SD=2.43), 7.54 (SD=6.65) and 16.66 (SD=0.61). Overexpression of Ki-67 was not found at all. In the whole examined group ($n=50$) the expression of this antigen at the level of 10 - 50% positive cells was noted in 13 (26%) cases and a negative result in 37 (74%) cases of aggressive fibromatosis. In abdominal desmoid Ki-67 expression was not observed in 20 of 27 (74.07%) cases, in extra-abdominal desmoid in 15 of 18 (83.33%) and in intra-abdominal desmoid in 2 of 5 (40%) cases. The results obtained in this study point to low proliferative activity of desmoid cells, which is in agreement with the results reported by other authors (Leithner et al., 2005).

Low proliferative activity of desmoid cells has also been confirmed by the results obtained in this study for MCM5 protein – a new proliferating antigen. No positive result was observed for this protein in 50 (100%) examined cases of aggressive fibromatosis. Single cells with positive nuclear staining for MCM5 were observed only in a few histological specimens. So far, in the available literature there is a lack of reports concerning studies on the expression of MCM family proteins, including MCM5, in desmoid cells. However, MCM5 was frequently used as a division activity index in the studies on other types of neoplasms. It was used, among others, for the detection of neoplastic cells in uterine cervix smear (Williams et al., 1998; Freeman et al., 1999), for diagnosis of primary and recurrent urinary bladder cancer (Stoeber et al., 1999, 2002), oesophageal cancer (Williams et al., 2004). The results obtained in our study, concerning MCM5 protein expression in aggressive fibromatosis cells are more proof confirming

low proliferative activity of desmoid tumors cells.

To conclude, the level of suppressor proteins pRb and p16 expression in desmoid cells observed in the studies reflects their function in cell cycle regulation. It should be presumed that the altered course of cell cycle, at least of the G1 phase, is not the cause of aggressive fibromatosis pathogenesis.

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Cell cycle proteins in desmoid tumors

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