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Expression pattern of MRPS18 family genes in medulloblastoma. An example of own practice

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Introduction. Medulloblastoma (MB) is one of the most frequently diagnosed common brain tumors in children. Due to alterations in the gene expression patterns, medulloblastomas display diversity in the transcriptional, genetic, and clinical markers. However, these markers are few. Hence, there is an urgent need for other molecular, preferentially, non-invasive markers to propose the personalized treatment. One of the putative markers can be the mitochondrial ribosomal protein MRPS18-2.

Purpose: to study expression of the MRPS18 family genes at mRNA and protein levels, in serum and tissue of the medulloblastoma.

Materials and methods. A real-time quantitative polymerase chain reaction (qPCR) was used to assess the relative expression of *RB* and MRPS18 family genes at mRNA levels in patient sera and tissues. Protein signals were detected by immunohistochemistry.

Results. The relative expression of MRPS18 genes was lower, when assessed in serum of the tumor patient compared with the control. Thus, *MRPS18-1* expression level, detected in serum, is up to 8.5 lower than in the control sample, while in tissue it is quite similar in both samples. The *MRPS18-2* gene was detected at up to 26 lower levels in the serum of the tumor patient. Importantly, *MRPS18-2* and *MRPS18-3* are elevated by 13 and 7.2 times, respectively, in the tumor tissue, compared to the control. Moreover, the MRPS18-2 protein signal is dramatically elevated in medulloblastoma cells, compared with the conditionally healthy brain tissue.

Concluding, the members of the MRPS18 protein family, especially MRPS18-2, are the putative candidates for molecular prognostic markers. More experiments should be done on a study on this family, and on a large cohort.

Key words: *embryonal tumors; medulloblastoma; MRPS18 family genes; proteins MRPS18-1, MRPS18-2 and MRPS18-3; RB gene; RB protein*

Introduction

Early diagnosis of malignant neoplasms is the cornerstone of the successful treatment of patients. Therefore, the molecular markers for diagnosis and prognosis of its course are still necessary and there are actively being carried out. Over the past decade, the progress was made in the field of timely detection and treatment of cancer patients, as well as in preventive measures to avoid such diseases.

Moreover, based on a molecular profile of tumors, it is possible, to create the "tailored", or personalized medicine for patients, suffering from even rare tumors. Embryonal brain tumors represent the rare diseases – an incidence rate is 4-5 cases 100 000 pediatric population [1-3]. And yet, the embryonal brain tumors of the central nervous system (CNS) are the most common solid neoplasms of childhood.

Medulloblastoma is one of the most frequently diagnosed brain tumors in children [4, 5]. Medulloblastoma is usually localized in the posterior cranial fossa, along the midline, sometimes spreading to the surface of the cerebellar hemispheres [6]. Medulloblastomas metastasize through the cerebrospinal fluid; metastases are usually located on the surface of the cerebellar hemispheres and along the spinal cord, less often - on the supratentorial surface [7, 8].

Due to alterations in the gene expression patterns, medulloblastomas display diversity in transcriptional, genetic, and clinical markers. Hence, medulloblastomas are divided into four subgroups, based on differences in gene expression profiles, namely I) with activated sonic hedgehog (SHH) pathway, II) with activated WNT pathway, III) non-WNT/non-SHH group 3, and Iv) non-WNT/non-SHH group 4. These groups were formed mainly by molecular profiles. Noteworthy, medulloblastomas in each group show additional layers of intertumoral heterogeneity [9, 10].

Tumors belonging to the SHH-activated group (i) are usually represented as large/anaplastic cells, sometimes they are desmoplastic/nodular. Tumors in this group

demonstrate the presence of chromosomal aberrations, for example, gain of 3q, 9p, loss of 9q, 10q, and 17p. *MYCN* (NM_001293228), *MYCL1* (*NM_001033081*) and GLI-Kruppel family member 2 (GLI2, *NM_001371271*) genes are also frequently amplified. Moreover, genes, such as *SUFU*, negative regulator of hedgehog signaling (*SUFU*, NM_016169) and *TP53* (NM_000546) are often mutated in the SHH-activated medulloblastomas. Mutations were also often found in genes, encoding isocitrate dehydrogenase 1 (*IDH1*, NM_005896), telomerase reverse transcriptase (*TERT*, NM_198253), and receptors Patchet 1 (*PTCH1*, NM_001083602) and SMO (NM_005631) as well [11].

Medulloblastomas of WNT-activated group (II) are characterized by classic histology and specific changes in the chromosomal, i.e., monosomy of chromosome 6 and mutations in the *SMARCA4* gene (NM_001128845), encoding one of the SWI/SNF family proteins, also known as BRG1. Mutations in other genes, such as *CNNB1* (NM_001904), encoding beta-catenin, Adenomatous polyposis coli (*APC,* NM_001127511), and *DDX3X* (NM_001356), encoding DEAD-box RNA helicase are common [11-13].

Morphology tissue medulloblastomas of tumors of group 3 non-WNT/non-SHH medulloblastomas (iii) could be either classic, or large/anaplastic. In these cells the chromosomal re-arrangements are often detected, such as deletions of chromosome regions 5q, 10q, 16q and loss of chromosome 11; insertions of 17q; gain of 1q. Several genes, namely, *MYC* (NM_002467), *MYC*N, *OTX2* (NM_021728); *GF1* (NM_00526) and *GF1B* (NM_004188) are usually overexpressed. Mutations are often registered in *SMARCA4*, *KBTBD4* (NM_018095), *KMT2D* (NM_00348), encoding lysine-specific methyltransferase 2D, and *CTDNEP1* (NM_015343), encoding CTD nuclear envelope phosphatase 1 [11].

Medulloblastomas of group 4 non-WNT/non-SHH (iv) are characterized by mainly classical morphology of tumor cells. Loss of 8p, 10q, chromosome X (for female) and gain of chromosome 7 are frequent events, along with detected insertions on 17q. In addition, *MYCN* and *CDK6* (NM_001259) are amplified, *SNAIP* (NM_001242935), encoding synuclein alpha interacting protein, is overexpressed. Mutations are found in genes, encoding lysine demethylase 6A (*KDM6A*, also known as *UTX*, NM_001291415), lysine N-methyltransferase 2C (*KMT2C*, also known as *MLL3*, NM_170606), and ZMYM3 (also known as *ZNF261*, NM_201599) [11].

Of course, above mentioned markers are few. Even so, they allow to clarify diagnosis and prognosis of the course of disease, thus, the treatment can be adjusted. Hence, there is an urgent need for other molecular, preferentially, non-invasive markers to propose personalized treatment.

As mentioned earlier, medulloblastoma belongs to embryonal brain tumors of the CNS, i.e., cells, destined to differentiate, instead proliferate. One of the reasons of this could be the imbalance between expression levels of retinoblastoma-associated protein, RB (NP_000312) and mitochondrial ribosomal protein MRPS18-2 (also known as MRPS18B, NP_054765) [14]. Most probably, these proteins can potentially be markers for predicting the course of the malignant process and choosing the appropriate treatment. We have recently shown that MRPS18-2 plays an important role in cell stemness through interaction in a multimeric complex with RB, namely enhancing the E3-ubiquitin ligase activity of RNF2 on histone H2A [15]. Moreover, various tumors show upregulation in the MRPS18-2 protein expression [16-18]. Importantly, other members of the MRPS18 protein family, MRPS18-1 (MRPS18C, NP_057151) and MRPS18-3 (MRPS18A, NP_060605) show the differential expression pattern in gliomas [19].

Based on the discussed above, we aimed to study expression of MRPS18 genes and RB, both on mRNA and protein levels, in serum and tissue of the medulloblastoma patient, in comparison with a conditionally healthy individual.

Materials and Methods

Medulloblastoma patient

In the present work, the tumor tissue sample of patient X (male, age 14) was studied. The tumor was located in the right hemisphere of the cerebellum and spread parastemically (with invasion into the brain stem). Subtotal tumor removal operation was performed, and cancer tissue was collected for this study. As the control, the brain tissue of the patient with the former atypical choroid papilloma (female, age 2 years and 9 months) was used. Firstly, at the age of 1 year 11 month she was operated on; the atypical choroid papilloma in the right lateral ventricle was totally removed. After 10 months, the tumor showed no signs of continued growth, according to MRI. Anyway, the CSF dysfunction clinic and a septostomy was performed and unaltered brain substance was taken for revision of the site of previous intervention. This tissue is considered as the conditionally normal tissue, designated as the Control in the manuscript. Parents of both patients have signed informed consents.

The use of the tumor tissue and serum was approved by the Ethics Committee of A.P. Romodanov Neurosurgery Institute of National Academy of Medical Sciences of Ukraine (NAMSU) and by the Institutional Review Board and Research Ethics Committee of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of National Academy of Sciences of Ukraine (NASU), according to the Declaration of Helsinki. Diagnosis was performed by experienced pathologists at A.P. Romodanov Neurosurgery Institute of NAMSU (head of the department of neuropathomorphology T. Malysheva). All experimental work was performed, according to approved protocols.

RNA isolation, cDNA synthesis and qPCR

For total mRNA extraction, tissue samples were homogenized in liquid nitrogen. The TRIzol isolation reagent (Thermo Fisher Scientific, USA) was added then, in the ratio of 500 ml of buffer per 2g of tissue.

In the case of mRNA extraction from sera, TRIzol was added directly to the tube, that contained sample

This article contains some figures that are displayed in color online but in black and white in the print edition

at the ratio of 1:1. Both samples were incubated at room temperature for 10 minutes.

After that, 0.5 mL of chloroform per 1 mL of TRIzol were added; after 2-3 minutes a reaction mixture was centrifuged for 20 minutes at 12000 RPM. An aqueous phase, that contained mRNA was transferred into a new tube, and isopropanol was added (1/3 of the aqueous phase volume). Samples were gently mixed and incubated for 30 minutes at -20℃ for mRNA precipitation. After centrifugation for 20 minutes at 12,000 RPM, precipitated mRNA was washed with 70% ethanol two times and then dissolved in RNase-free H_2O. RNA concentration was assessed, using a nanodrop "ND 1000" (Thermo Fisher Scientific).

The obtained mRNA (of the final concentration is 25 ng/μl) was used for a cDNA synthesis, using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer protocol.

Real-time quantitative polymerase chain reaction (qPCR) was used to assess the relative expression of *RB* and MRPS18 family genes at mRNA levels. The amplification reaction mixture consisted of 2μl of cDNA, 4μl of 5x HOT FIREPol EvaGreen qPCR Mix (Solis BioDyne, Estonia), 13μl of RNase-free H_2O and 1μl of the primer mix (reverse and forward, final concentration is 25 pM). A list of the primers, used in our study, is presented in ***Table 1***. Each reaction was performed in triplicate and normalized, according to *TBP* mRNA expression, that served as the internal control. Data were calculated, using $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

Immunohistochemistry for MRPS18-1-3 and RB performed on deparaffinized tissue sections. Paraffin was dissolved in xylol, and sections were rehydrated by a stepwise washing with ethanol in phosphate-buffered saline (PBS) (99%, 90%, 70% and 30% EtOH). Sections were then treated with the 2% solution of H2O2 in methanol at the room temperature for 30 min to reduce the background. Epitopes were exposed to hot citrate buffer (water bath, 92°C for 15 min). The rabbit antibodies against MRPS18 family proteins (Thermo fisher Scientific) and mouse monoclonal antibody against RB (Cell signaling, USA) were used for detection, diluted 1:200 in a blocking buffer (2% bovine serum albumin, 0.2% Tween-20, 10% glycerol, 0.05% NaN3 in PBS). EnVision system (DAKO, Denmark) was used in 30 min second-step incubation. After washing in phosphate-buffered saline, a peroxidase activity was assayed, using DAB. After counterstaining with hematoxylin for 1-2 min, sections were dehydrated and mounted in Canadian balsam.

Morphological features were evaluated on tissue sections, stained with hematoxylin and eosin (H+E). The stained slides were evaluated with a Leica microscope.

Statistical analysis

GraphPad Prism software (version 9, GraphPad Software, La Jolla, USA) was used to determine the means of the gene expression. The Kruskal-Wallis test was applied for non-parametric tests within groups for each of the genes.

Results and Discussion

Morphological features of medulloblastoma

The present embryonal brain tumor was characterized as medulloblastoma by localization (the cerebellum) and microscopic appearance. Histologically, the tumor represents the medulloblastoma classic histological variant 9470/3 (G IV). No atypical cells were detected in the lumbar cerebrospinal fluid; no macrometastases were detected after MRI of all parts of the spinal cord was performed.

Thus, tumor tissue demonstrates extremal cellularity, there are sheets of anaplastic cells. Moreover, tumor cells are quite small, and the cytoplasm is scant. Also, the cell nuclei in most tumors cells are elongated of have a shape of the half-moon. Specific rosettes, formed by tumor cells are also present. Such densely organized cellular masses, arranged in sheets with oval to elongated-shaped nuclei and minimal cytoplasm are characteristic for medulloblastoma ***(Fig. 1)***.

Expression of the MRPS18 and RB genes in serum and tumor tissue of medulloblastoma patient in comparison with conditionally healthy individual

The results of a qPCR analysis showed that genes of MRPS18 family differentially expressed at the mRNA levels in medulloblastoma cells (Patient X), compared with conditionally normal control brain cells. Importantly, all three members of the MRPS18 family showed different expression patterns.

In general, relative mRNA expression levels of MRPS18 genes in serum of the tumor patient are lower, than in the control. Thus, *MRPS18-1* expression level, detected in serum, is up to 8.5 lower than in the control sample ***(Fig. 2A)***. And yet, the relative *MRPS18-1* expression in tissue is quite similar in both, Patient X and control samples ***(Fig. 2B)***.

Table 1. The primers sequences, used in the present study

Gene	Forward primer 5´→3´	Reverse primer 3´→5´
TBP	TTTCTTGCCAGTCTGGAC	CACGAACCACGGCACTGATT
MRPS18-1	CAGGTATCCAGCAATGAGGACC	GCATCCAGTAAATGGAGAAACAAAC
MRPS18-2	CACAGCGGACTCGGAAGACATG	GCGCAGACAAATTGCTCCAAGAG
MRPS18-3	CATCTGCCGTTGGAACCTTGAAG	CTTGCGGTGTT CTTCCTGGCAT
RB	CAGAAGGTCTGCCAACACCAAC	TTGAGCACACGGTCGCTGTTAC

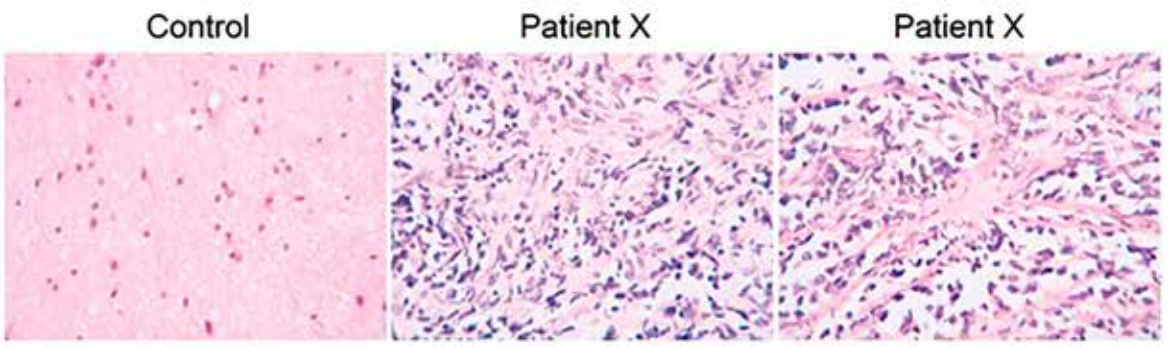

Fig. 1. The morphology of the studied tissues. The tissue sections were stained with hematoxylin and eosin, as described elsewhere. Tissue sample of the Patient X is diagnosed as medulloblastoma. Magnification is x400.

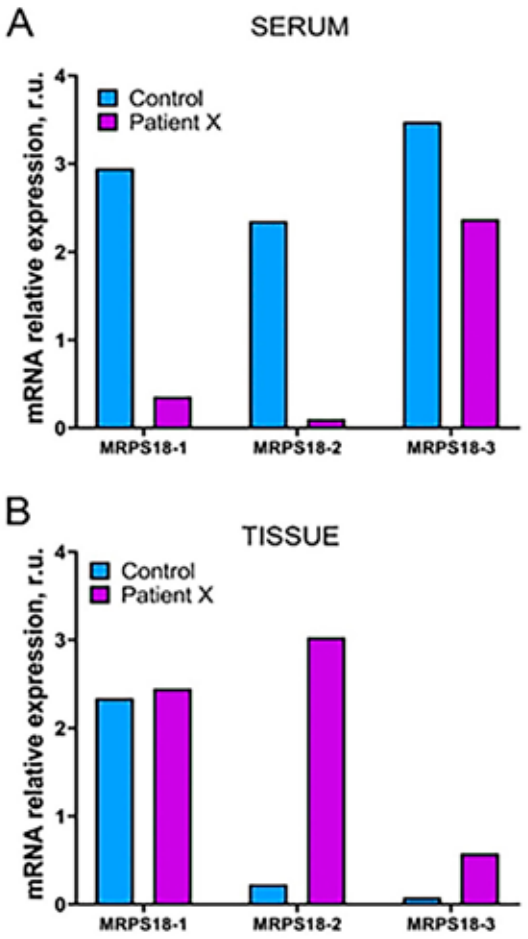

Fig. 2. mRNA expression levels of MRPS18 genes in patient sera and tissues, assessed by the qPCR.

Diminishing of the mRNA expression rates, evaluated for the serum, reaches up to 26 for *MRPS18-2* gene ***(Fig. 2A)***. At the same time, profiles of mRNA expression levels of *MRPS18-2* and *MRPS18-3* in the serum were inverted in tumor tissue sample ***(Fig. 2B)***. *MRPS18-2* and *MRPS18-3* in tumor tissue are elevated by 13 and 7.2 times, respectively, compared to the control ***(Fig. 2B)***.

Expression of *RB* at mRNA level was not detected in both, serum, and tissue samples of the control, as well as in the serum of the Patient X. Only in the tissue sample of the Patient X we could detect the mRNA of *RB*; however, this very low value was at a border line of the threshold level for the qPCR method.

Expression of the MRPS18 and RB proteins in medulloblastoma cells in comparison with conditionally normal brain tissue

Results of immunohistochemistry show that the expression pattern of MRPS18 family proteins in tumor tissue, compared with the conditionally normal brain tissue, is quite similar to one, obtained by the qPCR method ***(Fig. 3).***

Thus, MRPS18-1 is expressed at roughly the same levels in conditionally normal brain tissue and in medulloblastoma sample (Figure 3, an upper panel). MRPS18-3 is detected at the slightly higher level in medulloblastoma sample (Figure 3, the bottom panel). However, MRPS18-2 level are dramatically elevated in medulloblastoma cells, compared with the conditionally healthy brain tissue (Figure 3, the middle panel).

The obtained results once again confirm about importance of the MRPS18-2 in cancerogenesis.

Concluding, the members of the MRPS18 protein family, especially MRPS18-2, are the putative candidates for molecular prognostic markers.

The importance of a personalized medical approach is clearly demonstrated by intratumor heterogeneity. To solve a problem of individualized molecular profiling of each patient, the strong collaboration between clinical and basic research should be established. More experiments should be done on a study on the MRPS18 family genes, and on a larger cohort, in fruitful collaboration between our Institutions.

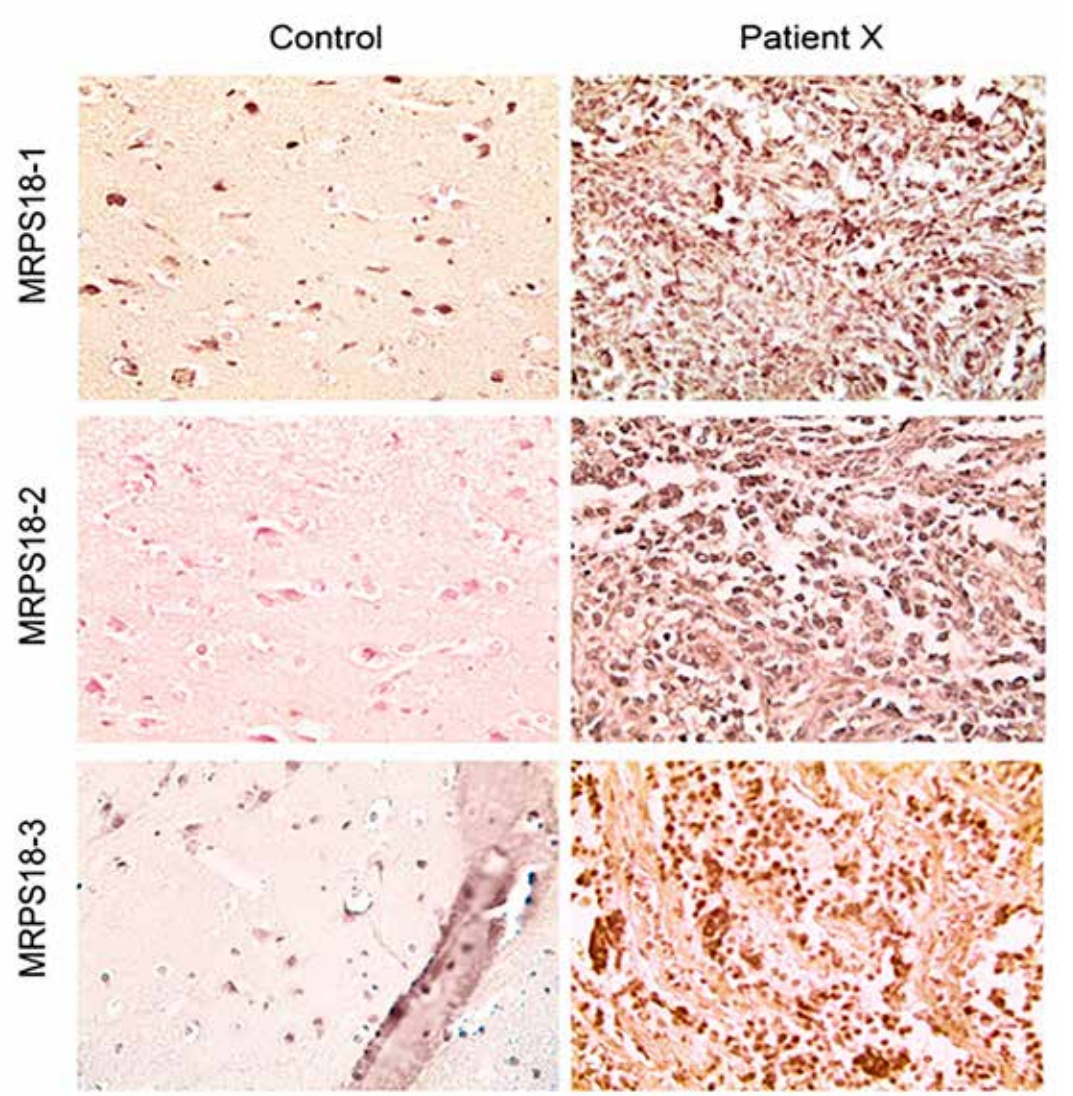

Fig. 3. Immunohistochemistry on tissue samples. Tissues were stained with the specific rabbit antibodies against MRPS18-1-3. Magnification is x400.

Disclosure

The authors declare no conflict of interest regarding the materials or methods used in this study, as well as conclusions presented in this paper.

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Author contribution

L.K., T.M. and E.K. designed research; L.K., V.S., T.M., S.K., and V.R. performed experiments; L.K., V.S., T.M., S.K., L.V., and E.K. analyzed data; and V.S., L.K., T.M., and E.K. wrote the paper.

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