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SMARCE1 mutation screening in classification of clear cell meningiomas

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Running title: SMARCE1 mutation in clear cell meningioma

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ABSTRACT

Aims: Clear cell meningioma is a rare subtype of meningioma and shows not only unusual histology, but also unique clinical features. Recently, SMARCE1 mutations have been shown to cause spinal and cranial clear cell meningiomas. We present 12 cases which were diagnosed with a clear cell meningioma in a single institution between 1997 and 2014, and investigate their SMARCE1 mutation status. Methods and results: To investigate the SMARCE1 mutation status of these tumours, we used a combination of Sanger sequencing and multiplex ligation dependent probe amplification analysis and also performed SMARCE1 immunohistochemical staining. We found both SMARCE1 mutational hits, including novel SMARCE1 mutations, in six out of eight tested patients. Immunohistochemical analysis showed loss of SMARCE1 protein staining in all but two cases. Two individuals who were originally diagnosed with clear cell meningioma were negative for SMARCE1 mutations, but tested positive for NF2 mutations. As a result, these two tumours were reanalyzed and eventually reclassified, as a microcystic and a mixed growth pattern meningioma with focal clear cell areas, respectively. Conclusions: These results expand the spectrum of pathogenic variants in SMARCE1 and show that mutation screening can help facilitate meningioma classification. This may have implications for prognosis and future clinical management of patients, since clear cell meningiomas are classed as grade II tumours, while microcystic and meningothelial meningiomas are classed as grade I.

Keywords: SMARCE1, meningioma, clear cell
INTRODUCTION

Meningiomas are the most common form of primary neoplasm in the adult central nervous system (CNS).\(^1,2\) Meningiomas account for 1.0-4.6% of childhood brain tumours.\(^3\) While most meningiomas have a good prognosis, high recurrence rates have been observed for WHO grade II and III meningiomas.\(^4\) Among grade II and III meningiomas, clear cell meningioma (CCM) is rare, consisting of only 0.2% to 0.81% of all meningiomas.\(^5\) Histologically, CCMs are characterized by patternless distribution of polygonal cells with a clear, glycogen-rich cytoplasm and blocky, perivascular, and interstitial collagen. CCMs show not only unusual histology, but also unique clinical features. They tend to occur in young patients, and are more likely to recur or metastasize than other common subtypes.\(^6\) Recurrence and CNS metastasis occurred in 37.8% and 8.1% of the cases reported in the literature, respectively.\(^6\)

Although the molecular genetic alterations occurring in meningiomas have not yet been fully elucidated,\(^5,7\) meningiomas exhibit a wide spectrum of molecular alterations. \(NF2\) mutations are the most common somatic genetic alteration found in sporadic meningiomas, and germline mutation of the \(NF2\) gene is known to predispose to multiple meningiomas as part of the neurofibromatosis type 2 (NF2) phenotype.\(^8\) Genomic sequencing of a subset of meningiomas lacking \(NF2\) alteration elucidated recurrent \(SMO\) and \(AKT1\) mutations, which have the potential to guide new therapeutic strategies.\(^9\) Furthermore, genomic changes are associated with histologic subtype and genomic data of various subtypes are expected to facilitate more accurate histologic grading and subtyping.\(^10-13\) In case of CCM, germline loss-of-function mutations in the \(SMARCE1\) chromatin remodeling factor were identified as a cause of both spinal and cranial CCM.\(^8,14\) To date, twelve families with \(SMARCE1\)-associated CCM have been reported (Table 1).\(^14-18\) \(SMARCE1\) mutations appear to be specific for the clear cell histological subtype, rather
than tumour location or syndromic presentation. Previously, intracranial CCM has also been reported in one child diagnosed with NF2 disease on clinical grounds and one adult, also on clinical grounds, while most meningiomas that occur in NF2 show a fibroblastic or transitional histology. In this study, we report 12 cases which were diagnosed as a CCM in a single institute of Korea during an 18-year of period. We investigated the involvement of SMARCE1 mutation in these tumours.

MATERIALS & METHODS

Patient population and data collection

A retrospective search of the pathology database at the Samsung Medical Center, using the term “Clear cell meningioma”, identified a total of 14 samples from 12 patients. All patients underwent surgery at the neurosurgery unit of the Samsung Medical Center, Seoul, South Korea, between 1997 and 2014. For patient 5 who underwent a two-staged operation, two surgical specimens (5-1 and 5-2) were included. Patient 6 underwent an initial operation at an outside hospital. The sample used in this study was a recurrent tumour with a nine year interval. The primary tumour sample for this patient was not available. For patient 11, who showed progression of disease, the initial tumour (11-1) and progressed tumour (11-2) with a three month interval, were analysed. A blood sample was also acquired from this patient. All patients were followed until October 2015, with a median follow-up time of 59 months. Formalin-fixed paraffin-embedded (FFPE) tissue was obtained from a retrospectively collected archive. Clinical information including age, tumour site, local and adjuvant therapy, recurrence and survival data were evaluated by reviewing the medical records. The study was approved by the institutional review board (IRB) at the Samsung Medical Center.
DNA extraction

Genomic DNA was extracted using a Qiagen DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After extraction, concentration, 260/280 and 260/230 nm ratio were measured by spectrophotometer (ND1000, Nanodrop Technologies, Thermo-Fisher Scientific, MA, USA).

Sanger sequencing

SMARCE1 exons were amplified from tumour DNA, using GoTaq G2 Green Master Mix (Promega, Southampton, UK). The products were purified using AxyPrep Mag PCR clean up beads (Appleton Woods, Birmingham, UK). Sequencing PCR was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI, Life Technologies, Paisley, UK). The products were purified using AxyPrep Mag DyeClean beads (Appleton Woods, Birmingham, UK) and analysed on an ABI 3730xl DNA Analyzer (ABI, Life Technologies, Paisley, UK).

Multiplex ligation dependent probe amplification (MLPA)

MLPA was carried out using SALSA MLPA kits, X072-X1 and P043-NF2 (MRC-Holland, Amsterdam, the Netherlands), according to the manufacturer’s instructions. Briefly, 100 ng DNA were used for hybridization, ligation and amplification of exon probes for control and test samples and the products were analyzed on an ABI 3100 sequencer (Applied Biosystems, Warrington, UK).

Immunohistochemistry

Paraffin embedded meningioma sections were immunostained for SMARCE1 protein as described previously. Briefly, 1:100 anti-SMARCE1 HPA003916 antibody (Sigma-Aldrich, St. Louis, MO), was used to stain sections, using an indirect peroxidase method on the Roche
Ultra IHC autostainer. This antibody is polyclonal and has been raised against the C-terminal end of SMARCE1 (amino acids 301-408).

RESULTS

Clinicopathologic feature of 12 patients diagnosed with a clear cell meningioma

The clinicopathologic information of the patients is summarized in Table 2. There were five spinal tumours and seven cranial tumours. The tumour sites were as follows: spinal cord, frontal lobe, cerebellomedullary cistern, cerebellopontine angle, and falx cerebelli, low cranial nerve. The sample from patient 6 was a recurrent tumour (nine years interval) and patient 11 showed disease progression (three months interval). However, to date, none of the patients have died due to tumour recurrence or progression. All patients denied any familial history or past history of brain or spinal tumour.

SMARCE1 status

Immunohistochemical analysis of paraffin embedded tissue from each tumour showed loss of SMARCE1 protein staining in 12 of 14 tumours (Figure 1, Table 2).

To investigate the SMARCE1 mutation status of these tumours, we used a combination of Sanger sequencing and MLPA analysis (Table 2). Four tumours were excluded from genetic analysis due to poor DNA quality. We found two SMARCE1 mutational hits in each of six out of eight tested patients. We identified two splice-site mutations, three nonsense mutations, three frameshift mutations, and one indel. These include three novel mutations located in exons 3 (c.23delC, p.(Pro9Hisfs*62)) and 10 ((c.831delA, p.(Lys277Lys*1) and (c.957delC, p.(Pro320Leufs*122)). The SMARCE1 exon 9 mutation, c.715C>T, p.(R239*), identified in one
patient has been seen previously in another cohort. In the current study this mutation was associated with a recurrent tumour.

For patient 11, showing tumour progression, an identical mutation, c.331G>T, p.(E111*), was found in both the original and progressed tumour samples (11-1 and 11-2). A blood sample was available for analysis and we were able to confirm the presence of this mutation in the germline. Two surgical specimens for the tumour from patient 5 (5-1 and 5-2) also showed identical mutations.

Tumours from three individuals contained two point mutations, rather than one point mutation and loss of heterozygosity (LOH). These included an exon 6 splice-site mutation and an exon 10 frameshift in the cranial CCM of a 16 year old male; two frameshift mutations involving exons 3 and 8 were identified in the spinal CCM of a 19 year old female. Finally, a nonsense mutation in exon 6 and a frameshift alteration in exon 10 were detected in the spinal CCM of a 10 year old female.

For the two tumours in which no SMARCE1 mutations were found (from patients 8 and 9), we analysed the NF2 gene and identified biallelic inactivating mutations in both tumours. The first contained a frameshift mutation, c.579delA, p.(Ala194Alafs*15) in exon 6, with LOH. The second tumour contained the missense mutation, c.755C>T, p.(Pro252Leu), in exon 8, also with LOH. The variant p.(Pro252Leu) is non-truncating, but was not found on ESP6500SI-V2 or ExAC and was predicted to be damaging by Polyphen2, SIFT, Mutation Taster and Align GVGD, suggesting that it is pathogenic.

**Histologic reclassification**
Since we identified two \( NF2 \) alterations in each of these last two tumours, SMARCE1 protein staining was positive, and we could not find \( SMARCE1 \) mutations in either of these tumours, the tissue was reassessed (by S.A., Y.S. and D.dP). It was determined that the major histology for the tumour from patient 8 was in fact microcystic (Figure 2). For patient 9, the tumour contained a focal clear cell component; however, the tumour was heterogeneous in composition, also containing meningotheial and small cell constituents (Figure 2).

After removing these two males without conventional CCM morphology, male to female ratio was 6:4 and cranial-to-spinal tumour ratio was equal (5:5). In this cohort, the mean and median ages at diagnosis of meningioma were not significantly different between males (27 and 24 years, respectively) and females (23 and 24.5 years, respectively).

**DISCUSSION**

Meningiomas consist of various histologic subtypes which appear to be determined by distinct genetic alterations. Recently, several large-scale genomic studies of meningioma extended our knowledge of somatic molecular alteration in various types of meningioma.\(^9,10,13,21,22\) These data can be used to fine-tune diagnostic accuracy, both subtyping and potentially grading.\(^11-13,21\) In CCM, germline loss-of-function mutations in the \( SMARCE1 \) chromatin remodeling factor were identified as a cause of both spinal and cranial CCM.\(^14,16\) To date, including the current study, pathogenic \( SMARCE1 \) mutations have been associated with CCM in individuals from 20 separate families. The majority of identified \( SMARCE1 \) mutations are nonsense or frameshift variants, predicted to lead to protein inactivation and complete loss of the protein product. Previous studies have shown two unrelated families to have a large multi-exon deletion, while one family
has an intraexonic inversion mutation and two families have a small exonic indel (Table 1). To date, missense mutations have not been associated with CCM. The majority of SMARCE1 nonsense and frameshift mutations have been found in exon 6. The functional significance of this region is further highlighted by three splice sites mutation that have been found in exons 5 and 6, with the potential to generate aberrantly spliced transcripts and therefore to perturb protein structure and function. However, immunohistochemical analysis of available tissue has shown loss of SMARCE1 protein in all SMARCE1 mutation positive tumours, suggesting that if any alternatively spliced products are generated, they are unstable.

Mutational analysis in the current cohort identified two SMARCE1 mutations in eight tumours from six people. No SMARCE1 mutations were identified in two remaining tumours, but two mutational hits were identified in NF2 (a point mutation and LOH) in each tumour. Each of these NF2-mutated tumours showed an unconventional morphology for CCM and subsequent reanalysis of their histology established one to be a microcystic meningioma (patient 8) and the other to have a mixed histology (patient 9). Microcystic meningiomas are known to be problematic to distinguish from CCMs. The tumour from patient 9 contained a focal clear cell component, however, the tumour was heterogeneous in composition, also containing meningothelial and small cell constituents (Figure 2).

CCMs are classed as grade II meningiomas and show unique clinical features. They tend to occur in young patients, and are more likely to recur or metastasize than other common subtypes.6 In this cohort the mean and median ages at diagnosis of meningioma were not significantly different between males (27 and 24 years, respectively) and females (23 and 24.5 years, respectively). This is in contrast to previous studies, indicating an earlier onset in males versus females. The reason for this is unknown. However, as the mutation status in tumours from
four adult males is unknown and no lymphocyte DNA was available for any of these patients to test germline status, it is possible that these are sporadic tumours occurring at a later age, while the younger male patients have a germline predisposition. The limitations of this study are that the follow-up period was relatively short for some patients and germline DNA was only available for one patient, limiting our ability to determine which cases were due to germline mutation and which were sporadic.

This study adds to the known spectrum of meningioma-associated variants in *SMARCE1* and the natural history of CCMs. To our knowledge, this is the first study examining *SMARCE1* mutation status in Asian patients with CCM. It also demonstrates the utility of genetic screening in accurate classification of meningiomas. This may have implications for future clinical management of patients, since CCMs are classed as grade II tumours, while microcystic are classed as grade I. The study findings also raise the prospect that meningiomas with focal clear cell components do not share the same genotype as conventional, “pure” CCM and may not, by implication, carry the same adverse prognosis. This, however, requires validation, requiring the genotyping of larger numbers of mixed meningiomas with focal clear cell areas.
Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest to declare.

Author contributions

Y.L.Suh conceived the idea and designed the study. Y.L.Suh, S.Ahn and D.plessis reviewed the slides. M.J.Smith, and M.Bulman conducted the experiment and analyzed the data. D.Plessis performed immunohistochemical staining and interpreted the results. J.I.Lee contributed by providing study material. S.Ahn and M.J.Smith wrote the main manuscript text. All authors reviewed the manuscript.
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13. Abedalthagafi MS, Merrill PH, Bi WL *et al.* Angiomatous meningiomas have a distinct genetic profile with multiple chromosomal polysomies including polysomy of chromosome 5. *Oncotarget* 2014; 5; 10596-10606.


Table 1. Clinical and genetic data on reported SMARCE1 mutated clear cell meningioma

<table>
<thead>
<tr>
<th>Study</th>
<th>Patient</th>
<th>Sex</th>
<th>Age of onset (years)</th>
<th>Location</th>
<th>Germline Mutation</th>
<th>Predicted protein alteration</th>
<th>Tumor mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smith et al. 2013</td>
<td>1</td>
<td>Female</td>
<td>27</td>
<td>Spinal</td>
<td>c.715C&gt;T</td>
<td>p.Arg239*</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Female</td>
<td>30</td>
<td>Spinal</td>
<td>c.237+2T&gt;C</td>
<td>p.Lys79_Val80ins3*</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p.Ala53_Lys79del</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Male</td>
<td>26</td>
<td>Spinal</td>
<td>c.311G&gt;A</td>
<td>p.Trp104*</td>
<td>Tumor 1: c.311G&gt;A heterozygous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tumor 2: c.311G&gt;A + LOH</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Female</td>
<td>17</td>
<td>Spinal</td>
<td>c.572insC</td>
<td>p.Thr191Thrfs*14</td>
<td>c.572insC heterozygous</td>
</tr>
<tr>
<td>Smith et al. 2014</td>
<td>5</td>
<td>Male</td>
<td>7</td>
<td>Spinal</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Hit 1: c.624_627delTGAG, p.(Ser208Argfs*26)</td>
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<tr>
<td></td>
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<td></td>
<td></td>
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<td>Hit 2: LOH</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Female</td>
<td>22</td>
<td>Cranial</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Hit 1: c.357C&gt;G, p.(Tyr119*)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>7</td>
<td>Male</td>
<td>10</td>
<td>Cranial</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Hit 1: c.688C&gt;T, p.(Gln230*)</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>Hit 2: LOH</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Male</td>
<td>8</td>
<td>Spinal</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Hit 1: Del promoter-E5/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hit 2: LOH</td>
</tr>
</tbody>
</table>
|               | 9†      | Male  | 2                    | Spinal   | c.275_276insA     | p.Leu93Valfs*17            | Hit 1: c.275_276insA, p.Leu93Valfs*17

† Indicates a case of familial meningioma.
<table>
<thead>
<tr>
<th>#</th>
<th>Gender</th>
<th>Age</th>
<th>Location</th>
<th>Genomic Change</th>
<th>Mutation</th>
<th>Notes</th>
</tr>
</thead>
</table>
| 10‡ | Female | 14  | Cranial  | c.374_395inv22 | p.(Glu125_Ala132delinsGlyLeuHisArgPhelleValLeu) | Hit 1: c.374_395inv22, p.(Glu125_Ala132delinsGlyLeuHisArgPhelleValLeu)  
Hit 2: c.267delT, p.(Asp90Thrfs*2) |
| 11  | Female | 17  | Spinal   | Del promoter-E5/6 | No protein product | Hit 1: del promoter to E5/6  
Hit 2: c.757C>T, p.(Gln253*) |
Hit 2: LOH |

† Discussed in more detail in Evans et al 2014  
‡Discussed in more detail in Raffalli-Ebezant et al 2014
Table 2. Clinical and genetic information of 12 patients diagnosed with a clear cell meningioma

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Age</th>
<th>Sex</th>
<th>Location</th>
<th>Treatment</th>
<th>Recur</th>
<th>Death</th>
<th>F/U period (month)</th>
<th>SMARCE1 protein staining</th>
<th>SMARCE1 Mutation 1</th>
<th>SMARCE1 Mutation 2</th>
<th>NF2 Mutation 1</th>
<th>NF2 Mutation 2</th>
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<tr>
<td>1</td>
<td>22</td>
<td>M</td>
<td>Spinal cord</td>
<td>GTR</td>
<td>No recur</td>
<td>Alive</td>
<td>106</td>
<td>Negative</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>2</td>
<td>39</td>
<td>M</td>
<td>CPA</td>
<td>STR and</td>
<td>No recur</td>
<td>Alive</td>
<td>157</td>
<td>Negative</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>M</td>
<td>CMC</td>
<td>STR and</td>
<td>No recur</td>
<td>Alive</td>
<td>18</td>
<td>Negative</td>
<td>Exon 6 c.238-1G&gt;A, p.(?)</td>
<td>Exon 10 c.957delC, p.(Pro320Leufs*122)</td>
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<tr>
<td>4</td>
<td>26</td>
<td>M</td>
<td>CPA</td>
<td>GTR</td>
<td>No recur</td>
<td>Alive</td>
<td>51</td>
<td>Negative</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5-1</td>
<td>11</td>
<td>M</td>
<td>CPA</td>
<td>STR</td>
<td>No recur</td>
<td>Alive</td>
<td>100</td>
<td>Negative</td>
<td>Exon6 c.369+1G&gt;C, p.(?)</td>
<td>LOH</td>
<td>Not tested</td>
<td>Not tested</td>
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<tr>
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<td>GTR</td>
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<td>Not tested</td>
<td>Not tested</td>
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<tr>
<td>6*</td>
<td>33</td>
<td>F</td>
<td>Spinal cord</td>
<td>GTR</td>
<td>Recurrent</td>
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<td>83</td>
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<td>LOH</td>
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<td>Not tested</td>
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<td>(L5-S2)</td>
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<tr>
<td>7</td>
<td>48</td>
<td>M</td>
<td>Spinal cord</td>
<td>GTR</td>
<td>No recur</td>
<td>Alive</td>
<td>59</td>
<td>Negative</td>
<td>ND</td>
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<td>#</td>
<td>Age</td>
<td>Gender</td>
<td>Location</td>
<td>Surgery</td>
<td>Recurrence</td>
<td>Status</td>
<td>Age</td>
<td>Histology</td>
<td>Exon</td>
<td>Mutation</td>
<td>LOH</td>
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<td>8#</td>
<td>58</td>
<td>M</td>
<td>Falx cerebelli</td>
<td>STR and RT</td>
<td>No recur</td>
<td>Alive</td>
<td>58</td>
<td>Positive</td>
<td>Negative</td>
<td>NF2 Exon 6 c.579delA, p.(Ala194Alafs*15)</td>
<td>LOH</td>
<td></td>
</tr>
<tr>
<td>9#</td>
<td>31</td>
<td>M</td>
<td>Frontal lobe</td>
<td>STR and RT</td>
<td>No recur</td>
<td>Alive</td>
<td>59</td>
<td>Positive</td>
<td>Negative</td>
<td>NF2 Exon 8 c.755C&gt;T, p.(Pro252Leu)</td>
<td>LOH</td>
<td></td>
</tr>
<tr>
<td>11-1**</td>
<td>30</td>
<td>F</td>
<td>Low cranial nerve</td>
<td>STR and RT</td>
<td>Progressive</td>
<td>Alive</td>
<td>30</td>
<td>Negative</td>
<td>Exon 6 c.331G&gt;T, p.(E111*)</td>
<td>LOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-2**</td>
<td></td>
<td></td>
<td></td>
<td>STR and RT</td>
<td>Negative</td>
<td>Same as no.11-1</td>
<td>Same as no.11-1</td>
<td>Negative</td>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>F</td>
<td>Spinal cord (L1-2)</td>
<td>GTR</td>
<td>No recur</td>
<td>Alive</td>
<td>16</td>
<td>Negative</td>
<td>Exon 6 c.313C&gt;T, p.(R105*)</td>
<td>Exon 10c.831delA, p.Lys277Lys*1</td>
<td></td>
<td></td>
</tr>
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CPA, Cerebellopontine angle; CMC, Cerebellomedullary cistern; GTR, gross total resection; STR, subtotal resection; RT, radiotherapy; ND, not determined due to poor DNA quality;

*, This case was studied on the recurrent tumor because of previous operation at outside hospital. ; #, Tumors were reclassified as microcystic meningioma (tumor 8) and mixed pattern of meningothelial meningioma with focal clear cell component (tumor 9).; **, Germline mutation was identified in the blood sample.
Figure Legends

Figure 1. Representative images of immunohistochemical staining of meningiomas for SMARCE1 (all at x400 magnification). A) Case 1 and B) 11 show typical loss of nuclear staining with positive staining restricted to endothelial and leukocytic nuclei plus mucoid/glycogen content of tumour cells. C) Case 8 and D) 9 show retained SMARCE1 staining.

Figure 2. A) H&E stained section for case 5-1 showing a patternless growth of clear cells and prominent perivascular and interstitial collagen, typical of clear cell meningioma (x200). B) H&E stained section for case 9 (region 1) showing a heterogeneous histology with focal clear cell meningioma features. C) H&E stained section for case 9 (region 2) indicating a meningotheelial growth pattern.