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The Expression of miR-155-5p and Local Matrix Gla Protein in Meningiomas

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Abstract

Meningiomas are classified by the World Health Organization (WHO) in three grades, based on morphological features. Independent of this grading, the presence of calcification in meningiomas influences their growth rate. The messenger RNA of matrix Gla protein (MGP), an extra-hepatic protein with different conformations involved in the homeostasis of ectopic calcification has been found in meningiomas and was shown to be regulated in breast cancer by miR-155-5p, a specific micro RNA. Therefore, we investigated the expression of miR-155-5p and its relationship with local MGP conformations in different grade meningiomas. According to the WHO classification, our 41 samples of meningiomas were stratified in groups WHO I and WHO II. Using real time polymerase chain reaction, we observed a higher miR-155-5p expression in group WHO I versus group WHO II [with a fold change (FC) of 3.83, p=0.027)]. Moreover, the expression of miR-155-5p was higher in calcified tumors compared to non-calcified tumors in all samples (FC=3.01, p=0.047) and in group WHO I (FC=3.65, p=0.048). Utilizing immunohistochemistry, we determined the concurrent presence of all MGP conformations in calcified meningiomas. This study was the first to establish higher miR-155-5p expression in grade WHO I and calcified meningiomas, which could

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improve molecular classification and targeted therapy and also the presence of all MGP conformations in calcified meningiomas, confirming the existence of an anti-calcification mechanism in meningiomas..

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Introduction

Meningiomas are the most common primary tumors of the central nervous system, often accidentally diagnosed (1). Originating from the arachnoid cap cells found in the meninges and with a pathognomonic histology represented by whorls of meningothelial cells (2), they can develop in various locations. The symptomatology is usually nonspecific and greatly dependent on the location of the tumor. The World Health Organization (WHO) has classified meningiomas into three grades based on their histological characteristics (3). The majority are grade I (benign), representing 80%, approximately 17% are grade II (atypical), while grade III (anaplastic) meningiomas account for a very small percentage of the cases (4). Grade II and III meningiomas have an unpredictable outcome due to their aggressive behavior, while grade I tumors are more idle (5). It was also demonstrated that calcified meningiomas, regardless of their WHO grade, have a lower growth rate, while the absence of calcification was associated with rapid tumor growth (6). An important extra-hepatic vitamin K dependent protein, matrix Gla protein (MGP) is involved in the inhibitory mechanism of ectopic calcification (7) and can present different conformations: uncarboxylated MGP (ucMGP), carboxylated MGP (cMGP), desphosphorylated MGP (dp-MGP), phosphorylated MGP (pMGP), or combinations thereof. To the best of our knowledge, only one study (8) has been published regarding MGP in meningiomas, more specifically, it reported the expression of MGP mRNA in the vascular smooth muscle cells of the blood vessels of the tumor, but the local presence of the protein was not assessed.

A previous study investigating the regulation of MGP in breast cancer found that MGP was significantly influenced by a specific micro RNA (miRNA), identified as miR-155-5p, leading to cell proliferation and invasiveness (9). miRNAs are short noncoding RNA molecules consisting of approximately 22 nucleotides which modulate several paramount biological processes, such as proliferation, differentiation and apoptosis, as well as tumor development and metastasis (10). Out of 200 miRNAs analyzed, miR-155-5p and 13 other miRNAs were labeled as the meningioma miRNA "fingerprint" because of their significantly different expression compared with normal adjacent tissue (11).

Based on the existing literature which reported the expression of MGP mRNA and miR-155-5p in meningiomas and the down-regulation of MGP by miR-155-5p in breast cancer, our purpose was to assess the presence of all MGP conformations and to determine the expression of miR-155-5p in calcified/non-calcified and different WHO grade meningiomas, as well as to establish whether miR-155-5p regulates the local presence of MGP in these tumors.

Therefore, we conducted the first pilot study to establish whether miR-155-5p chaperones the local presence of MGP in meningiomas.

Materials and Methods

Samples of tumor tissue from 41 patients undergoing surgery for tumor resection at the "Bagdasar-Arseni" Clinical Emergency Hospital were collected between 2006 and 2012 while demographic information was obtained from patient records. We enrolled subjects with a postopera-

tive histopathological diagnosis of meningioma who did not undergo treatment with vitamin K antagonists. The histopathological grading was performed following the WHO classification (3). Subjects diagnosed with grade I meningioma were included in group WHO I, while those with grade II meningioma were assigned to group WHO II. Additionally, regardless of the WHO grade, the subjects were divided in calcified and non-calcified groups based on the presence or absence of tumor calcification. The study was conducted according to the guidelines enclosed in the Declaration of Helsinki and all procedures involving human subjects were approved by the Medical Ethics Committee of "Iuliu Haţieganu" University of Medicine and Pharmacy. Written informed consent was obtained from all subjects before the enrollment in the study.

Immunohistochemical tissue staining and evaluation

From the paraffin embedded tissue sample blocks, with the help of a microtome, 4 μm thick sections were cut and mounted on glass slides. Next, after tissue deparaffinization and rehydration, the slides were stained with hematoxylin-eosin. Each slide was examined and evaluated by a pathology specialist identifying the following types of meningiomas: meningothelial, transitional, and atypical.

For the immunohistochemical staining of the MGP conformations, we used specific monoclonal antibodies against ucMGP (residues 35–49), cMGP (residues 35–54), dpMGP (residues 3–15), and pMGP (residues 3–15) provided by VitaK BV (Maastricht, The Netherlands). After tissue rehydration, samples were heated in a 0.2% citrate bath for antigen retrieval and incubated with diluted antibodies against ucMGP (0.9 μg/ml), cMGP (1.0 μg/ml), dpMGP (1.0 μg/ml), or pMGP (0.75 μg/ml). The specific antibodies were diluted in blocking reagent (Roche Diagnostics, Germany). After adding the primary antibody to the slides, they were incubated overnight at 4ºC. The following day, horse radish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Denmark), diluted 1:100 was used as the secondary antibody. We used NovaRED substrate kit (Vector Laboratories, USA) in order to expose the antibodies. For the cell nuclei staining, we used hematoxylin and preserved the samples with coverslips mounted with Entellan (Merck, Germany). Negative controls were obtained by omitting the primary antibody.

For the identification of calcification, we used the von Kossa staining by which the sample slides were deparaffinized and rehydrated, followed by 5 minutes incubation with 1% silver nitrate. After washing, we applied sodium thiosulfate and sodium formaldehyde for 1 minute to eliminate the excess of silver nitrate. We used nuclear fast red as a counterstain and covered the samples with coverslips.

Sample slides were evaluated by a specialist blinded to the sample and subject information. Staining pattern for MGP conformations and von Kossa was defined as: negative – absence of staining throughout the sample, and positive – presence of staining in at least one microscopic field.

miR-155-5p detection process

Total RNA was extracted from the 41 tissue samples mounted on slides after xylene deparaffinization using the miRNeasy FFPE kit (Qiagen) according to manufacturer protocol. The total RNA was eluted in 14 μL RNAse/DNAse free water. Quantitation was performed with the Qubit 2.0 Fluorometer (ThermoFisher) with the RNA BR Assay Kit (ThermoFisher). The mean RNA concentration was 48 ng/ μL (range 1 – 454 ng/ μL). cDNA synthesis for specific miRNA detection was carried out using miScript II RT kit (Qiagen) and qPCR reactions were performed in triplicate on an ABI 7900 HT real time PCR machine (ThermoFisher), using the miScript

Sybr green PCR kit (Qiagen) and miScript Primer assays (Qiagen) for miR-155-5p and RNU6 as housekeeping gene. An initial 15 min polymerase activation step was followed by 40 cycles (94 °C/15 s, 55 °C/30 s, 70 °C/35 s). The miR-155-5p expression was calculated using the comparative ΔCt method relative to the RNU6 (12). The fold change (FC) was calculated based on the following formula: $FC = 2^{(\Delta Ct\ group\ 2\ -\Delta Ct\ group\ 1)}$.

Statistical analysis

For statistical analysis we used SPSS 15.0 (SPSS, Chicago, USA) and statistical significance was considered on two-tailed tests at p values < 0.05. The distribution of continuous variables was assessed with the Kolmogorov-Smirnoff test. The variables were normally distributed and were expressed as mean ± standard deviation (SD). We reported the Spearman's Rho coefficient for the association between nominal variables. Student t-test was used to assess the differences between groups.

Results

The study group included a total of 41 subjects, 18 males and 23 females with the average age of 53±13 (mean±SD). Based on the histopathology of the tumor tissue, we diagnosed three types of meningiomas: meningothelial, transitional, and atypical. According to the WHO classification, the first two are considered grade I meningiomas, while the latter is a grade II meningioma. Taking into consideration this classification, we comprised two study groups: WHO I (n=29) and WHO II (n=12). Regarding the presence of local MGP, out of all 41 samples, 22 were positive for all protein conformations, 16 belonging to WHO I and 6 to WHO II. The specific immunohistochemical staining for all MGP conformations was consistent with the presence of calcification in the tumors.

When analyzing all the samples (n=41), there was a significant difference in ΔCt in group WHO I versus group WHO II (5.77±2.55 vs. 7.71±2.1), with a FC of 3.837 (p=0.027) in miR-155-5p expression between the two groups.

Furthermore, we divided the study group into tumors with calcification and tumors without calcification, regardless of their WHO grade, as shown in Table 1.

The analysis of the immunohistochemical samples revealed that all calcified tumors were positive for all MGP conformations. The specific staining for MGP was observed to be extracellular, around the calcified regions, bordering or coating the mineral accumulations. A caption showing all MGP conformations around the calcified area is presented in Figure 1. The non-calcified samples were negative for any MGP conformations.

Finally, we performed correlations between ΔCt, WHO classification, and the presence of local MGP conformations. We found a positive correlation between miR-155-5p expression and

Table 1. ΔCt values and FC of calcified versus non-calcified samples.

	All n=41		**WHO I** n=29		**WHO II** n=12	
	Calcified n=22	**Non-calcified** n=19	**Calcified** n=16	**Non-calcified** n=13	**Calcified** n=6	**Non-Calcified** n=6
ΔCt	5.60±2.32	7.19±2.65	4.93±2.22	6.80±2.63	7.37 ± 1.64	8.05 ± 2.74
FC(p-value)	3.01 (0.047)		3.65 (0.048)		1.6 (0.615)	

The data are presented as mean ± SD or number, as appropriate. Abbreviations: ucMGP, uncarboxylated matrix gla protein; cMGP, carboxylated matrix gla protein; dpMGP, desphospho matrix gla protein; pMGP, phosphorylated matrix gla protein; ΔCt, delta cycle threshold; FC, fold change.

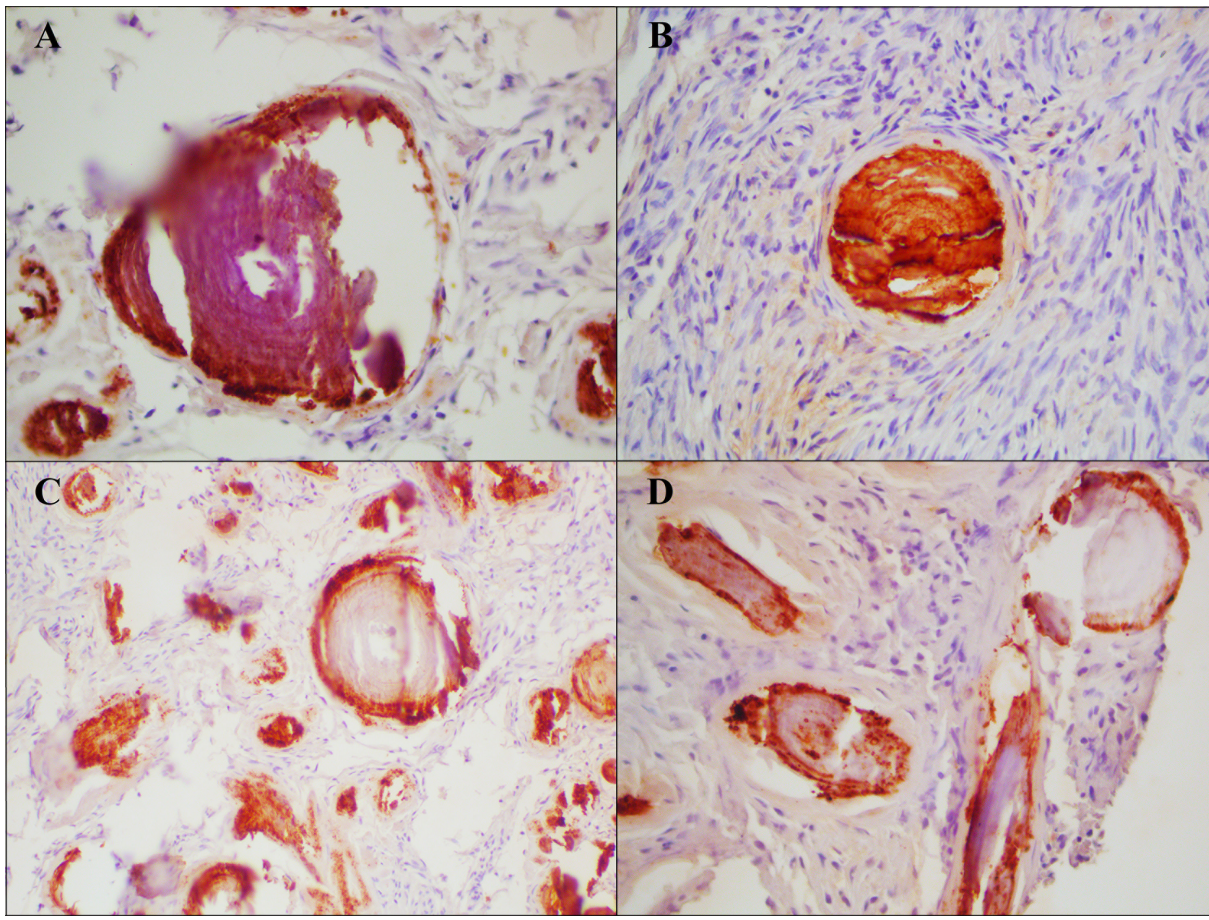

Fig. 1. Immunohistochemical images of MGP conformations in calcified meningiomas: A – ucMGP; B – cMGP; C – dpMGP; D – pMGP. The local MGP conformations are represented as dark red extracellular deposits bordering or coating the calcifications; magnification x400 for all images.

WHO classification (r=0.344, p=0.027), but miR-155-5p did not correlate with the local deposits of MGP conformations (r = -0.289, p = 0.067).

Discussion

Our study was the first to establish an increased expression of miR-155-5p in WHO grade I and calcified meningiomas compared with WHO grade II and non-calcified tumors. Furthermore, it was the first study to identify the local presence of all MGP conformations in calcified meningiomas. All MGP conformations were present concurrently in the calcified meningiomas, but were absent in non-calcified meningiomas. The expression of miR-155-5p was inversely correlated with the increase in WHO grading, but no correlation was found between miR-155-5p and the presence of local MGP in meningiomas. We established a positive correlation between ΔCt and WHO classification along with a higher ΔCt in group WHO II compared with WHO I. The expression of miR-155-5p is inversely proportional to ΔCt, thus the expression of miR-155-5p is higher in group WHO I compared with

WHO II. The FC is used to measure the change in the expression level of a gene and we found that miR-155-5p is almost 4 times more expressed in the tumors pertaining to grade I compared with those from grade II. The positive correlation between ΔCt and WHO classification strengthens the observation that ΔCt increases with grading, resulting in a decrease in miR-155-5p expression as the grade of the meningioma increases.

Additional to the previous study (11) reporting the overexpression of miR-155-5p in meningiomas, our research was able to determine that miR-155-5p expression is different when the tumors are divided according to the tumor grade. The higher expression of miR-155-5p in grade I meningiomas can contribute to the slow growing rate of these tumors compared with grade II where miR-155-5p expression is decreased, as it was shown that the up-regulation of miR-155-5p in head and neck tumors leads to the repression of tumor growth (13). We could hypothesize that up-regulating miR-155-5p expression in patients with grade II meningioma, could decelerate the growth of the tumor which is known to have a more aggressive behavior. Confirmation of this hypothesis requires follow-up studies on a larger number of subjects in order to introduce miR-155-5p in the molecular grading of meningiomas, as well as in the molecular targeted therapy of grade II tumors.

Moreover, we found a significantly higher ΔCt in tumors without calcification compared with those with calcification in all patients. Due to the inverse proportionality between ΔCt and miR-155-5p, we can state that tumors with calcification have a higher expression of miR-155-5p compared with tumors without calcification. The same observation was made when analyzing calcified versus non-calcified tumors within group WHO I. In both situations, the expression of miR-155-5p was approximately 3 times higher in calcified tumors. Although the sample size of group WHO II was small (n=12) and the difference in ΔCt was not statistically significant, we observed the same pattern of miR-155-5p expression as in overall samples and group WHO I, as well. The higher expression of miR-155-5p could be a factor in hindering the growth of calcified meningiomas, as it was demonstrated that calcified meningiomas have a slower growth rate compared with the non-calcified entities (6).

In our previous study (14), we were able to identify local accumulations of both ucMGP and cMGP around the calcification sites in all calcified meningiomas, while the non-calcified meningiomas were negative for the MGP immunohistochemical staining. The present study confirms the previous findings and, additionally, demonstrates that dpMGP and pMGP are also present at the border of calcified regions in all calcified meningiomas and absent in those without calcifications, regardless of the meningioma grade. Our observations were also consistent with the findings of Spronk et al. (15) which identified local MGP around ectopic calcification in arteries, although, at that time, it was impossible to differentiate between the conformations of MGP bordering the calcification areas.

The presence of cMGP and pMGP in calcified meningiomas could be explained by the increased excretion of cMGP to inhibit the development of the calcification by binding to the calcium crystals while the phosphorylation facilitates the protein excretion in the extracellular matrix (16,17). The presence of the inactive forms, ucMGP and dpMGP could be explained by an increased rate of synthesis and subsequent extracellular secretion at a higher rate than post-translational reactions.

Although there is a previous study showing the presence of MGP mARN in meningioma (8), this is the first study to report the local expression of the protein around the calcified regions of this tumor. The presence of MGP in meningiomas certifies that meningiomas possess calcification inhibitory mechanisms.

We did not find any correlation between miR-155-5p and local MGP deposits. However, we observed that miR-155-5p has a higher expression in calcified tumors which are consequently positive for local MGP conformations. This finding is in contrast with the previous study (9) which reported that miR-155-5p suppresses MGP in breast cancer cells. Nonetheless, a few miRNAs have been shown to switch from a down regulatory role to one of activation of their targeted molecules (18). Therefore, in calcified meningiomas, an overexpression of miR-155-5p could up-regulate local MGP excretion in order to minimize the effects of the ongoing calcification process and to inhibit the prospective mineral accumulation.

Our current study should be interpreted within the context of its possible limitations. The sample size, notably of the grade II meningiomas, was small, whereas grade III meningiomas were not enrolled in the study. For future studies, to assess the influence of miR-155-5p expression on circulating levels of MGP could be of great interest, as well as of miR-155-5p and MGP in grade III malignant meningiomas.

In our study, we established a higher miR-155-5p expression in grade I versus grade II and in calcified versus non-calcified menigiomas, additionally confirming the presence of MGP at the tumor level, while evaluating the relationship between mir-155-5p and local MGP conformations in meningiomas.

Conclusions

This is the first study showing that the expression of miR-155-5p was higher in WHO grade I and calcified meningiomas compared with WHO grade II and non-calcified meningiomas. All MGP conformations were present in calcified tumors and absent in non-calcified tumors, suggesting the existence of a suppressive mechanism of ectopic calcification in meningiomas. Although miR-155-5p did not correlate with the local deposits of MGP identified by immunohistochemistry, we found a positive correlation between miR-155-5p expression and WHO classification. This study could be a starting point in introducing miR-155-5p in the molecular classification of meningiomas.

Abbreviations

BMP-2: Bone morphogenetic protein-2
cMGP: Carboxylated matrix Gla protein
dpMGP: Desphosphorylated matrix Gla protein
ΔCt: Delta cycle threshold
FC: Fold change
MGP: Matrix Gla protein
mRNA: messenger RNA
miRNA: Micro RNA
pMGP: Phosphorylated matrix Gla protein
ucMGP: Uncarboxylated matrix Gla protein
WHO: World Health Organization.

Author Contributions

SRG - Conceptualization; Investigation; Writing-original draft preparation
CM - Formal Analysis; Investigation; Writing – review and editing
LGT - Investigation; Resources; Writing – review and editing
AD - Investigation; Resources; Writing – review and editing
CV - Resources; Supervision; Writing – review and editing
SNC - Formal Analysis; Validation; Writing – review and editing
AMC - Project administration; Funding acquisition; Writing – review and editing
All authors have read and agreed to the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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