The expression of cytoskeleton regulatory protein Mena in colorectal lesions

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Abstract
The actin regulatory proteins Ena/VASP (Enabled/Vasodilator stimulated phosphoprotein) family is involved in the control of cell motility and adhesion. They are important in the actin-dependent processes where dynamic actin reorganization is necessary. The deregulation of actin cycle could have an important role in the cells’ malignant transformation, tumor invasion or metastasis. Recently studies revealed that the human orthologue of murine Mena is modulated during the breast carcinogenesis. In our study, we tried to observe the immunohistochemical expression of mammalian Ena (Mena) in the colorectal polyps and carcinomas. We analyzed 10 adenomatous polyps (five with dysplasia) and 36 adenocarcinomas. We used the indirect immunoperoxidase staining. BD Biosciences have provided the Mena antibody. We observed that Mena was not expressed in the normal colorectal mucosa neither in polyps without dysplasia, but its expression was very high in polyps with high dysplasia. In colorectal carcinomas, Mena marked the tumoral cells in 80% of cases. In 25% of positive cases, the intensity was 3+, in 60% 2+ and in the other 15% 1+. The Mena intensity was higher in the microsatellite stable tumors (MSS) and was correlated with vascular invasion, with intensity of angiogenesis marked with CD31 and CD105 and with c-erbB-2 and p53 expression. This is the first study in the literature about Mena expression in colorectal lesions.

Keywords: colorectal carcinomas, Mena immunostain, microsatellite instability, angiogenesis.

Introduction
Cell motility is very important for physiological tissue formation and development of organisms. To realize this motility, the cells use the actin cytoskeleton, considered the engine of cellular migration [1].

The following proteins promote the actin nucleation and polymerization: N-WASP/Arp2/3 (Wiskott Aldrich syndrome protein/Actin related protein), DRFs (Diaphanous-related formins) and Ena/VASP proteins (Enabled/Vasodilator stimulated phosphoprotein). The Ena/VASP family members are mammalian Ena (Mena), VASP and Ena-VASP-like (EVL) [2].

The actin regulatory proteins Ena/VASP family, involved in the control of cell motility and adhesion are a structurally conserved family found in vertebrates, invertebrates and Dictyostelium [3]. They are important in the actin-dependent processes where very dynamic actin reorganization is necessary, such as axon guidance, neural tube closure, attenuation of platelet aggregation, phagocytosis, T-cell activation, fibroblasts’ motility and the intracellular movement of the bacterial pathogen Listeria monocytogenes and Shigella flexneri [4–7]. These proteins play an important role in linking signaling pathways to remodeling the actin cytoskeleton but the exact mechanism is not known [3]. In mouse tissues, Mena expression was observed in brain, stomach, intestine, blood vessels, glomerular mesangial kidney cells and cellular membranes of epithelial cells. The mouse tissues Mena expression decreases from neonatal to adult animals [8].

The deregulation of actin cycle cause aberrant cell motility. In the embryo, the consequence is deregulation of organogenesis. At the adult, this process could have an important role in cells’ malignant transformation, tumor invasion and metastasis [1].

Recent studies revealed that Mena is modulated during the breast carcinogenesis. The human orthologue of murine Mena (hMena, ENAH) is overexpressed in 70% of primary breast cancers, 90% of metastatic breast tumors, but also in 66% of atypical hyperplasia or atypical papilloma, known like the two high-risk benign breast lesions [9, 10]. The treatment with Herceptin down-modulates hMena expression and neuregulin-1 up-regulates its expression, it suggesting that hMena couples tyrosine kinase receptor signaling to the actin cytoskeleton [10]. hMena could be an early marker of breast carcino genesis and could help at the treatment indication [12].
The role of Mena in colorectal carcinogenesis, tumor invasion and its possible prognostic and predictive role in colorectal carcinomas (CRC) was not studied yet. In this paper, we analyzed the Mena immunostain in colorectal polyps and carcinomas.

In CRC, we correlated the Mena expression with classical prognostic factors, microsatellite instability, the angiogenesis and other immunohistochemically markers (bcl-2, c-erbB-2, p53, Ki67, E-cadherin).

Material and methods

Surgical specimens from 46 patients (12 females and 34 males) with colorectal polyps and carcinomas diagnosed in the Department of Pathology of Emergency Hospital Targu Mures, Romania, were used for IHC staining. The median age was 66, range 35–77. We analyzed 10 polyps (5 with dysplasia) and 36 carcinomas (15 non-mucinous, 10 mucinous adenocarcinomas and three signet-ring-cell carcinomas). From all carcinomas, 24 cases were in pT3, six in pT2 and six in pT4, according to pTNM staging. Twelve cases presented lymph node involvement. Eighteen cases were in B, 12 in C and six in D stage, according to Dukes-MAC staging. Twenty cases were from left colon, the other 26 being located in the right colon.

For the immunohistochemical staining, we used the following antibodies, provided by LabVision: CD31 clone JC/70A, CD105 clone SN6h, VEGF clone VG 1, p53 clone DO-7, Ki67 clone Ki-S5 and E-cadherin clone NCH-38. c-erbB-2 provided by DAKO. The murine Mena antibody, izotype mouse IgA, clone NCH-38 was provided by BD Biosciences.

We used the immunoperoxidase staining, in formalin-fixed, paraffin-embedded sections. The deparaffinized sections were incubated at 100°C in citrate solution, pH 6.0 (CD31, p53, Ki67, E-cadherin, c-erbB-2, bcl-2) or in EDTA, pH 9 (CD105, p53) and the primary antibody was applied for 60 minutes.

For Mena we used the indirect immunoperoxidase staining. After incubation at 100°C in citrat solution pH 6.0, hydrogen peroxide incubation and incubation with primary antibody for 60 minutes, the sections were incubated with Primary Antibody Enhancer Solution for 20 minutes and with Large Volume HRP Polymer Solution for other 30 minutes.

For all sections, the development was performed with substrate-chromogen solution 3,3′-diaminobenzidine dihydrochloride (DAB) for 3–5 minutes. The nuclei were stained with Mayer’s Hematoxylin.

The Mena immunostaining was observed in the cytoplasm of the tumoral cells. The intensity of Mena was scored according to the following criteria: score 0, no staining; score 1+, weak diffuse cytoplasmic staining in <10% of cells; score 2+, moderate cytoplasmic staining in 10% to 70% of cells; score 3+, strong cytoplasmic staining in >70% of the cells. The same criteria for intensity quantification was used for c-erbB-2, bcl-2, VEGF and E-cadherin. 15% of positive nuclei were the cutoff for p53 and Ki67.

The intensity of angiogenesis evidentiated with CD31 and CD105 was morphometrical counted. To determine the endothelial area we used the pictures by “hot-spot” regions, at 400 high power fields. The pictures were realized with Nikon 800E microscope and were made in the intratumor surface. The count was made using NIH’s ImageJ program-Trial Version. We measured the positive vessels’ area (endothelial area) versus total tissue area ratio.

To determine the microsatellite instability, DNA was extracted from formalin-fixed, paraffin-embedded tissues. We used the Real-Time PCR (Roche GmbH, Mannheim, Germany), melting point analysis method and the microsatellite mononucleotide markers BAT25 and BAT26 by Roche.

For statistical analysis, we used the Statistical Program GraphPad InStat 3-Trial Version. We used the t-test, chi square test and the contingency tables, Fischer’s test, One way Anova test, determining the values of p and chi. We considered the significant association when p<0.05, with 95% confidence interval.

Results

Mena immunostain was not observed in the normal colorectal mucosa neither in the polyps without dysplasia. In polyps with dysplasia Mena was overexpressed in all cases with 3+ intensity (Figure 1).

In all carcinomas, Mena marked the tumoral cells in 80% of cases. In 25% of positive cases, the intensity was 3+, in 60% 2+ and in the other 15% 1+.

We did not observe statistically significant differences between the grade of differentiation and Mena intensity (p = 0.17) (Figure 2).

All signet-ring-cell carcinomas had the Mena intensity 2+ (Table 1).

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>No. of cases</th>
<th>Mena positive</th>
<th>Mena negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyps without dysplasia</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Polyps with dysplasia</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinomas well differentiated</td>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Adenocarcinomas moderately differentiated</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinomas poorly differentiated</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Mucinous carcinomas</td>
<td>12</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Signet-ring-cell carcinomas</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Neither lymph node involvement nor level of tumoral penetration was correlated with Mena intensity (p = 0.34, respectively 0.92). The gender and age of patients did not present differences regarding Mena expression.

One interesting aspect was that Mena immunostain was significantly higher in carcinomas with vascular invasion than in that without vascular invasion. It was not observed a difference between Mena expression in CRC with and without lymphatic vessels invasion (p = 0.15).

Table 1 – The Mena expression in different colon lesions
The expression of cytoskeleton regulatory protein Mena in colorectal lesions

Figure 1 – Adenomatous polyp: the Mena is overexpressed in glands with dysplasia but is not expressed in the normal glands; ob. 4× (A) and ob. 10× (B)

Figure 2 – The Mena expression does not present difference between well (A) or poorly differentiated adenocarcinomas (B); ob. 10×

Figure 3 – The Mena expression was higher in MSS (A) than in MSI carcinomas (B). This difference was more evidently between MSS (C) and MSI mucinous carcinomas (D); ob. 10× (A, B) and ob. 4× (C, D)
In the MSS (microsatellite stable) tumors Mena intensity was significantly higher than in MSI (microsatellite instability) (Figure 3, A and B). This difference was specially observed in the mucinous adenocarcinomas. Therefore, in the MSI mucinous adenocarcinomas, the Mena intensity was significantly lower than in the MSS mucinous adenocarcinomas (Figure 3, C and D).

Regarding the angiogenesis, in the CRC Mena was not correlated with VEGF expression \( (p = 0.45) \). The intensity of Mena was significantly higher in the tumors with a higher endothelial area marked with CD31 \( (p = 0.02) \) but also with CD105 \( (p = 0.04) \). The intensity of Mena immunostain was correlated with c-erbB-2 \( (p = 0.0001) \) and p53 \( (p < 0.0001) \). Therefore, in the cases where the intensity of these two markers was higher, Mena was also overexpressed. Mena was not correlated with E-cadherin \( (p = 0.11) \) neither with Ki67 \( (p = 0.14) \) or bcl-2 immunostain \( (p = 0.46) \).

### Discussion

It is known that the deregulation of actin cytoskeleton can determine disorders in the tissues' development but in the same time, it may be associated with the cells' malignant transformation [13]. The exact process is not known yet. In our paper, we tried to find if the Mena immunostain is expressed in the premalignant and malignant colorectal lesions. We found that Mena is not expressed in the normal colorectal mucosa neither in the polyps without dysplasia but is overexpressed in the polyps with high dysplasia. In the CRC, its expression was correlated with vascular invasion, c-erbB-2 and p53 immunostain was not correlated with VEGF expression \( (p = 0.45) \).

These statistical correlations prove that Mena overexpression could be an early event in colorectal dysplasia and cancer development. As in the breast lesions and tumors, revealed by Di Modugno F et al. [10], in our study c-erbB-2 was correlated with the intensity of Mena immunostain. Many paper revealed that the p53 negative tumors with MSI had a better prognosis [14]. In our study, the p53 negative carcinomas with MSI had also lower intensity of Mena expression.

Regarding the angiogenesis, it was demonstrated that a high neoangiogenesis is an unfavorable prognostic factor for CRC, it being necessary to be reduced with the antiangiogenetic treatment [15, 16]. In our study Mena intensity was correlated with the CD31 and CD105 expression being overexpressed in the cases with a higher endothelial area. Therefore, the Mena expression could be expressed in the early stages of CRC and seemed to be correlated with that factors which showed an unfavorable prognosis.

### Conclusions

Our study demonstrated that the Mena overexpression in colorectal polyps could be associated with a high risk for malignant transformation. In the malignant tumors, a very bad prognostic it seems to have the tumors with overexpression of Mena \( (2^+, 3^+) \), with vascular invasion, with a high percent for p53 positive nucleus, MSS tumor status and with a high angiogenesis. The p53 negative tumors with MSI and a lower Mena intensity \( (1^+) \) or Mena negative may have a better prognosis.

The small number of cases do not allow yet to state definite conclusions. Because the Mena intensity was correlated with some prognostic factors of colorectal carcinomas, we believe that it is necessary to enlarge the researches about Mena to demonstrating its role in the development of premalignant and malignant colorectal lesions. This is the first study in the literature about Mena expression in colorectal lesions.

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