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RESEARCH ARTICLE

Cribriform neuroepithelial tumor: molecular characterization of a SMARCB1-deficient non-rhabdoid tumor with favorable long-term outcome

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Keywords
atypical teratoid/rhabdoid tumor, copy number alterations, DNA methylation profiling, SMARCB1/INI1, tyrosinase.

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Abstract

Rhabdoid phenotype and loss of SMARCB1 expression in a brain tumor are characteristic features of atypical teratoid/rhabdoid tumors (ATRT). Rare non-rhabdoid brain tumors showing cribriform growth pattern and SMARCB1 loss have been designated cribriform neuroepithelial tumor (CRINET). Small case series suggest that CRINETs may have a relatively favorable prognosis. However, the long-term outcome is unclear and it remains uncertain whether CRINET represents a distinct entity or a variant of ATRT. Therefore, 10 CRINETs were clinically and molecularly characterized and compared with 10 ATRTs of each of three recently described molecular subgroups (i.e. ATRT-TYR, ATRT-SHH and ATRT-MYC) using Illumina Infinium HumanMethylation450 arrays, FISH, MLPA, and sequencing. Furthermore, outcome was compared to a larger cohort of 27 children with ATRT-TYR. Median age of the 6 boys and 4 girls harboring a CRINET was 20 months. On histopathological examination, all CRINETs demonstrated a cribriform growth pattern and distinct tyrosinase staining. On unsupervised cluster analysis of methylation data, all CRINETs examined exclusively clustered within the ATRT-TYR molecular subgroup. As ATRT-TYR, CRINETs mainly showed large heterozygous 22q deletions (9/10) and *SMARCB1* mutations of the other allele. In two patients, *SMARCB1* mutations were also present in the germline. Estimated mean overall survival in patients with CRINETs was 125 months (95% confidence interval 100–151 months) as compared to only 53 (33–74) months

in patients with ATRTs of the ATRT-TYR subgroup (Log-Rank $P < 0.05$). In conclusion, CRINET represents a SMARCB1-deficient non-rhabdoid tumor, which shares molecular similarities with the ATRT-TYR subgroup but has distinct histopathological features and favorable long-term outcome.

INTRODUCTION

Rhabdoid phenotype and loss of SMARCB1 (also known as hSNF5/INI1) protein expression are characteristic features of atypical teratoid/rhabdoid tumors (ATRT). Apart from genetic alterations affecting the *SMARCB1* region on chromosome 22q, ATRT show stable genomic profiles without further recurrent chromosomal alterations (9). On an epigenetic level, however, ATRT has recently been shown to be a heterogeneous disease comprised of three different molecular subgroups (i.e. ATRT-TYR, ATRT-SHH and ATRT-MYC), which are characterized by distinct methylome profiles, enhancer landscapes and subgroup-specific regulatory networks (13). The same holds true for histopathologic features encountered in ATRT, which are remarkably diverse. In addition to rhabdoid tumor cells, areas with primitive neuroectodermal, mesenchymal and epitheloid features are commonly encountered (3, 7). For rare non-rhabdoid brain tumors showing a cribriform growth pattern and loss of SMARCB1 expression the term cribriform neuroepithelial tumor (CRINET) has been coined (8). Small case series and individual case reports suggest that CRINETs may have a relatively favorable prognosis (2, 4, 8, 11). As yet, however, little is known on long-term outcome of CRINET and there is uncertainty whether CRINET represents a distinct entity or a variant of ATRT. We thus aimed to further characterize the clinical and molecular features of CRINET as compared with ATRT. Here we show that CRINET is a tumor with distinct histopathologic features, molecular similarities with the ATRT-TYR subgroup and favorable long-term outcome.

MATERIALS AND METHODS

Samples and patients

Formalin-fixed paraffin-embedded (FFPE) samples of 10 CRINETs were collected from the archives of the Institute of Neuropathology Münster and by contacting institutions, which had previously published CRINET cases (2, 11, 19). Our tumor bank received local ethical committee approval (Ethics committee of the University Hospital Münster) and parents had given informed consent for scientific use of the archival samples. Follow-up information for all patients was obtained by contacting treating physicians. For clustering analyses, available clinical and molecular data of 10 ATRTs of each of three recently described molecular subgroups (i.e. ATRT-TYR, ATRT-SHH and ATRT-MYC) (13) were evaluated (for characteristics see Supporting Information Table S1). Furthermore, outcome of patients with CRINET was compared to a larger cohort of 27 patients with ATRT-TYR (including eight of the ATRT-TYR cases used for clustering analyses), for which information on overall survival was available. Protein expression of SMARCB1 and tyrosinase was examined using immunohistochemistry (13, 14). Fluorescence *in situ* hybridization (FISH) analyses of the *SMARCB1* region and *SMARCB1* sequencing were performed as described previously (17) and Multiplex ligation-dependent probe amplification (MLPA) was carried out using the SALSA MLPA P258 (SMARCB1) kit (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's protocol.

DNA methylation array processing

For DNA methylation profiling of CRINETs, we used Illumina Infinium HumanMethylation450 Bead Chip arrays according to the manufacturer's instructions and protocols at the German Cancer Research Center (DKFZ) Genomics and Proteomics Core Facility. DNA methylation data were generated from FFPE tissue samples using 250 ng of DNA as input material. All DNA methylation analyses were performed in R version 3.2.0 (R Development Core Team, 2015). The following criteria were applied to filter the data: removal of probes targeting sex chromosomes (n = 11 551), removal of probes containing a single nucleotide polymorphism (dbSNP132 Common) within five base pairs of and including the targeted CpG-site (n = 24 536), and probes not uniquely mapping to the human reference genome (hg19) allowing for one mismatch (n = 9993). In total, 438 370 probes were kept for analysis. Unsupervised hierarchical clustering of the samples was performed using the 5000 most variably methylated probes across the dataset and the 1-Pearson correlation coefficient as the distance measure. Data were compared to 450k methylation data generated in 30 ATRTs of each of three recently described molecular subgroups (i.e. ATRT-TYR, ATRT-SHH and ATRT-MYC) (13) deposited in GEO (accession number GSE70460). The cluster dendrogram was formed by using average linkage as agglomeration method. To reorder probes for the heatmap visualization, probes were clustered by agglomerative hierarchical clustering using 1-centered Pearson correlation as distance measure and average linkage as agglomeration method. Copy-number variation (CNV) analysis from 450k methylation array data was performed using the conumee Bioconductor package version 1.0.0 (http://bioconductor.org/packages/release/bioc/html/conumee.html). Scoring of chromosomal gains and losses was performed by manual inspection of each profile.

Statistical analysis

Continuous and categorical variables were compared using Mann–Whitney-U-Test and Chi square test, respectively. Survival analysis was performed using Kaplan-Meier estimation for survival curves and the Log-rank test using IBM SPSS 23 software (release 23.0). Overall survival time was defined as the time from the date of diagnosis to the date of death. For all analyses, $P < 0.05$ was considered to be significant.

Table 1. Patient characteristics. Clinical and molecular characteristics of 10 patients harboring a CRINET (CR = complete remission; DOD = dead of disease; WT = wildtype).

#	Age at diagnosis (months)	Sex	Tumor location	Copy number alterations on analysis 450k data	*SMARCB1* region FISH	*SMARCB1* region MLPA	*SMARCB1* sequencing	Germ line status	Adjuvant therapy	Follow-up (months)	Status	Comments
1	26	male	Infratentorial (fourth ventricular region)	large heterozygous deletion 22q	heterozygous deletion	heterozygous deletion (delTBX1_*SMARCB1*)	c.367>T p.Gln123* (Exon 4)	not available	CPT-SIOP-2000 (etoposide, cyclophosphamide, and vincristine)	86	CR	
2	10	male	Infratentorial (fourth ventricular region)	large heterozygous deletion 22q	not available	heterozygous deletion (delTBX1_*SMARCB1*)	c.492duplCCTT p.Pro165Leufs*6 (Exon 4)	not available	CPT-SIOP-2000 (etoposide, cyclophosphamide, and vincristine), radiotherapy (54G)	139	CR	Case 1 reported by Hasselblatt *et al* (8)and Gessi *et al* (6)
3	21	male	Infratentorial (fourth ventricular region)	large heterozygous deletion 22q	heterozygous deletion	heterozygous deletion + duplEx6 *SMARCB1* (reported by Ibrahim *et al*)	duplEx6 *SMARCB1* (reported by Ibrahim *et al*)	duplEx6 *SMARCB1* [reported by Ibrahim *et al* (11)]	COG-99703 (cisplatin, etoposide, cyclophosphamide and vincristine) followed by three rounds of high-dose chemotherapy (carboplatin, thiotepa) with stem-cell rescue.	68	CR	Case reported by Ibrahim *et al*. (11)
4	27	female	Supratentorial (third ventricular region)	large heterozygous deletion 22q	not available	delTBX1_NIPSNAP1 + delEx7-9 *SMARCB1*	WT	not available	HIT-SKK-2000 (including methotrexate, cyclophosphamide, and vincristine)	124	CR	Case 2 reported by Hasselblatt *et al* (8) and Gessi *et al* (6)
5	11	female	Supratentorial (third ventricular region)	large heterozygous deletion 22q	heterozygous deletion	delTBX1_NIPSNAP1 + delEx7 *SMARCB1*	WT	not available	no information available	10	CR	
6	20	female	Supratentorial (third ventricular region)	large heterozygous deletion 22q	heterozygous deletion	not available	WT	WT	EU-RHAB protocol,radiotherapy (proton-radiation)	78	CR	
7	20	male	supratentorial	large heterozygous deletion 22q	not available	not available	not available	not available	CPT-SIOP-2000 (etoposide, cyclophosphamide, and vincristine)	36	CR	
8	14	male	Supratentorial (lateral ventricle)	large heterozygous deletion 22q	heterozygous deletion	heterozygous deletion (delTBX1_NIPSNAP1)	c.986 + 1G>T (Intron 7)	WT [reported by Arnold *et al* (2)]	COG-99703 (cisplatin, etoposide, cyclophosphamide and vincristine) followed by three rounds of high-dose chemotherapy (carboplatin, thiotepa) with stem-cell rescue.	43	CR	Case reported by Arnold *et al* (2)
9	14	male	Supratentorial (lateral ventricle)	absent	not available	no copy number alterations	homozygous c.367>T p.Gln123* (Exon 4)	not available	Patient died from respiratory failure before therapy could be initiated	1	DOD	Case reported by Park *et* al (19)
10	129	female	Supratentorial (third ventricular region)	large heterozygous deletion 22q	heterozygous deletion 22q	heterozygous deletion (delTBX1_NIPSNAP1)	c.1142C>G p.Thr381Arg(Exon 9)	c.1142C>G (Exon 9)	EU-RHAB protocol,radiotherapy	16	CR	Case 3 reported by Gessi *et al* (12)

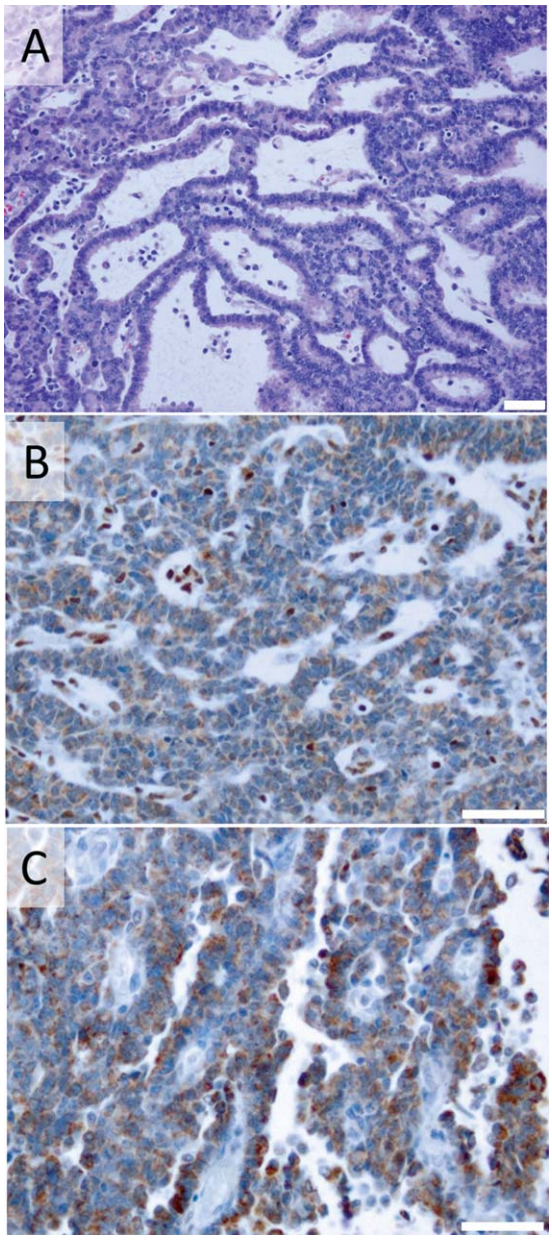

Figure 1. *Histopathology of CRINET*. Hematoxylin and eosin staining (**A**), immunohistochemistry for SMARCB1 (**B**) and tyrosinase (**C**) in a representative CRINET case. Note loss of nuclear SMARCB1 staining in the tumor cells, which is retained in the nuclei of non-neoplastic cells (internal positive control) as well as distinct staining of tumor cells for tyrosinase. Scale bars denote 50 μm.

RESULTS

The median age of the 6 boys and 4 girls harboring a CRINET was 20 months (range 10–129 months). CRINETs were either located supratentorially [midline in the vicinity of the third ventricle (4/10), near the lateral ventricles (2/10)], or infratentorially in the posterior fossa (4/10 cases; see Table 1 for detailed patient characteristics). Based on neuroradiological findings and clinical features, the possibility of a choroid plexus tumor had been considered initially in the majority of patients (6/10). On histopathological examination, all CRINETs were characterized by the presence of cribriform strands and ribbons. In more compact areas, small lumina were also present, but rhabdoid tumor cells showing eosinophilic cytoplasms and eccentric nuclei with prominent nucleoli were absent (Figure 1A). All CRINETs showed loss of tumoral SMARCB1 protein expression (Figure 1B). The median Ki67/MIB1 proliferation index was 29% (range: 15%–35%). Furthermore, 10/10 CRINETs exhibited staining for tyrosinase (Figure 1C), which was also observed in 9/10 ATRTs of the ATRT-TYR subgroup, but only in 1/10 tumors of the ATRT-SHH and ATRT-MYC subgroup, respectively, (Chi-square: 29.2, df:3; $P < 0.00001$). Tyrosinase staining in CRINETs was cytoplasmic and heterogeneous as observed in ATRTs of the ATRT-TYR subgroup.

On unsupervised cluster analysis of methylation profiles using the 5000 most differentially methylated CpG sites across all samples, all eight CRINETs for which sufficient DNA was available for examination, exclusively clustered within the ATRT-TYR molecular subgroup (Figure 2A). Copy-number profiles as derived from intensity measures of the methylation probes indicated 22q losses affecting the *SMARCB1* region as the only recurrent alteration in CRINETs with a pattern very similar to that seen in ATRT-TYR tumors (Figure 2B).

FISH and/or MLPA analyses confirmed the presence of large heterozygous 22q deletions affecting the *SMARCB1* region in 9/10 CRINET cases. The only CRINET without a large heterozygous 22q deletion showed loss of heterozygosity for a truncating *SMARCB1* mutation affecting exon 4 (c.367C > T p.Gln123*). In the remaining CRINET cases, a truncating *SMARCB1* mutation affecting exon 4 (c.367C > T p.Gln123*), an exon 4 mutation resulting in a frameshift (c.492duplCCTT p.Pro165Leufs*6) and a mutation affecting intron 7 (c.986 + 1G > T) were identified as a "second hit," while two cases showed additional small *SMARCB1* deletions on MLPA (delEx7, delEx7-Ex9, Figure 3). Furthermore, an exon 9 missense mutation (c.1142C > G) expected to result in disturbed splicing and an exon 6 duplication were encountered in two CRINETs, both mutations also being present in the germline of the respective patients. In the boy harboring a germline exon 6 duplication, the mutation was inherited from the apparently healthy mother and grandmother. The grandmother's brother had died due to a brain tumor. The grandmother's first cousin and her 3 children all carry the same mutation. The two daughters both had SMARCB1-negative pediatric brain tumors initially interpreted as "ependymoma with monosomy 22" (22). One survived, the other one passed away as a teenager. The son never developed a tumor. The surviving daughter has 3 children, two of whom carry the mutation. Her oldest son presented with a CRINET in 2013 [reported by Dunham (4) but not included in the present series and is in complete remission.

Importantly, except for one child, who died one month postoperatively from respiratory failure, to our knowledge all patients with CRINET of the present series are alive and well. On Kaplan Meyer analysis of survival data, mean overall survival was 125 months (95% confidence interval 100-151 months) and thus longer as compared to the patients of the ATRT groups used for genetic profiling and methylation clustering [ATRT-TYR: 37 (18–56) months, ATRT-SHH:16 (8–25) months, ATRT-MYC: 13 (5–22) months, Log-Rank $P < 0.05$]. Given the molecular similarities of CRINET and ATRT-TYR, we next examined outcome of patients with

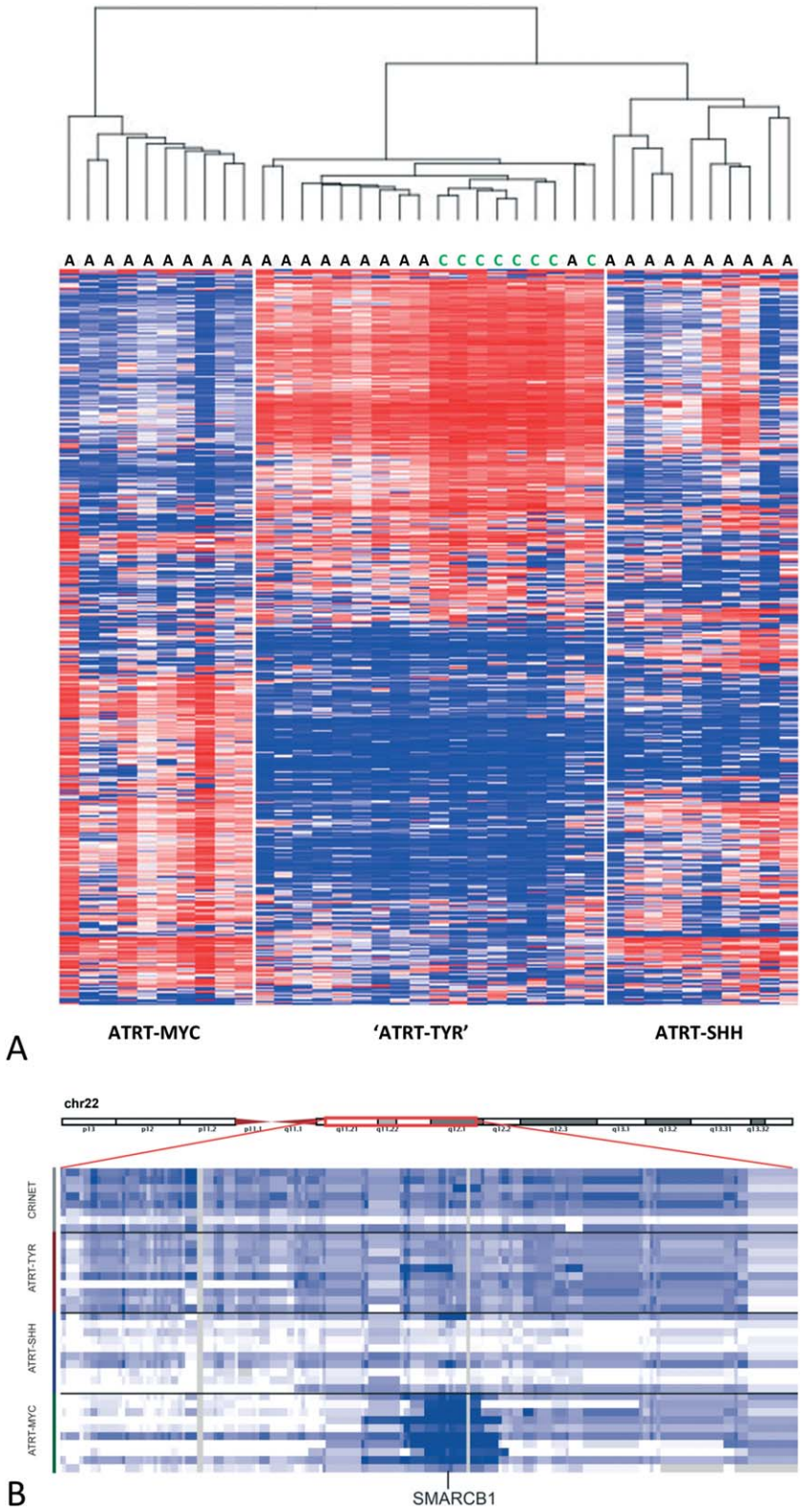

Figure 2. *Molecular profiling of CRINET vs. ATRT*. Heatmap showing unsupervised clustering of methylation profiles of 38 samples using the 5000 most variable probes (**A**) as well as copy number alterations affecting chromosome 22q (**B**) of 8 CRINETs and ATRTs of the molecular subgroups TYR, SHH and MYC (n = 10 each). Note that all CRINETs cluster within the ATRT-TYR subgroup and (like ATRT-TYR) show large heterozygous deletions of 22q affecting the *SMARCB1* locus.

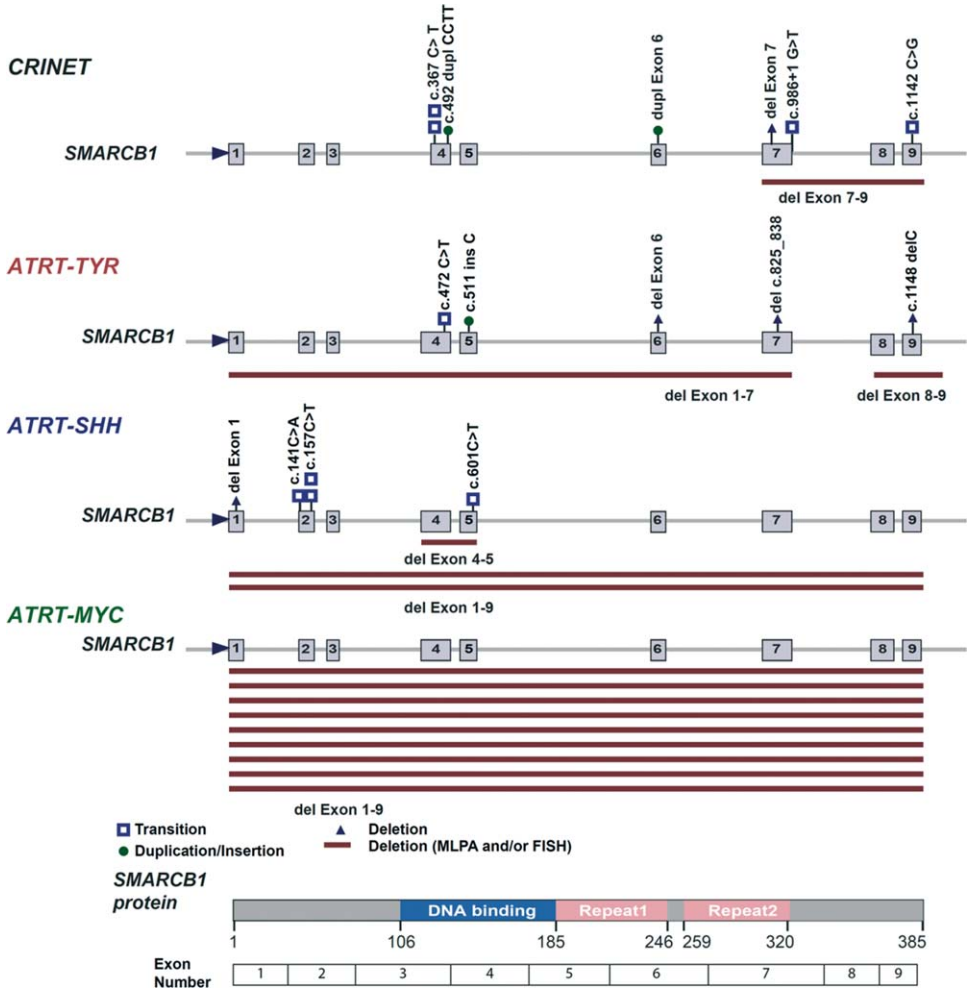

Figure 3. Spectrum of *SMARCB1* mutations in CRINET vs. ATRT. *SMARCB1* mutations encountered on FISH, MLPA and sequencing in CRINETs as compared to ATRTs of the molecular subgroups TYR, SHH and MYC (n = 10 each). Note similar distribution of mutations across the *SMARCB1* gene in CRINET and ATRT-TYR. Plotted are only hits affecting the second *SMARCB1* allele. For more details, see Table 1 and Supporting Information Table S1.

CRINETs as compared to a larger cohort of 27 patients harboring ATRTs of the ATRT-TYR subgroup. Here, significantly longer overall survival as compared to ATRT-TYR patients [53 (33–74) months] could be confirmed (Log-Rank $P < 0.05$, Figure 4).

DISCUSSION

The key finding of our study is that genetic and epigenetic profiles of CRINET are highly similar to those of ATRT-TYR, representing one of the three recently described molecular subgroups of ATRT (13). On gene expression profiling, ATRT-TYR is characterized by overexpression of melanosomal markers, such as tyrosinase and MITF. Another characteristic feature of this subgroup is the overexpression of genes involved in ciliogenesis (13). The observation that protein expression of tyrosinase was not only present in ATRT-TYR, but also in CRINET, suggests similarities between CRINET and ATRT-TYR also at gene expression level. Another common feature of CRINET and ATRT-TYR is the presence of large heterozygous 22q deletions affecting the *SMARCB1* region, which are relatively rare in ATRT of the SHH and MYC subgroups (13). This finding could well point to a role of gene dosage of other genes on 22q in the biology of CRINET and ATRT-TYR.

Importantly, despite the highly similar (epi)genetic profiles of CRINETs and ATRT-TYR tumors, patients harboring CRINETs experience relatively favorable long-term outcomes and do much better than patients with ATRT-TYR tumors. This situation is reminiscent of desmoplastic medulloblastomas, which are also genetically and epigenetically indistinguishable from classic medulloblastomas of the SHH subtype, but still show a difference in outcome (15, 16) and provides another example that information obtained on histopathologic analysis and molecular profiling is not redundant, but rather complementary.

Taking into account the limitations of this retrospective series, which combines outcome data of patients treated at various institutions across several countries, our data suggest that CRINET responds well to chemotherapy protocols commonly employed for the treatment of malignant brain tumors and ATRT. What makes

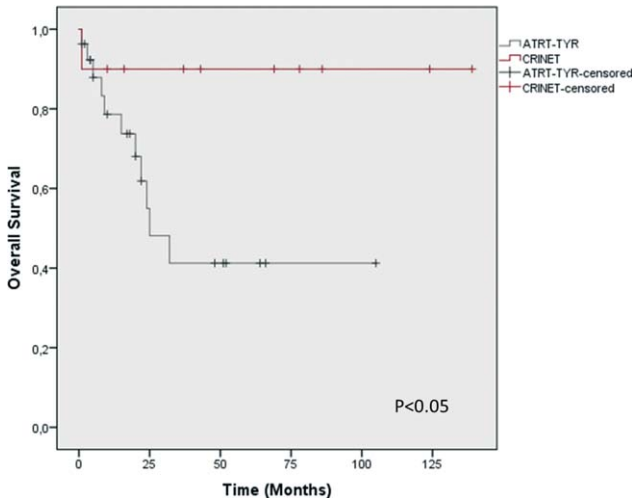

Figure 4. Prognosis of CRINET vs. ATRT-TYR. On Kaplan Meier analysis of survival data, the 10 patients with CRINET show longer overall survival as compared to a cohort of 27 patients harboring ATRT-TYR, $P < 0.05$ Log rank test).

the difference between the relatively favorable biological behavior of CRINET and the dismal outcome of ATRT-TYR? The absence of recurrent genomic alterations apart from 22q losses in CRINET (also a characteristic feature of ATRT-TYR) (6, 9, 12, 21) argues against a role of losses or gains of other chromosomal regions in the biology of CRINET. Even though the possibility of point mutations of genes putatively modifying the detrimental effects of SMARCB1-deficiency cannot be excluded, it is tempting to speculate that the type of *SMARCB1* mutation may determine tumor phenotype and prognosis.

The distribution of mutations across the *SMARCB1* gene encountered in CRINET seems to be quite similar to that of ATRT-TYR, but distinct from those seen in ATRT-SHH or ATRT-MYC. Some mutations encountered in CRINET such as c.367C > T have also been described in ATRT (5), but histopathological features and molecular subgroup information of these published cases are unknown. Interestingly, the somatic mutation affecting intron 7 in the CRINET of patient #8 (c.986 + 1G > T) has been previously described in a family with rhabdoid tumor predisposition syndrome and been shown to result in the exclusion of exon 7 on RNA level (5). A similar mutation in the donor splice site of exon 7 has been reported in a family with pediatric posterior fossa brain tumors diagnosed as ATRT or choroid plexus carcinoma (24). Germline duplications affecting exon 6 of the *SMARCB1* gene have also been reported in a family with rhabdoid tumor predisposition syndrome and schwannomatosis (23). The family of the CRINET patient harboring a *SMARCB1* germline exon 6 duplication, however, rather shows similarities to a previously described Dutch family (1). In this family, two patients survived for an unusually long time. Of note, both tumors had been initially reported as "anaplastic ependymomas" and showed growth in strands and ribbons, i.e. histopathological features reminiscent of CRINET (18) and a *SMARCB1* mutation affecting a splice site (c.500 + 1G > A) could be demonstrated in the patients and their unaffected fathers (1). In schwannomatosis patients, it has been suggested that synthesis of an altered SMARCB1 protein (either by translation re-initiation or encoded by missense, splice-site mutations or in-frame deletions) may prevent the development of malignant rhabdoid tumors (10). Neither exon 1 mutations nor the mosaic SMARCB1 staining pattern typical for schwannomatosis-associated schwannomas (20) were encountered in CRINET patients. Nevertheless, as the BAF47 antibody is directed against a relatively C-terminal epitope (amino acids 257-359), the possibility that a truncated SMARCB1 protein with some residual function could be responsible for the less aggressive biological behavior of CRINET cannot be entirely excluded and warrants attention in future studies. Furthermore, the potential prognostic role of molecular subgrouping in ATRT will need to be determined in the carefully characterized patient cohorts of large international registries.

In conclusion, CRINET represents a SMARCB1-deficient non-rhabdoid tumor, which shares molecular similarities with the ATRT-TYR subgroup but has distinct histopathological features and favorable long-term outcome.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Characteristics of the AT/RT control group. Characteristics of 30 patients harboring AT/RT that were employed for as controls for clustering analyses and molecular profiling (WT = wildtype).