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Maspin and Nm23-H1 expression in colorectal cancer

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The aim of the study was to analyze the expression of Nm23-H1 and maspin proteins in a series of colorectal adenocarcinoma and to assess their applicability as prognostic factors in this type of cancer. 102 specimens of colorectal carcinoma were analyzed by immunohistochemistry with the use of anti-Nm23-H1 and anti-maspin monoclonal antibodies. Cytoplasmic expression of Nm23-H1 and maspin was found in 90 of all investigated cases. In 60 cases maspin protein was found also in nucleus. Medium/high Nm23-H1 cytoplasmic expression level was associated with tubular type of adenocarcinoma with deeper invasion of cancer into intestinal wall (T3, T4) and presence of vascular invasion. Medium/high expression level of maspin was connected uniformly with bad prognostic features: low differentiation of tumors (G3), deeper invasion of cancer (T3, T4) presence of nodular and distant metastases, higher Astler-Coller stage (C1, C2, D) and presence of vascular invasion. No statistically significant associations between presence of nuclear maspin expression and any clinicopatological and biological features were stated. Cytoplasmic medium/high expression level of maspin but no Nm23-H1 and no presence of maspin nuclear expression was found as independent bad prognostic factor in the investigated group of patients. Measurement of level and cellular pattern of maspin expression could be valuable for predicting disease course in patients suffering from colorectal cancer.

Key words: colorectal cancer, immunohistochemistry, prognosis, survival rate, maspin, Nm23-H1 nucleoside diphosphate kinase.

Despite progress in diagnostic and treatment procedures during the last years, colorectal cancer remains one of the most frequent and deadly neoplasms. It is also the second leading cause of cancer death in the western world. In recent decades researchers have tried to identify biological markers that appear to be involved in tumor progression and development of metastasis [1] and which can be useful in predicting clinical outcome [2]. Among them, there are two promising tumor and metastasis suppressing proteins, maspin and Nm23-H1.

Maspin (mammary serine protease inhibitor, SERPIN-B5) was initially identified in human breast carcinoma cells while searching for tumor suppressor gene candidates [3]. It has been shown to inhibit tumour cell motility and invasion in mammary and prostate carcinoma cell lines in vitro [4] and to reduce the capacity of cancer cell lines for tumorigenesis and metastasis in animal models [5]. In concordance with this findings, downregulation or loss of maspin expression has been shown to correlate with increased aggressiveness in breast [6], prostate [7] and colorectal [8] cancers. Surprisingly, opposing data were also published, even for the same types of cancer. Overexpression of the protein has been reported to be associated with aggressive phenotype in mammary ductal carcinoma [9] and poor prognosis in non-small cell lung [10] and ovarian [11] cancers. Recently, an imposing list of protein-binding partners of maspin was uncovered [12]. Among them, there are collagen I, collagen III, urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator receptor (uPAR), glutathione S-transferase (GST), β-catenin and early growth response protein 1 (EGR1). It suggests that maspin could be involved in a multitude of processes including matrix adhesion, protein degradation, oxidative stress response, transcription regulation and its function depends on cellular localization [12].

In 1988, Steeg and colleagues described the first metastasis suppressor gene, Nm23-H1 which expression was reduced in highly metastatic melanoma cell lines and rat mammary carcinomas [13]. The gene is coding for NDP kinase, an ubiquitously distributed enzyme catalyzing the phosphorylation

of nucleotide diphosphate to the corresponding nucleoside triphosphate [14]. It was postulated that granzyme A activates DNA nicking via DNAse NM23-H1 which has an important role in cancer prevention throught the induction of tumour cell apoptosis [15]. Expression of both Nm23-H1 mRNA and protein has been shown to be reduced in different human cancers [16–18]. Decreased expression of Nm23-H1 was connected with aggressive behavior and inversely related to the metastatic capacity of colon cancer, gastric cancer, melanoma of skin and breast cancer [14]. Similarly to maspin, adverse findings were also presented. High level of Nm23-H1 was correlated with high proliferation rate of thyroid cancer [19] and higher risk of death in colorectal cancer patients [20]. Probably, the role of Nm23-H1 may vary between tissue types because of different regulatory mechanism.

Considering insufficient and conflicting data about Nm23-H1 and maspin in colorectal cancer, in the present study, expression of both proteins was analyzed by means of immunohistochemistry in a series of colorectal cancer specimen. To assess the utility of Nm23-H1 and maspin as potential prognostic markers, their expression was compared with some clinicopathological features of tumour and survival time of patients.

Materials and methods

Patients and tissue samples. Total of 102 patients of the Oncological Center of Łódź, Poland suffering from colorectal carcinomas were enrolled in the study. Tissue samples were obtained from the patients during surgical removal of tumor. All experiments were carried out with the local ethical committee approval (No KE/813/07) and patient's informed consent.

Immunohistochemistry. 4μm thick sections of formaline–fixed, paraffin embedded tissue were placed on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). These were deparaffinized in xylenes and rehydrated through graded alcohol. Then, the sections were microwaved in 0.01M sodium citrate buffer, pH 6.0, twice for 10 minutes at 360W to epitope retrieval. The slides were then washed with TRIS buffered saline, pH 7.4, and incubated for 1 hour at room temperature with the primary monoclonal antibody anti-Nm23-H1 (clone 37.6, 1:500 dilution, Leica Biosystems, Newcastle, UK), and anti-maspin (clone EAW24, 1:50 dilution, Leica Biosystems, Newcastle, UK) and processed with EnVision+ (DAKO, Glostrup, Denmark) system. Sections were counterstained with haematoxylin, dehydrated with ethanol and cleared in xylene.

Staining pattern analysis. For Nm23-H1 expression analysis, all cases were divided into four classes: 0% of cells stained – negative cases, 1-30% of cells stained – low level, 31-60% – medium level, 61-100% – high level, as previously described [20]. According to Umekita at al. [9], expression level of six-stage classification was assumed for maspin: 0% of cells stained – negative cases, 1–5% of cells stained – very low level, 6–25% – low level, 26–50% – medium level, 51–75% – high level, 76–100% – very high level.

Statistical analysis. To estimate relationships between clinicopatological and biological characteristics and the expression of maspin and Nm23-H1 chi-squared and V-square tests were used. Dependences between the above mentioned features and overall survival were assessed (univariate analysis) using a Kaplan-Meier estimator. Overall survival was determined as the interval between surgery and death. Median overall survival time was 71 months (minimum – 1 month, maximum – 110 months). Statistical significance of the dependences was estimated using a log-rank test. To identify the independent prognostic factors, Cox proportional hazard regression model (multivariate analysis) was created employing variables that showed p value lesser than 0.1 in univariate analysis. P-value <0,05 was assumed as significant in all conducted tests.

Results

Immunohistochemical proteins expression. Nm23-H1 cytoplasmic expression (Figure 1A) was detected in 90 out of 102 investigated samples. In 23 and 29 cases there were low and medium NM23 expression level, respectively. 38 samples were defined as having a high expression level.

In all tested samples cytoplasmic maspin expression (Figure 1B) was detected, but in 18 and 24 cases on very low and low level, respectively. 20 cases were determined as having medium level of maspin expression. In only 9 samples the maspin expression was high, whereas in 31 samples the expression was stated as very high. Nuclear maspin expression (Figure 1C) was observed in 60 cases.

Statistical dependences between Nm23-H1 and maspin expression and clinicopathological and biological features. With regard to a relatively low number of examined patients, we classified samples as "absent/low" and "medium/high" expression level, showing up and above 30% of cells with Nm23-H1 positive cytoplasmic reaction, respectively. For maspin, samples showing up and above 25% of cells with positive cytoplasmic reaction were included into classes named "low" and "medium/high", respectively.

In the investigated population median age at the time of diagnosis was 63 years (30-86 years). There were no statistically significant differences between patients of age up and above 60 with regard to Nm23-H1 (p=0.938) and maspin (p=0.740) expression levels. No associations were also found between gender and expression levels of both examined proteins (p=0.236 for Nm23-H1, p=0.868 for maspin). Expressions of both Nm23-H1 and maspin were not connected with tumor localization (p=0.331 for Nm23-H1, p=0.153 for maspin). There was a statistically significant association between Nm23-H1 expression level and histological type of tumor (p=0.005). Medium/high expression level of Nm23-H1 was stated in 62 of tubular adencarcinoma and only 5 of mucinosus adencarcinoma cases. No link between maspin expression level and histological type of tumor was found (p=0.219). There was no dependence between tumor grading and Nm23-H1 expression level (p=0.294), whereas medium/high expression level of

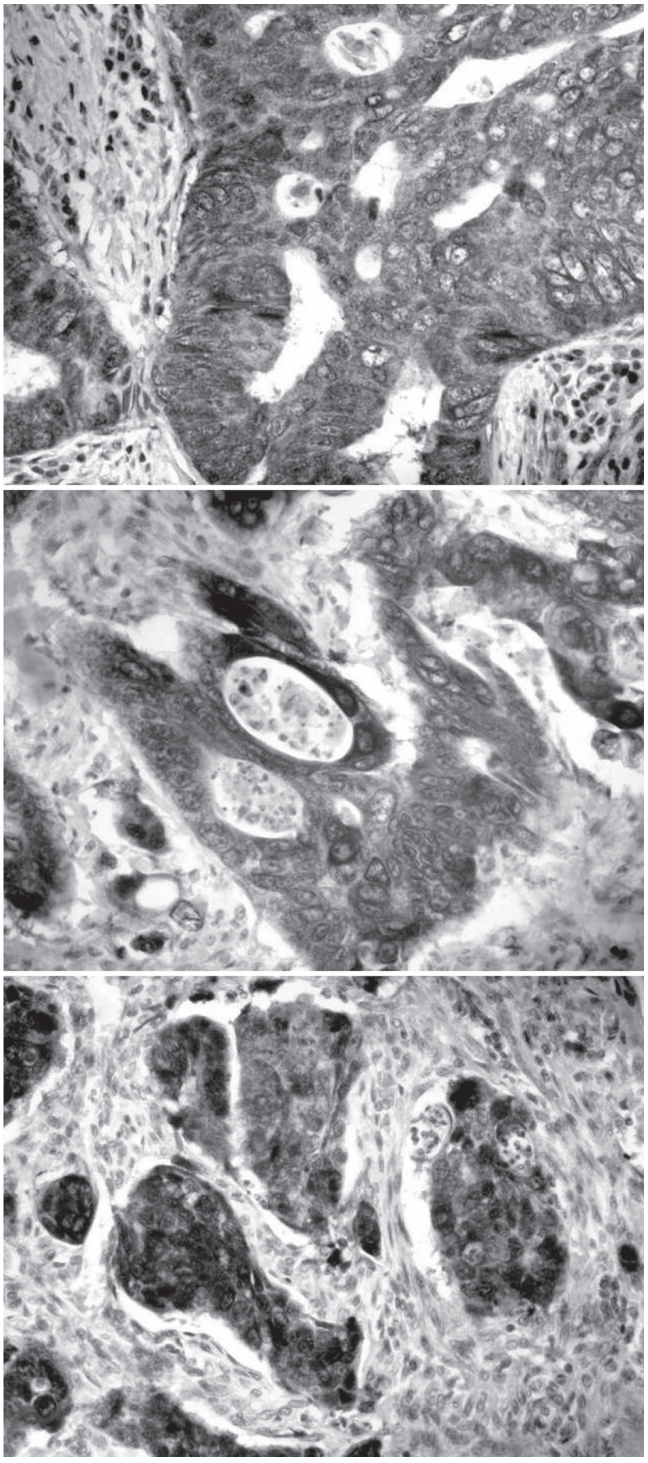

Figure 1. Positive immunohistochemical reaction with antibodies against Nm23-H1 (A-cytoplasmic) and maspin (B-cytoplasmic, C-nuclear) in a case of colorectal cancer (magnification 400x)

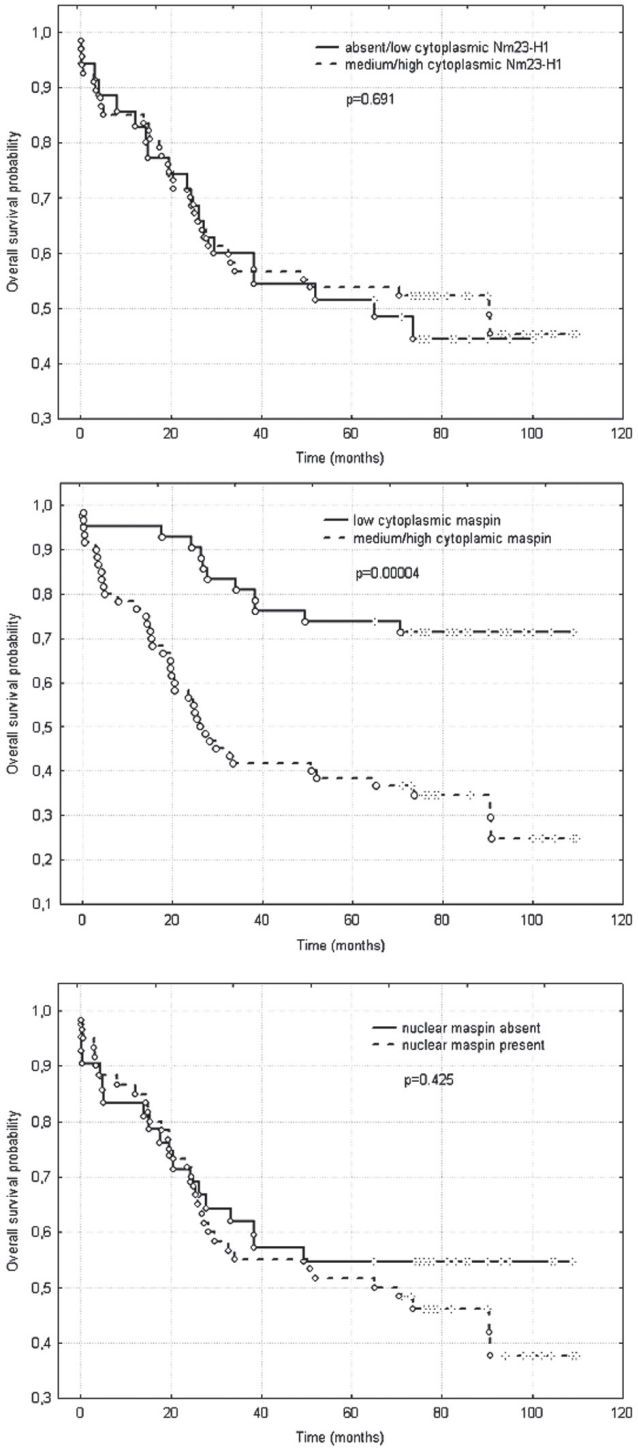

Figure 2. Overall survival probability according to cytoplasmic expression level of Nm23-H1 (A), maspin (B) and presence of nuclear maspin expression (C).

maspin was significantly associated with poorly differentiated tumors (G3, p=0.001).

Secondly, expression level of both investigated proteins and some clinical staging features including depth of tumor invasion, lymph nodes metastases, distant metestases, stage according Astler-Coller classification were compared. There was a statistically significant dependence between the depth of tumor invasion and Nm23-H1 expression level (p=0.048). Medium/high levels of the expression were stated more frequently in tumors T3 and T4. No significant connections between the remaining characteristics and Nm23-H1 expression level were found (Table 1). Dependencies between all the above mentioned staging features and maspin cytoplasmic expression level reached statistical significance. Medium/high levels of the expression were associated with deeper invasion of intestine wall (T3,T4, p=0.006), presence of lymph node (N1,N2, p=0.002), and distant metastases (M1, p=0.032). It was proved by the existence of statistically significant connection between expression level of this protein and Astler-Coller stage (p=0.001).

The expression levels of both proteins were also compared to the presence of lymphocytes in tumor and vascular invasion. The Nm23-H1 and maspin expression levels were not connected with the presence of lymphocytes infiltration, a good prognostic feature (p=0.545 and 0.551, respectively). There were statistically significant dependencies between Nm23-H1 as well as maspin expression levels and vascular invasion that is connected with poor prognosis (p=0.018 and 0.002, respectively).

Additionally, dependencies between the existence of nuclear maspin expression and all the above mentioned clinicopatological and biological features were assessed, but no statistically significant dependence was noted (Table 1).

Survival analysis according to clinicopathological and biological features, NM23 and maspin expression. In univariate analysis, the lower depth of tumor invasion (T1,T2, p=0.041), absence of lymph nodes (N0, p=0.0004) and distant (M0, p=0.00001) metastasis, lower Astler-Coller stage (B1,B2, p=0.0003) and low cytoplasmic maspin expression level (p=0.0004) were all significantly associated with a longer sur-

Table 1. Statistical dependencies between Nm23-H1 and maspin expression and clinicopathological features

Feature (number of cases)		Nm23-H1			Maspin					
		Cytoplasmic expression level			Cytoplasmic expression level			Nuclear expression		
		absent/ low	medium/ high	p value	low	medium/ high	p value	absent	present	p value
Age	Up to 60 yrs (49)	17	32	0.938 [a]	21	28	0.740 [a]	19	30	0.636 [a]
	Over 60 yrs (53)	18	35		21	32		23	30	
Gender	Female (52)	15	37	0.236 [a]	21	31	0.868 [a]	26	26	0.065 [a]
	Male (50)	20	30		21	29		16	34	
Localization	Rectum (40)	16	24	0.331 [a]	13	27	0.153 [a]	15	25	0.545 [a]
	Colon (62)	19	43		29	33		27	35	
Histological type	Tubular aca (87)	25	62	0.005 [b c]	38	49	0.219 [b]	37	50	0.506 [b]
	Mucinosus aca (15)	10	5		4	11		5	10	
Histological grade	G1 (12), G2 (60)	27	45	0.294 [a]	37	35	0.001 [a c]	31	41	0.550 [a]
	G3 (30)	8	22		5	25		11	19	
Depth of tumor invasion	T1 (4), T2 (27)	15	16	0.048 [a c]	19	12	0.006 [a c]	16	15	0.157 [a]
	T3 (60), T4 (11)	20	51		23	48		26	45	
Lymph nodes metastasis	N0 (62)	25	37	0.112 [a]	33	29	0.002 [a c]	29	33	0.153 [a]
	N1 (19), N2 (21)	10	30		9	31		13	27	
Distant metastasis	M0 (85)	29	56	0.926 [b]	39	46	0.032 [b c]	35	50	1.000 [b]
	M1 (17)	6	11		3	14		7	10	
Astler-Coller stage	B1 (29), B2 (32)	25	36	0.084 [a]	33	28	0.001 [a c]	28	33	0.237 [a]
	C1 (2), C2 (24), D (15)	10	31		9	32		14	27	
Lyphocyte infiltration	Absent (57)	21	36	0.545 [a]	22	35	0.551 [a]	23	34	0.849 [a]
	Present (45)	14	31		20	25		19	26	
Vascular invasion	Absent (42)	20	22	0.018 [a c]	25	17	0.002 [a c]	21	21	0.130 [a]
	Present (60)	15	45							

[a] χ^2 test, [b] V^2 test; [c] statistically significant differences

Table 2. Overall survival analysis according to clinicopathological features, Nm23-H1 and maspin expression

	Univariate analysis		Multivariate analysis	
	number of deaths (%)	p value (log-rank test)	hazard ratio (95% confidence interval)	p value (Cox regression model)
Age (yrs)				
Up to 60	21 (42.9)	0.057	1.00	0.021 [a]
Over 60	32 (60.4)		2.09 (1.12 – 3.91)	
Gender				
Female	26 (50.0)	0.519	---	---
Male	27 (54.0)		---	
Localization				
Rectum	25 (62.5)	0.188	---	---
Colon	28 (45.2)		---	
Histological type				
Tubular adencarcinoma	44 (50.6)	0.391	---	---
Mucinosus adenocarcinoma	9 (60.0)		---	
Histological grade				
G1, G2	35 (48.6)	0.161	---	---
G3	18 (60.0)		---	
Depth of tumor invasion				
T1, T2	12 (38.7)	0.041 [a]	1.00	0.268
T3, T4	41 (57.8)		1.48 (0.74 – 2.95)	
Lymph nodes metastasis				
N0	25 (40.3)	0.0004 [a]	1.00	0.319
N1,2	28 (70.0)		2.87 (0.36 – 22.89)	
Distant metastasis				
M0	37 (43.5)	0.00001 [a]	1.00	0.00007 [a]
M1	16 (94.1)		4.53 (2.15 – 9.58)	
Astler-Coller stage				
B1,B2	24 (39.3)	0.0003 [a]	1.00	0.498
C1,C2,D	29 (70.7)		0.47 (0.05 – 4.18)	
Vascular invasion				
Absent	34 (59.7)	0.086	1.00	0.880
Present	19 (42.2)		0.94 (0.44 – 2.02)	
Lyphocyte infiltration				
Absent	34 (59.7)	0.083	1.000	0.103
Present	19 (42.2)		0.609 (0.335 – 1.11)	
Maspin – cytoplasmic expression level level				
Low	12 (28.6)	0.00004 [a]	1.00	0.003 [a]
Medium/high	41 (68.3)		2.89 (1.42 – 5.85)	
Maspin – nuclear expression				
Absent	19 (45.2)	0.425	---	---
Present	34 (56.7)		---	
NM23 – cytoplasmic expression level				
Low	19 (54.3)	0.691	---	---
Medium/high	34 (50.8)		---	

[a] statistically significant differences

vival time (Table 2). Moreover, trends toward a longer survival time in patients with absence of vascular invasion and presence of lymphocytes in tumor tissue were observed, even though the dependencies were not statistically significant (p=0.086 and 0.083, respectively). Likewise, age up to 60 years was associated with better survival rate despite an absence of statistical significance (p=0.057). Overall survival probability according to NM23 and maspin cytoplasmic expression levels and the

presence of nuclear maspin expression were shown in Figure 2A, B and C), respectively.

As independent prognostic factors, age (p=0.021), distant metastases (p=0.00007) and maspin cytoplasmic medium high expression level (p=0.003) were identified. Hazard ratios with their 95% confidence intervals and p-values were summarized in Table 2.

Discussion

Currently, conventional clinical and pathomorphological features like tumor stage or grade remain the best tools for predicting subsequent disease course in colorectal cancer. However, clinical outcome often vary considerably among patients with the same remaining characteristics. It is likely that there are some other biological factors that could influence tumour progression and metastasis and because of that be responsible for the observed changeability.

In this study, the expression of Nm23-H1 and maspin, proteins proposed as tumor and metastasis suppressor were analysed in a series of colorectal adenocarcinomas. To assess their applicability as prognostic factors in this type of cancer, the expression of both proteins with established prognostic features and survival time of patients suffered from colorectal cancer was compared.

Most of investigated specimen were Nm23-H1 positive, which correlates with the results obtained by Lee et al. [22]. Similarly to the others [22, 23] it was found that positive immunostaining for Nm23-H1 was primarily confined to the cytoplasm. Medium or high expression level of Nm23-H1 in cytoplasm of cancer cells was stated significantly more frequent in tumor invading deeper layers of the intestine wall (T3, T4). Despite the presence of blood vessels invasion, one of the crucial steps in metastasis process was remarkably connected with medium or high expression of Nm23-H1, no link between the expression level and presence of lymph node or distant metastases was observed. Higher expression of the protein was not connected with a more advanced Astler-Coller stage. Due to the presence of metastases and higher staging influencing prognosis negatively, the Nm23-H1 expression do not affect survival time of investigated patients. However, when examined, the cases that revealed up to and above 60% of cells stained in IHC were stratified as absent/low/medium and high expression level, respectively, the dependence between the level and stage reached statistical significance. High expression level of Nm23-H1 was connected with tumors of C1, C2 and D according to Astler-Coller (data not shown). In spite of this, lack of influence of Nm23-H1 on presence of metastasis and survival time remained unchanged. Therefore, it seems possible that Nm23-H1 affects local aggressiveness of colorectal cancer but only when its expression is high enough and is not essential for metastatic process.

We found the medium or high expression of Nm23-H1 was significantly more frequent in tubular than in mucinous colorectal adencarcinoma, which confirm results obtained by Dursun et al. [24]. Since the tubular type of the adenocarcinoma is connected with better prognosis than the mucinous type, it could – at least in part – eliminate the impact of Nm23-H1 on local invasiveness of cancer and explain the lack of effect of the protein on survival probability.

Maspin was detected in cytoplasm in all tested samples, but in almost one fifth of cases at a very low level. Considering this fraction of specimen as negative, the percentage of positive reaction would be comparable with findings published so far [8]. The obtained results indicate that higher expression level of maspin is associated only with bad prognostic features. Medium or high cytoplasmic expression level of maspin was connected with poorly differentiated tumours (G3) which have high proliferation rate. Accordingly, the statistically significant association between the level of the examined protein and depth of tumor invasion (T3, T4) was shown. Moreover, the level of maspin was associated with the presence of nodal (N1, N2) and distant metastases (M1), vascular invasion and higher Astler-Coller stage (C1, C2, D). This data suggest that maspin could play a role in both local invasion of cancer and metastases formation. Consequently, the level of protein expression was connected with lower survival probability of patients suffering from colorectal cancer independently of any established prognostic factors. Cox regression revealed that patients with medium or high expression of maspin have almost three times higher risk of death compared to those with low expression.

Previously it was suggested that maspin may act as a transcription factor if it is expressed in nucleus [12]. Two studies prior to this one, showed that nuclear maspin expression could be associated with either tumors with mutator phenotype [25] or a shorter overall survival time [26] in colorectal cancer patients. In this study, nuclear maspin expression was observed in almost 60 percent of cases. However, there was no statistically significant association between the presence of nuclear expression of the protein and any clinicopathological features. Similarly, maspin localized in nucleus had no impact on survival probability of patients. In the light of the presented results, the role played by maspin in nucleus is not crucial for disease progression and prognosis in colorectal cancer.

Since higher expression level of both Nm23-H1 and maspin proteins was found to be connected mostly with adverse prognostic features, like depth of tumor or vascular invasion, it can be suggested that both investigated proteins show rather oncogenic than suppressor activity.

Measurements of level and cellular pattern of maspin expression could be valuable for predicting disease course in patient suffering from colorectal cancer. Considering contradictory findings with regard to Nm23-H1, some further studies are needed.

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