In vitro examination of childhood medulloblastoma

PhD Thesis Outlines

Tímea Pócza

Doctoral School of Pathological Sciences
Semmelweis University

Supervisor: Dr. Péter Hauser MD, Ph.D.

Official reviewers:
Dr. Ágnes Jermendy MD, Ph.D.
Dr. Gábor Nagy MD, Ph.D.

Head of the Final Examination Committee:
Dr. Janina Kulka MD, Ph.D.

Members of the Final Examination Committee:
Dr. Lilla Reiniger MD, Ph.D.
Dr. Attila György Bagó MD, Ph.D.

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Introduction

Medulloblastoma is the most frequent malignant brain tumor in childhood and is responsible for a substantial percentage of cancer-related death. It arises commonly in cerebellar vermis, rarely in hemispheres. It is thought to originate from cerebellar granule neuron precursor cells in which signalling pathways participating in the development of cerebellum (WNT, SHH, Notch, etc.) are dysregulated. Medulloblastoma has different histological subtypes; according to the WHO, there are classic, desmoplastic, extensive nodular, anaplastic and large cell variants. Life expectancy depends on the different histological subtypes. The current treatment consists of surgery, radiation- and chemotherapy. The intensity of treatment is influenced by clinical risk stratification of patients, by their age, the histology of the tumor, the extent of resection, and the presence of metastases. Serious side effects, like neurocognitive impairment, psychological and behavioural disturbances, secondary malignancies and endocrinopathies are often the consequences of therapy. As a result of improved treatment strategy, 70–90% five-year overall survival could be achieved in low-risk patients, which is worse in high-risk patients. New diagnostic tools and targeted strategies may improve survival and the long-term quality of life. Based on recent extensive investigations a molecular based classification was accepted. Four molecular subgroups were created (SHH, WNT, Group 3 and 4) with different life expectancy according to the aberrant signalling pathway characteristic for each group. WNT subgroup is associated with the best survival, whereas Group 3 and 4 indicate worse prognosis.

Burning questions of the treatment of medulloblastoma are concerning the following topics, what kind of unknown factors may influence tumor formation and the survival of patients with medulloblastoma? How could be reduced the side effects of the treatment?

As an important player in the regulation of protein synthesis, mammalian target of rapamycin (mTOR), and participants of epigenetic regulation processes, namely DNA methyltransferases (DNMTs) has not been investigated yet as prognostic marker for medulloblastoma.

The central role of the mTOR pathway in the regulation of cell growth and metabolism is well established. mTOR protein is a serine-threonine kinase, which

participates in two types of complexes, mTORC1 and mTORC2. Dysfunction of the mTOR pathway could result several defects leading to malignant phenotype. The mTORC1 mediated pathway stimulates the translation of proteins related to cell cycle progression, cell survival and metastasis formation. Cell proliferation is also facilitated by increased ribosome biogenesis providing protein synthesis executors. Additionally, there is more and more evidence of mTORC2 involvement in tumor initiation. Mutations in the components of the mTOR pathway are described in many types of tumors (e.g. breast and colorectal tumors, glioblastoma, leukaemia, etc.). Former investigations described abnormal regulation of AKT and ERK pathways, upstream regulators of mTOR, in some cases of medulloblastoma. Deletion of tuberous sclerosis 1 (TSC1) gene was found in a small proportion of medulloblastoma patients. Since TSC1 indirectly inhibits mTOR, lack of this could lead to abnormal activation of the mTOR pathway. The relation between insulin-like growth factor (IGF) - mTOR and SHH pathway was described in cerebellar granule precursors, which promotes tumor formation.

Contribution of mTOR pathway to tumor development raises the question of targeting this pathway with pharmacoligcal inhibitor. The first known mTOR inhibitor is rapamycin, which inhibits the mTORC1 complex. A new group of mTOR inhibitors act on both mTORC1 and mTORC2 complexes. The similarity of the catalytic domains of mTOR and phosphatidylinositol 3-kinase (PI3K) led to the development of so-called dual-kinase inhibitors (simultaneous inhibition of mTORC1, mTORC2 and PI3K). Dual-inhibitors achieved promising results in several types of cancers. Rapamycin and its derivatives are effective in some medulloblastoma cell lines, which was also confirmed by *in vivo* experiments.

Emerging data suggest that epigenetic changes contribute to tumorigenesis. Epigenetics covers molecular mechanisms, that heritably affect functionality of genes without changing the sequence of DNA. During tumor formation and development the epigenome undergoes many changes, aberrant methylation patterns are developed, and the structure of nucleosomes and histone modification are altered. These different epigenetic processes are in close connection, and this altered gene expression promote tumor evolution. The simultaneous presence of genetic and epigenetic processes in tumor development could be detected at all stages.

DNA methylation provides the appropriate gene expression. The DNA methylation is achieved by DNMTs, which catalyze transfer of a methyl group from S-adenosyl-L-methionine to the 5'-position of cytosine residues. Methylation of a promoter region of a gene inhibits its expression. In mammals, three types of enzymes – DNMT1, DNMT3A and DNMT3B – are known to be involved in the process of DNA methylation. DNMT3A and DNMT3B enzymes are de novo methyltransferases, which are responsible for formation of initial methylation pattern, and are highly expressed in embryonic tissue and germ cells. DNMT1 sustains methylation pattern in later phase of life, recognizes hemi-methylated DNA strand and copies methylation pattern during DNA replication, and shows a preference towards the hemi-methylated DNA strand.

Altered methylation pattern is frequently observed in tumor cells, where DNA is globally hypomethylated and promoter regions are hypermethylated. Hypomethylation of repetitive DNA sequences and hypermethylation of tumor suppressor genes contribute to abnormal gene expression and chromosomal instability that facilitates tumor formation. Furthermore, DNA repair genes could be repressed by hypomethylation. In addition, sustain of chromosomal stability could be disturbed by lack of repression of repeat and transposable elements, thus increasing the probability of insertional mutations. Methylation of repetitive elements protects from chromosomal instability by inhibiting homologous recombination. Hypermethylation of tumor suppressor genes has been observed in many types of cancers, like breast and colon cancer, brain tumors, such as medulloblastoma and gliomas. In tumor cells, hundreds or thousands of genes can be hypermethylated. The overexpression of DNMTs has been observed in many types of tumors, both at mRNA and at protein level. Increased DNMT1 expression was detected in gastric cancers, increased DNMT1 and DNMT3A in breast and pancreatic cancer, and increased DNMT1 and DNMT3B in glioma.

DNA methylation is a reversible process, and consequently, inhibition of DNMT enzymes also provides possible therapeutic target. DNMT inhibitors have been evaluated in clinical trials in several types of cancer, however, to date only haematological malignancies showed promising results, their anti-tumor effect in solid tumors has not been proven yet. DNMT inhibitors were tested *in vitro* and *in vivo* experiments, which suggest that combined inhibition of DNMTs, histone deacetylase (HDAC) and tyrosine kinase may have effect on medulloblastoma.

Objectives

Medulloblastoma shows heterogeneity in terms of clinical and molecular biological characteristics. There is an ongoing widespread research to classify different molecular subgroups, in order to determine personalized targeted therapy for individual patients. Through development of molecular biology research focuses on possible molecular therapy. Our research goal was to investigate new molecular markers, to further refine and clarify the biological background of medulloblastoma and find new, drugable prognostic markers.

The following issues were investigated:

a) whether primary human medulloblastoma samples show mTORC1 activation
b) whether the expression of p-mTOR and p-S6 (component of mTORC1 complex) correlates with clinical and pathological characteristics of patients and could be used as prognostic markers
c) if there is any correlation between mTORC1 activation and WNT, SHH and non-WNT/SHH subgroups of medulloblastoma (determined by β-catenin and SFRP1)
d) to test the effect of mTOR pathway inhibitors – rapamycin (inhibiting mTORC1), and NVP-BEZ235 (inhibiting mTORC1 and mTORC2 complexes) – on proliferation of medulloblastoma cell lines, and in combination with cytostatic drugs used in the treatment of medulloblastoma
e) whether presence of characteristic proteins of mTORC1 and mTORC2 influences the sensitivity of medulloblastoma cell lines to various inhibitors of mTOR
f) whether three active members of DNMTs (DNMT1, DNMT3A and DNMT3B) are expressed in primary human medulloblastoma
g) whether the expression of DNMTs correlates with clinical and pathological characteristics of patients and could be used as a prognostic marker
h) if there is any correlation between the expression of DNMTs and WNT, SHH and non-WNT/SHH subgroups of medulloblastoma

Methods

Formalin-fixed paraffin-embedded medulloblastoma tissues of 44 patients were examined (ethical approval: TUKEB No. 100/2012 and No. 30/2015). Tissue microarray (TMA) blocks were created and 4 μm sections were cut for immunohistochemistry. To examine mTORC1 activation, p-S6 and p-mTOR-specific antibodies were used. DNMTs were detected with DNMT1, DNMT3A and DNMT3B-specific antibodies. WNT and SHH pathway activation were determined by anti-β-catenin and anti-SFRP1 (secreted frizzled-related protein 1) antibodies, respectively. Only cytoplasmic reaction of p-mTOR and p-S6 staining and the nuclear reaction of DNMTs were considered for evaluation.

Effect of mTOR pathway inhibitors (rapamycin and NVP-BEZ235) were tested on medulloblastoma cell lines (Daoy, UW228-2) in monotherapy and in combination with cytostatic drugs applied in the clinical treatment of medulloblastoma. Inhibition of cell proliferation was examined by methyl tetrazolium (MTT) assay after a 72-hour treatment period. Immunocytochemical assay was performed in both cell lines to detect characteristic proteins of mTOR complexes: p-mTOR (mTORC1 and 2), p-S6 and Raptor (mTORC1), and Rictor (mTORC2).

Results

Median age of 44 patients was 8.5 years (range 1.1-28.7 years), rate of male patients was 55%. 84.1%, 11.4% and 4.5% of patients showed classic, desmoplastic and large cell/anaplastic (L/A) subtype, respectively. Survival data were available in 40 patients (90.9%). Median follow-up time of patients was 5.6 years (range 0-9.7 years). Dysregulated signalling pathways were examined by immunohistochemical reaction. Only one patient (2.3%) showed presence of nuclear β-catenin with weak staining, typical for WNT-subgroup. There were 12 (27.3%) patients assigned to SHH subgroup by their cytoplasmic, membranous or secreted SFRP1 expressions and lack of nuclear ß-catenin expression.

To determine the activation of mTORC1 pathway, the phosphorylated forms of mTOR protein (p-mTOR) itself and its target molecule (p-S6) were tested. As ribosomal S6 protein is phosphorylated via mTORC1 complex, activation of mTORC1 pathway could be presumed by the co-expression of these two proteins. Forty samples

could be evaluated. Rate of the expression of p-mTOR and p-S6 was equal, 32.5%, their simultaneous expression was present in 9 samples (22.5%). Presence of p-mTOR and p-S6 was observed in 30.3% and 27.3% of samples with classic subtype, and 20.0% and 40.0% in desmoplastic samples, respectively. Expression of p-mTOR and p-S6 showed strong correlation (R=0.55; p=0.0002). All large cell/anaplastic samples were positive for p-mTOR and p-S6. Due to the limitation of the cohort correlations of these proteins with histological subtype only in classic and desmoplastic subtypes could be evaluated. Similar reasons WNT subgroup were omitted from statistical analysis. Nor the expression of p-mTOR or p-S6 showed correlation with age, gender, histology or molecular subtypes. We investigated the effect of mTORC1 activation on the survival of the patients. Patients were divided into two groups whether mTORC1 pathway was activated (p-mTOR and p-S6 co-expression (N=9), or non-activated (N=27). Kaplan-Meier estimated survival shows that patients with activated mTORC1 have lower overall survival rate compared to patients with non-active mTORC1 pathway (5 year estimated survival: 44.4% vs. 77.0%) without statistical significance (p=0.13).

Inhibitory effect of rapamycin and dual inhibitor NVP-BEZ235 were tested in two MB cell lines (Daoy and UW228-2). UW228-2 compared to Daoy cell line shows higher sensitivity at 50 ng/µl rapamycin (proliferation rate: 50% vs. 92%) and at 1 µM NVP-BEZ235 (proliferation rate: 36% vs 61%). mTOR inhibitors were also combined with cisplatin (1 µM) and etoposide (0.1 µM in UW228-2, 1 µM in Daoy). Rapamycin (5 ng/ml) and NVP-BEZ235 (1µM) significantly enhanced their cytostatic effect in both cell lines, especially to cisplatin in Daoy cell line (cisplatin plus rapamycin: 34% in Daoy vs. 59% in UW228-2, cisplatin plus NVP-BEZ235: 11% in Daoy vs. 36% in UW228-2, etoposide plus rapamycin: 63% in Daoy vs. 42% in UW228-2, etoposide + NVP-BEZ235: 53% in Daoy vs. 30% in UW228-2, $p<0.05$). Immunocytochemical analysis of p-mTOR and p-S6, Raptor and Rictor were performed in both MB cell lines. Daoy cells showed strong p-mTOR, moderate p-S6, weak Raptor and strong Rictor expression. UW228-2 cells showed strong expression of all examined proteins, except moderate expression of Raptor. These results indicate weak mTORC1 and elevated mTORC2 activity in Daoy cells, whereas activity of both complexes were elevated in UW228-2 cells.

Based on our results, the presence of p-mTOR and p-S6 protein in tumor samples has no prognostic significance, although lower survival rate was observed among patients with mTORC1 activation. However, the available small cohort could have biased our results. Our experiments with cell lines confirm that the inhibition of the mTOR pathway may have been used as therapeutic target in a subset of patients. Several of mTOR inhibitors have been developed, their efficacy would be influenced by the presence of activated mTORC1 and mTORC2 complexes according to our experiments. Theoretically, in case of their therapeutic application, the measurement of activation of different mTOR complexes is proposed.

Expression of DNMTs (DNMT1, DNMT3A and DNMT3B) was tested and analyzed in terms of pathological data. Forty-four samples could be analyzed. Elevated (moderate/strong) expression of DNMT1, DNMT3A and DNMT3B was observed in 63.6%, 68.2% and 72.7% of patients, respectively. The correlation of histology and DNMT expressions was evaluated in classic and desmoplastic type. Moderate/strong expression of DNMT1 can be detected in 62.2% of classic and 60.0% of desmoplastic cases, and DNMT3A in 70.3% of classic and 60.0% of desmoplastic, and DNMT3B in 70.3% of classic and 80.0% of desmoplastic samples. Two samples of L/A subtype showed moderate/strong expression for DNMT1 and DNMT3B, but only one of them for DNMT3A. There was not any correlation between the expression of DNMT1, DNMT3A and DNMT3B and the age at disease onset, gender or histological subtype. Kaplan-Meier curves, based on different expression of DNMTs did not show significant difference in overall survival. None of the DNMTs could be used as a prognostic marker for medulloblastoma in our cohort. DNMT1 and DNMT3A, or DNMT1 and DNMT3B or DNMT3A and DNMT3B did not show co-expression patterns. The expression of DNMT1 in the SHH subgroup compared to non-WNT/non-SHH subgroup of patients was significantly higher ($p=0.02$), whereas expression of DNMT3A and DNMT3B did not differ significantly in the subgroups. We examined the correlation between the expression of DNMT1 and SFRP1 at mRNA level used dataset from gene expression database (R2) to confirm our finding. No relation was found between the expression of two genes at mRNA level ($p = 0.88$) in comparison with the SHH and non-SHH group.

Based on our experiments the expression of DNMTs did not affect the survival of medulloblastoma patients, however, its increased expression raises the possibility of therapeutic application of DNMT inhibitors.

Conclusions

This was the first time to analyse the prognostic role of mTORC1 complex and DNMTs in medulloblastoma.

a) a small group of patients with medulloblastoma showed mTORC1 pathway activity
b) mTORC1 pathway activation is associated with a poorer prognosis of disease without statistical significance. mTORC1 pathway activation does not correlate with the tested clinical or molecular characteristic of the patients
c) mTORC1 pathway activation does not correlate with WNT, SHH and non-WNT/SHH subgroups of medulloblastoma
d) our *in vitro* results confirm the earlier studies, and suggest that inhibition of mTOR pathway with rapamycin (mTORC1 inhibitor) or NVP BEZ235 (mTORC1/mTORC2/PI3K inhibitor) can increase the efficacy of chemotherapy in medulloblastoma patients showing activated mTOR pathway
e) our *in vitro* studies showed that expression of components of the mTOR pathway are in accordance with the sensitivity of different mTOR inhibitors
f) elevated expression of DNMT1, DNMT3A and DNMT3B shown in most of human medulloblastoma samples propose the therapeutic application of DNMT inhibitors
g) DNMTs did not show any correlation with clinical outcome, patient's age or gender or the histological type of the tumor
h) DNMT1 showed elevated expression in SHH subgroup, in contrast, DNMT3A and DNMT3B did not show relation with any subgroup of medulloblastoma

Publications

Publications related to the PhD thesis

1. **Pócza T**, Sebestyén A, Turányi E, Krenács T, Márk Á, Sticz TB, Jakab Z, Hauser P. (2014) mTOR pathway as a potential target in a subset of human medulloblastoma. Pathol Oncol Res, 20: 893-900.
IF: 1.855

2. **Pócza T**, Krenács T, Turányi E, Csáthy J, Jakab Z, Hauser P. (2016) High expression of DNA methyltransferases in primary human medulloblastoma. Folia Neuropathol, 54: 105-113.
IF: 1.233

Independent publications

1. Himer L, Csóka B, Selmeczy Z, Koscsó B, **Pócza T**, Pacher P, Németh ZH, Deitch EA, Vizi ES, Cronstein BN, Haskó G. (2010) Adenosine A2A receptor activation protects CD4+ T lymphocytes against activation-induced cell death. FASEB J, 24: 2631-2640.
IF: 6.515

2. Hauser P, Vancsó I, **Pócza T**, Schuler D, Garami M. (2013) Antiangiogenic treatment of pediatric CNS tumors in Hungary with the Kieran schedule. Magyar Onkológia, 57: 259–263.
IF: -

3. Thompson EM, Hielscher T, Bouffet E, Remke M, Luu B, Gururangan S, McLendon RE, Bigner DD, Lipp ES, Perreault S, Cho YJ, Grant G, Kim SK, Lee JY, Rao AA, Giannini C, Li KK, Ng HK, Yao Y, Kumabe T, Tominaga T, Grajkowska WA, Perek-Polnik M, Low DC, Seow WT, Chang KT, Mora J, Pollack IF, Hamilton RL, Leary S, Moore AS, Ingram WJ, Hallahan AR, Jouvet A, Fèvre-Montange M, Vasiljevic A, Faure-Conter C, Shofuda T, Kagawa N, Hashimoto N, Jabado N, Weil AG, Gayden T, Wataya T, Shalaby T, Grotzer M, Zitterbart K, Sterba J, Kren L, Hortobágyi T, Klekner

A, László B, **Pócza T**, Hauser P, Schüller U, Jung S, Jang WY, French PJ, Kros JM, van Veelen MC, Massimi L, Leonard JR, Rubin JB, Vibhakar R, Chambless LB, Cooper MK, Thompson RC, Faria CC, Carvalho A, Nunes S, Pimentel J, Fan X, Muraszko KM, López-Aguilar E, Lyden D, Garzia L, Shih DJ, Kijima N, Schneider C, Adamski J, Northcott PA, Kool M, Jones DT, Chan JA, Nikolic A, Garre ML, Van Meir EG, Osuka S, Olson JJ, Jahangiri A, Castro BA, Gupta N, Weiss WA, Moxon-Emre I, Mabbott DJ, Lassaletta A, Hawkins CE, Tabori U, Drake J, Kulkarni A, Dirks P, Rutka JT, Korshunov A, Pfister SM, Packer RJ, Ramaswamy V, Taylor MD. (2016) Prognostic value of medulloblastoma extent of resection after accounting for molecular subgroup: a retrospective integrated clinical and molecular analysis. Lancet Oncol, 17: 484-495.

IF: 26.509