

Implication of active Erk in the classic type of human medulloblastoma

Pawel K. Wlodarski¹, Anna Boszczyk¹, Wieslawa Grajkowska², Marcin Roszkowski³, Jaroslaw Jozwiak¹

¹Department of Histology and Embryology, Centre for Biostructure Research, Medical University of Warsaw, Warsaw, Poland;

²Department of Pathology, The Children's Memorial Health Institute, Warsaw, Poland; ³Department of Neurosurgery, The Children's Memorial Health Institute, Warsaw, Poland

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Abstract

Molecular pathways underlying medulloblastoma (MB), the most common malignant brain tumour in children, are still under scrutiny. The mammalian target of the rapamycin (mTOR) pathway is one of the kinases that was recently found to be implicated in a number of human tumours. Also in the case of MB it is suspected that mTOR dysregulation may play an important role in pathogenesis. Active mTOR leads to translation of several proteins, some of which affect cellular proliferation. On the other hand, Akt/PKB (protein kinase B) and Erk (extracellular signal-regulated kinase, also called mitogen-activated protein kinase, MAPK) are two protein kinases whose hyperactivity leads to a number of downstream effects, including activation of mTOR.

In our previous report we found that indeed Akt and Erk are variably activated in human MBs. However, because MBs are a highly heterogeneous group of tumours, we were unable to associate Akt or Erk activation with all the cases of MB. In this paper we evaluated six cases of MB, only of the classic subtype. We found that elements of the Erk pathway are hyperactive in all six tumours. Thus, we postulate that in classic type of MB, growth factor stimulation may lead to Erk upregulation and mTOR-dependent protein translation, causing malignant growth.

Key words: classic medulloblastoma, Erk, mTOR.

Introduction

Medulloblastoma (MB) is one of the most fatal CNS tumours among children, with five-year survival rates ranging from 30% to 70%. The tumour has a tendency to metastasize and disseminate throughout the CNS. Current standard treatment involves surgery and combined chemotherapy/radiotherapy. Unfortunately, because of the influence of

radiation on the developing CNS, therapy results in severe side effects, i.e. memory, attention, motor functioning, language and visuospatial deficits [20]. It is postulated that further research could help determine the most reliable prognostic factors which would be used for the stratification of patients, which in turn could allow adjustment of treatment radicality to prognosis [5,17,18].

Communicating author:

Jaroslaw Jozwiak, PhD, Department of Histology and Embryology, Centre for Biostructure Research, Medical University of Warsaw, Chalubinskiego Str. 5, 02-004 Warsaw, Poland, Email: jjozwiak@atdv.com.pl

In the current WHO classification three main variants of MB are recognized: classic, desmoplastic and large cell/anaplastic [13]. Molecular research is currently underway, but determination of pathways implicated in MB development seems very difficult, e.g. because of the heterogeneity of this group of tumours. Also the precise origin of MB has not been clarified, although it is suspected that at least the desmoplastic variant originates from cerebellar neural precursor cells, i.e. external granular layer (EGL) cells, although progenitor cell populations outside the EGL are also likely the cells of origin for a subset of medulloblastomas [4].

So far no single factor has been identified to contribute to all cases of MB. Probably because of the high heterogeneity of MB subtypes several signalling pathways have been associated with MB development [8]. One of them, WNT (human homologues of Wingless in *Drosophila*), belongs to the family of embryonic growth factors. WNTs, controlling β -catenin transcription factor, are responsible for the regulation of genes modulating cell proliferation, such as cyclin D1, c-Myc and N-Myc [7,31]. It is estimated that mutations in proteins of the WNT pathway may be responsible for about 15% of sporadic MBs [6]. The status of β -catenin phosphorylation and its translocation to the nucleus seems to favour WNT implication in some MBs [22]. Also it has recently been found that inappropriate histone modifications might deregulate expression of Dickkopf-1 (DKK1), a Wnt antagonist, in MB tumorigenesis and block its tumour-suppressive activity [27].

Sonic hedgehog (Shh), liberated by the Purkinje cells located between the molecular and granular layers of the cerebellum, is a critical mitogen for granule cell precursors. Under the influence of Shh EGL cells proliferate and enter the inner granular layer (IGL), where they stop dividing and start differentiating. Also activation of the Shh pathway has been associated with MB growth, as evidenced by stimulation of Gli transcription factors [11]. It is hypothesized that Shh promotes MB through inhibition of apoptosis, as overexpression of Gli1 and Gli2 in MB cultures increased Bcl-2 protein levels [1].

Other signalling pathways associated with MB formation include mTOR-related molecules. mTOR controls translation of around 10% of all intracellular proteins, including those responsible for proliferation control, such as cyclin D1 and ornithine decarboxylase [21,24]. mTOR engagement has been demonstrated in several neoplasms, including those developing in the brain. It

is postulated that mTOR upregulation may be caused by stimulation of Akt/PKB (protein kinase B) and Erk (extracellular signal-regulated kinase). Indeed, this mechanism seems to be valid e.g. in subependymal giant cell astrocytomas [9]. In our previous study we showed that Akt and Erk are variably associated with growth of all subtypes of MB [30]. In this study, we aimed at focusing on a more homogeneous group of tumours. Thus, we selected six cases of classic MB and evaluated signalling status upstream and downstream of Akt and Erk. We found that the Erk pathway was upregulated in five of six classic MBs, while the status of Akt does not seem to be significantly associated with MB progression.

Material and Methods

Tissue samples

Samples of six human MBs from six 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 and whole brain lysate of mouse.

Cell culture

CHO (Chinese Hamster Ovary) cells, whose proliferation depends on Akt/Erk stimulation [12], were cultured in Dulbecco Modified Eagle's Medium supplemented with 10% FCS, L-glutamine and antibiotics (Invitrogen, Carlsbad, CA).

Tissue lysis

Tissues were homogenized with a tissue grinder in 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 the sample preparation procedure, all the samples were processed at the same time, in the same conditions. Before WB analysis tissue lysates were stored at -80°C .

Western blot

Antibodies against phosphorylated forms Akt (S473) (p-Akt), Akt (T308) (p-Akt), PDK1 (S241) (p-PDK1), Erk

(Y204) (p-Erk), MEK1/2 (S217/221) (p-MEK1/2), p90 RSK1 (S380) (pRSK1) and against tubulin 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) (p-PTEN), c-Raf (S338) (p-c-Raf), Elk (S383) (p-Elk) and GSK3 β (S9) (p-GSK3 β) were purchased from Cell Signaling Technology (Beverly, MA, USA). 20 μ g of protein extracted from tissues or cell culture 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, Rocford, IL).

Results

Lack of stimulation upstream of Akt

PTEN (phosphatase and tensin homologue) is a tumour suppressor protein which is found mutated in many human sporadic cancers and in hereditary cancer syndromes, such as Cowden disease. The major substrate of PTEN is phosphatidylinositol-3,4,5-trisphosphate PIP₃, a second messenger molecule produced following PI3K (phosphatidylinositol 3-kinase) activation induced by a variety of stimuli. When PTEN is inactive, PIP₃ activates Akt through the mediation of 3-phosphoinositide dependent protein kinase-1 (PDK-1).

In a preliminary experiment we tested the level of PTEN phosphorylation, as a measure of its activity. We did not find any differences between MBs and control brain tissues (Fig. 1). Also, the amount of active PDK1 was not elevated in any of six cases compared to control tissues.

Weak upregulation of Akt is accompanied by phosphorylation of GSK3 β in three cases

Active PDK1 translocates to the plasma membrane, where Akt becomes phosphorylated in the catalytic domain at T308. In addition, Akt is phosphorylated in the regulatory domain at S473 through mechanisms not completely understood. Phosphorylation of Akt at both T308 and S473 is required for full kinase activity [28]. Consistently with lack of PDK1 activation, in the six eva-

luated cases of MB we did not find increased levels of Akt phosphorylated at T308, although in two cases (MB1 and MB6), phosphorylation at S473 was slightly higher than in control brain.

Glycogen synthase kinase 3 β (GSK3 β) is usually thought to be a direct effector of active Akt. Thus, we measured the amount of phosphorylated GSK3 β in order to confirm lack of Akt activation. In three cases (MB1, MB3 and MB5) GSK3 β was upregulated in comparison to control (Fig. 1). This fact may be explained by the existence of other kinases for GSK3 β , such as Erk [16].

Ras/c-Raf upregulation was present in five of six cases

The canonical pathway for Ras activation begins with dimerization and autophosphorylation of the receptor tyrosine kinases that bind growth factors. As a result,

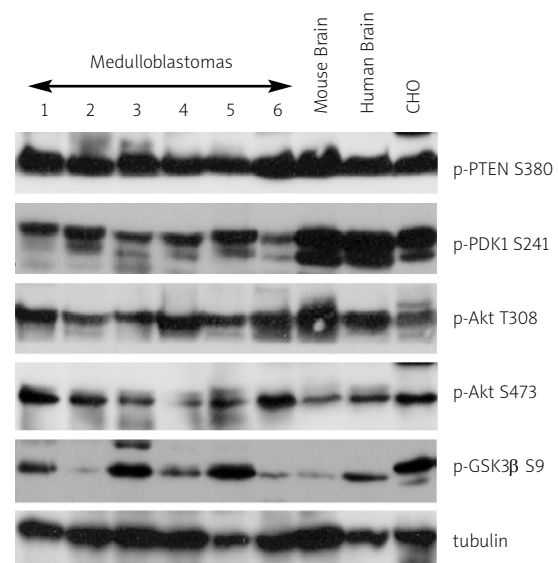


Fig. 1. Detection of proteins belonging to the Akt pathway by Western blot. Six classic cases of MB along with two negative (control brain tissue) and one positive control (CHO cell line) were probed with a panel of antibodies directed against the active forms of proteins. PDK1, the direct activator of Akt, was not upregulated in MB samples, while two of six MBs showed very weak stimulation of Akt. Protein loading was verified by tubulin detection

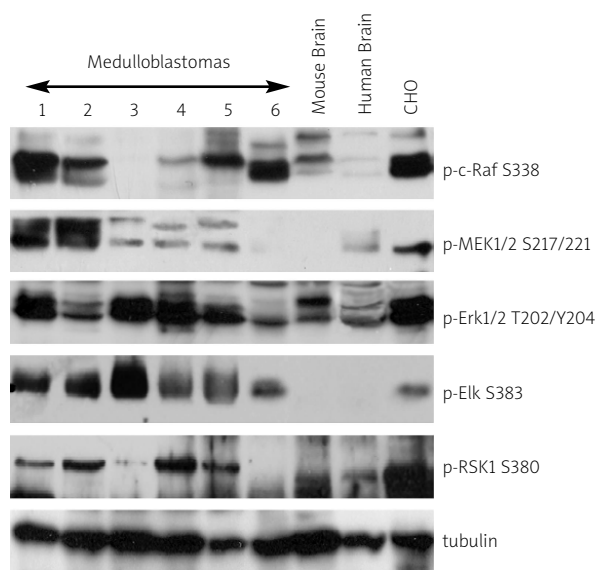


Fig. 2. Detection of active forms of proteins of the Erk pathway by Western blot. Erk stimulation is pronounced in four of six cases. Biological activity of Erk is confirmed by two specific phosphorylations of downstream effectors: Elk (S383) and RKS1 (S380). Protein loading was verified with tubulin detection

activated Ras can bind c-Raf, a serine/threonine-specific kinase being MAP kinase kinase kinase (MAP3K) and functioning downstream of the Ras family of membrane-associated GTPases. Once activated, c-Raf can phosphorylate dual specificity protein kinase MEK (or MAP2K), which in turn phosphorylates Erk.

We determined the level of c-Raf activation in MB and found that in five (MB1, MB2, MB4-6) of six cases c-Raf was upregulated, although the level of activation varies between cases.

Erk and its downstream effectors were hyperactive

Finally, we aimed at confirming whether activation of c-Raf is biologically significant and leads to recruitment and phosphorylation of Erk.

We found that MEK, the classical and direct activator of Erk, is indeed active in five cases (M1-5). As a consequence, also upregulation of Erk was noticed in four cases (MB1, MB3-5). In the two remaining cases (M6) Erk was also active, but at a level similar to control brain tissue. We revealed that active Erk phosphorylates its

transcription factor Elk at S383. However, interestingly, Elk phosphorylation was demonstrated in all six cases, not only in those where Erk itself was upregulated.

Similarly, RSK1, another effector of Erk, was phosphorylated in four cases (MB1-2, MB3-4).

Discussion

MBs pose a great threat to the paediatric population, affecting 1 in every 50,000 people. As the tumour is very aggressive, it requires radical treatment which, in view of the young age of patients, has a destructive impact on developing CNS. Unfortunately, the heterogeneity of this group of tumours complicates reliable stratification of patients that could allow better adjustment of treatment radicality to actual aggressiveness of the tumour.

From the three main mechanisms postulated to influence the progress of the disease, namely involving Shh, WNT and Akt/Erk, we decided to take a closer look at the latter one. Both of 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. Indeed, Kurihara et al. [14] found an increased amount of IGF-IR in a sample of MB. Also, other authors noted the presence of IGF-IR protein and IGF-I mRNA in MB-derived cell lines. Importantly, such cells stimulated with IGF-I proliferate thanks to the activation of the Erk pathway, as shown by suppression of this effect by IGF-IR blocking antibodies and by PD 98059, an Erk pathway inhibitor [19]. There have been contradictory reports concerning upregulation of PDGF (platelet-derived growth factor) receptors in MB [2,25], 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 [3,23].

In order to increase homogeneity of the sample, we analyzed six cases of the classic type of MB and evaluated their Akt/Erk status. We found that the kinases participating in the Akt pathway and Akt itself were slightly upregulated. At the same time, most of the elements of the Erk pathway showed significant potentiation.

According to the present hypothesis, activation of Akt and Erk in MB rescues cells from death [29]. Both Akt and Erk have been shown to phosphorylate tuberin, a protein forming an intracellular complex with hamartin and exerting its control over mTOR (mammalian target of rapamycin), a central regulator of protein translation. Activation of Erk, found in biopsies of classic MBs, abolishes control over mTOR and leads to translation of cell cycle regulating proteins, such as cyclin D1 [26]. Also, it has

recently been hypothesized that TSC complex can associate with the β -catenin degradation complex, participating in the WNT pathway, and negatively affecting gene transcription [10]. Phosphorylation of Erk in MB inhibits formation of the TSC complex and increases β -catenin-dependent transcription of genes encoding cell cycle-regulating proteins such as c-Myc, N-Myc and cyclin D1 [15]. Thus, our report supports the hypothesis that Erk may be a significant signal for progression of the classic type of MB.

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References

- Bar EE, Chaudhry A, Farah MH, Eberhart CG. Hedgehog signaling promotes medulloblastoma survival via Bc/ll. *Am J Pathol* 2007; 170: 347-355.
- Black P, Carroll R, Glowacka D. Expression of platelet-derived growth factor transcripts in medulloblastomas and ependymomas. *Pediatr Neurosurg* 1996; 24: 74-78.
- Bodey B, Kaiser HE, Siegel SE. Epidermal growth factor receptor (EGFR) expression in childhood brain tumors. *In Vivo* 2005; 19: 931-941.
- Eberhart CG. In search of the medulloblast: neural stem cells and embryonal brain tumors. *Neurosurg Clin N Am* 2007; 18: 59-69.
- 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.
- Gilbertson RJ. Medulloblastoma: signalling a change in treatment. *Lancet Oncol* 2004; 5: 209-218.
- 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.
- Jozwiak J, Grajkowska W, Wlodarski P. Pathogenesis of medulloblastoma and current treatment outlook. *Med Res Rev* 2007; 27: 869-890.
- Jozwiak J, Jozwiak S, Wlodarski P. Possible mechanisms of disease development in tuberous sclerosis. *Lancet Oncol* 2008; 9: 73-79.
- Jozwiak J, Wlodarski P. Hamartin and tuberin modulate gene transcription via beta-catenin. *J Neurooncol* 2006; 79: 229-234.
- Kimura H, Stephen D, Joyner A, Curran T. Gli1 is important for medulloblastoma formation in Ptc1+/- mice. *Oncogene* 2005; 24: 4026-4036.
- Kleihues P, Cavenee WK. *Pathology & Genetics. Tumours of the Nervous System*, IARC press, Lyon 2000; pp. 123-140.
- 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.
- Mak BC, Kenerson HL, Aicher LD, Barnes EA, Yeung RS. Aberrant beta-catenin signaling in tuberous sclerosis. *Am J Pathol* 2005; 167: 107-116.
- Medina MG, Ledesma MD, Domínguez JE, Medina M, Zafra D, Alameda F, Dotti CG, Navarro P. Tissue plasminogen activator mediates amyloid-induced neurotoxicity via Erk1/2 activation. *EMBO J* 2005; 24: 1706-1716.
- 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.
- Misaki 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.
- Patti R, Reddy CD, Georger 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.
- 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.
- Rosenwald IB, Kaspar R, Rousseau D, Gehrke L, Leboluch P, Chen JJ, Schmidt EV, Sonenberg N, London IM. Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels. *J Biol Chem* 1995; 270: 21176-21180.
- Salaroli R, Russo A, Ceccarelli C, Mina GD, Arcella A, Martinelli GN, Giangaspero F, Capranico G, Cenacchi G. Intracellular distribution of beta-catenin in human medulloblastoma cell lines with different degree of neuronal differentiation. *Ultrastruct Pathol* 2007; 31: 33-44.
- 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.
- Shantz LM, Pegg AE. Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. *Cancer Res* 1994; 54: 2313-2316.
- 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.
- 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.
- Vibhakar R, Foltz G, Yoon JG, Field L, Lee H, Ryu GY, Pierson J, Davidson B, Madan A. Dickkopf-1 is an epigenetically silenced candidate tumor suppressor gene in medulloblastoma. *Neuro Oncol* 2007; 9: 135-144.
- Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002; 2: 489-501.

28. 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.
29. Włodarski P, Grajkowska W, Łojek M, Rainko K, Józwiak J. Activation of Akt and Erk pathways in medulloblastoma. *Folia Neuropathol* 2006; 44: 214-220.
30. Zurawel RH, Chiappa SA, Allen C, Raffel C. Sporadic medulloblastomas contain oncogenic beta-catenin mutations. *Cancer Res* 1998; 58: 896-899.