

Favourable prognosis in medulloblastoma with extensive nodularity is associated with mitogen-activated protein kinase upregulation

Jarosław Józwiak¹, Iwona Sontowska¹, Barbara Bikowska¹, Wiesława Grajkowska^{2,3}, Ryszard Galus¹, Marcin Roszkowski⁴

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

²Department of Pathology, The Children's Memorial Hospital, Warsaw, Poland, ³Department of Clinical and Experimental Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences, ⁴Department of Neurosurgery, The Children's Memorial Hospital, Warsaw, Poland

Folia Neuropathol 2011; 49 (4): 257-261

Abstract

Medulloblastoma with extensive nodularity (MBEN) is the only type of medulloblastoma (MB), an aggressive CNS tumour of childhood, that is connected with favourable prognosis. In patients with MBEN tumour resection and chemotherapy are sometimes sufficient.

While development of other types of MB is usually connected with activation of the wingless pathway, sonic hedgehog pathway or mammalian target of rapamycin (mTor) pathway, little is known about the molecular basis of MBEN pathophysiology. In the present paper we evaluated activation of the mTor pathway and kinases upstream of mTor, mitogen-activated protein kinase (MAPK/Erk) and protein kinase B (PKB/Akt) in an MBEN sample.

Using western blot technique with antibodies directed against active, phosphorylated forms of proteins, we found upregulation of mTor, Akt and Erk. Thus we postulate that the mTor pathway, often implicated in the development of CNS tumours, is also responsible for MBEN progression. Especially interesting seems implication of Erk and other kinases belonging to the same pathway: mitogen-activated protein kinase kinase (MEK-1) or phospho-ribosomal S6 kinase-1 (p90 RSK1), whose activity we usually demonstrate in more benign neoplasms. However, it remains to be clarified whether Erk pathway activation is actually prognostic for benign tumour development.

Key words: medulloblastoma with extensive nodularity, mTor, mitogen-activated protein kinase.

Introduction

Medulloblastomas (MBs) are a heterogeneous group of tumours, with currently unclear pathogenesis. They are the most common type of primary CNS

tumours in children, comprising around 20% of all primary CNS tumours of childhood [10]. Because of its aggressive nature, prognosis for patients is grim: in spite of standard therapy including surgery and

Communicating author:

Jarosław Józwiak, Department of Histology and Embryology, Center for Biostructure Research, Medical University of Warsaw, ul. Chalubinskiego 5, 02-004 Warsaw, Poland, e-mail: jaroslaw.jozwiak@gmail.com

irradiation followed by chemotherapy, only 50-80% 5-year survival is achieved. One should also remember about long-term side-effects connected with irradiation of the neuroaxis in very young children. Most of such patients are eventually psychosocially disabled, which is one of the main negative impacts of the therapy.

The last (2007) WHO classification lists four types of MB: desmoplastic/nodular MB, MB with extensive nodularity (MBEN), anaplastic MB, and large-cell MB. Interestingly, different types of MB are associated with different prognosis, e.g. large-cell MB has especially poor prognosis [14], while MBEN is a rare MB type and the only one with good prognosis [9]. In fact, numerous patients with MBEN are treated by tumour resection and chemotherapy, without radiotherapy, and have a favourable outcome [4]. Association of MBEN with nevoid basal cell carcinoma syndrome (Gorlin's syndrome) has been hypothesized [1].

As far as pathophysiology of MB is concerned, there are currently several hypotheses contributing to our understanding of this tumour. Although numerous cell lines and animal models have been derived for the study of genetic alterations in MB, their anatomical location, progression and, probably, signalling pathways are different than those present in human tumours. In general, however, three pathways are usually associated with MB development: hedgehog, regulating cerebellar development [15]; wingless, also implicated in embryogenesis as well as normal epithelial differentiation [5,17]; and the insulin-like growth factor-1 pathway [11]. Precise significance of these genes/growth factors and their contribution to MB is unknown.

In the present paper, we evaluated activation of the mTor (mammalian target of rapamycin) pathway and kinases upstream of mTor, mitogen-activated protein kinase (MAPK/Erk) and protein kinase B (PKB/Akt), in MBEN. Both of these kinases can be triggered by insulin-like growth factor-1 and are often implicated in tumorigenesis [6,7,16].

Material and methods

Tissue samples and sample preparation

The MBEN tumour sample excised from the patient as well as control tissues (normal human brain and subependymal giant cell astrocytoma, SEGA) were retrieved from the Department of Pathology, Children's Memorial Hospital, Warsaw, Poland.

The SEGA sample was retrieved from a TS patient diagnosed clinically according to the criteria of Roach [12]. Control brain tissue consisted of periventricular regions of patients operated on for epilepsy.

In order to perform electrophoresis, 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) and Phosphatase Inhibitor Cocktail I (Sigma-Aldrich, St. Louis, MO). In order to minimize differences in the sample preparation procedure, all the samples were processed at the same time, in the same conditions. Lysates were stored at -80°C .

Histopathological preparation

Serial sections obtained from the tumour were embedded in paraffin and subsequently stained according to a routine procedure with haematoxylin and eosin.

Western blot

20 μg of protein extracted from tissues or cells were subjected to SDS PAGE in a 10% polyacrylamide gel. 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, Rockford, IL). Even protein loading was verified by Ponceau S staining. Antibodies against phosphatase and tensin homolog (p-PTEN), phospho-S6 kinase 1 (p-S6K1) T421/S424, phospho-extracellular regulated kinase (p-Erk) T202/Y204, phosphoprotein kinase B (p-Akt) S473, insulin receptor α (InR α), phospho-ribosomal S6 kinase-1 (p-RSK1) T348 and secondary antibodies (HRP-goat anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-phosphoinositide-dependent kinase-1 (p-PDK1) S241, phospho-glycogen synthase kinase 3 β (p-GSK-3 β) S9, phospho-mitogen-activated protein kinase kinase (p-MEK) S217/S221, phospho-S6 ribosomal protein (p-S6rp) S235/S236 were from Cell Signaling Technologies (Beverly, MA).

Results

Histopathological findings

Histologically MBEN displays an extreme degree of nodular architecture (Fig. 1A) with large elongated reticulin-free pale islands (Fig. 1B). These areas are rich in neuropil-like tissue and contain rows and streaming of small round neurocyte-like cells with nuclei containing granular chromatin and small nucleoli (Fig. 1C). The internodular component may be markedly reduced.

Strong upregulation of mTor effectors

The MBEN sample showed significantly increased staining for phospho-S6 ribosomal protein (p-S6) and phospho-eukaryotic translation initiation factor 4E binding protein 1 (p-4E-BP1), compared to the control brain sample. The level of phosphorylation was similar to the SEGA sample, where implication of the mTor pathway in tumour progression is well evidenced (Fig. 2, upper panel).

Akt pathway is hyperactive in MBEN

Protein kinase B, also known as Akt, is often regarded as a classic activator of mTor. Thus, in view of the fact that mTor effectors were overly active, it seemed reasonable to test potentiation of Akt. Phosphoinositide-dependent kinase-1 (PDK1), which is typically phosphorylated at S241, after insulin receptor stimulation was found to be hyperactive (as shown by overt phosphorylation). Also Akt itself was more active than the control. On the other hand, phosphatase and tensin homologue (PTEN), an antagonist of the Akt pathway, was only slightly upregulated and could not abolish the signal conferred to Akt. In order to confirm biological significance of phospho-Akt, we measured the level of phospho-glycogen synthase kinase 3 β (p-GSK-3 β), which is phosphorylated at S9 by Akt. Indeed, the signal was strong and thus confirmed our expectations (Fig. 2, middle panel).

Consistent activation of all Erk upstream regulators

We also evaluated the possibility of mTor signal transmission through Erk. In response to receptor stimulation (e.g. insulin-like growth factor receptor 1, epidermal growth factor receptor, platelet-derived

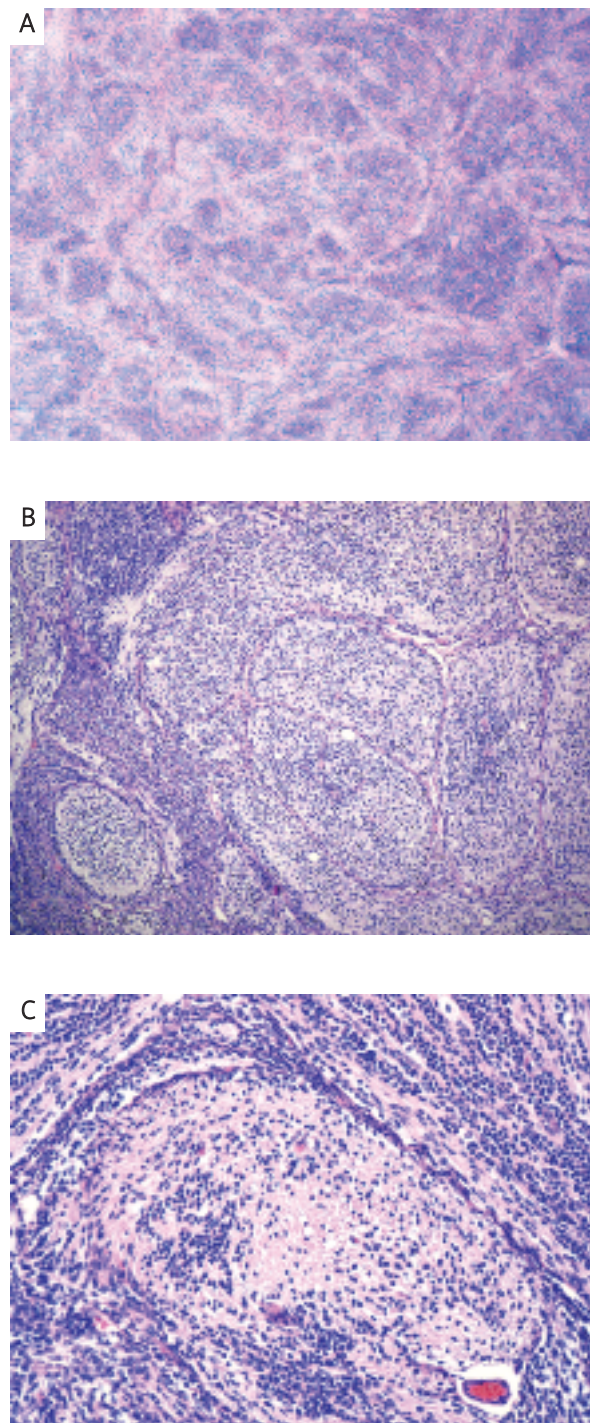


Fig. 1. Microscopic features of medulloblastoma with extensive nodularity. **(A)** Nodular pattern. **(B)** Elongated pale islands. **(C)** The streaming pattern of neurocyte-like cells in reticulin-free zones.

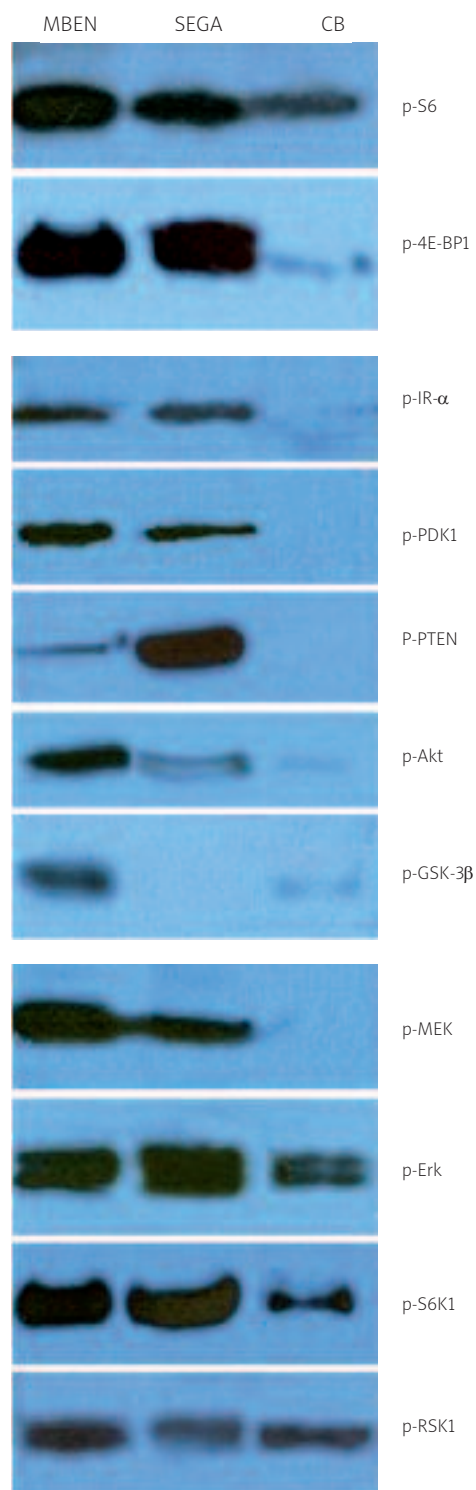


Fig. 2. Western blot analysis of protein lysates from MBEN, subependymal giant cell astrocytoma (SEGA) and control brain (CB). Assayed antibodies were pooled into three groups, from the top: mTor effectors, antibodies implicated in Akt pathway and Erk pathway.

growth factor receptor), mitogen-activated protein kinase kinase (p-MEK) is phosphorylated at S217/S221. In our sample, MEK showed stronger phosphorylation at this site than the positive control, i.e. the SEGA sample. Also Erk was hyperactive and demonstrated its effect on two downstream targets: ribosomal protein S6 kinase 1 (p90 RSK1) and protein S6 kinase 1 (p70 S6K1).

Discussion

Medulloblastoma with extensive nodularity (MBEN) is a distinct medulloblastoma subset associated with younger age and better prognosis. The gyriform, nodular mass in the child's posterior cranial fossa that is hyperdense on unenhanced CT, and markedly enhancing on MRI, may strongly suggest medulloblastoma with extensive nodularity [2].

Medulloblastoma with extensive nodularity occurs mainly in infants and accounts for less than 3% of medulloblastomas. The tumour is very advanced in nodularity and reveals signs of neuronal differentiation [8]. However, unlike other medulloblastomas, MBEN is connected with a favourable outcome, which is probably related to spontaneous neurocytic (as well as astrocytic) differentiation of this type of medulloblastoma [4,13]. It has also been noted that sometimes MBEN, after treatment, undergoes complete maturation into a tumour resembling ganglioglioma [3].

In the present study we aimed to analyse the status of mTor, a kinase centrally regulating the fate of the cell. In favourable conditions, when the cell is stimulated by external stimuli and has sufficient levels of resources (e.g. oxygen, energy in the form of ATP or nutrients), mTor phosphorylates and thus triggers its downstream effectors, eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1), which in turn intensify translation of proteins participating in cell cycling. On the other hand, when external stimuli are absent or necessary resources are lacking, the cell concentrates on survival and differentiation and does not enter the cell cycle.

Activation of mTor is often found in CNS tumours. In previous studies we and other authors detected elevated concentration of active kinases upstream of mTor in subependymal giant cell astrocytoma [7], medulloblastoma [16] and atypical teratoid/rhabdoid tumour [6]. Thus, we wanted to determine whether the same kinases may be impli-

cated in MBEN and whether the level of active Erk, a kinase usually upregulated in more prognostically favourable neoplasms, is actually higher in MBEN.

We started our study by testing the levels of phosphorylated, and thus active, forms of mTor kinase effectors. mTor is sometimes called the “central regulator of cell well-being”, as different growth factor pathways focus on mTor, triggering its activity and mediating protein translation. Among such effectors are S6 ribosomal protein (S6) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), phosphorylated in response to mTor activity. Their role is to recruit ribosome subunits and initiate the translation process. In the evaluated sample of MBEN p-S6 and p-4E-BP1 we demonstrated elevated amounts of active forms of both proteins.

Although we found that both pathways triggering mTor, i.e. Akt and Erk, were upregulated in MBEN, the hyperactivity of Erk may be especially interesting. As we have shown before, e.g. in SEGA and other benign tumours, Erk is usually upregulated in neoplasms with lower proliferative potential. Also in this case such a relation was found, although it remains to be clarified whether this fact is actually prognostic for benign tumour development.

Acknowledgements

The study was supported by the grant R13001106/2009 from the Polish Ministry of Education.

References

- Aliani S, Brunner J, Graf N, Altmeyer K, Niedermayer I, Strowitzki M. Medulloblastoma with extensive nodularity in nevroid basal cell carcinoma syndrome. *Med Pediatr Oncol* 2003; 40: 266-267.
- Bekiesińska-Figatowska M, Jurkiewicz E, Uliasz M, Mazurczak T, Barszcz S, Grajkowska W, Roszkowski M. Medulloblastoma with extensive nodularity: US, CT and MRI findings. *Pol J Radiol* 2008; 73: 32-35.
- Chelliah D, Mensah Sarfo-Poku C, Stea BD, Gardetto J, Zumwalt J. Medulloblastoma with extensive nodularity undergoing post-therapeutic maturation to a gangliocytoma: a case report and literature review. *Pediatr Neurosurg* 2010; 46: 381-384.
- Giargaspero F, Perilongo G, Fondelli MP, Brisigotti M, Carollo C, Burnelli R, Burger PC, Garré ML. Medulloblastoma with extensive nodularity: a variant with favorable prognosis. *J Neurosurg* 1999; 91: 971-977.
- 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.
- Jóźwiak J, Bikowska B, Grajkowska W, Sontowska I, Roszkowski M, Galus R. Activation of Akt/mTOR pathway in a patient with atypical teratoid/rhabdoid tumor. *Folia Neuropathol* 2010; 48: 185-189.
- Jozwiak J, Grajkowska W, Kotulska K, Jozwiak S, Zalewski W, Zajaczkowska A, Roszkowski M, Slupianek A, Wlodarski P. Brain tumor formation in tuberous sclerosis depends on Erk activation. *Neuromolecular Med* 2007; 9: 117-127.
- Louis ND, Ohgaki H, Wiestler OD, Cavenee WK. WHO Classification of Tumours of the Central Nervous System (3rd Edition). IARC, Lyon 2007; pp. 132-140.
- McManamy CS, Pears J, Weston CL, Hanzely Z, Ironside JW, Taylor RE, Grundy RG, Clifford SC, Ellison DW; Clinical Brain Tumour Group. Nodule formation and desmoplasia in medulloblastomas – defining the nodular/desmoplastic variant and its biological behaviour. *Brain Pathol* 2007; 17: 151-164.
- Packer RJ. Chemotherapy for medulloblastoma/primitive neuroectodermal tumors of the posterior fossa. *Ann Neurol* 1990; 28: 823-828.
- 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.
- Roach ES, Sparagana SP. Diagnosis of tuberous sclerosis complex. *J Child Neurol* 2004; 19: 643-649.
- Suresh TN, Santosh V, Yasha TC, Anandh B, Mohanty A, Indiradevi B, Sampath S, Shankar SK. Medulloblastoma with extensive nodularity: a variant occurring in the very young – clinicopathological and immunohistochemical study of four cases. *Childs Nerv Syst* 2004; 20: 55-60.
- von Hoff K, Hartmann W, von Bueren AO, Gerber NU, Grotzer MA, Pietsch T, Rutkowski S. Large cell/anaplastic medulloblastoma: outcome according to myc status, histopathological, and clinical risk factors. *Pediatr Blood Cancer* 2010; 54: 369-376.
- Vorechovský I, Tingby O, Hartman M, Strömberg B, Nister M, Collins VP, Toftgård R. Somatic mutations in the human homologue of *Drosophila* patched in primitive neuroectodermal tumours. *Oncogene* 1997; 15: 361-366.
- Wlodarski PK, Boszczyk A, Grajkowska W, Roszkowski M, Jozwiak J. Implication of active Erk in the classic type of human medulloblastoma. *Folia Neuropathol* 2008; 46: 117-122.
- Yokota N, Nishizawa S, Ohta S, Date H, Sugimura H, Namba H, Maekawa M. Role of Wnt pathway in medulloblastoma oncogenesis. *Int J Cancer* 2002; 101: 198-201.