

## Activation of Akt/mTOR pathway in a patient with atypical teratoid/rhabdoid tumor

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*Folia Neuropathol* 2010; 48 (3): 185-189

### Abstract

Atypical teratoid/rhabdoid tumor (AT/RT) is a highly malignant childhood brain tumor. Most AT/RTs are shown to contain chromosome 22 mutation in the region of *hSNF5/INI1* gene, whose protein product participates in chromatin remodeling. Although the presence of this mutation is well described, molecular pathways underlying AT/RT development are poorly, if at all, understood.

In the current paper we evaluate a case of AT/RT with special consideration of two pathways often implicated in tumor development: protein kinase B (PKB or Akt) and extracellular signal-regulated kinase (Erk). First, we confirmed expression of typical protein pattern being unique for AT/RT, including epithelial membrane antigen, S-100 and glial fibrillary acidic protein. In molecular analyses we tested the sample for activity of Akt and Erk kinase cascade.

We found that Erk pathway signaling in the tumor was not upregulated. Neither *c-Raf*, MAPK nor Erk were hyperphosphorylated. On the other hand, we detected significant phosphorylation of Akt, phosphoinositide-dependent kinase-1 (PDK1) and glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ). At the same time, inhibitor of Akt pathway, phosphatase and tensin homolog (PTEN), was not upregulated. These results strongly support the hypothesis that Akt pathway contributes to chromatin remodeling disruption, promoting malignant transformation of AT/RT.

**Key words:** brain tumors, Erk, Akt, AT/RT.

### Introduction

Atypical teratoid/rhabdoid tumor (AT/RT) is a highly malignant childhood brain tumor, almost inevitably associated with dismal prognosis [17]. AT/RT can occur anywhere in the central nervous system (CNS), including the spinal cord. About 60% of tumors are located in the posterior cranial fossa (especially in the cerebel-

lum). Most rhabdoid tumors were shown to contain chromosome 22 mutation in the region of *hSNF5/INI1* gene [2]. The protein encoded by *INI1/hSNF5* is a component of the chromatin remodeling SWI/SNF complex, and is a critical tumor suppressor biallelically inactivated in rhabdoid tumors [9]. Identification of *INI1* as a tumor suppressor in AT/RT has facilitated diagnosis of rhabdoid tumors.

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Although the presence of *hSNF5/INI1* mutations in rhabdoid tumors is now well documented, little is known of actual pathophysiology of these neoplasms. However, in a recent study performed on cell lines originating from AT/RT and malignant rhabdoid tumor, Arcaro *et al.* [1] evaluated the status of several receptor tyrosine kinases. We found that insulin receptor (InR) and insulin-like growth factor-I receptor (IGFIR) are overexpressed in AT/RT cell lines.

This observation turned our attention to the mTOR (mammalian target of rapamycin) pathway. mTOR is a central regulator of cellular state, whose function largely depends on the state of well-being of the cell. If the cell lacks energy, oxygen or nutrients, the kinase inhibits protein synthesis and stops proliferation. On the other hand, when all these elements are available, cell cycle is triggered with eventual division. Because of its central regulatory role, mTOR kinase is often implicated in tumorigenesis [3,10,18]. As mTOR is directly activated by Akt [4] and Erk [15], we decided to evaluate these two kinases and their pathways in AT/RT.

As material we used tumor mass from the area of left cerebellopontine angle excised from a 3-year old boy with histopathological confirmation of AT/RT.

## Material and methods

### Tissue samples and sample preparation

AT/RT sample of the tumor 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. SEGA sample was retrieved from a TS patient diagnosed clinically according to the criteria of Roach. Control brain tissue consisted of periventricular regions of patients operated on for epilepsy.

For electrophoresis, tissues were homogenized in 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 1 × Complete Protease Inhibitor (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail I (Sigma-Aldrich, St. Louis, MO). In order to minimize differences in sample preparation procedure, all the samples were processed at the same time, in the same conditions. Lysates were stored at  $-80^{\circ}\text{C}$ .

## Western blot

20  $\mu\text{g}$  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, Rocford, IL). Even protein loading was verified by Ponceau S staining. Antibodies against: phosphatase and tensin homolog (PTEN), phospho-S6K1 T389, phospho-Erk Y204, phospho-Akt S473, insulin receptor  $\alpha$  (InR $\alpha$ ) 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, phosphoglycogen synthase kinase  $3\beta$  (p-GSK- $3\beta$ ) S9, phospho-c-Raf (p-c-Raf) S338, 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).

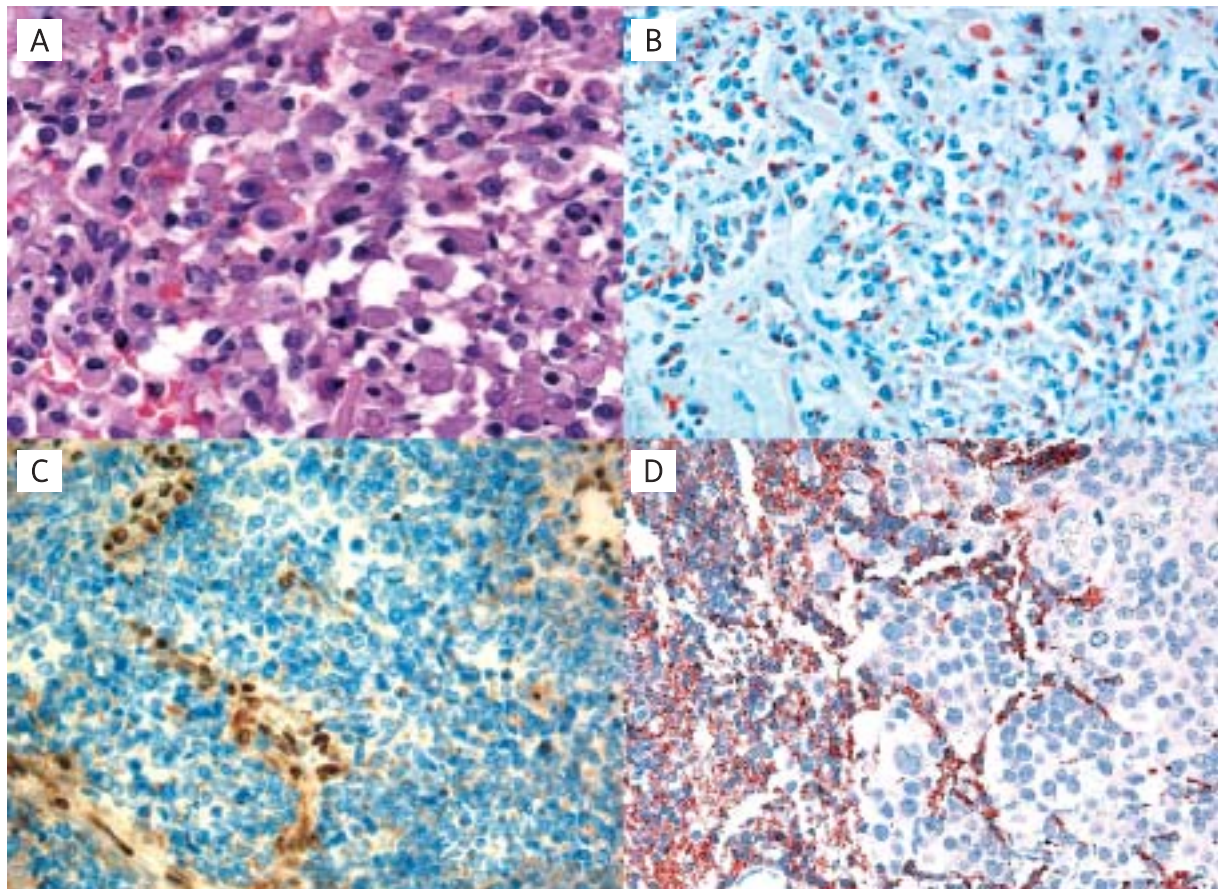
## Immunohistochemistry

The paraffin sections were routinely stained with haematoxylin-eosin (H&E) and Gomori's method. Immunohistochemical analyses were performed on paraffin-embedded specimens according to the labelled streptavidin-biotin complex method with DAB as chromogen. S100 protein, epithelial membrane antigen (EMA), glial fibrillary acidic protein (GFAP), synaptophysin and secondary antibodies (HRP-goat anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal to SNF5 (ab42503) was purchased from Abcam. AE1/AE3 cytokeratin produced by DAKO was used too.

## Results

### Histopathologic findings

The tumor consisted of poorly differentiated cells with high mitotic activity. Some cells with abundant pale eosinophilic cytoplasm and eccentrically situated nuclei containing prominent nucleoli were found. The cytoplasm of these cells revealed the presence of globular inclusions. The tumor contained also spindle – cell component and a cell small embryonal element. Large areas of necrosis were encountered.



**Fig. 1.** Histopathological evaluation of AT/RT case. **A)** rhabdoid cells, hematoxylin-eosin staining; **B)** immunodetection with anti-cytokeratin antibodies AE1/AE3; **C)** loss of expression of SNF5 (Sucrose NonFermenting 5) in nuclei of tumor cells; **D)** immunopositivity of synaptophysin.

### AT/RT tumor shows unique protein expression pattern

First we evaluated histopathological status of the tumor. We detected extensive staining for S-100 protein normally found in cells of neural crest origin. Then we showed that the tumor also stained well for EMA, whose expression is found in AT/RT by some authors [20], while others do not detect it [12]. Some cells revealed immunopositivity for cytokeratin and GFAP. The focal expression of synaptophysin was also observed. All tumour cells showed loss of expression of SNF5 in nuclei. The intratumour blood cells were positive for SNF5.

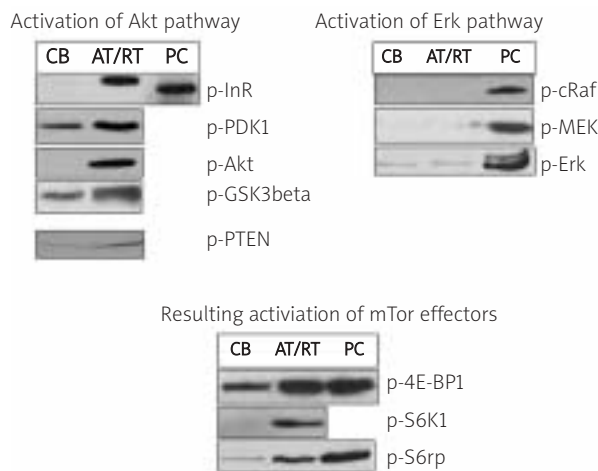
### Activation of Akt by insulin receptor pathway

Our primary goal was to evaluate whether AT/RT tumor development might be potentiated by the

upregulation of mTOR pathway and two kinases leading to mTOR phosphorylation i.e. Akt and Erk. We found that both Akt and its effector, GSK-3 $\beta$ , are markedly upregulated. Also PDK1, upstream from Akt, is activated. As Akt pathway is usually triggered by the stimulation of growth factor receptors, we checked and confirmed increased levels of InR $\alpha$ . On the other hand, we found that PTEN phosphatase, inhibiting signal transduction to Akt, was not elevated and did not influence activity of this pathway.

### Normal levels of phosphorylated Erk

Another kinase sometimes implicated in mTOR-dependent tumor development is Erk. In our case, however, we were unable to confirm activation of any of kinases belonging to Erk cascade. Neither c-Raf, MEK nor Erk itself were active and could lead to mTOR phosphorylation.



**Fig. 2.** Molecular analysis of Akt and Erk pathways potentially leading to mTOR activation. Activation was evaluated in the form of detection of phosphorylated forms of proteins. As far as Akt pathway elements were consistently activated, none of the proteins of Erk pathway was upregulated. Nevertheless, Akt triggered phosphorylation of mTOR effectors, i.e. 4E-BP1 (eIF4E-binding protein 1), S6K1 (S6 kinase 1) and S6rp (S6 ribosomal protein); CB – control brain, PC – positive control.

### Strong signaling to mTOR leads to protein synthesis

As mTOR is a central regulator of protein synthesis, phenomenon responsible for mTOR implication in tumorigenesis, we tested activity of its downstream effectors. Assembly of multisubunit eukaryotic translation initiation factor (eIF4F) complex is inhibited by a family of repressor polypeptides, eIF4E-binding proteins (4E-BPs). 4E-BP1 (also known as PHAS-1) binds eIF4E, inhibiting cap-dependent translation. Increased phosphorylation of 4E-BP1 at S65/T70 disrupts this binding and allows cap-dependent translation. In our AT/RT case we demonstrated upregulation of phosphorylated 4E-BP1. S6K1 kinase phosphorylates S6 ribosomal protein, which is thought to be required for biosynthesis of the translational apparatus. In the tumor both S6K1 and S6rp were hyper-phosphorylated compared to normal human brain.

### Discussion

Although it seems that unique disruption of cellular pathways characterising AT/RT may already be

established and connected with chromatin remodeling by the SWI/SNF (SWItch/Sucrose NonFermentable) complex, we are still far from understanding all the events necessary for AT/RT development. It is thought that one of the targets of SWI/SNF complex, including INI1 protein (whose gene is often mutated in AT/RT), may be cyclin D1, responsible for the control of G1/S transition. Normal activity of SWI/SNF complex disrupts cyclin D1 transcription. According to others, SWI/SNF complex activates transcription of p16 protein, an inhibitor of cyclin D1. In both cases, when the complex does not work, this leads to uncontrolled acceleration of cell cycle.

Tumorigenic transformation is never a result of a single event. Thus, in the current study we focused on activation of mTOR pathway, which is often found implicated in other brain tumors of the childhood, like medulloblastoma, glioblastoma subependymal giant-cell astrocytoma. Indeed, we found that downstream effectors of mTOR, including 4E-BP1 and S6K1, are upregulated and lead to translation of proteins. As mTOR controls synthesis of essential proteins involved in cell cycle progression, such as cyclin D1 [14], ornithine decarboxylase [16] or survival, like c-Myc [15], its inhibition was found to suppress proliferation of cells in all those applications that involved local or systemic control of cell multiplication. mTOR-inhibitor therapies are currently introduced into pre-clinical models of brain tumors [11].

mTOR is controlled by several upstream mechanisms, responding to stimuli like hypoxia, nutrient availability and energy insufficiency. However, all these factors lead to mTOR inhibition and cannot participate in neoplastic transformation. On the other hand, growth factors and mitogens signal to mTOR through PI3K/Akt or Ras/Erk pathways, which potentiate mTOR. In our previous studies we demonstrated that active Erk may trigger tumor development in subependymal giant-cell astrocytoma or medulloblastoma [10,19]. Also in other brain tumors upregulation of Erk is well-documented [6-8]. In view of our negative results, this study is the first one to show that Erk pathway probably does not play a role in AT/RT development.

Also PI3K/Akt are often implicated in tumorigenesis, especially affecting CNS [13]. Akt, a downstream effector of PI3K, is a typical activator of mTOR-dependent protein translation, to this extent, that numerous authors write about the “PI3K/mTOR” pathway. Thanks to its strong anti-apoptotic and pro-

proliferative functions, Akt is found implicated in the pathogenesis of various human malignancies. Here we report consistent upregulation of Akt pathway elements in the case of AT/RT, showing that targeting this kinase may bring favorable outcome in future molecular therapies.

## Acknowledgements

This work was supported by the institutional project 159/06 from The Children's Memorial Institute and the project R13001106/2009 from Polish Ministry of Science and Higher Education.

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