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(Article begins on next page)

# Primary intracranial spindle cell sarcoma with RMS-like features share a highly distinct methylation profile and DICER1 mutations

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DICER1; TP53; MAPK; CNS; NGS; DNA methylation profiling; EPIC array

## **Abstract**

Patients with *DICER1* predisposition syndrome have an increased risk to develop pleuropulmonary blastoma, cystic nephroma, embryonal rhabdomyosarcoma and several other rare tumor entities. In this study we identified 22 intracranial sarcomas, including 18 in pediatric patients, with a distinct methylation signature detected by array-based DNA-methylation profiling. In addition, two uterine cervix rhabdomyosarcomas sharing identical features were identified. Gene panel sequencing of the 22 intracranial sarcomas revealed the almost unifying feature of *DICER1* hotspot mutations (21/22; 95%) and a high frequency of co-occurring *TP53* mutations (12/22; 55%). In addition 17/22 (77%) of the sarcomas in our series exhibited alterations in the mitogen-activated protein kinase (MAPK) pathway most frequently affecting the mutational hotspots of *KRAS* (8/22; 36%) and mutations or deletions of *NF1* (7/22; 32%), followed by mutations of *FGFR4* (2/22; 9%), *NRAS* (2/22; 9%) and amplification of *EGFR* (1/22; 5%). A germline *DICER1* mutation was detected in two of five cases with constitutional DNA available. In contrast to the genetic findings, the morphological features of these tumors were less distinctive, although rhabdomyoblasts or rhabdomyoblast-like cells could retrospectively be detected in all cases. The identified combination of genetic events indicates a relationship between the intracranial tumors analysed and *DICER1* predisposition syndrome-associated embryonal rhabdomyosarcoma and anaplastic sarcomas of the kidney. However, the intracranial tumors in our series were initially interpreted to represent various tumor types but rhabdomyosarcoma was not typically among the differential diagnoses considered. Given the rarity of intracranial sarcomas this molecularly clearly defined group comprises a considerable fraction thereof. We therefore propose the designation “spindle cell sarcoma with RMS-like features, *DICER1* mutant” for this intriguing group.

## **Introduction**

Intracranial mesenchymal tumors mostly affect the leptomeningeal coverings of the CNS. The predominant tumors are meningioma and hemangiopericytoma / solitary fibrous tumor, both of which are well defined tumor entities [16]. However, less commonly a great variety of other sarcomas can manifest either primarily or secondarily in the brain. More often, malignant gliomas undergo mesenchymal transition and subsequently are diagnosed as gliosarcoma [16].

Mesenchymal brain tumors predominantly arise in adult patients. However, they rarely also affect children and in this setting they are often associated with syndromic disorders [14,17,24]. In general, pediatric intracranial mesenchymal tumors not clearly falling into the group of meningioma or hemangiopericytoma / solitary fibrous tumor pose a diagnostic challenge.

Recent omics-based approaches have demonstrated great promise for the classification of tumors. Methylation analysis has proven of high reproducibility allowing precise classification of known and identification of novel brain tumor entities [1,29]. The methylation based concept relies on the observation that promoter methylation patterns capture a reminiscence of discrete time points in developing cells (i.e. putative cells of origin) and that methylation patterns are fairly stable throughout tumor progression [21]. Methylation-based classification is currently expanded to include sarcomas and for instance has shown great power in distinguishing mesenchymal small blue round cell tumors mimicking Ewing sarcoma [12].

We here report on a significant group of intracranial pediatric sarcomas, which histologically exhibit considerable heterogeneity but by molecular omics analyses emerge as a homogenous entity sharing a characteristic methylation profile and a set of highly recurrent mutations.

## **Material and Methods**

### **Sample selection and DNA extraction**

We performed unsupervised clustering of methylation data generated from primary intracranial tumors and peripheral sarcomas analyzed at the German Cancer Research Center (DKFZ) and focused on clusters not matching those of previously established methylation groups [1,12,13]. Additional samples for further methylation testing were supplied by the Peruvian national cancer center and its cooperating institutes. Source material for DNA extraction was formalin-fixed and paraffin-embedded (FFPE) tumor tissue. Representative tumor tissue with highest available tumor content was chosen for DNA extraction. The Maxwell® 16 FFPE Plus LEV DNA Kit or the Maxwell® 16 Tissue DNA Purification Kit (for frozen tissue) was applied on the automated Maxwell device (Promega, Madison, WI, USA) according to the manufacturer's instructions. All tumor DNAs had a total amount of >100 ng and were suitable for the array-based DNA-methylation analysis. Germline information was available from five patients that had been enrolled in the MNP2.0 or INFORM trial. Tissue collection, processing and data collection were in compliance with local ethics regulation and approval.

### **Genome-wide DNA-methylation data generation and pre-processing**

All tumors were subjected to Illumina Infinium HumanMethylation450 (450k) BeadChip or the successor EPIC/850k BeadChip (Illumina, San Diego, USA) analysis at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ) Heidelberg or at the Institute for Neuropathology at the University Medical Center Hamburg-Eppendorf. DNA methylation data were normalized by performing background correction and dye bias correction (shifting of negative control probe mean intensity to zero and scaling of normalization control probe mean intensity to 20000, respectively). Probes targeting sex chromosomes, probes containing multiple single nucleotide polymorphisms and those that could not be uniquely mapped were removed. Probes from the EPIC array were excluded if the predecessor Illumina Infinium 450k BeadChip did not cover them, thereby making data generated by both 450k and EPIC feasible for subsequent analyses. In total, 438370 probes were kept for analysis.

### **Unsupervised clustering, t-SNE analysis and cumulative copy number plotting**

For unsupervised hierarchical clustering, we selected 10000 probes that showed the highest median absolute deviation (MAD) across the beta values. Samples were hierarchically clustered using the Euclidean distance and Ward's linkage method. Methylation probes were reordered by hierarchical clustering using Euclidean distance and complete linkage. The unscaled methylation levels were shown in a heat map from unmethylated state (blue color) to methylated state (red color). For unsupervised 2D representation of pairwise sample correlations, dimensionality reduction by t-distributed stochastic neighbor embedding (t-SNE) was performed using the 10000 most variable probes, a perplexity of 20 and 2500 iterations. Novel methylation groups were tested for stability by varying the number of the most variable probes. Copy-number assessment was done based on methylation array data using the R-package conumee.

### **Gene panel next generation sequencing**

A customized SureSelect XT technology (Agilent) panel covering the coding regions of 130 genes was applied. Library preparation, quality control, sequencing on a NextSeq sequencer (Illumina) and data processing were performed as previously described [25]. Reads were aligned against the reference genome hg19. Gene panel sequencing data were automatically annotated using ANNOVAR software [33]. Synonymous and stoploss variants, variants with a frequency exceeding 1% in the healthy population as well as variants described as known polymorphisms in the single nucleotide polymorphism database were not considered.

### **Immunohistochemistry**

A representative block was chosen for immunohistochemistry. 4-micron paraffin sections were dried at 80°C for 15min and stained on a Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ, USA) using standard techniques. The following antibodies were applied:  $\alpha$ -Smooth Muscle Actin (Dako, Carpinteria, CA, USA, mouse monoclonal, clone 1A4), Desmin (Dako, mouse monoclonal, clone D33) and Myogenin (Cell Marque, Rocklin, CA, USA, mouse monoclonal, clone F5D).

## **Results**

### **Distinct methylation signature in a significant proportion of CNS sarcomas NOS**

We identified 11 intracranial tumors forming a previously undetected methylation group. These tumors had previously been diagnosed as CNS sarcoma NOS (n = 4), intracranial malignant tumor NOS (n = 2), and a single case each diagnosed as glioblastoma, gliosarcoma, extraskeletal mesenchymal chondrosarcoma, primitive neuroectodermal tumor (PNET) and embryonal rhabdomyosarcoma. Interestingly, two embryonal rhabdomyosarcoma of the uterus also fell in this methylation group. Subsequent methylation analysis of 11 pediatric intracranial sarcomas NOS from Peru demonstrated similarity with the same novel methylation group.

Next, we selected from our pool of tumors 26 morphologically and epigenetically defined entities to be distinguished from our novel group. The 26 entities covered the spectrum of differential diagnoses to the initial diagnosis of our tumor series, among them *DICER1*-related tumors such as pineoblastoma and medulloepithelioma of the eye. The defined entities and the number of tumors included are provided in **Supplementary Table 1**.

Unsupervised clustering (**Figure 1a**) and t-SNE analysis (**Figure 1b**) of these 26 entities together with our novel group confirmed characteristic and distinctive methylation patterns.

### **Clinical characteristics and morphological features**

All cases were located intracranially apart from the two uterine tumors. With two exceptions in the cerebellopontine angle and the cerebellum the intracranial tumors were supratentorially located (20/22). The age distribution of the 22 intracranial cases ranged from 0 to 76 years with a median age of 6 years. The gender distribution was almost equal with a female (n = 12) to male (n = 10) ratio of 1.2:1. Basic clinical information of the study cases is summarized in **Table 1**.

Histologic features could be assessed in 20/22 intracranial cases (**Figure 2**). The predominant tumor growth was patternless in 15 cases (15/20; 75%) and fascicular in five cases (5/20; 25%). Overall, the cellularity was variable in most cases with a patternless architecture, but markedly increased in cases with a predominant fascicular growth pattern. The mitotic activity was brisk in all cases. Atypical mitotic figures were observed in 14 cases (14/20; 70%). Focal areas with coagulative



necrosis were present in 13 cases (13/20; 65%). Rhabdomyoblasts or rhabdomyoblast-like cells were observed in all cases. These were most obvious in areas with lower cellularity, but single cells reminiscent of rhabdomyoblasts could also be identified in highly cellular tumors. Cartilaginous differentiation was seen only in one primary intracranial case (1/20; 5%), which morphologically presented highly variable (**Supplementary Figure 1**). However, cartilaginous nodules were present in one of the two uterine cases.

The immunophenotype could be assessed in 17/22 intracranial cases (**Figure 3**). Smooth muscle actin was detected in all 17 cases, desmin in 14 cases (14/17; 82%) and myogenin was positive in five cases (5/17; 29%). However, myogenin-positive cells were sometimes sparsely scattered. The histologic features and the immunophenotype are listed in **Supplementary Table 2**.

### **Highly recurrent *DICER1* mutations concomitant with MAPK pathway and *TP53* mutations**

We next investigated the cohort for molecular alterations by DNA sequencing and copy number profiling (**Figure 4**). Next-generation panel-sequencing revealed 28 *DICER1* mutations in 23 tumors. 23 missense mutations were located in mutational hot-spots within exons 24 and 25, both coding for the RNase IIIb domain. In addition, five cases had a second nonsense mutation, four of which resulted in a premature stop codon and one in a frameshift mutation. We were able to examine germline DNA from five patients. Two of these contained both, a somatic hotspot missense mutation and a germline nonsense mutation.

We additionally detected recurrent mutations in the MAPK pathway. *KRAS* hotspot mutations were seen in eight, *NF1* mutations in seven with one case showing a homozygous deletion, *NRAS* hotspot mutations in two, *RAF1* and *FGFR4* mutations in two each and *EGFR* amplification in one of the tumors. Four tumors exhibited two MAPK pathway mutations resulting in 17 tumors (17/22; 77%) overall being affected in this pathway.

Furthermore, four cases harbored mutations either in *SMO* (2/22; 9%) or in *SUFU* (2/22; 9%), both known for their pivotal role in activating the hedgehog pathway. Three cases with a hedgehog pathway mutation also had mutations in the MAPK pathway.

*TP53* mutations were found in 13/24 (54%) cases. Unfortunately, germline information was unavailable in these *TP53* mutant cases.

All variants being called and the respective distributions are depicted in **Figure 4**.

Sequencing data are provided in **Supplementary Table 3**.

## **Discussion**

We here report a series of 22 intracranial sarcomas in predominantly pediatric patients, which closely resemble embryonal rhabdomyosarcomas with *DICER1* mutations in terms of a distinct methylation signature and the presence of *DICER1* mutations [2-5,8].

We propose the designation “spindle cell sarcoma with RMS-like features, *DICER1* mutant” (SCS-RMSlike-DICER1) for this group. The methylation signature of SCS-RMSlike-DICER1 was distinct compared to 26 tumor subtypes with established methylation groups representing differential diagnoses to the initial diagnoses allotted to the tumors in our series. Both, unsupervised cluster and t-SNE analyses clearly demonstrate a strict separation of SCS-RMSlike-DICER1 from the other tumors. The combination of a unique methylation signature, a defining mutation in more than 95% of cases and the predominant occurrence in the pediatric age group strongly argue that SCS-RMSlike-DICER1 constitutes a distinct sarcoma entity.

In contrast to the unequivocal genetic features, the morphology of SCS-RMSlike-DICER1 is highly variable. For this reason, the tumors in our series initially have been diagnosed as sarcoma NOS, chondrosarcoma, glioblastoma, gliosarcoma or PNET. This is not surprising, since the tumor architecture in these tumors is highly diverse and the tumor cell appearance quite heterogeneous. Common to all SCS-RMSlike-DICER1 is high cellularity, brisk mitotic activity and presence of intermingled rhabdomyoblasts or rhabdomyoblast-like cells. While these cells in some instances focally predominate or are quite obvious, they may be infrequently encountered in most of the cases. Approximately 30% of SCS-RMSlike-DICER1 exhibited focal expression of myogenin.

Previous studies have reported intracranial rhabdomyosarcomas [6,18,22,23,30]. Most of the tumors in these studies localized to the cerebellum. This contrasts the supratentorial localization of the tumors in our series. While this may indicate separate groups, the *DICER1* status of cerebellar rhabdomyosarcomas has not been determined. The two cases in our SCS-RMSlike-DICER1 series occurring in the posterior fossa were highly similar to the supratentorial cases. The relation of those previously reported cerebellar rhabdomyosarcomas and SCS-RMSlike-DICER1, therefore, currently is unresolved. Notably, in the course of this investigation we also identified embryonal and alveolar rhabdomyosarcoma primarily manifesting in the

CNS, which histologically and epigenetically matched with their peripheral counterparts. Thus, these cases were not further addressed here (data not shown). A fraction of extracranial embryonal rhabdomyosarcoma has been reported with *DICER1* mutations. *DICER1* mutations appear to be restricted to embryonal rhabdomyosarcoma of the uterine cervix [4,9]. Three studies employing whole exome sequencing on 102 fusion-negative rhabdomyosarcomas did not detect *DICER1* mutations at all [11,27,28]. However, none of them analyzed tumors originating from the uterine cervix. Interestingly, morphological features of rhabdomyosarcoma of the uterine cervix included cartilaginous nodules in nearly half of the cases (6/14), contrasting the single case (1/22) in our series of intracranial SCS-RMSlike-*DICER1* [4]. However, one of the two uterine cervix rhabdomyosarcoma in our series also exhibited cartilaginous nodules.

In our series of 22 SCS-RMSlike-*DICER1*, germline DNA was available from five patients. Analysis of constitutional DNA revealed germline *DICER1* mutations in two of these five patients. This may indicate a considerable proportion of all SCS-RMSlike-*DICER1* having a hereditary background – a hypothesis that needs to be addressed in future studies. Germline mutations were accompanied by two findings: First, the type of germline mutations differed from that of somatic *DICER1* mutations. Both germline mutations were of the nonsense type, resulting in a premature stop codon and protein truncation. Second, both patients with a *DICER1* germline mutation carried an additional somatic missense mutation. The co-occurrence of two mutations in *DICER1* was previously described in tumors arising in patients with *DICER1* syndrome [10]. Three other patients in our series harbored two simultaneous *DICER1* mutations. Two of these exhibited the combination of nonsense together with missense mutations while one patient carried two missense mutations. Although we cannot provide proof for lack of constitutional DNA in these three patients, we speculate on a hereditary background. This may indicate that at least 20% of SCS-RMSlike-*DICER1* develop on a genetic predisposition background. The *DICER1* mutational screening of the coding sequence including the exon-intron junction failed to detect any variant in a single SCS-RMSlike-*DICER1*. This case might harbor a deep intronic variant with pathogenic potential. Such variants have recently been described in *DICER1*-related tumors and often remain hidden by conventional NGS approaches [26,31].

The second most frequent gene affected by mutations in SCS-RMSlike-DICER1 was *TP53*, observed in 12/22 (55%) of the patients. Notably, the *TP53* mutation frequency in SCS-RMSlike-DICER1 is much higher compared to previous findings in pediatric patients with rhabdomyosarcomas [19,20,27]. It is known that rhabdomyosarcoma may arise in the setting of Li-Fraumeni syndrome caused by germline mutations in *TP53*. Due to the lack of constitutional DNA we could not determine the germline *TP53* status in our cases. A very rare pediatric renal neoplasm referred to as anaplastic sarcoma of the kidney was first recognized in 2007 [32]. These tumors predominantly occur in female patients and exhibit histologic features reminiscent of pleuropulmonary blastomas. Interestingly, a recent study focusing on molecular alterations in these tumors revealed recurrent mutations in *DICER1* and *TP53* [34]. This may indicate a close relation between anaplastic sarcomas of the kidney and SCS-RMSlike-DICER1, although SCS-RMSlike-DICER1 seems to have an equal gender distribution.

Mutations in the MAP-kinase pathway occurred in 17/22 (77%) of SCS-RMSlike-DICER1 (**Figure 4**). Most frequently affected was *KRAS* followed by *NF1*, *NRAS*, *FGFR4* and *EGFR*. MAPK pathway gene mutations have also been observed in rhabdomyosarcoma without *DICER1* mutations, albeit at a lower frequency [27]. Four patients carried mutations in *SUFU* or *SMO*, both involved in the sonic hedgehog pathway. Recently a mouse model based on *SMO*-induced activated hedgehog pathway was shown to prompt transdifferentiation of non-myogenic endothelial progenitor cells by aberrant expression of myogenic specification factors resulting in fusion-negative rhabdomyosarcoma [7]. This may be of interest because Gorlin Syndrome, caused by constitutional mutations in the sonic hedgehog pathway gene *PTCH1*, can be accompanied by embryonal rhabdomyosarcoma [15].

It is not yet known for certain how SCS-RMSlike-DICER1 clinically behave. Limited follow-up data being available at this point do not allow reliable conclusions to be drawn so far, although preliminary data leads us to suspect an aggressive clinical course. Further investigations regarding biological and clinical aspects are necessary and warranted for a comprehensive understanding of this molecularly distinct sarcoma group.

In conclusion, we describe a set of predominantly pediatric intracranial spindle cell sarcomas exhibiting rhabdomyosarcoma-like features with a highly characteristic and

diagnostic epigenetic and genetic profile. We therefore propose this intriguing group be called “spindle cell sarcoma with RMS-like features, *DICER1* mutant”.

### **Conflict of Interest**

The authors state no conflict of interest.

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**Table 1****Clinical characteristics of the study cohort****FIGURE LEGENDS****Figure 1: DNA-methylation profiling indicates a novel tumor methylation group**

Unsupervised hierarchical clustering (**a**) and t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis (**b**) of DNA-methylation data from intracranial tumors not otherwise specified and a reference set of prototypical brain tumors and soft tissue sarcomas. The arrows point out the novel methylation group. Abbreviations: aMen - anaplastic meningioma °III; ASPS - alveolar soft part sarcoma; CMN – congenital mesoblastic nephroma; CNS EFT-CIC – CNS Ewing family tumor with CIC alteration; con – control; mCS - mesenchymal chondrosarcoma; DFSP - dermatofibrosarcoma protuberans; ES - epithelioid sarcoma; EwS - Ewing sarcoma, GBM / GS IDH / H3 wild-type – glioblastoma °IV / gliosarcoma °IV, IDH1/2 and H3 wild-type; GBM H3 G34 – glioblastoma °IV, Histone 3 G34 mutant; DMG H3 K27 mutant – diffuse midline glioma °IV; CNS HGNET-BCOR – CNS high-grade neuroepithelial tumor with BCOR alteration; CNS HGNET-MN1 – CNS high-grade neuroepithelial tumor with MN1 alteration; IFS - infantile fibrosarcoma; LMS – leiomyosarcoma; MEPL – medulloepithelioma of the eye; MPNST – malignant peripheral nerve sheath tumor; MRT - malignant rhabdoid tumor; SCS-RMSlike, DICER1 mutant – spindle cell sarcoma with RMS-like features, DICER1 mutant; CNS NB-FOXR2 – CNS neuroblastoma with FOXR2 activation; PB – pineoblastoma °IV; RMS (ALV) – alveolar rhabdomyosarcoma; RMS (EMB) - embryonal rhabdomyosarcoma; SFT – solitary fibrous tumor; SySa – synovial sarcoma; UPS – undifferentiated pleomorphic sarcoma

**Figure 2: Histologic features of cases assigning to the novel methylation group**

Most primary intracranial tumors presented with spindle-shaped cells and exhibited rhabdomyosarcoma-like features. Rhabdomyoblasts or rhabdomyoblast-like cells were present, although variable in number and mostly sparsely scattered (indicated by arrows in **a**, **b** and **e**). The predominant growth of these tumors was patternless (**a** - **c**). Some cases exhibited a prominent fascicular growth sometimes being reminiscent of a solitary fibrous tumor (**d**). Some cases showed, at least in some areas, a myxoid stroma matrix (**e**). Many cases showed signs for anaplasia with hyperchromatic enlarged and pleomorphic tumor cell nuclei (**f**) and/or atypical mitotic figures. The infiltration boarder was sometimes sharply demarcated, although small tumor cell islands infiltrated the adjacent brain parenchyma (**c**). Other cases showed a more diffuse infiltration pattern (**h**). Some cases focally exhibited areas with more differentiated rhabdomyoblasts (**i**). Scale bars equal 100  $\mu\text{m}$ .

**Figure 3: Immunophenotype compatible with myogenic lineage differentiation**

The figure indicates a representative staining (left panel) and the summarized distribution pattern (right panel) of myogenin,  $\alpha$ -smooth muscle actin (SMA) and desmin in 17 SCS-RMSlike-DICER1. Nuclear expression of myogenin in a case scored with focal distribution (**a**). SMA immunohistochemistry in a case with focal expression (**b**). Desmin stains focally in some cases (**c**). Scale bars equal 100  $\mu\text{m}$ .

**Figure 4: Molecular features of spindle cell sarcoma with RMS-like features**



## Supplementary data

### Supplementary Figure 1: Case with an outstanding heterogeneous morphology

This triple positive case (myogenin, desmin,  $\alpha$ -smooth muscle actin) exhibits a striking variation in morphological patterns. This case exhibits areas with increased cellularity of polygonal to spindle-shaped tumor cells (**a**) and areas with less cellularity and a myxoid tumor matrix, occasionally accompanied with a perivascular accentuation of tumor cells (**b**). In these areas, some cells are suspicious for rhabdomyoblasts (arrows). The tumor focally exhibits a hyaline collagenous matrix (**c**). A substantial proportion of the tumor bulk is composed of prominent cartilaginous differentiation (**d**). Insets show the myogenin expression in the corresponding area shown in the H&E. Scale bars equal 100  $\mu$ m.

### Supplementary Table 1: Clinical data

### Supplementary Table 2: Histologic features and immunophenotype

### Supplementary Table 3: Next-generation sequencing results

Variants are depicted in a two-tier ranking separated in probably (red colored) and possibly (blue colored) clinically relevant.

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