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Matrix Gla Protein in Meningiomas: An Immunohistochemical Study

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Abstract. *Calcified meningiomas have a slower growth rate and a better outcome than non-calcified meningiomas. Matrix Gla Protein (MGP) acts as an inhibitor of soft tissue calcification. Depending on its carboxylation status MGP may occur in two conformations: uncarboxylated (ucMGP) or carboxylated (cMGP). Low levels of serum ucMGP have been described to be a sign of tissue calcification. In calcified tumoral tissue samples we identified both ucMGP ($r = 0.957$, $p<0.001$) and cMGP ($r = 1$, $p<0.001$), while non-calcified tumors were negative for both MGP conformation deposits. The concentration of serum t-ucMGP in patients with calcified and non-calcified meningiomas were not significantly different (3499±1388 vs. 3882±2558).*

Keywords: *calcification, uncarboxylated matrix gla protein, carboxylated matrix gla protein, immunohistochemistry*

1. Introduction

Meningiomas represent the most common primary tumors of the central nervous system (CNS) with a slow growth-rate and usually with benign characteristics [1]. In adults, they represent approximately 35% of all CNS tumors, and more than 50% of the benign cerebral neoplasms [1]. On the other hand, meningiomas rarely occur in children and adolescents [2]. The prevalence amplifies with age, with a reported 65.2 median age at diagnosis, and is two-fold higher in women than in men [3].

The heterogeneous clinical manifestations of these tumors can emerge spontaneously or in the context of various syndromes [4], in different areas of the CNS, from cells of the outer layer of the arachnoid. This particularity leads to a frequent accidental diagnosis, approximately 3% being discovered only in autopsies [5]. In addition, calcified meningiomas have been found to present a slower growth rate [6] and the lack of tumoral calcification was associated with less favorable outcome [7] and may require a closer observation and repeated follow-ups [6].

Matrix Gla protein (MGP), is one of the extrahepatic vitamin K dependent proteins and is known to act as a strong local inhibitor of soft tissue calcification [8]. Its calcification inhibitory function is only acquired after a posttranslational vitamin K-dependent γ-carboxylation reaction [9]. Besides this primary role of MGP, later studies also showed an important implication of the protein in tumor angiogenesis, as a protein produced by arterial endothelium cells [10]. Depending on the γ-carboxylation status, MGP may occur in two conformations: undercarboxylated MGP (ucMGP) and carboxylated MGP (cMGP). Low levels of serum ucMGP were found to be associated with tissue calcification [11].

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To our knowledge, only one study reporting MGP mRNA expression in meningiomas has been published [12] and none regarding the presence of local MGP in tumoral tissue or circulating ucMGP levels. Granted that MGP mRNA expression was demonstrated in meningiomas, the main purpose of our study was to determine if ucMGP and cMGP are locally present in meningiomas and whether there is a difference in distribution between the two MGP conformations in calcified and noncalcified tumoral tissue. Our second objective was to assess and evaluate serum t-ucMGP levels in patients with and without calcified meningioma.

2. Method and materials

Tissue sample collection and preparation

Tumoral tissue samples were obtained from patients undergoing neurosurgery at the "Bagdasar-Arseni" Clinical Emergency Hospital in Bucharest, after receiving every subject's written consent. The study was approved by the University Medical Ethics Committee and was in accordance with the declaration of Helsinki. After the histopathological diagnosis of meningioma was confirmed by pathologists from the aforementioned hospital, we collected 42 tissue samples. After fixing in formaldehyde, the tissue samples were embedded in paraffin blocks and then placed on a cooling console. Next, with the help of a microtome, we cut sections of 4 μm and mounted them on glass slides. For each subject we prepared 10 sample slides.

Immunohistochemical staining

The immunohistochemical staining for the two MGP conformations was performed using monoclonal mouse antibodies against ucMGP and cMGP (VitaK, The Netherlands), diluted in a reagent containing 0.1% Tween, Tris-buffered saline and 1% bovine serum albumin. The slides were incubated overnight at 4ºC. For the secondary antibody we used 1:100 horse radish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Denmark). NovaRED substrate kit (Vector Laboratories, USA) was applied to expose the antibodies. Cell nuclei were stained with hematoxylin and coverslips were mounted with Entellan (Merck, Germany).

Von Kossa (VK) staining

Sample slides were incubated with 1% silver nitrate for 5 minutes, after they had been deparaffinized and rehydrated. Sodium formaldehyde was applied for 1 minute after washing, and sodium thiosulfate to eliminate the excess silver nitrate. Nuclear fast red counterstained the tissue samples, after which coverslips were applied.

Evaluation of staining intensity score

After performing the immunohistochemical and VK staining, the absence and presence of staining was microscopically evaluated by a pathologist with no prior knowledge of the subjects' clinical background. After a thorough screening of each sample, representative fields of vision were chosen and the staining intensity score was determined by two specialized and independent observers, as follows: 0 = absent, 1 = weak, 2 = moderate and 3 = intense.

Serum sample collection and t-ucMGP assay

After overnight fasting, venous blood was collected by venipuncture from all patients before surgical removal of the tumor. Prior to analysis, the samples were centrifuged and aliquots of serum were stored at -80ºC. Serum t-ucMGP levels were measured by a competitive mono-antibody ELISA kit (VitaK, Maastricht University, The Netherlands) as described previously [13].

Statistical analysis

For statistical analysis, SPSS 15.0 was used (SPSS, Chicago, USA) and statistical significance was based on two-tailed tests at p values < 0.05. The Kolmogorov-Smirnoff test was performed in order to

assess the distribution of continuous variables. The normally distributed variables were expressed as mean ± standard deviation (SD). For the correlations of nonparametric variables, Spearman's Rho coefficient was reported. Student t-test and U Mann-Whitney were performed for differences between groups.

3.Results and discussions

The study population (n=42) was divided in two groups, based on the absence or presence of tumoral calcification. The characteristics of the study population are presented in table 1.

Table 1. Characteristics of the study population

	All (n=42)	**Tumoral calcification**		
		Absent (n=18)	**Present** (n=24)	**p-value**
Demographics				
Age (years)	53 ± 13	53 ± 10	54 ± 15	NS
Gender (m/f)	18/24	9/9	9/15	NS
Local staining of MGP conformations				
ucMGP *n(%)*	22(52)	0(0)	22(92)	<0.05
cMGP *n(%)*	23(55)	0(0)	23(96)	<0.05
Serum t-ucMGP [nM]	3718±2123	3499±1388	3882±2558	NS

The data are presented as mean ± SD or number (percentage), as appropriate. The p-value is given for the the difference between two tumoral groups: absent and present calcification.Of all (n=42) the subjects, 18 were males and 24 were females with ages of 53 ± 13 years. In the Absent group (n=18), subjects without tumoral calcification, both genders were equally represented with 53 ± 10 years of age. Participants with tumoral calcification were included in group Present (n=24). In this group we had 9 males and 15 females with ages of 54 ± 15 years. The gender, age and serum t-ucMGP levels were not significantly different between the two groups.Regarding the local staining of MGP conformations, 22 samples were positive for local ucMGP and in 23 samples we identified local cMGP. All these positive samples were identified as being in the Present group. None of the samples included in the Absent group had local staining of MGP conformations.

We found a strong positive association between calcification (defined as positive VK staining) and the presence of ucMGP ($r = 0.957$, $p<0.001$) and cMGP ($r = 1$, $p<0.001$), respectively.

Furthermore, the analysis of the intensity score of local tissue staining, in the overall meningioma group, demonstrated a positive correlation between VK intensity score and ucMGP intensity score ($r = 0.592$, $p<0.001$), as well as with cMGP intensity score ($r = 0.681$, $p<0.001$). A tissue sample without calcification (left) and one with positive calcification (right) are presented in figure 1. Tissue calcification is represented by the black areas in the right sample. The absence of calcification in the left sample is confirmed by the lack of black staining.

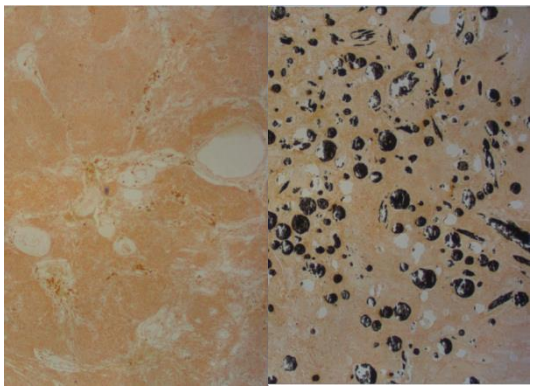

Figure 1. Negative (left) and positive black VK staining (right) in two samples of meningioma tissue (magnification 10x). On the left we present a tissue sample of a non-calcified meningioma. On the right we present a tissue sample of a calcified meningioma. The calcifications are represented by the black areas in the tissue, stained with silver nitrate using the von Kossa method

The calcifications are surounded by collagen and elastin fibers localized in the extracellular matrix of the tumor, they are not calcifications of the blood vessels. From this immunohistochemical staining it is clear that ucMGP was localized around the areas of calcification, as shown in figure 2. The same location was observed for cMGP deposits, as depicted in figure 3.

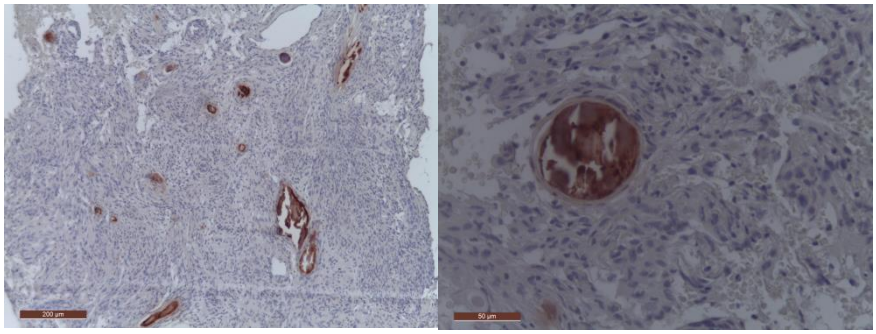

Figure 2. ucMGP (dark pink) around the calcification sites (magnification 10x on the left and 40x on the right). Two parallel captions depicting the deposition of ucMGP on the calcifications present in the extracellular matrix of a meningioma. The depositions are represented by the dark pink areas in the calcified tissue

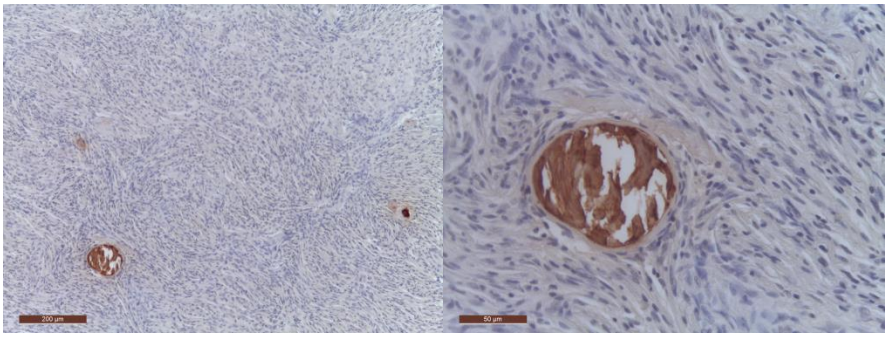

Figure 3. cMGP (dark pink) around the calcification sites (magnification 10x on the left and 40x on the right). Two parallel captions depicting the deposition of cMGP on the calcifications present in the extracellular matrix of a meningioma. The depositions are represented by the dark pink areas in the calcified tissue

Noncalcified tumoral tissue was negative for MGP immunohistochemical staining. Both MGP conformations had a positive staining in calcified meningioma only, and were strongly associated with spots of calcification. There were no correlations between calcified\noncalcified meningiomas and age or gender. Finally, analysis of serum t-ucMGP levels did not show differences between gender, age or immunohistochemical intensity score of the patients.

A search of the literature demonstrated that scientists focused especially on MGP's role in vascular calcification, for example in patients with chronic kidney disease [14], with type 2 diabetes [15] or with minor carotid stenosis [16]. In contrast, we found only two studies concerning MGP and tumoral calcification, one in human meningioma [12] and one in human breast cancer [17], both demonstrating the expression of MGP mRNA in tumoral tissue. In none of these studies a difference is made between cMGP and ucMGP. Therefore, our major objective was to identify both local MGP conformations in meningiomas with and without calcification, as well as serum t-ucMGP levels in this pathology.

Meningiomas usually present calcifications in the form of calcareous structures presenting concentric lamellas, called psammoma bodies with a specific architecture and mechanism, found in the extracellular matrix [18,19], as summarized in figure 4. The mineral deposition occurs without the disruption of calcium metabolism and with normal serum calcium levels [20].

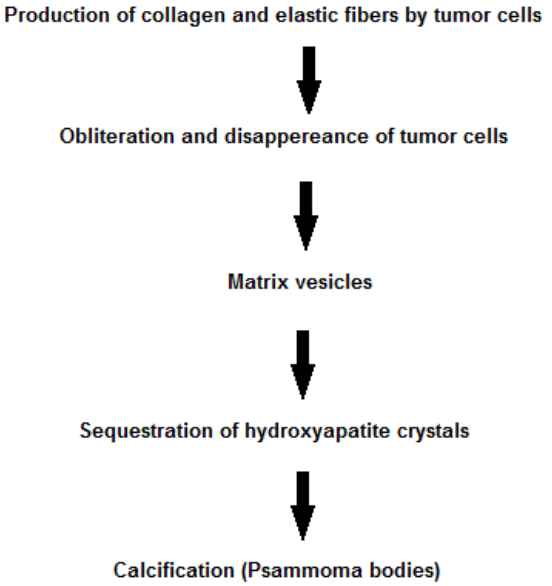

Figure 4. Psammoma body development. The figure represents the mechanism of psammoma body development in meningioma tissue

We were able to confirm that the calcifications observed in our tissue samples are not located in the lumen of blood vessels, but rather they are found in the extracellular matrix, surrounded by collagen and elastin fibers. We assume that the mechanism of calcification in our samples is consistent with the one depicted in figure 4 and it is not one of vascular nature.

We have identified a positive association between calcification and both ucMGP and cMGP in the study population. This is consistent with the idea that calcified deposits in soft tissues strongly induce local MGP synthesis. The fact that part of the MGP was found to be in the uncarboxylated form demonstrates that the local vitamin K status is insufficient to support full carboxylation of all MGP formed around the calcified meningiomas. The only study regarding MGP and meningiomas [12] reported that MGP mRNA was expressed in human meningioma tissue but had no correlation with the calcification sites. We were able to identify the local MGP deposits consistent with the calcifications by using specific monoclonal antibodies against ucMGP and cMGP, which were developed after the aforementioned article was published.

The mechanism through which MGP inhibits local calcification is not yet entirely elucidated. In order for MGP to exert its inhibitory role, it needs to go through a posttranslational γ-carboxylation reaction of glutamate residues, dependent of vitamin K [21]. One theory would be that the carboxylated form binds to calcium crystals inhibiting their growth [22], as summarized in figure 5.

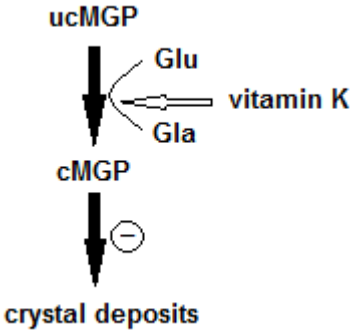

Figure 5. Formation of cMGP and its effect on crystal deposits. The activation of ucMGP through a carboxylation reaction dependent of vitamin K and the inhibitoy effect of cMGP directed towards the calcification

Our study has shown that, while almost all calcified meningiomas were positive for both ucMGP and cMGP, noncalcified meningiomas did not present immunohistochemical staining for the two MGP conformations. We found that only part of the MGP present around the calcified areas is in its active form as cMGP. This raises the question whether vitamin K supplementation or, on the contrary, the use of vitamin K-antagonists (oral anti-coagulants) might have an impact on the progress of the disease.

Another theory postulates that cMGP inhibits the activity of bone morphogenetic protein-2 (BMP-2), a protein demonstrated to induce calcification in the arterial wall [23,24]. One study showed that BMP-2 contributes to the development of calcification in ovarian cancer [25] while, in a later study, positive immunohistochemical staining for BMP-2 was observed around calcifications in meningiomas [26]. In our study, the presence of cMGP at sites of calcification in menigiomas might be explained by the fact that cMGP is secreted into the extracellular matrix in order to inhibit the activity of BMP-2.

Due to the fact that our study design did not include a control group of healthy subjects, we decided to compare the values for the serum assessment with publications that defined a reference range for circulating MGP. The most recent article [27] reported a reference range for total MGP, which was not suitable for comparison, but Cranenburg [13] had established a reference range for serum t-ucMGP. The mean ±SD obtained in our study population for serum t-ucMGP was not significantly different than the mean±SD of t-ucMGP determined for the healthy population in the aforementioned article.

The literature states that decreased serum t-ucMGP levels were found to be associated with increased risk for vascular calcification development [11]. Unfortunately, we were not able to confirm this statement in tumoral calcification, nor were we able to find any differences between subjects without tumoral calcification and those with calcified meningiomas. Therefore, we hypothesize that the circulatory pool of t-ucMGP is not influenced by meningioma calcification.

Local deposits of ucMGP and cMGP were found to be associated with calcifications in meningiomas, while noncalcified tumoral tissue was negative for both protein conformations. The presence of cMGP could be due to its binding to calcium crystals in the calcification or an increased secretion in the extracellular matrix to inhibit the activity of BMP-2. A local vitamin K deficiency could explain the presence of ucMGP in calcified meningiomas. Tissue ucMGP does not influence the serum t-ucMGP levels.

4.Conclusions

Local deposits of ucMGP and cMGP were found to be associated with calcifications in meningiomas, while noncalcified tumoral tissue was negative for both protein conformations. The presence of cMGP could be due to its binding to calcium crystals in the calcification or an increased secretion in the extracellular matrix to inhibit the activity of BMP-2. A local vitamin K deficiency could explain the presence of ucMGP in calcified meningiomas. Tissue ucMGP does not influence the serum t-ucMGP levels.

Strengths and limitations of the study

Although we were unable to find a correlation between circulating t-ucMGP levels and the presence of MGP conformations in tumoral tissue, the major strength of our work is that this is the first study that demonstrates the presence of local MGP conformations in calcified meningiomas and the absence of the protein in noncalcified meningiomas.

As for the drawbacks of the study, we could mention the lack of information regarding subject comorbidities which may have influenced the assessment of circulating t-ucMGP and the fact that no plasma was available, so that we were unable to determine the patients' extrahepatic vitamin K status by dp-ucMGP, a marker that can only be assessed in plasma.

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Future recommendations

Future studies should focus on the association between vitamin K status and the development or progress of meningiomas.

Also, a study directed towards evaluating the vitamin K status and identifying MGP conformations in malignant tumors of the CNS would be of great interest.

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