Chapter 13

PATHOLOGICAL BIOMINERALIZATION AS A PROSPECTIVE MARKER FOR THE DIAGNOSIS OF MENINGIOMA

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INTRODUCTION

Biomineralization is the process of formation of biominerals, which is widespread among living organisms. Pathological biomineralization refers to the deposition of calcium compounds outside the tissues of the skeleton and teeth[1]. In the central nervous system, this phenomenon can manifest age-related changes or pathological processes (tumour growth, dystrophy, metabolic disorders, inflammation, intoxication, congenital pathology, etc.)[2].

Biomineralization is characteristic of meninges both in a state of relative normality (in 12.5% of patients according to computer tomography data, up to 72% according to autopsy data)[3] and during tumour growth (up to 100% in psammomatous meningiomas)[4]. Biomineralization can total or manifest itself in forming lamellar calcifications or psammoma bodies[5].

According to the World Health Organization (WHO) classification in 2021, psammomatous meningiomas are benign and characterized by slow growth and an asymptomatic course[6]. The danger is that the manifestation

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occurs with large tumours when they are no longer operable, and therefore, differential diagnosis in the early stages is critically important[7].

However, calcifications in meningiomas and TMO differ, which can help in differential diagnosis. We believe calcification can be an early marker for detecting a tumour process in the meninges.

Therefore, a detailed study of the structural features of pathological biominerals in meningiomas will help to improve the accuracy of early diagnosis and treatment of patients with brain tumours.

1. Epidemiology, etiology, classification and diagnostics of meningiomas

Epidemiology. According to the report of The Central Brain Tumor Registry of the United States (from October 5, 2019) for 2012-2016, meningiomas accounted for 37.6% of all CNS tumours. They were detected with a frequency of 8.83 cases per 100,000 population. They were 19 times more likely localized in the brain's membranes than in the spinal cord. Meningiomas were diagnosed in women 2.32 times more often than in men, and the number of cases increased with age (65.5 years on average). Most meningiomas were histologically classified as benign (80.5%), while the rest showed signs of atypicality (17.7%) and malignancy (1.8%). Ten-year survival for patients with malignant meningiomas was 61.7% and with benign, 83.7%[8].

According to the latest report of The Central Brain Tumor Registry of the United States (from October 4, 2023), for the years 2016-2020, the prevalence of meningiomas has increased to 40.8% of all CNS tumours (9.73 cases per 100,000 population). The ratio of intracranial tumours to spinal tumours (19.8:1), the number of women relative to men (2.21:1), and the average age (67 years) did not change significantly. Histologically, most meningiomas were benign (80.1%), others were atypical (18.3%) or malignant (1.6%). Ten-year survival remained almost unchanged at 60.1% for patients with malignant meningiomas and 83.4% with benign ones[9].

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Although meningiomas are more common in the elderly, they also occur in children (<1% of all meningiomas)[10]. Children's meningiomas are characterized by more aggressive behaviour and an even ratio between boys and girls[11], and spinal cord localization is observed 3 times more often than in adults and accounts for 10% of all CNS tumours in children[12]

It is not possible to determine the prevalence of meningiomas in the territory of Ukraine according to the data of the National Cancer Registry due to the combined statistics for all brain tumours.

Etiology. A large number of risk factors for the development of CNS tumours have been studied. However, the etiology of meningiomas remains poorly understood[13]. Among the problems that arise when studying this nosology, we can note the relative rarity of the disease, a long latent course (20-30 years or more), subclinical morbidity (the diagnosis is established for the first time at autopsy), false detection using imaging methods[14].

To date, the only reliable risk factor for the development of meningiomas is exposure to ionizing radiation (which increases the risk by 6-10 times)[15]. Thus, meningiomas occupy the first place among secondary brain tumours arising after radiation therapy of the skull[16]. Therefore, if ionizing radiation is used to treat a primary focus in the CNS, it is necessary to consider the risk of

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secondary meningiomas and to conduct long-term follow-ups of these patients[17].

Hereditary predisposition can also be noted, as the presence of a brain tumour in a close family member (including parents, children, and siblings) increases the risk by approximately twofold[18].

A reliable correlation between the development of meningioma and hormones (exogenous or endogenous), head injuries, use of mobile phones, professions, diets and allergies has not been established yet. However, these factors require further study[19].

Classification. By localization, meningiomas are at the skull's base, along the brain's sickle and the cerebellum's tent, and around the cerebral hemispheres[20]. They are usually attached broadly to the dura mater, which causes their spherical or hemispherical shape[21]. Plaque-like meningiomas characterized by infiltrative growth have also been described[22].

According to the classification of the World Health Organization (WHO) in 2021, meningiomas are divided into three degrees of malignancy (benign, atypical, malignant), and 15 histological subtypes are distinguished: meningothelial, fibrous, transitional, psammomatous, angiomatous, microcystic, secretory, with pronounced by lymphoplasmacytic infiltration and metaplastic, chordoid, clear cell, atypical, papillary, rhabdoid, anaplastic[23].

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Diagnostics. Clinically, tumours of the meninges can manifest themselves as headache, epilepsy, behavioural disorders, and other neurological and cognitive disorders that lead to disability and mortality[24].

Spinal meningiomas usually manifest before intracranial meningiomas due to anatomical features (intraspinal meningiomas are much smaller than intracranial ones)[25] and, depending on the location, can manifest themselves as neurological disorders in the limbs, problems with urination and defecation[26].

However, most meningiomas are asymptomatic and are discovered incidentally using modern neuroimaging methods[27] or during autopsy[28].

2. Features of pathological biomineralization of meningiomas

The phenomenon of pathological biomineralization is one of the features of meningiomas. With intracranial localization, calcification is observed in 20-25% of cases[29] and with spinal localization in 1-5%[30].

Currently, there is no single concept of pathological biomineralization. Regarding meningiomas, there is an assumption about the role of tumour cell

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degeneration in dystrophic calcification and its possible antitumor barrier value[31].

Another popular opinion is that calcification is associated with the immaturity and high malignancy of tumour cells and is a manifestation of secondary changes in the tumour[32].

One of the current theories of biomineralization is that hemi-stem cells can differentiate into osteoblast cells and produce biomineral deposits and elements of bone tissue[33].

Psammoma bodies can be observed during the staining of meningioma tissue with hematoxylin-eosin inside concentric structures. They are often associated with cell debris and foci of necrosis, as well as collagen fibres. With the help of X-ray structural analysis, scanning and transmission electron microscopy, energy dispersive X-ray spectroscopy and Raman spectroscopy, it was found that the main mineral component of psammoma bodies are calcium hydroxyapatite crystals $Ca_{10}(PO_4)_6(OH)_2$[34].

Surgical tactics for calcified meningiomas may differ from classical ones[35], as they require additional time and lead to more significant blood loss and a more extended postoperative period[36].

3. Materials and methods

The Commission approved this study on Compliance with Bioethics in conducting experimental and clinical research at the Academic and Research Medical Institute of Sumy State University (protocol No. 2/12, dated December 8, 2022). All studies were performed by the Declaration of Helsinki

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(6th edition, revised 2008, Seoul) and the Universal Declaration of Bioethics and Human Rights (2006).

The research was carried out on tissues obtained after surgical interventions in the neurosurgical departments of the Sumy Regional Clinical Hospital and the Central City Clinical Hospital, as well as on tissues obtained during autopsies in the pathology department of the Sumy Regional Clinical Hospital. (Sumy, Ukraine). Thirty samples of meningiomas with signs of biomineralization (group MEN-I) and 30 samples without these signs (group MEN-II) were examined. Group MEN-I contained 24 samples from female patients and six from male patients. The MEN-II group included 18 samples from female patients and 12 from male patients.

Histological and histochemical study. *For histological examination (hematoxylin-eosin staining)*, meningioma biological material was fixed in 10% buffered formalin solution (CAS No. 50-00-0) for 24 hours. Subsequently, pieces of 1.0x1.0x0.5 cm were cut out, dehydrated and filled with paraffin in the ATM-4M carousel-type apparatus (Ukraine). Tissue sections 7 micrometres thick were made from paraffin blocks using a Shandon Finesse 325 rotary microtome (Thermo Scientific, Waltham, MA, USA). After that, the paraffin sections were stained with hematoxylin-eosin. All photos were taken using a Zeiss Primo Star microscope with a Zeiss Axiocam ERc 5s camera and "Zen 2.0" software (Carl Zeiss, Jena, Germany).

Histochemical examination of meningioma tissue samples by the von Koss method began with deparaffinization (twice for 5 min in a solution of xylene (CAS No. 95-47-6)) and dehydration (twice for 5 min in a solution of 96% ethyl alcohol (CAS No. 64– 17-5), 10 min in a solution of 70% ethyl alcohol (CAS No. 64-17-5)) to distilled water. Then, the samples were placed in a beaker with a 5% solution of silver nitrate (CAS No. 7761-88-8) under intense illumination (in front of a 60-watt lamp, having previously wrapped the beaker in foil) for 1 hour. The samples were washed three times with distilled water to remove silver nitrate residues and placed in a sodium thiosulfate solution (CAS No. 10102-17-7) for 5 min. The samples were washed with tap and distilled water. The samples were then stained in a 0.1% solution of nuclear fast red (CAS No. 6409-77-4) for 5 minutes. The samples were washed with tap and distilled water. Dehydration, illumination and medium coverage were then carried out.

The division into groups was based on the results of a histological examination, during which the histological subtype, according to the 2021 WHO classification, and the presence and type of calcification were considered.

Immunohistochemical study. Serial sections four μm thick, made from prepared histological paraffin blocks, were applied to SuperFrost adhesive slides (Thermo Scientific) and dried at 37°C for 18 hours. Deparaffinized

sections were subjected to unmasking of antigens by the thermal method by heating the sections in a citrate buffer (pH 6.0) at 95–98°C. To visualize the IHC results, the research used the detection system "UltraVision Quanto Detection System HRP Polymer" (Thermo Scientific), which involved blocking the endogenous peroxidase activity with 3% hydrogen peroxide, blocking non-specific background staining using "Ultra V Block", strengthening the reaction "Primary Antibody Amplifier Quanto". Diaminobenzidine (DAB) was used as a chromogen. The following panel of antibodies was used in the studies: OPN (Thermo Fisher Scientific, PA5-34579, dilution 1:300) and SPARK (Abcam, ab203284, dilution 1:300).

Active (use of tissue, with a predetermined positive and negative reaction) and passive control of the obtained results were carried out as quality control of IHC research.

Morphometric studies of constituent elements of micropreparations were carried out using the "SEO Scan Lab 3.0" program's morphometric program. In the environment of the specified program, using morphometric tools, circular fields of view with a diameter of 1,000 μm were selected, inside which the number of immunopositively stained cells was counted. In each micropreparation, six fields of vision were analyzed, which did not overlap. Obtaining and storing images of preparations was carried out using the ZEN digital image system for microscopes "Sarl Zeis" (Germany).

Physico-chemical research methods. *Scanning electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (EDX)* required the following preliminary sample preparation: meningioma biological tissue was fixed in 10% buffered formalin (CAS No. 50-00-0), embedded in a paraffin block. Then, histological sections with a thickness of 12 μm were made from the paraffin block of tissue, which were placed on a table of spectrally pure graphite. For maximum attachment of the biological material to the microscope stage and melting of the paraffin, the sections were kept in a thermostat at a temperature of 60°C for 30 minutes. To remove paraffin, the samples were covered with xylene (CAS No. 95-47-6) three times for 3–4 min, then with 96% ethanol (CAS No. 64-17-5) three times for 5–6 min and rinsed with distilled water. After that, the sample of biological material was additionally grounded with conductive tape wrapped around the stage. The prepared preparations were examined on a scanning microscope SEO-SEM Inspect S50-B (SEO, Sumy, Ukraine) with an energy dispersive spectrometer AZtecOne with an X-MaxN20 detector (Oxford Instruments plc, Abingdon, UK).

Transmission electron microscopy (TEM) with electron diffraction (ED) required subsequent preliminary heat treatment of the samples in an electric furnace (in air) at 200 °C for an hour. At the same time, the destruction of the organic part of the deposit and the removal of free water occurred while

preserving the unchanged structure of the mineral. After such low-temperature annealing, in most cases, the solid particles of the mineral could be easily separated mechanically from the ash of organic tissues. The study was carried out on a TEM-125K microscope (SELMI, Ukraine). The mineralized tissue in powder was treated with ultrasound in distilled water using the UZDN-A unit (SELMI, Ukraine). The specific power of the installation is 15-20 W/cm^2 at the emitter frequency of 22 kHz. The obtained suspension (several drops) was applied to the vertically upward ultrasonic emitter UZDN-A and sprayed for 2-3 seconds, changing the installation's power. The sprayed aerosol was caught on a thin carbon film (10-20 nm) located on the copper grid of the sample holder. ED images and photomicrographs were obtained at a U(injector) = 90 kV.

Statistical analysis. The results of morphometric measurements and immunohistochemical examination were checked for normal distribution using the Shapiro-Wilk test. In the case of the non-normal distribution of digital indicators, the Mann-Whitney test was used to assess statistical significance.

If the data samples had a correct distribution, they were compared using the Student's parametric t-test. The results were considered statistically significant with a probability of more than 95% ($p<0.05$). Graphic presentation of the statistical analysis results was performed using GraphPad Prism 8.0.

4. Results

Macroscopic study. Meningiomas were represented by solid spherical or plaque-like formations tightly fused with the dura mater. Their size varied from 0.5 to 2.2 cm; the colour was from light grey to dirty grey, and the surface was smooth or granular. Some of them were pretty dense and cut with a characteristic crunch.

Histological examination. Histologically, the meningioma tissue was represented by relatively uniform medium-sized cells of the endothelial phenotype, which are morphologically very close to the endothelium of meninges. The cells were closely adjacent and had an oval or polygonal shape. The nuclei were round-oval in shape; nucleoli are small with indistinct contours. Mitoses were rarely observed. The stroma, in some cases, was not expressed (represented by blood vessels and connective tissue), and in others, it was characterized by the formation of incomplete connective tissue membranes. A peculiar concentric arrangement of flattened tumour cells was also observed, resembling a bulb in cross-section (Figs. 1A, 2A). The presence of psammoma bodies (specific rounded formations with a layered structure) was typical. In some cases, most of the tumour tissue was replaced by

psammoma bodies. The presence of damaged psammoma bodies and their fragments can be explained by mechanical damage by a microtome knife during the preparation of histological samples (Fig. 1A).

Histochemical study. Von Koss staining of meningioma samples was performed to detect and visualize calcium phosphate compounds. At the end of the study, the biomineral deposits acquired a brown colour (variations from light brown to dark brown), which confirms the presence of calcium phosphate compounds in their composition. It is also possible to note the unevenness of dye saturation (Fig. 1B). Staining of meningioma samples without signs of pathological biomineralization confirmed the absence of calcium phosphate compounds in the tissue samples studied, i.e., an adverse reaction was observed (Fig. 2B).

Immunohistochemical study. Immunohistochemical examination of the tissue of the MEN-I group with antibodies against osteopontin (OPN) showed a marked presence of this protein. Its most significant accumulation was observed in calcifications and surrounding tissues. OPN expression in meningioma samples with pathological biomineralization amounted to 137.23 ± 7.16 cells in a field of view with a diameter of 1 mm (Fig. 1C).

Immunohistochemical examination of tissues of the MEN-II group with antibodies against osteopontin (OPN) showed a positive reaction with low intensity in tumour cells. OPN expression in meningioma samples without pathological biomineralization was 57.43 ± 5.20 cells in a field of view with a diameter of 1 mm (Fig. 2C).

Immunohistochemical examination of the tissue of the MEN-I group with antibodies against SPARC showed a general moderate presence of this protein. A pronounced positive reaction was observed in the cells of the tumour microenvironment. An increased level of background chromogenic staining (DAB) was also observed. SPARC expression in meningioma samples with pathological biomineralization was 73.60 ± 5.88 cells in a field of view with a diameter of 1 mm (Fig. 1D).

Immunohistochemical examination of the tissue of the MEN-II group with antibodies against SPARC showed a general moderate presence of this protein. A pronounced positive reaction was observed in the cells of the tumour microenvironment. An increased level of background chromogenic staining (DAB) was also observed. SPARC expression in meningioma samples without pathological biomineralization was 61.80 ± 2.95 cells in a field of view with a diameter of 1 mm (Fig. 2D).

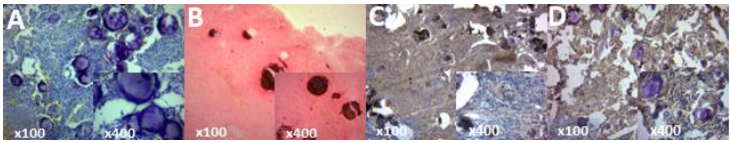

Fig. 1. Examination of meningioma tissue with signs of pathological biomineralization (MEN-I group). A: hematoxylin-eosin staining. B: von Kossa staining. C: IHC study with anti-OPN antibodies. D: IHC study with anti-SPARC antibodies. The magnification of each image is indicated in the lower left corner

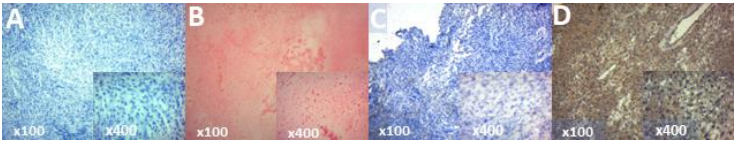

Fig. 2. Examination of meningioma tissue without signs of pathological biomineralization (MEN-II group). A: hematoxylin-eosin staining. B: von Kossa staining. C: IHC study with anti-OPN antibodies. D: IHC study with anti-SPARC antibodies. The magnification of each image is indicated in the lower left corner

Scanning electron microscopy with energy-dispersive X-ray spectroscopy. SEM examination of the tissue of the MEN-I group revealed the presence of many bright white-grey objects in the form of oval and round formations. Their sizes ranged from several tens to hundreds of micrometres. Separate fragments in the form of "blocks" and "plates" were also visualized, which most likely formed as a result of the destruction of large biomineral deposits (Fig. 3A).

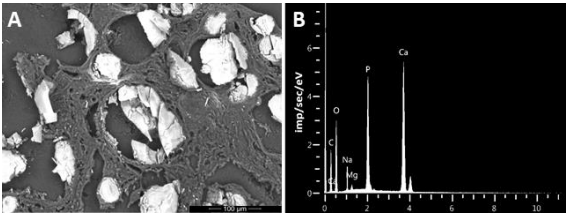

Fig. 3. Scanning Electron Microscopy with EDX of meningioma tissue with calcifications (group MEN-I). A – SEM of the calcified area of the meningioma, magnification in the lower right corner 100 μm, B – EDX spectrum

According to the EDX analysis of samples of the MEN-I group, pronounced lines of calcium (Ca) and phosphorus (P), as well as moderately pronounced lines of oxygen (O) and carbon (C), the ratio of which, most likely, corresponds to calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (Fig. 3B).

According to EDX mapping of samples of the MEN-I group, it was established that calcium, phosphorus, and oxygen were accumulated in the locations of biomineral deposits, as well as the absence of carbon accumulation in these areas, despite its uniform distribution throughout the scanning field (the result of shielding the graphite table from electronic bundle with biomineral deposits). These data confirm that the mineral of the deposits is calcium phosphate of apatite composition (Fig. 4).

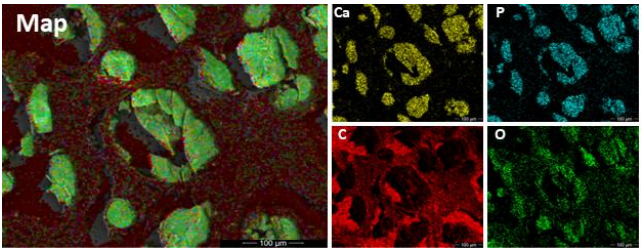

Fig. 4. EDX mapping of meningioma tissue with signs of pathological biomineralization (MEN-I group): calcium (Ca) ions are marked in yellow, phosphorus (P) – blue, carbon (C) – red, and oxygen (O) – green. The magnification of each image is indicated in the lower right corner

Transmission Electron Microscopy. Studies of the microstructure show that the samples are conglomerates of crystals 1–5 μm in size, bound by an amorphous substance (Fig. 5 A, B, C). Crystalline particles have sizes from 10 to 300 nm. Heating with an electron beam activates recrystallization processes, which are accompanied by reorientation of crystallites.

Electron diffraction from conglomerates shows weak rings from crystalline particles (Fig. 5D) and a low-intensity halo from the amorphous component. Selected area electron diffraction (SAED) with relatively large (80–200 nm) crystals (Fig. 5F – corresponds to the central part of Fig. 5C) shows an increase in the intensity of reflections from the crystals. At the same time, a decrease in the brightness of the halo from the amorphous component is observed.

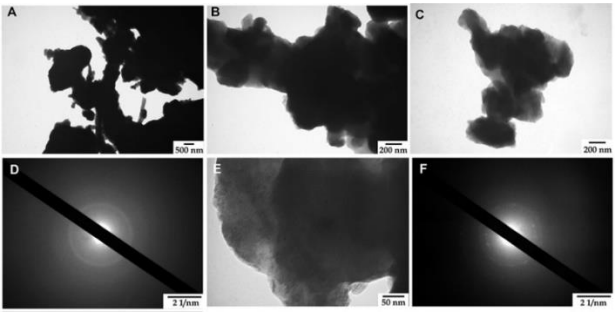

Fig. 5. TEM (A-C, E) and ED (D, F) meningioma with signs of pathological biomineralization (MEN-I group). The magnification of each image is indicated in the lower right corner

CONCLUSIONS

Meningiomas mainly originate from growths of the arachnoid endothelium of the meninges. Usually, meningiomas are benign neoplasms with expansive growth that are closely connected to the dura mater[37].

It is appropriate to start differential diagnosis with a narrow circle of extracerebral tumours, such as neuroma. They have a similar histological pattern ("vortices", "convolutions", "concentric structures") but differ precisely due to the presence of calcification[38]. Characteristic localization also distinguishes meningiomas from intracerebral cancer metastases of various locations[39].

It is also essential to differentiate between meningiomas because they have different proliferative potential. However, it is sometimes quite tricky due to the presence of morphological patterns of several subtypes in one sample simultaneously.

In this work, we investigated pathological biomineralization of meningiomas using histological, histochemical, and immunohistochemical methods and methods of applied materials science – SEM and TEM.

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The earliest signs of biomineralization can be detected even during macroscopic examination (characteristic crunch). During histological examination, psammoma bodies were a reliable marker of biomineralization. The von Koss method of identifying calcium compounds easily detects even small calcifications and makes it possible to narrow down the range of possible biominerals to calcium phosphate.

SEM and TEM data indicate that biomineral deposits in meningiomas are represented by calcium hydroxyapatite with an average crystal size of 20-40 nm. The deposits' main mineral is calcium phosphate of the apatite type.

In our previous work, we studied biomineralization in the relatively normal dura mater of the brain. We can conclude that these biominerals have a chemical composition similar to psammoma bodies (calcium hydroxyapatite), but a different morphology (greater variation in size, irregular shape, diffuse confluent type of location[40]. The different morphology of these biominerals allows us to separate relatively normal tissue meninges from meningioma tumour tissue.

Osteopontin (OPN) is a glycosylated phosphoprotein that participates in the processes of tumour growth and regulates osteogenesis[41]. In some studies, OPN expression was higher in meningiomas than non-neoplastic meningeal tissue and prevailed in atypical and malignant meningiomas compared to benign ones[42]. In our study, biomineralization is positively correlated with OPN overexpression, but it is not yet clear how this is related to clinical prognosis. Because according to the 2021 WHO classification, psammomatous meningiomas are benign, and OPN overexpression is characteristic of both calcified and more malignant tumours[43]. We believe a significant amount of OPN is present in connection with remodelling in the calcification process, as this acidic glycoprotein is attracted to hydroxyapatite crystals[44].

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Research by other scientists found that the expression of SPARC (secreted protein acidic and cysteine-rich), also termed osteonectin, positively correlates with the degree of malignancy of meningiomas and is also higher in recurrent tumours[45]. In our study, we found no significant difference in the expression level of SPARC between groups with and without calcification. This may be because osteonectin is present in meningioma tissue, and its role in pathological biomineralization is uncertain. Perhaps SPARC does not play a significant role in the calcification processes of meningeal tumours.

Based on the results of the IGH study, we can conclude that the primary mechanism of the formation of pathological biominerals is the development of dystrophic and necrotic changes in the tumour tissue with the subsequent formation of calcifications.

The obtained results make it possible to distinguish between calcifications in meningiomas and in the dura mater (similar in chemical composition but different in structure and morphology), which is a promising marker for differential diagnosis.

SUMMARY

Biomineralization is the process of formation of biominerals, which is widespread among living organisms. Biomineralization is characteristic of meninges both in a state of relative normality (in 12.5% of patients according to computer tomography data, up to 72% according to autopsy data) and during tumour growth (up to 100% in psammomatous meningiomas). However, there is a difference between calcifications in meningiomas and meninges that can help in differential diagnosis. We believe calcification can be an early marker for detecting a tumour process in the meninges. We examined 30 samples of meningiomas with signs of biomineralization (group MEN-I) and 30 samples without signs of biomineralization (group MEN-II). To study pathological biomineralization, we used the following methods: macroscopic, histological, histochemical and immunohistochemical, scanning electron microscopy, and transmission electron microscopy. The SEM study confirmed that the biomineral part of the meningioma samples consisted mainly of apatites. The Ca/P ratio corresponds to hydroxyapatite. According to TEM, the sizes of single crystals lie in a wide range, from 10 to 300 nm. A significantly higher level of OPN and the absence of fluctuations in SPARC expression may indicate a dystrophic type of development of biomineral deposits in meningiomas.

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