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ORIGINAL PAPER

*AKT1*E17K mutations cluster with meningothelial and transitional meningiomas and can be detected by SFRP1 immunohistochemistry

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Abstract The activating E17K mutation in the *AKT1* gene has been detected in several tumor entities. Currently several clinical studies with specific AKT1 inhibitors are under way. To determine whether *AKT1* mutations are involved in human tumors of the nervous system, we examined a series of 1,437 tumors including 391 primary intracranial brain tumors and 1,046 tumors of the coverings of the central and peripheral nervous system. *AKT1*E17K mutations were exclusively seen in meningiomas and occurred in 65 of 958 of these tumors. A strong preponderance was seen in the variant of meningothelial meningioma WHO grade I of basal and spinal localization. In contrast, *AKT1*E17K mutations were rare in WHO grade II and absent in WHO grade III meningiomas. In order to more effectively detect this mutation, we tested for immunohistochemical markers associated with this alteration. We observed strong upregulation of SFRP1 expression in all meningiomas with *AKT1*E17K mutation and in HEK293 cells after transfection with mutant *AKT1*E17K, but not in meningiomas and HEK293 cells lacking this mutation.

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Introduction

The protein kinase v-Akt murine thymoma viral oncogene homolog 1 (AKT) also termed protein kinase B (PKB) comprises three isoforms AKT1, AKT2 and AKT3 which have physiological roles in metabolism, translation, proliferation, cell survival and angiogenesis [12]. AKT is frequently activated in different human cancer types which in the majority of instances is consequence of mutations in upstream regulators such as *PI3K* [21] and *PTEN* [22]. Structural alterations of the *AKT* isoforms most frequently are amplifications of *AKT2*. Point mutations are less common, however, have been detected in the *AKT1* gene in breast, colorectal and ovarian cancer [5, 7, 23]. The majority of *AKT1* mutations is characterized by substitution of glutamic acid by lysine in amino acid position 17 (E17K) and localizes to the pleckstrin homology domain resulting in pathological association of AKT1 with the plasma membrane [5]. Given the frequent activation of AKT in human cancer, small molecule inhibitors targeting the pleckstrin domain have been developed [13].

Several brain tumors frequently exhibit mutations in genes involved in upstream regulation of AKT. *PTEN* and *PIK3CA* mutations are frequent in glioblastoma [10, 11] and consequently these tumors have been analyzed for the presence of *AKT1*E17K mutations [3] [2], however, without detecting this alteration. On the other hand, recent studies based on exome sequencing detected *AKT1*E17K mutations in a fraction of meningiomas [4, 6]. Meningiomas are among the most common intracranial and intraspinal tumors [14, 15, 25]. The prime therapeutic approach for meningiomas is complete resection. Although predominantly slow growing, the location frequently hampers complete resection. Incomplete resection, however, is an independent risk factor for recurrence [8, 14]. Thus, a fraction of patients with incomplete meningioma resection might benefit from upcoming therapies targeting the AKT pathway. In fact, compounds inhibiting mutant AKT1 have already entered clinical trials (e.g. NCT01226316, clinicaltrials.gov). Importantly, identification of the exact driver of the AKT/PI3K/mTOR activation is needed to select the appropriate compound, since *AKT1*E17K confers resistance to certain inhibitors and increases sensitivity to others [1].

In the present study, we aimed at exploring the potential role of *AKT1*E17K mutations in human brain tumors with special focus on meningiomas of different subtypes and WHO grades. Furthermore, we intended to develop an immunohistochemistry-based assay for rapid detection of this alteration in formalin fixed and paraffin-embedded tissues.

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Materials and methods

Samples

Tumor tissue was obtained from the archives of the Institutes and Departments of Neuropathology at the University Hospitals Münster, Hannover, Frankfurt, Tübingen, Heidelberg (Germany), Vienna (Austria) and Cambridge (United Kingdom). Research use of tissues and anonymization of data were in accordance with local ethical approvals.

Sanger sequencing

Fragments spanning codon 17 of *AKT1* were amplified using 20 ng each of the forward primer 5′ACA TCT GTC CTG GCA CAC C and the reverse primer 5′CTC ACG TTG GTC CAC ATC CT. Primer design was based on accession number NM_001014431.1.

For PCR, 20 ng of DNA and TaqPlus 2× PCR Master Mix (Qiagen, Hilden, Germany) were employed. PCR was performed in a total volume of 30 µl, and included initial denaturation at 95 °C for 180 s, followed by 35 cycles with denaturation at 95 °C for 30 s, annealing at 56 °C for 25 s and extension at 72 °C for 40 s. Two microliters of

the amplification product was submitted to bidirectional sequencing using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were determined using a ABI 3500 Genetic Analyzer (Applied Biosystems) and the Sequence Pilot version 4.0.1 (JSI-Medisys, Kippenheim, Germany) software.

SFRP1 immunohistochemistry

Immunohistochemistry was performed on sections cut to 4 µm. A Ventana BenchMark XT® immunostainer (Ventana Medical Systems, Tucson, AZ, USA) was employed. The Ventana staining procedure included pretreatment with cell conditioner 2 (pH 6) for 60 min, followed by incubation with anti-SFRP1 (abcam, ab4193, Cambridge, UK, 1:50) antibody at 37 °C for 32 min. Antibody incubation was followed by incubation with OptiView HQ Universal Linker for 12 min, incubation with OptiView HRP Multimer for 12 min, OptiView Amplification (catalog number 760-099; setting of OptiView Amplifier and OptiView Amplifier Multimer both for 12 min), and incubation with hematoxylin and Bluing reagent for 4 min each.

AKT1E17K transfection and *SFRP1* immunoblotting

HEK293T cells were obtained from ATCC (Manassas, VA, USA) and transfected with the human mutant ORF clone *AKT1*E17K (HEK E17K) (Origene, Rockville, MD, USA) applying Fugene transfection reagent (Promega, Madison, WI, USA). After incubation for 72 h, protein was extracted using NucleoSpin protein extraction kit (Machery-Nagel, Duren, Germany). HEK293T control cells (HEK ctrl) were transfected with empty vector only.

For immunoblot analysis, anti-SFRP1 antibody (3,534, Cell Signaling, Danvers, MA, USA) and standard protocols were applied.

Results and discussion

*AKT1*E17K mutations in tumors of the nervous system

1,437 tumors (Table 1) of the central and peripheral nervous system were analyzed for mutations in exon 2 of *AKT1* spanning the site of the E17K mutation. This mutation has been established as an important cause of AKT1 activation in breast, colorectal and ovarian cancer. We neither detected the E17K mutation in 174 low- and in 189 high-grade primary tumors of the CNS nor in 28 pituitary adenomas. This confirms previous studies on glioblastomas also failing to detect this alteration [2]. Likewise, none of the 59 tumors of the peripheral nerve contained an *AKT1*E17K mutation. However, E17K was detected in 65 of 958 meningiomas thus confirming a role for AKT1 activation in a fraction of these tumors.

Table 1 1,437 tumors of the nervous system tested for *AKT1*E17K mutations

Tumor type	*n*
Primary CNS tumors	
Pilocytic astrocytoma WHO grade I	42
Diffuse astrocytoma WHO grade II	36
Anaplastic astrocytoma WHO grade III	26
Glioblastoma WHO grade IV	111
Oligodendroglioma WHO grade II	19
Anaplastic oligoastrocytoma WHO grade III	11
Oligoastrocytoma WHO grade II	10
Anaplastic oligodendroglioma WHO grade III	20
Pleomorphic xanthoastrocytoma WHO grade II	11
Subependymal giant cell astrocytoma WHO grade I	3
Ganglioglioma WHO grade I	21
Myxopapillary ependymoma WHO grade I	11
Ependymoma WHO grade II	8
Anaplastic ependymoma WHO grade III	6
Central neurocytoma WHO grade II	13
Medulloblastoma WHO grade IV	15
Peripheral nerve sheath tumors	
Schwannoma WHO grade I	29
Neurofibroma WHO grade I	18
Malignant peripheral nerve sheath tumor WHO grade IV	12
Sellar tumors	
Pituitary adenoma	28
Meningeal tumors	
Meningiomas WHO grade I	705
Meningiomas WHO grade II	144
Meningiomas WHO grade III	96
Meningioma radiation induced[a]	13
Hemangiopericytomas/solitary fibrous tumors	29

All listed tumors were analyzed for the *AKT1*E17K mutation by direct sequencing

[a] Among the radiation-induced meningiomas were 7 grade I, 4 grade II and 2 grade III tumors, none of them harboring an *AKT1*E17K mutation

*AKT1*E17K mutations in meningioma subtypes

Two recent studies identified *AKT1*E17K mutations in meningiomas [4, 6]. Meningiomas are currently divided in 15 subgroups by the WHO classification (Table 2) [14]. We, therefore, set out to determine whether all or only distinct subgroups of meningiomas carry this alteration. *AKT1*E17K clearly grouped with distinct morphologic subgroups of meningiomas: the mutation was detected in 30/231 (13.0 %) meningothelial meningiomas WHO grade

Table 2 Number of *AKT1*E17K mutations, localizations and subtypes in 945 meningiomas

		All localizations	Convexity	Intra-ventricular	Posterior fossa	Basal	Spinal	Ambiguous
All meningiomas	mut/n	65/945	11/270	0/18	2/59	34/286	6/57	12/255
Meningothelial	mut/n	30/231 (13.0 %)	4/62	0/1	1/6	15/118	6/9	4/35
Transitional	mut/n	28/205 (13.6 %)	4/59	0/3	1/18	18/69	0/10	5/46
Fibroblastic	mut/n	0/97	0/41	0/4	0/19	0/18	0/2	0/13
Psammomatous	mut/n	0/55	0/7	0/2	0/1	0/15	0/27	0/3
Secretory	mut/n	0/56	0/6	0/0	0/1	0/21	0/0	0/28
Angiomatous	mut/n	1/28 (3.5 %)	0/8	0/0	0/0	0/5	0/1	1/14
Metaplastic	mut/n	2/8	0/0	0/0	0/0	1/2	0/1	1/5
Microcystic	mut/n	0/25	0/8	0/0	0/1	0/4	0/0	0/12
Atypical	mut/n	4/109 (3.7 %)	3/46	0/8	0/8	0/13	0/2	1/32
Chordoid	mut/n	0/29	0/14	0/0	0/1	0/4	0/1	0/9
Clear cell	mut/n	0/6	0/0	0/0	0/0	0/4	0/0	0/2
Anaplastic	mut/n	0/86	0/17	0/0	0/4	0/13	0/1	0/51
Rhabdoid	mut/n	0/9	0/2	0/0	0/0	0/0	0/3	0/4
Papillary	mut/n	0/1	0/0	0/0	0/0	0/0	0/0	0/1

Tumors were analyzed for the *AKT1*E17K mutation by direct sequencing. The variant of lymphoplasmacyte-rich meningioma was not analyzed by sequencing for its extraordinary high content of constitutional DNA. However, two cases examined by SFRP1 immunohistochemistry did not show evidence for an *AKT1*E17K mutation. All tumor localizations involving more than one of the designated localizations were termed ambiguous. The 13 radiation-induced meningiomas were omitted from this table

I and in 28/205 (13.6 %) transitional meningiomas WHO grade I. In contrast, *AKT1*E17K occurred in only 4/109 (3.7 %) atypical meningiomas WHO grade II, 1/28 angiomatous meningiomas WHO grade I and in 2/8 from the rare and ill-defined subgroup of metaplastic meningioma WHO grade I. In all other subtypes, including the frequent fibroblastic variant and all grade III meningiomas, no *AKT1*E17K mutation was detected (Table 2). Thus, there was a significant association of E17K mutations with meningothelial and transitional differentiation ($p < 0.0001$, Fisher's exact test).

The detection of 61 mutations in 705 meningiomas of WHO grade I but only of 4 mutations in 240 meningiomas of WHO grades II and III clearly associates *AKT1*E17K with meningiomas of low malignancy potential ($p < 0.0001$, Fisher's exact test). It should be noted that one of the major criteria for separating WHO grade I meningiomas from atypical meningiomas WHO grade II is mitotic count with 4–20 mitoses per 10 high power fields resulting in the diagnosis of atypical meningioma. Anaplastic meningiomas WHO grade III exhibit frank features of high malignancy. Thus, the presence of E17K mutations in a low fraction of atypical meningiomas may be attributed to the not fully convincing criteria currently applied for grading of these tumors. Consequently, identifying strong correlations of molecular markers with malignancy may support future grading systems.

In the present series, there was a strong association of *AKT1*E17K mutations with basal and spinal meningioma localization. Among 343 tumors arising from the skull base meninges or spinal, 40 (11.7 %) turned out to harbor an *AKT1* mutation. In contrast, of 270 tumors designated as falx or convexity meningiomas, only 11 (4 %) presented with the alteration ($p = 0.0006$, Fisher's exact test). This is of particular interest since meningiomas of basal location frequently are difficult to resect completely and, therefore, have a higher tendency to recur. Thus, the high prevalence of a "druggable" aberration in skull base meningiomas as reported here might offer a target for adjuvant therapy. The strong correlation of subtype and localization is even more apparent when focusing on meningothelial meningioma of the spinal meninges: Among 57 spinal meningiomas, six harbored an *AKT1*E17K mutation. Interestingly, these six mutations occurred in six of nine meningothelial meningiomas while all spinal psammomatous, metaplastic and high-grade meningiomas were wild-type. Conversely, certain localizations did not harbor a mutation in any case, particular all 18 intraventricular meningiomas were devoid of *AKT1*E17K.

In order to assess the correlation of *AKT1* mutation and other aberrations in meningioma, we tested 185 meningiomas with established *AKT1* status for presence of the most frequent mutation in this entity, *NF2* mutations [18, 20, 24]. While only 3/39 (7 %) *AKT1* mutant cases concurrently carried a *NF2* mutation, 65/146 (44 %) *AKT1* wild-type cases revealed a *NF2* mutation ($p < 0.0001$, Fisher's exact test). Moreover, we tested for correlation of *TRAF7*, *KLF4* and *AKT1* mutations in 88 cases with established

TRAF7 and *KLF4* status [17]: *TRAF7* mutations are most frequently described in secretory meningiomas and *KLF4* mutations are exclusively found in this subtype. In contrast, no *AKT1* mutation was detected in secretory meningioma. Consequently, *KLF4* and *AKT1* were mutually exclusive and only two *AKT1*E17K mutations occurred in 23 *TRAF7* mutant cases.

Surrogate marker *SFRP1* identifies meningiomas with *AKT1*E17K mutations

To facilitate assessment of the *AKT1*E17K status, we searched for a marker that can be detected on tissue sections. A previous study addressed this successfully on single cases with a commercially available AKT1E17K antibody [6]. Alas, we could not establish reproducible results for this antibody in a more extensive series of cases. In fact, this reagent is not recommended for immunohistochemistry by the distributor. Stathmin shown to be upregulated in *AKT1*-mutated tumors also turned out not to be a reliable marker for the *AKT1*E17K mutation in our hands as well as in other studies [4]. However, AKT has been demonstrated to augment sonic hedgehog (SHH) signaling [19] and SHH has been demonstrated to induce the expression of secreted frizzled-related protein 1 (SFRP1) [9], a marker also employed in the subclassification of medulloblastoma [16]. Thus, we also performed SFRP1 immunohistochemistry on 43 meningiomas with *AKT1*E17K mutation and on 50 meningiomas with wild-type status in this position. Moderate to strong staining was observed in 42/43 meningiomas harboring E17K while all wild-type meningiomas did not bind the antibody (Fig. 1; Suppl. Fig 1a–d). Of note, single infiltrating leukocytes presented with positive staining in both mutant and wild-type cases (Suppl. Fig. 1e). The single *AKT1*E17K-mutated meningioma not exhibiting SFRP1 immunopositivity was severely altered by thermal exposure at resection, which rendered this case unsuitable for immunohistochemistry but still allowed for detection of the mutation by sequencing (Suppl. Fig 1f). The usual SFRP1 staining pattern was a diffuse cytoplasmic positivity. We were able to detect SFRP1 positivity in samples shelved up to 22 years, however, staining intensity decreased with storage time of the blocks. In very old blocks, immunoreactivity was mainly observed in meningioma whorls (Suppl. Fig. 1g–j). Next, we performed Western Blot analysis to confirm up-regulation of SFRP1 protein in concordance with immunohistochemistry in *AKT1*-mutated meningiomas. A distinct signal of 35 kD was detected employing extracts from three meningioma with *AKT1*E17K mutation whereas no signal was detected in three meningiomas without this mutation (Fig. 2a). Thus, SFRP1 immunoreactivity in meningioma is tightly associated with the presence of an *AKT1*E17K mutation and proofed to be a reliable surrogate marker for the detection of this specific alteration ($p < 0.0001$, Fisher's exact test). While the link between the activation of wild-type AKT and SFRP1 already has been suggested [9, 19], this has not been tested for *AKT1*E17K. In order to also demonstrate SFRP1 up-regulation upon presence of the *AKT1*E17K mutation, we performed in vitro analyses employing a cell culture model. HEK293

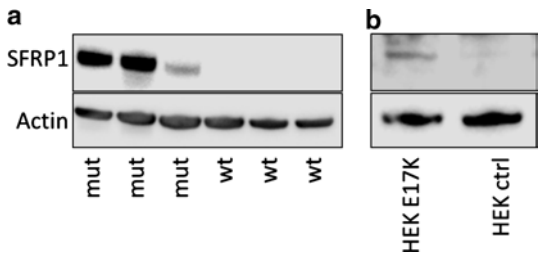

Fig. 2 Western blot with anti-SFRP1 antibody of three meningioma extracts each with (mut) and without (wt) *AKT1*E17K mutation. SFRP1 expression is restricted to meningiomas with *AKT1*E17K mutation (**a**). Western blot with anti-SFRP1 antibody of HEK cell extracts with ectopic expression of *AKT1*E17K (HEK E17K) and without (HEK ctrl). SFRP1 expression is restricted to HEK cells transfected with *AKT1*E17K (**b**)

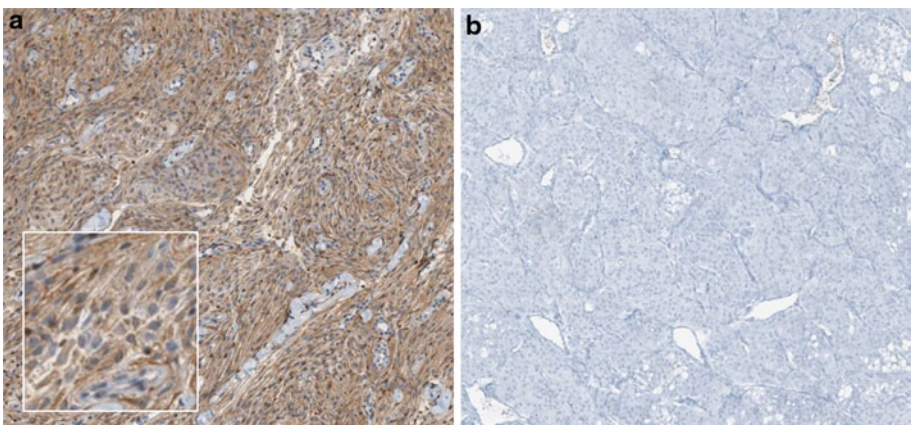

Fig. 1 Representative immunohistochemistry for SFRP1 in an *AKT1*E17K mutant case (**a**, with *inset* in higher magnification) and a wild-type meningioma (**b**)

cells were transfected with mutant *AKT1*E17K (HEK E17K). Consecutive Western Blot analyses demonstrated induced SFRP1 expression only in HEK E17K but not in HEK ctrl cells (Fig. 2b).

Conclusions

In conclusion, we identified *AKT1*E17K mutations in specific subsets of meningiomas with highest incidence in meningothelial and transitional meningiomas WHO grade I in spinal and basal localization. Further, we established SFRP1 immunohistochemistry as a reliable surrogate marker for detection of *AKT1*E17K mutations in meningioma.

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