

Activation of Akt and Erk pathways in medulloblastoma

Paweł Włodarski¹, Wiesława Grajkowska², Magda Łojek¹, Kamila Rainko¹, Jarosław Józwiak¹

¹Department of Histology and Embryology, Centre for Biostructure Research, Medical University of Warsaw, Poland; ²Department of Pathology, The Children's Memorial Health Institute, Warsaw, Poland

Folia Neuropathol 2006; 44 (3): 214-220

Abstract

Medulloblastoma (MB) is the most common malignant brain tumour in children. Its aetiology is unknown, although several signalling pathways controlling cell proliferation are thought to participate in the progress of the neoplasm. Mutations of the genes encoding proteins participating in the pathways triggered by embryonic growth factors like Sonic hedgehog (Shh) or WNT are often found in MB.

Another model of MB development is overexpression or mutation of several types of growth factor receptors, including IGF-IR, EGF-R and PDGFR, that have the ability to activate cellular kinases responsible for promoting cell proliferation. In order to test this hypothesis, in the current paper we tested the activation of two kinases, Akt/PKB (protein kinase B) and Erk (extracellular signal-regulated kinase) and their substrates in 10 sporadic medulloblastoma cases. We show that MBs are a highly heterogeneous group of tumours that show upregulation of various signalling pathways. Nevertheless, both Akt and Erk may contribute to the progression of MB, triggering, at least in some cases, the mTOR (mammalian target of rapamycin) pathway, controlling translation of several cell cycle-related proteins. We hypothesize that Akt and Erk activation may also be associated with downregulation of protein phosphatase 2A (PP2A).

Key words: medulloblastoma, Akt, Erk, PP2A.

Introduction

Medulloblastoma (MB), a malignant tumour of the cerebellum, is one of the most fatal CNS tumours among children. MB has a tendency to metastasize and disseminate throughout the CNS early in its course. The current treatment of choice includes surgery and subsequently combined chemotherapy/radiotherapy. However, because of serious complications connected with irradiation of the developing CNS, i.e. memory, attention, motor functioning, language and visuospatial deficits [22], current research focuses on determining the most reliable prognostic factors that

could be used for the stratification of tumour-related prognosis [4,5,18,19]. Thanks to such prognostic factors in the case of the highest-risk patients irradiation doses could be maintained, while in better-fairing cases irradiation could be limited or even completely replaced with chemotherapy alone.

Current WHO classification of tumours of the CNS distinguishes three main variants of MB: classic, desmoplastic and large cell/anaplastic MB [15]. So far the precise origin of MB has not been clarified, although it is suspected that at least the desmoplastic variant originates from cerebellar neural precursor

Communicating author:

Jarosław Józwiak, PhD, Department of Histology and Embryology, Centre for Biostructure Research, Medical University of Warsaw, 02-004 Warszawa, ul. Chatubińskiego 5, e-mail: jjozwiak@atdv.com.pl

cells, i.e. external granular layer (EGL) cells. It is also unclear which processes implicated in cell cycle control contribute to formation of the tumour. Several signalling molecules are associated with MB development. It has been established that tumour growth is connected with activation of Sonic hedgehog (Shh) [21], a critical mitogen for cerebellar granule cell precursor cells. Shh is liberated by the Purkinje cells located at the border of molecular and granular layers. Under the influence of Shh EGL cells proliferate and enter the inner granular layer (IGL), where they stop dividing and start differentiating.

Also, another embryonic growth factor family is found upregulated in MB: WNTs (human homologues of Wingless in *Drosophila*), thanks to their control over β -catenin transcription factor, are regarded as responsible for the regulation of genes modulating cell proliferation, such as cyclin D1, c-Myc or N-Myc [10,28]. It is estimated that mutations in proteins of the WNT pathway may be responsible for about 15% of sporadic MBs [7].

In this research we focused on the third mechanism that is suspected to control cell proliferation in MB. Insulin-like growth factors (IGFs) and their receptors (IGF-IR and IGF-IIR) are known to promote cell proliferation and inhibit cell death by apoptosis [3]. IGF-IR is present in the brain and granular layer of the cerebellum during embryonic and early postnatal development, decreasing significantly during adolescence [8]. After activation, IGF-IR binds a number of substrates, including IRS-1, Shc, PI3K. In turn, IRS-1 and PI3K may trigger downstream phosphorylation events through the Akt (also known as protein kinase B, PKB) or Erk (extracellular signal-regulated kinase) pathways [9]. In the current paper we evaluated activation of Akt and Erk pathways, as well as their participants, in 10 sporadic MB cases. We show that Akt and Erk are indeed implicated, although to a variable extent, in MB progression.

Materials and methods

Tissue samples

Samples of ten human MBs from ten different patients as well as control brain tissue were excised during elective surgery and retrieved from the Department of Pathology, Children's Memorial Hospital, Warsaw, Poland. Control brain tissue consisted of periventricular regions of a patient

operated on for epilepsy. Serving as positive control was a sample of tuberous sclerosis-associated subependymal giant cell astrocytoma (SEGA) surgically resected during elective surgery resulting from cerebrospinal fluid flow obstruction.

Light microscopy

Specimens were fixed in 10% buffered formalin, at room temperature, for 24 h, fixed in paraffin and processed according to routine techniques of light microscopy. Slices 5 to 8 μ m thick were stained with hematoxylin and eosin.

Tissue lysis

Tissues were homogenized in a tissue grinder with RIPA lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) with 50 mM sodium fluoride and 1 mM sodium orthovanadate, supplemented with 1x Complete Protease Inhibitor (Roche, Indianapolis, IN, USA) and Phosphatase Inhibitor Cocktail I (Sigma-Aldrich, St. Louis, MO, USA). In order to minimize differences in sample preparation procedure, all samples were processed at the same time, in exactly the same conditions. Lysates were stored at -80°C .

Western blot

Antibodies against phosphorylated forms Akt (S473) (pAkt), Erk (Y204) (pErk), p70 S6K1 (T421/S424) (pS6K1), p-4E-BP1 (S65/T70) (p4E-BP1), p-p90 RSK1 (S380) (pRSK1) and against PP2A as well as secondary antibodies (HRP-goat anti-rabbit or HRP-bovine anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phosphorylated PTEN (S380) and pGSK3 β (S9) were purchased from Cell Signalling Technology (Beverly, MA, USA). 20 μ g of protein extracted from tissues were subjected to SDS PAGE in a 12% polyacrylamide gel. Afterwards, the gels were transferred onto PVDF membranes. After blocking with 5% non-fat dry milk in TBST (Tris buffered saline, 0.05% Tween), the blots were incubated with respective primary and secondary (HRP-conjugated) antibodies. Membranes were washed in TBST buffer and proteins were detected by West Pico chemiluminescence substrate (Pierce, Roford, IL).

Results

Light microscopy

All the cases of MB were histologically confirmed and determined according to current classification [15] (Table I and Fig. 1).

Akt and Erk are upregulated in most MBs

In a preliminary experiment we tested the activity of Akt and Erk, measured as the degree of phosphorylation of these kinases. We found that all except two (M8 and M10) MBs demonstrated

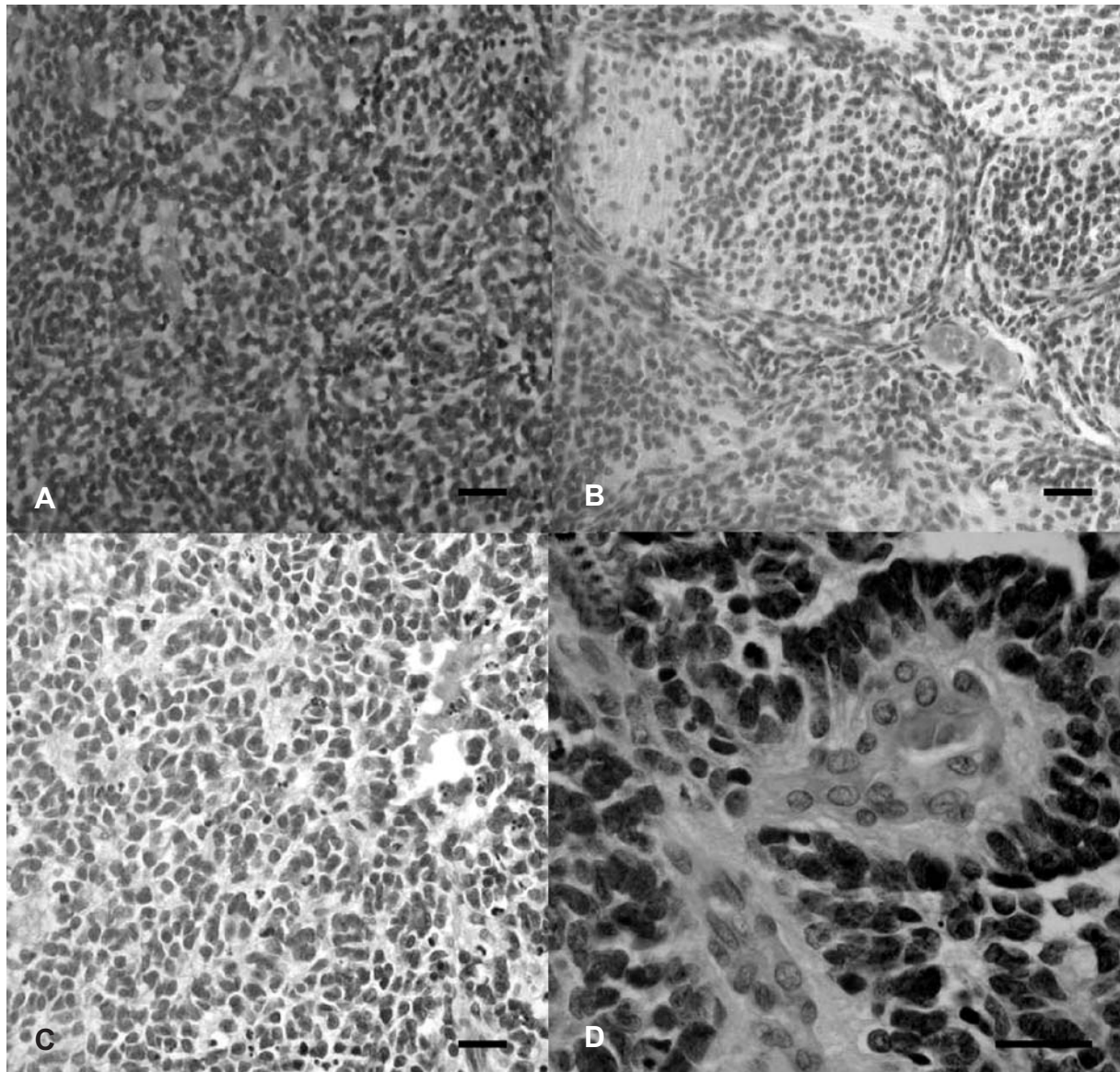


Fig. 1. Representative images of MBs included in the research. A: M1 – Classic medulloblastoma. Typical hypercellular tumour composed of small, round cells; B: M5 – Desmoplastic nodular variant. Clear islands with the characteristics of medulloblastoma surrounded by dark, collagen-rich areas containing small tumour cells; C: M10 – Classic variant; D: M6 – Classic variant with Homer-Wright rosettes. Bars represent 50 μ m

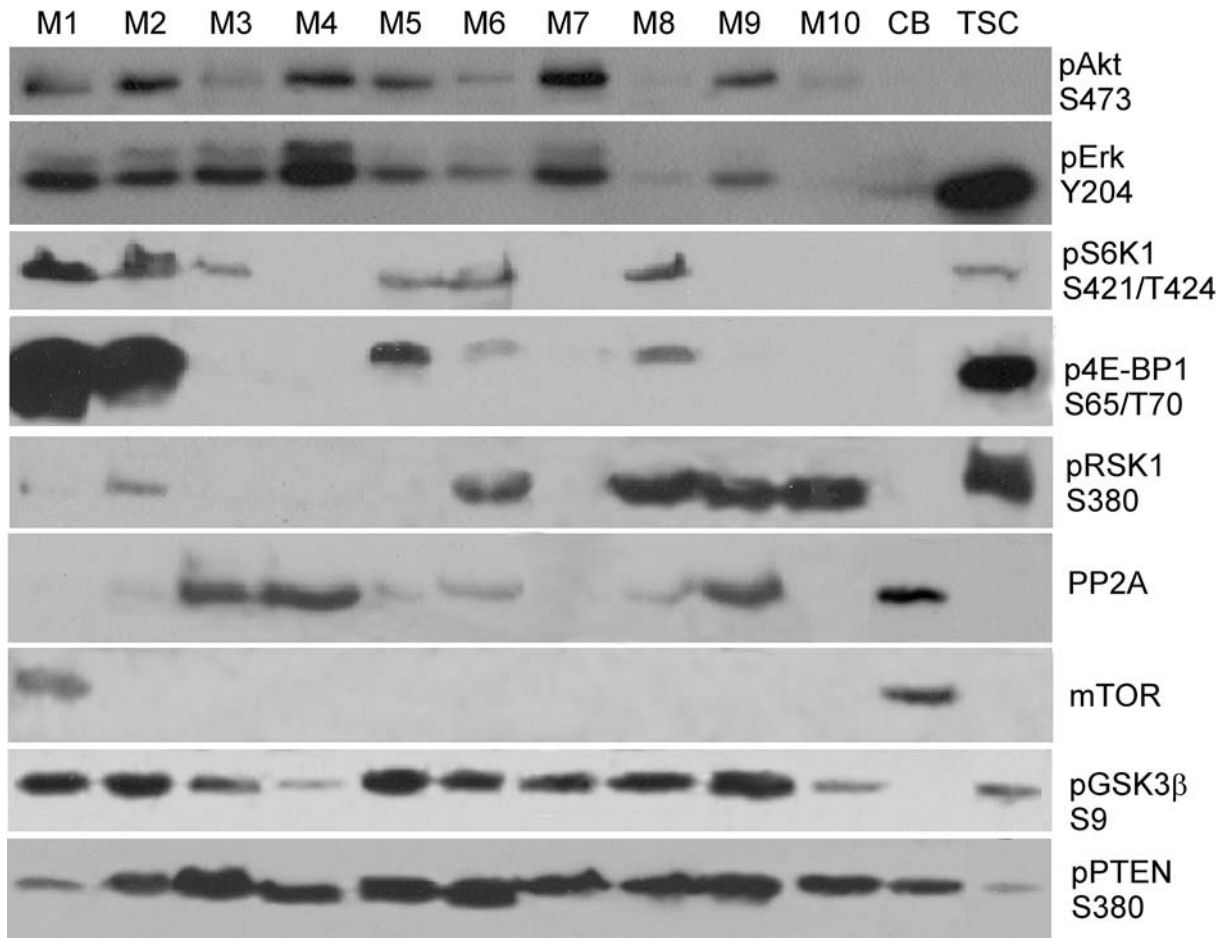


Fig. 2. Western blot of MB samples with antibodies directed against respective (activated) kinases participating in Akt and/or Erk pathway. M1-M10: MB cases; CB: control brain; TSC: tuberous sclerosis-associated SEGA

Table I. Variants of medulloblastoma included in the research

Case no.	Variant
M1	classic
M2	classic
M3	classic with anaplasia
M4	classic with Homer-Wright rosettes
M5	desmoplastic
M6	classic with Homer-Wright rosettes
M7	classic with Homer-Wright rosettes
M8	desmoplastic
M9	classic with anaplasia
M10	classic

hyperphosphorylation of both kinases. We compared the result with SEGA, where upregulation of pErk is documented (manuscript in preparation). Erk hyperphosphorylation in MBs was not as pronounced as in SEGA, but significantly higher than in the control brain (Fig. 2).

Activation of Akt is accompanied by phosphorylation of GSK3β

Active Akt phosphorylates and deactivates GSK3β. Non-phosphorylated GSK3β is active and phosphorylates β-catenin, leading not however a result of a decreased amount of PTEN which, in normal cells, inhibits Akt activation (Fig. 2).

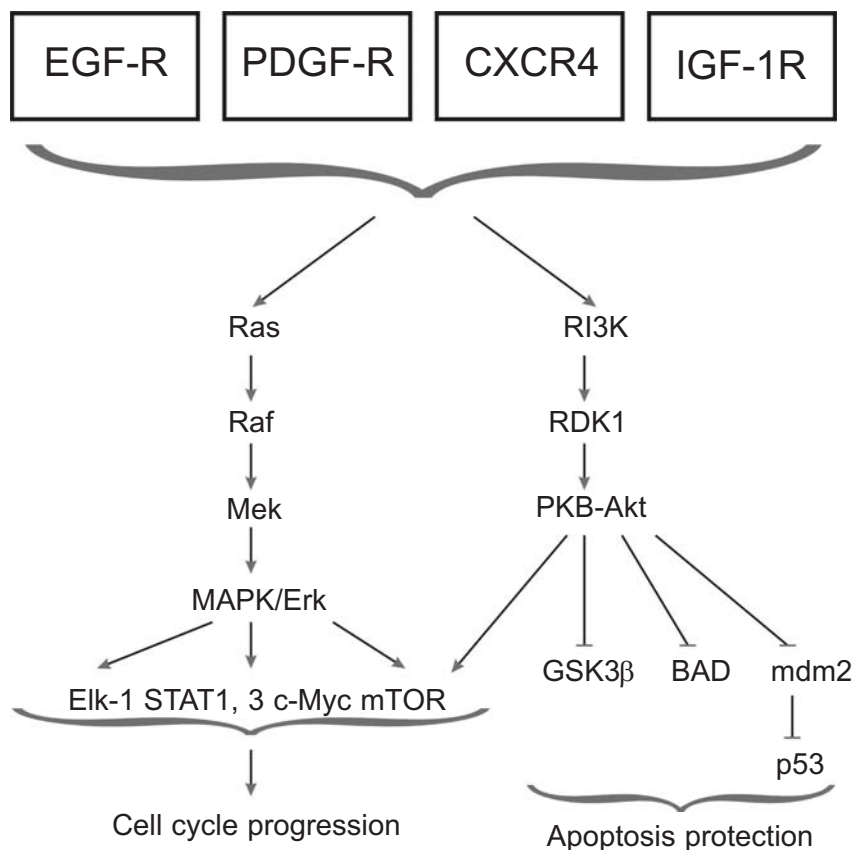


Fig. 3. Growth factor receptors known to trigger activation of Akt and Erk pathways

Erk activation sometimes involves upregulation of the mTOR pathway and may be accompanied by phosphorylation of 4E-BP1

In order to confirm whether Erk phosphorylation is biologically significant, we measured phosphorylation of two other kinases used by activated Erk as substrates: p70 S6K1 and p90 RSK1 [11,23]. Phosphorylation of S6K1 and RSK1 was in fact potentiated; however, to our surprise, correlation of S6K1 and RSK1 hyperphosphorylation with the activity of Erk was very low or even absent. According to our previous observations, such a correlation may be found in SEGA (Fig. 2).

At the same time, we showed that phosphorylation of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1), known to be mediated by the mTOR pathway and

directly controlling translation of numerous proteins, is potentiated compared to controls. In two MBs (M1 and M2) 4E-BP1 hyperphosphorylation was significantly stronger than in SEGA, where 4E-BP1 is known to mediate the cell cycle promoting effect [12]. Interestingly, the level of mTOR kinase seems to be lower than in normal controls in all except one case (M1). The same case of MB shows the strongest hyperphosphorylation of 4E-BP1.

Most MBs show elevated levels of PP2A

Upregulation of Erk may be explained by at least two mechanisms. The first, classical mechanism requires activity of MEK-1, being a classical upstream kinase for Erk. The second, recently formulated hypothesis points to mitochondrial reactive oxygen species that could activate Erk [6]. As we have

recently shown that both of these hypotheses do not fully explain Erk activation (manuscript in preparation), we set out to test whether Erk upregulation could be connected with attenuation of PP2A, acting as a gatekeeper of Akt and Erk activity [14]. For this purpose, we measured by Western blot the level of PP2A in MBs, control tissue and SEGA. We found that most MBs and SEGA show decreased levels of PP2A, while the protein band in controls is strong. Interestingly, in the two MBs with very pronounced hyperphosphorylation of 4E-BP1, the level of PP2A was practically undetectable.

Discussion

The numerous ambiguities concerning pathophysiology of MB prevent precise stratification of patients, which is the first requirement of a more effective and, especially in the case of this disease, lower toxicity of radiotherapy. Three main mechanisms postulated to have an influence on the progress of the disease are under close observation by many research groups: Shh, WNT and growth factor receptor overexpression. In the current research we aimed to determine whether and which proteins participating in the Akt and Erk pathways could be activated in MB. These kinases are known to be triggered by growth factors and their respective receptors, including IGF-IR, ErbB2 (EGF family receptor) and chemokine receptor CXCR4.

The evidence of IGF-IR overexpression in MB comes from studies on MB-derived cell lines and MB tissue samples. Kurihara et al. [16] found for the first time increased amount of IGF-IR in a sample of MB. Other authors noted the presence of IGF-IR protein and IGF-I mRNA in MB-derived cell lines. Such cells stimulated with IGF-I proliferate thanks to activation of the Erk-1/2 pathway, as shown by suppression of this effect by IGF-IR blocking antibodies and by PD 98059, an Erk pathway inhibitor [20]. There have been contradictory reports concerning upregulation of PDGF (platelet-derived growth factor) receptors in MB [1,25], although it seems that PDGFRB (PDGF receptor β) is present in metastatic MB. Also ErbB2 and CXCR4 have been found to be upregulated in MB [2,24] (Fig. 3).

It is a well known fact that IGF-IR stimulation triggers cell proliferation and rescues cells from death. Activation of Akt and Erk in MB certainly contributes to this phenomenon [27]. Both Akt and Erk have been shown to phosphorylate tuberin, a protein forming

intracellular complex with hamartin and exerting control over mTOR (mammalian target of rapamycin), a central regulator of protein translation. Activation of Akt and/or Erk, found in our MB biopsies, abolishes control over mTOR (which is documented by phosphorylation of 4E-BP1 in our experiments) and leads to translation of cell cycle regulating proteins, such as cyclin D1 [26]. On top of that, it has recently been hypothesized that the TSC complex can associate with the β -catenin degradation complex, participating in the WNT pathway, and negatively affecting gene transcription [13]. When Akt and/or Erk are phosphorylated, formation of the TSC complex is inhibited, which increases β -catenin-dependent transcription of genes encoding cell cycle-regulating proteins such as c-Myc, N-Myc or cyclin D1 [17].

In the current paper we also show that upregulation of Akt and Erk may be connected with lower levels of protein phosphatase 2A (PP2A). PP2A is known to act as a phosphatase controlling the level of phosphorylation of a number of kinases, including Akt and Erk [14]. Thus, we hypothesized that hyperphosphorylation of Akt and Erk in MB may be a result of PP2A downregulation. Indeed, in the present paper we demonstrate that PP2A is downregulated in MB. However, a precise mechanism explaining how PP2A may be attenuated requires further research.

References

1. Black P, Carroll R, Glowacka D. Platelet-derived growth factor expression and stimulation in human meningiomas. *J Neurosurg* 1994; 81: 388-393.
2. Bodey B, Kaiser HE, Siegel SE. Epidermal growth factor receptor (EGFR) expression in childhood brain tumors. *In Vivo* 2005; 19: 931-941.
3. Bozyczko-Coyne D, Glicksman MA, Prantner JE, McKenna B, Connors T, Friedman C, Dasgupta M, Neff NT. IGF-I supports the survival and/or differentiation of multiple types of central nervous system neurons. *Ann N Y Acad Sci* 1993; 692: 311-313.
4. Eberhart CG, Kratz J, Wang Y, Summers K, Stearns D, Cohen K, Dang CV, Burger PC. Histopathological and molecular prognostic markers in medulloblastoma: c-myc, N-myc, TrkC, and anaplasia. *J Neuropathol Exp Neurol* 2004; 63: 441-449.
5. Ellison DW, Onilude OE, Lindsey JC, Lusher ME, Weston CL, Taylor RE, Pearson AD, Clifford SC; United Kingdom Children's Cancer Study Group Brain Tumour Committee. beta-Catenin status predicts a favorable outcome in childhood medulloblastoma: the United Kingdom Children's Cancer Study Group Brain Tumour Committee. *J Clin Oncol* 2005; 23: 7951-7957.
6. Finlay GA, Thannickal VJ, Fanburg BL, Kwiatkowski DJ. Platelet-derived growth factor-induced p42/44 mitogen-activated

- protein kinase activation and cellular growth is mediated by reactive oxygen species in the absence of TSC2/tuberin. *Cancer Res* 2005; 65: 10881-10890.
7. Gilbertson RJ. Medulloblastoma: signalling a change in treatment. *Lancet Oncol* 2004; 5: 209-218.
 8. Glick RP, Unterman TG, Blaydes L, Hollis R. Insulin-like growth factors in central nervous system tumors. *Ann N Y Acad Sci* 1993; 692: 223-229.
 9. Hartmann W, Koch A, Brune H, Waha A, Schuller U, Dani I, Denkhau D, Langmann W, Bode U, Wiestler OD, Schilling K, Pietsch T. Insulin-like growth factor II is involved in the proliferation control of medulloblastoma and its cerebellar precursor cells. *Am J Pathol* 2005; 166: 1153-1162.
 10. Huang H, Mahler-Araujo BM, Sankila A, Chimelli L, Yonekawa Y, Kleihues P, Ohgaki H. APC mutations in sporadic medulloblastomas. *Am J Pathol* 2000; 156: 433-437.
 11. Iijima Y, Laser M, Shiraishi H, Willey CD, Sundaravadivel B, Xu L, McDermott PJ, Kuppuswamy D. c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. *J Biol Chem* 2002; 277: 23065-23075.
 12. Jozwiak J, Jozwiak S, Grzela T, Lazarczyk M. Positive and negative regulation of TSC2 activity and its effects on downstream effectors of the mTOR pathway. *Neuromolecular Med* 2005; 7: 287-296.
 13. Jozwiak J, Włodarski P. Hamartin and tuberlin modulate gene transcription via beta-catenin. *J Neurooncol* 2006; 79: 229-234.
 14. Van Kanegan MJ, Adams DG, Wadzinski BE, Strack S. Distinct protein phosphatase 2A heterotrimeric modulate growth factor signaling to extracellular signal-regulated kinases and Akt. *J Biol Chem* 2005; 280: 36029-36036.
 15. Kleihues P, Cavenee WK. *Pathology & Genetics. Tumours of the Nervous System*, IARC press, Lyon, 2000: 123-140.
 16. Kurihara M, Tokunaga Y, Ochi A, Kawaguchi T, Tsutsumi K, Shigematsu K, Niwa M, Mori K. Expression of insulin-like growth factor I receptors in human brain tumors: comparison with epidermal growth factor receptor by using quantitative autoradiography. *No To Shinkei* 1989; 41: 719-725.
 17. Mak BC, Kenerson HL, Aicher LD, Barnes EA, Yeung RS. Aberrant beta-catenin signaling in tuberous sclerosis. *Am J Pathol* 2005; 167: 107-116.
 18. Mendrzyk F, Radlwimmer B, Joos S, Kokocinski F, Benner A, Stange DE, Neben K, Fiegler H, Carter NP, Reifemberger G, Korshunov A, Lichter P. Genomic and protein expression profiling identifies CDK6 as novel independent prognostic marker in medulloblastoma. *J Clin Oncol* 2005; 23: 8853-8862.
 19. Masaki K, Marukawa K, Hayashi Y, Fukusato T, Minamoto T, Hasegawa M, Yamashita J, Fujisawa H. Correlation of gamma-catenin expression with good prognosis in medulloblastomas. *J Neurosurg* 2005; 102: 197-206.
 20. Patti R, Reddy CD, Geoerger B, Grotzer MA, Raghunath M, Sutton LN, Phillips PC. Autocrine secreted insulin-like growth factor-I stimulates MAP kinase-dependent mitogenic effects in human primitive neuroectodermal tumor/medulloblastoma. *Int J Oncol* 2000; 16: 577-584.
 21. Raffel C, Jenkins RB, Frederick L, Hebrink D, Alderete B, Fults DW, James CD. Sporadic medulloblastomas contain PTCH mutations. *Cancer Res* 1997; 57: 842-845.
 22. Ribi K, Rely C, Landolt MA, Alber FD, Boltshauser E, Grotzer MA. Outcome of medulloblastoma in children: long-term complications and quality of life. *Neuropediatrics* 2005; 36: 357-365.
 23. Roux PP, Richards SA, Blenis J. Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. *Mol Cell Biol* 2003; 23: 4796-4804.
 24. Schuller U, Koch A, Hartmann W, Garre ML, Goodyer CG, Cama A, Sorensen N, Wiestler OD, Pietsch T. Subtype-specific expression and genetic alterations of the chemokine receptor gene CXCR4 in medulloblastomas. *Int J Cancer* 2005; 117: 82-89.
 25. Smits A, van Grieken D, Hartman M, Lendahl U, Funa K, Nister M. Coexpression of platelet-derived growth factor alpha and beta receptors on medulloblastomas and other primitive neuroectodermal tumors is consistent with an immature stem cell and neuronal derivation. *Lab Invest* 1996; 74: 188-198.
 26. Takuwa N, Fukui Y, Takuwa Y. Cyclin D1 expression mediated by phosphatidylinositol 3-kinase through mTOR-p70(S6K)-independent signaling in growth factor-stimulated NIH 3T3 fibroblasts. *Mol Cell Biol* 1999; 19: 1346-1358.
 27. Wang JY, Del Valle L, Gordon J, Rubini M, Romano G, Croul S, Peruzzi F, Khalili K, Reiss K. Activation of the IGF-IR system contributes to malignant growth of human and mouse medulloblastomas. *Oncogene* 2001; 20: 3857-3868.
 28. Zurawel RH, Chiappa SA, Allen C, Raffel C. Sporadic medulloblastomas contain oncogenic beta-catenin mutations. *Cancer Res* 1998; 58: 896-899.