Maspin and Ezrina - Biomarker Molecules in Colorectal Cancer

Correlative immunohistochemical study

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The results of the recent years researches support the need for personalized therapeutic of cancer by completing the clinical, imagistic and histopathological diagnosis with molecular studies to identify new useful biomarkers for diagnosis, prognosis and tumor progression. Maspin is a non-inhibitory serine protease having a proapoptotic activity, suppressor of tumor invasion, metastasis and angiogenesis. Ezrin is a member of Ezrin/Radixin/Moesin (ERM) family, involved in cellular adhesion mechanisms, motility and invasiveness of tumor cells. In colorectal tumors, there is a heterogeneity of research results regarding the clinical significance of the maspin due to a possible partnership with other molecules with which it interacts through the same signaling pathways. Our study investigated the two molecule's immunoreactivity (IR) in 92 colorectal tumors highlighting an inverse correlation between ezrin's and maspin's expression, suggesting the fact that ezrin's overexpression could influence maspin's tumoral suppressor role. Furthermore there was observed a difference of the molecules IR within the same tumoral stage, suggesting their utility regarding the treatment protocol of these tumors.

Keywords: ezrin, maspin, colorectal tumors, biomarkers, correlation

The major goal of recent research on carcinogenesis in various tumor types has been both to identify and clarify the molecular mechanisms and to find new predictive and prognostic biomarkers. The transfer of data obtained from experimental research to clinical management of these tumors is often difficult due to the molecular interrelations at cell level, tissue context and genetic background of each individual; moreover, in the case of some molecules, the heterogeneity in the results among these studies on the same tumor type is also added.

Such an example is represented by the results of studies aimed at evaluating maspin's and ezrin's expression in different trumor types. Maspin is a non-inhibitory serin protease which suppresses tumor invasion, metastasis and angiogenesis [1,2]. Multiple studies shows that maspin is a molecule whose expression is modulated by p53, being the only serpin involved in apoptosis regulation [3,4]. The functions of the molecules belonging to the serpin family are due to a structural feature known as the serpin reactive site loop (RSL). These molecules exhibit intracellular localization but can also function extracellularly. Most of them are inhibitory proteases, except maspin, having structural similarities to ovalbumin [5,6]. Maspin, at molecular level, has some specific features that support its inclusion in the non-inhibitory serine protease category: the presence of a hydrophobic reactive site loop (RSL) shorter than that of the rest of the serpins, which is thought to decrease the stability of the molecule and justify the inability to make the transition from stressed to relaxed state. However, research has shown that the RSL peptide alone is sufficient to induce the adhesion of malignant breast tumors cells to fibronectin [5,6]. Maspin's crystal structure analysis has identified a new domain specific to it - the G á helix area or the P1 position of the RSL that is capable of presenting a relaxed/stressed configuration by redistributing the charged residues. This conformational modification of the molecule's region is specific to maspin, and research indicates that this domain is important for binding molecules from the extracellular matrix (ex: á1 and â1 integrin), thus being essential for maspin's involvement in cell migration [5,6]. Considering these elements, it was expected that studies on maspin expression in various tumor types would provide clinically relevant and applicable information [1].

However, the results of maspin's expression evaluation in different tumor types are heterogeneous and do not

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always demonstrate its suppressive effect, as well as studies that have found no correlation between its level of expression and the invasive phenotype of tumor cells [3,5,7,8]. Thus, several studies correlate maspin overexpression and/or predominantly nuclear expression with good prognosis and increased survival rate while others highlight a positive correlation between its overexpression and the aggresive phenotype of some tumors [9]. Sometimes, heterogeneous results are reported for the same cancer type [1].

According to the studies by Dzinic et al. and Locket et al., these conflicting results may be due to the modulation of maspin subcellular localization by some molecular partners that in their turn are influenced by the activation of signaling pathways specific to malignant transformation [3,10].

The other studied molecule, considered the most important member of the ERM (ezrin/radixin/moesin) family, is expressed into a variety of epithelial or non-epithelial malignant tissues. Usually it is distributed at cytoplasmic level in an inactive state and presents an N-terminus and a C-terminus end which contains a FERM respectively an ERM domain. Ezrin's activation depends on binding of PIP2 (phosphatidylinositol 4, 5 biphosphate) to the N-terminus end and threonine T567 phosphorylation in the C-terminus domain. The active state's stability it is given by binding the actin filaments (via C-terminus) and to membrane protein membranes (ex. EBP50, CD44, ICAM-2) via the N-terminus end. In the active state, N-terminus end binds NHERF (Na+/H+ exchanger regulatory factor) [11].

Ezrin plays a role in the modulation of many cellular mechanisms, including intercellular adhesiveness, motility, being involved in the development and evolution of malignant tumors. A series of studies associate its overexpression with the metastatic process and the poor prognostic for digestive system tumors, HNSCC, osteosarcomas, gynecological tumors [12] while studies of Andersson et al, Palou et al, Moilanen et al, Palmerini et al have opposite results [13-16].

In view of these aspects the present study evaluated ezrin immunohistochemical expression correlated with maspin's expression in colorectal carcinomas in order to highlight possible interrelationships between these two molecules and to indentify a possible molecular pattern specific to colorectal cancer.

Experimental part

Material and methods

Between February 2017 and April 2018, at the Iasi Regional Cancer Institute and Iasi Railways Hospital normal and tumor tissue fragments were collected from 92 patients aged 49 to 75 years, diagnosed anatomo-patologically with CRC in the following stages: T2N0M0 - 17 patients, T3-4N0M0 – 54 patients and T3-T4N0-2M0-1-21 patients, who went for primary tumor resection. Informed consent was obtained from each patient prior to surgery.

None of these patients underwent chemotherapy, radiotherapy or any other anti-tumoral treatment prior to sampling and all of them survived more than 6 months after diagnosis.

After the usual H&E staining aimed in order to obtain a histopathological diagnosis, from the same tumoral fragments, some of them were processed using the immunohistochemical technique using Anti-Maspin mouse monoclonal Antibody C-8, Santa Cruz Biotechnology® in order to evidentiate maspin's immunoreactivity (IR) and others were processed using the same technique but using Anti-Ezrin antibody produced in rabbit, by SIGMA-ALDRICH®. Also, normal colonic tissue fragments were procesessed through the same immunohistochemical technique, using the same antibodys for the control group. The sections were placed on SuperFrost® Excell microscope slides, incubated with Endogenous Enzyme Block, then the primary antibodies were applied (Anti-Maspin Antibody in a 1:120 dilution and Anti-Ezrin antibody in a 1:100 dilution), application of secondary antibodies type Dako EnVisionTM + Dual Link System-HRP0, TBS wash, DAB application followed by TBS wash, hematoxylin for counterstaining and Entellan® as mounting medium.

Slides were then examined using an Olympus BX40 microscope with Olympus E330 camera at the Department of Cell and Molecular Biology of the Grigore T. Popa University of Medicine and Pharmacy, Iasi, Romania.

Assessment of maspin and ezrin immunoreactivity

Immunohistochemical expression of maspin and ezrin was considered positive when we detected immunoreactivity in more than 10% of all tumor cells, and lymphocytes were used as reference for the intensity of immunoreactivity: +++ (strong) similar to lymphocyte staining intensity, ++ (moderate), + (weak) when still distinct from basic staining and - (negative) when present only in 0-10% of the total tumor cells.

Statistical analysis

Associations between clinical-pathological variables (tissue type, tumor stage, dissemination, and presence of metastases) were analyzed using Chi-square tests and the occurrence frequency of events was compared using IBM SPSS Version 18.0 for Windows (SPSS Inc, Chicago, IL).

Results and discussions

In ezrin-labeled samples, examination of normal human colon tissue fragments revealed a moderately positive (++) membrane immunoreactivity (IR), negative (-) cytoplasmic IR and weakly positive (+) nuclear IR (Figure 1). As to maspin, normal tissue showed negative (-) membrane IR, moderately positive (++) cytoplasmic and weakly positive (+) nuclear IR (Figure 2).

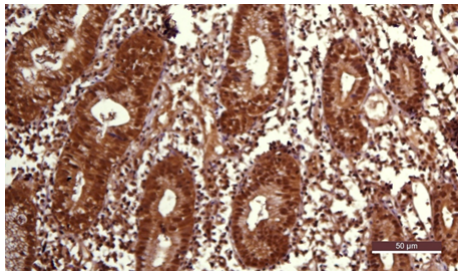

Fig. 1. Ezrin IR in normal colon tissue

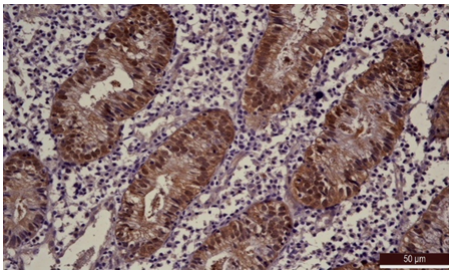

Fig. 2. Maspin IR in normal colon tissue

Of the T2-3N0M0 stage tumors, in 68 of them ezrin IR was weakly positive (+) in membranes, moderately positive (++) in the cytoplasm and negative (-) in nuclei (Figure 3, Tabel 1), while 65 of these tumors showed for maspin, a weakly positive (+) IR at membrane level, intensely positive (+++) cytoplasmic, and moderately positive (++) at nuclear level (fig. 4, Tabel 2). Having categorical data, we used Chi-square tests to compare frequencies of occurrence of events, with statistically significant results for comparing ezrins's and maspin's subcellular IR with the TNM stage ($p < 0.01$) (Tabel 3,4). In 3 of the cases, ezrin IR was negative (-) in membranes, intensely positive (+++) in the cytoplasm and negative (-) in nuclei, as in the 21 tumors in advanced stages T3-4N0-2M0-1 (Figure 5, Tabel 1).

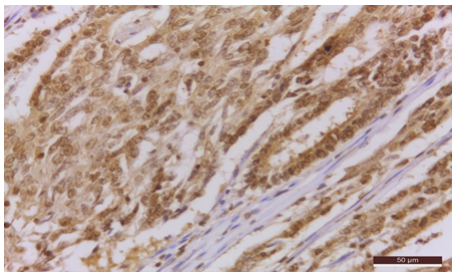

Fig.3. Ezrin IR in T2-3N0M0 tumors

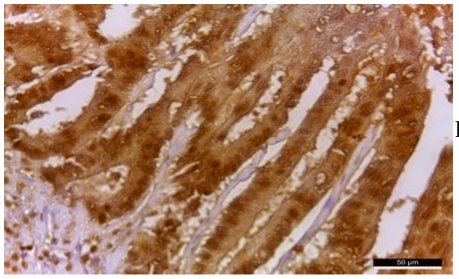

Fig. 4. Maspin IR in T2-3N0M0 tumors

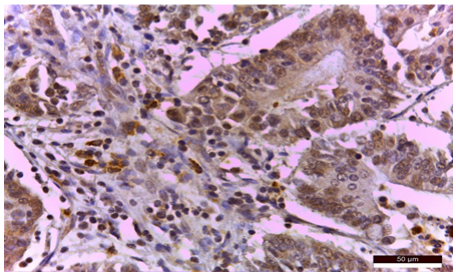

Fig. 5. Ezrin IR in T3-4N0-2M0-1 tumors

In 6 of the T2-3N0M0 tumors, maspin IR was negative (-) in membranes, moderately positive (++) in the cytoplasm, and only weakly positive (+) in nuclei, similar to the results obtained in advanced stage tumors T3-4N0 - 2M0-1 (Figure 6, Tabel 2). The results indicate for T3-4N0-2M0-1 tumors an increased cytoplasmic ezrin expression (+++) and absence of membrane and nuclear IR, while for maspin a similar membrane IR (-) accompanied by a moderately positive cytoplasmic expression (++) and weakly positive (+) nuclear expression were found (Tabel 1,2).

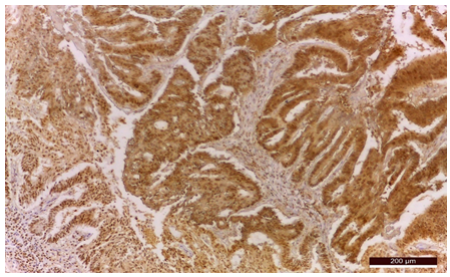

Fig. 6. Maspin IR in T3-4N0-2M0-1 tumors

The results regarding these two molecules IR revealed in most localized stage tumors the existence of an inverse correlation between their level of expression: moderately positive (++) cytoplasmic and negative (-) nuclear ezrin IR compared to intensely positive (+++)) cytoplasmic and moderately positive (++) nuclear maspin IR, with relevance for their values as predictive indices.

The goal of the experimental studies in the latest years was to highlight the molecular changes occuring during tumorigenesis by identifying the expression of some molecules essential in the functioning of certain signaling pathways and cellular mechanisms. These can lead to improvement of screening tests and to establishing relevant biomarkers for the tumor progression, with prognostic value and useful in designing personalized therapy.

Recently, ezrin and maspin were identified as being among the molecules whose expression was found to be altered in tumor cells and which could potentially represent predictive and prognostic biomarkers in several tumors with different locations [17-19]. At present, there is a heterogeneity in the results among the studies on the expression of maspin and its prognostic value in various tumor types compared to its clearly suppressive effects

		NR.	EZRIN IMMUNOREACTIVITY		
			MEMBRANE	CYTOPLASMIC	NUCLEAR
COLORECTAL TISSUE	Normal Tissue	92	++	-	+
	Tumoral Tissue	71 (77,2%) T2N0M0	68 +	68 ++	-
		T3N0M0	3 -	3 +++	
		21(22,8%) T3-4N0-2M0-1	-	+++	-

Tabel 1
EZRIN IMMUNOREACTIVITY AND THE TUMOR STAGE.

		NR.	MASPIN IMMUNOREACTIVITY		
			MEMBRANE	CYTOPLASMIC	NUCLEAR
COLORECTAL TISSUE	Normal Tissue	92	-	++	+
	Tumoral Tissue	71 (77,2%) T2N0M0	65 +	65 +++	65 ++
		T3N0MO	6 -	6 ++	6 +
		21 (22,8%) T3-4N0-2M0-1	-	++	+

Tabel 2
MASPIN IMMUNOREACTIVITY AND THE TUMOR STAGE

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
TNM_Stage * Membrane	92	100.0%	0	0.0%	92	100.0%

Stadiu_TNM * Membrane Crosstabulation

			Membrane		
			-	+	Total
TNM_Stage	T2N0M0 / T3N0M0	Count	3	68	71
		% within TNM_Stage	4.2%	95.8%	100.0%
	T3-4N0-2M0-1	Count	21	0	21
		% within TNM_Stage	100.0%	0.0%	100.0%
Total		Count	24	68	92
		% within TNM_Stage	26.1%	73.9%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	77.099[a]	1	.000		
Continuity Correction[b]	72.211	1	.000		
Likelihood Ratio	80.754	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	92				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.48.

b. Computed only for a 2x2 table

TabeL 3a

COMPARISON BETWEEN EZRIN MEMBRANE IR AND TNM STAGE

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
TNM_Stage * Cytoplasmic	92	100.0%	0	0.0%	92	100.0%

Stadiu_TNM * Cytoplasmic Crosstabulation

			Cytoplasmic		
			++	+++	Total
TNM_Stage	T2N0M0 / T3N0M0	Count	68	3	71
		% within TNM_Stage	95.8%	4.2%	100.0%
	T3-4N0-2M0-1	Count	0	21	21
		% within TNM_Stage	0.0%	100.0%	100.0%
Total		Count	68	24	92
		% within TNM_Stage	73.9%	26.1%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	77.099[a]	1	.000		
Continuity Correction[b]	72.211	1	.000		
Likelihood Ratio	80.754	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	92				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.48.

b. Computed only for a 2x2 table

Tabel 3b

COMPARISON BETWEEN EZRIN CYTOPLASMIC IR AND TNM STAGE

identified by in vitro studies and in normal tissues. There are also a series of questions raised by some studies [3,4] regarding on possible interactions with other cytoplasmic and/or membrane-associated protein molecules that could justify these contradictory results. For example, studies by Umekita et al., Hirai et al. and Sood et al. found an association between maspin overexpression and an aggressive phenotype in ductal carcinomas and a poor prognosis in ovarian and lung cancer [20-22], similar to the findings of Pasz-Walczak et al. who associated moderate or elevated cytoplasmic IR with poorly differentiated tumors and increased proliferation rate [23]. Pföhler et al. correlated maspin expression in tumor cells at the invasive margins

of melanomas with their aggressiveness [24]. Also, some of these studies suggest that only the nuclear maspin nuclear expression has a tumor suppressor role, acting as a transcription factor, at least in breast and ovarian carcinomas [25,26].

For maspin, a molecule belonging to the serpin (serine protease inhibitors) family, the only one classified as non-inhibitory serpin, our immunohistochemical study revealed in the normal colorectal tissue a moderately positive (++) cytoplasmic and weakly positive (+) nuclear IR, in agreement with literature results.

Subcellular localization of maspin in normal tissues depends on the cell type and was identified at both cytoplasmic and nuclear levels, but according to a number of studies also at the level of secretory vesicles bound to the external face of plasma membrane and even in the extracellular matrix as a molecule secreted on non-traditional ways [4,5]. Due to these findings, it is believed to be involved in numerous mechanisms such as cell adhesion, a bridge between the plasminogen activator system and â1 integrin, modulation of some integrine types

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
TNM_Stage * Membrane	92	100.0%	0	0.0%	92	100.0%

TNM_Stage * Membrane Crosstabulation

			Membrane		
			-	+	Total
TNM_Stage	T2N0M0 / T3N0M0	Count	6	65	71
		% within TNM_Stage	8.5%	91.5%	100.0%
	T3-4N0-2M0-1	Count	21	0	21
		% within TNM_Stage	100.0%	0.0%	100.0%
Total		Count	27	65	92
		% within TNM_Stage	29.3%	70.7%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	65.509[a]	1	.000		
Continuity Correction[b]	61.168	1	.000		
Likelihood Ratio	70.234	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	92				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.16.
b. Computed only for a 2x2 table

Tabel 4a
COMPARISON BETWEEN MASPIN MEMBRANE IR AND TNM STAGE

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
TNM_Stage * Cytoplasmic1	92	100.0%	0	0.0%	92	100.0%

Stadiu_TNM * Cytoplasmic1 Crosstabulation

			Cytoplasmic1		
			++	+++	Total
TNM_Stage	T2N0M0 / T3N0M0	Count	6	65	71
		% within TNM_Stage	8.5%	91.5%	100.0%
	T3-4N0-2M0-1	Count	21	0	21
		% within TNM_Stage	100.0%	0.0%	100.0%
Total		Count	27	65	92
		% within TNM_Stage	29.3%	70.7%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	65.509[a]	1	.000		
Continuity Correction[b]	61.168	1	.000		
Likelihood Ratio	70.234	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	64.797	1	.000		
N of Valid Cases	92				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.16.
b. Computed only for a 2x2 table

Tabel 4b
COMPARISON BETWEEN MASPIN CYTOPLASMIC IR AND TNM STAGE

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
TNM_Stage * Nuclear1	92	100.0%	0	0.0%	92	100.0%

TNM_Stage * Nuclear1 Crosstabulation

			Nuclear1		
			+	++	Total
TNM_Stage	T2N0M0 / T3N0M0	Count	6	65	71
		% within TNM_Stage	8.5%	91.5%	100.0%
	T3-4N0-2M0-1	Count	21	0	21
		% within TNM_Stage	100.0%	0.0%	100.0%
Total		Count	27	65	92
		% within TNM_Stage	29.3%	70.7%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	65.509[a]	1	.000		
Continuity Correction[b]	61.168	1	.000		
Likelihood Ratio	70.234	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	64.797	1	.000		
N of Valid Cases	92				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.16.
b. Computed only for a 2x2 table

Tabel 4c
COMPARISON BETWEEN MASPIN NUCLEAR IR AND TNM STAGE

expression and fibronectin binding at this molecules, cell motility, inhibition of peritumoral angiogenesis, increasing the sensitivity to pro-apoptotic factors only in tumor cells [5]. According to a meta-analysis of Zheng et al., in most tumor cell types maspin has a suppressor effect on the TGF-β/β-catenin/E-cadherin pathway thus contributing to the disruption of cell coupling areas [9].

In the case of tumor cells, maspin study is considered to be a challenge due to the absence of a molecular pattern of its localization and multifaceted biological functions [3]. In the tumors considered in this study, we found positive membrane, cytoplasmic and nuclear IR of different intensities in most T2-3N0M0 stage cases (Tabels 1,2), which represents a change in cellular expression from normal tissue and also an index of a less aggressive phenotype of tumor cells in the context of its tumor suppressing effect revealed by some of the existing studies. This aspect regarding tumor aggressiveness was also suggested by the pattern of ezrin expression, revealed by this study.

However, cytoplasmic and/or nuclear expression of maspin has been shown to have different clinical and prognostic significances in malignant tumors with various localizations, the gastrointestinal ones included [3-9], and the presence of membrane IR suggests, according to the studies by Khalkhali-Ellis et al., its role in tissue remodeling and adhesiveness [27]. According to Zheng's meta-analysis, absence of maspin expression or its subexpression is a potential biomarker of aggressiveness of gastric, esophageal, breast, prostate and oral squamous cell tumors [9]. According to the above- mentioned studies, maspin expression must be correlated with the expression of other molecular partners and/or regulatory molecules with which it interacts.

In colorectal tumors, Song et al. found that those with maspin-positive IR showed a lower peritumoral microvascular density compared to those with maspin-negative IR, and Zhang et al. suggested that maspin acts directly on endothelial cells via VEGF, thus limiting their proliferation [28,29].

According to the results of the present study, 6 of stage T2-3N0M0 tumors showed only cytoplasmic and nuclear maspin IR, similar to that of stage T3-4N0-2M0-1 tumors (Tabel 1,2), which, according to most existing studies, could indicate a more aggressive phenotype of tumor cells, aspect also suggested by ezrin IR.

As to ezrin, member of ezrin/radixin/moesin (ERM) family, predominantly expressed in certain regions of plasma membrane, it is involved in connecting the membrane cytoskeleton to cell membrane through signaling molecules and signal transduction pathways at this level [29]. Studies have shown that it has a role in regulating cell adhesion, proliferation, migration processes, the functioning of some important signaling pathways essential for these cellular mechanisms [29].

In the last decade, the results of both *in vivo* and *in vitro* studies indicate that ezrin plays an essential role in the process of tumor metastasis in osteosarcomas, breast, nasopharyngeal, prostate, ovarian and gastric tumors, as well as an association between ezrin overexpression and a poor prognosis of these tumors [29]. The same studies revealed a change in ezrin subcellular localization in tumor cells. For example, ezrin cytoplasmic expression is associated with increased invasive potential in breast tumors and its cytoplasmic overexpression is a marker of aggressiveness in squamous cell carcinomas compared to a series of premalignant lesions such as actinic keratosis, keratoacanthomas, seborrheic keratosis and Bowen's disease [30,31]. These findings are consistent with the results of our research team unpublished yet.

The study by Wei et al. shows that ezrin is overexpressed in 93% of gastrointestinal tumors and is associated with a low survival rate [32]. In colorectal tumors, some heterogeneity is observed with respect to the prognostic and predictive value of ezrin expression and subcellular localization, but not on the response of tumor cells to 5-FU treatment, but there is a consensus on the correlation between ezrin subexpression or even absence and a better tumor response to 5-FU treatment [33,34].

The results of the present study are similar with those of other studies on ezrin subcellular expression and localization in the normal colon tissue and for tumor tissue reveals a correlation between tumor stage and ezrin subcellular expression and localization. Based on these

results, a stratification of tumors in the same stage of development was noticed. Thus, a moderately positive (++) cytoplasmic ezrin IR with the preservation of an weakly positive (+) membrane IR in most localized stage tumors (68), compared to the 3 cases same stage tumors but with cytoplasmic ezrin overexpression (+++) and negative membrane and nuclear IR (-) may be an important indicator for the clinician to establish a more personalized therapeutic strategy. The invasive tumor cell phenotype suggested by ezrin cytoplasmic overexpression is due, among others, probably to the activation of the NF-kB signaling pathway by a mechanism involving this cytoskeletal protein necessary for the phosphorylation of pIkB (inhibitory protein of kB nuclear factor), knowing the fact that the activation of this signaling pathway is involved in a series of cellular changes in the case of immune response, inflammation and oncogenesis [35].

Ezrin cytoplasmic overexpression detected in some of the studied tumors may also indicate increased expression of activated c-Src according to the studies by Elliot et al. They also showed the in breast carcinomas a correlation between activated c-Src expression, ezrin overexpression, and disassembly of cadherin-based cell-cell adhesion by enhanced β-catenin phosphorylation [36]. Li et al. reported the identification of a correlation between ezrin cytoplasmic and E-cadherin overexpression in breast carcinomas followed by metastases compared with the less invasive ones [30]. Studies by Elliot et al. revealed that integrin signaling is directed through ezrin and c-Src to the cytoskeleton thereby modulating the invasive tumor cell phenotype [36]. All of these aspects suggest complex molecular interactions of ezrin in the malignantly transformed cells.

As it results from the immunohistochemical study, the expression of these two molecules is inversely correlated with the differences within the same tumor stage - weak (+) and moderately positive (++) ezrin IR/intensely positive (+++) cytoplasmic maspin IR, intensely positive (+++) cytoplasmic ezrin IR/moderately positive (++) cytoplasmic for maspin IR (Tabels 1,2) – suggestive for a more personalized therapeutic strategy. Establishing a personalized therapy in the early stages of tumors with similar histopathological features is a challenge for clinicians, and in this context the correlation of expression of these two molecules brings important information. Also, in these tumors, localization of these two molecules IR indicates, according to literature studies, a good response to 5-FU therapy [17,26,37,38].

The contradictory results of *in vivo* studies mainly related to maspin expression call into question its relevance as a clinical and prognostic biomarker on its own, and we believe that the hypotheses regarding the existence of some partner molecules that influence maspin's tumor suppressor role are warranted. A series of studies have shown that maspin expression in the tumor cell membrane causes changes in the expression of some proteins associated with the actinic cytoskeleton, resulting in a reduction in their metastatic potential. Maspin's RSL appears to mediate the binding of some membrane proteins through which it is realised the cell adhesion to type I collagen and to the fibronectin from the extracellular matrix. Also, maspin regulates cell invasiveness and modifies the integrin profile of the tumor cell indicates. This facts indicate the existence of a possible reciprocal influence between maspin and ezrin [5,6]. This hypothesis is supported by the results of research that showed that the functionality of integrins, whose expression is influenced by the maspin, within the signaling pathways, is directly modulated and directed via ezrin [36]. Given all these aspects and the localization of both molecules at membrane, cytoplasmic and nuclear levels in the case of colorectal tumor cells, the existence of an inverse correlation between their expression revealed by our study, their involvement in the activation and functioning of some signaling pathways with a role in colorectal oncogenesis, cytoskeleton-membrane connections, and implication in mantaining the integrity of cellular coupling zones [29], we believe that a molecular partnership between ezrin and maspin in colorectal tumor progression is likely. At this level, the expression and localization of ezrin along with the presence of its phosphorylation mechanism could influence the functionality of maspin expression or viceversa (maspin's expression at membrane and cytoplasmic level can influence ezrin's status or/and it s functionality at actin citoskeleton level) and may be one of the reasons for the contradictory results on the relationship between maspin expression and tumor progression.

We believe that further molecular studies on large series of patients are needed to correlate the molecular details with the expression and localization of these two molecules and the tumors evolution.

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