Molecular analysis of meningioma increases prognostic power: A methylation-based classification and grading system

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Summary

Background
The World Health Organization (WHO) classification of brain tumors describes 15 subtypes of meningioma. Nine of these are allotted to WHO grade I, and three each to grade II, and WHO grade III, respectively. Grading is purely based on histology, molecular markers are lacking. While the current classification and grading approach is of prognostic value, it harbors shortcomings such as ill-defined parameters for subtypes and grading criteria prone to arbitrary judgment.

Methods
We investigated genome-wide DNA methylation patterns of 479 meningiomas to identify distinct methylation classes (MC) of meningioma. The MCs were further characterized by DNA copy-number analysis, mutational profiling and RNA sequencing. We validated our findings in an independent cohort of 140 meningiomas.

Findings
DNA methylation profiling distinguished six distinct MCs associated with typical mutational, cytogenetic, and gene expression patterns. Meningioma MCs exhibit a more homogeneous clinical course and allow prognostication with significantly higher power than the current morphology-based WHO classification. Meningioma MCs more accurately identify patients at high risk of recurrence among tumors with WHO grade I histology, and patients at lower risk of recurrence among WHO grade II tumors. DNA methylation-based classification and grading reduces the number of meningioma subtypes from 15, as historically defined by histology, to six clinically relevant MCs, each with a characteristic molecular profile.

Interpretation
DNA methylation-based meningioma classification captures biologically more homogenous groups and has a higher power for predicting tumor recurrence than the current WHO classification. The approach presented here is highly useful for stratifying meningioma patients for observation or post-surgery treatment groups. We consider epigenetic tumor classification highly relevant for future diagnosis and treatment of meningioma.

Funding
This work was supported by the German Cancer Aid (110670, 110983) and the Else Kröner-Fresenius Foundation [A_60]. We thank the DKFZ-Heidelberg for funding by HIPO H033.
Introduction

The meninges exert a protective function for the entire central nervous system (CNS). During development, their precursor cells merge from mesodermal structures and the neural crest, actively contributing to the differentiation of the brain\textsuperscript{1-3}. However, meningeal cells may transform to initiate tumors, which represent the most frequent primary intra-cranial and spinal tumors\textsuperscript{4}. While 80\% of meningiomas show benign clinical behaviour and can be cured by resection alone, about 20\% recur and need additional treatment such as repeated surgery, irradiation and systemic therapy\textsuperscript{4,5}. Histopathological evaluation aims at identification of cases at risk for recurrence. The histological differentiation classification into subtypes initially dates back to the 19\textsuperscript{th} century. In the first internationally recognized classification approach in 1928, Bailey and Cushing distinguished a meningotheelial, fibroblastic, and angiomatous subtypes\textsuperscript{6}, and to this day, allocation to subtype is purely based solely on histological findings. The current WHO classification recognizes 15 subtypes and three grades of malignancy\textsuperscript{4}, but some of the diagnostic criteria are vaguely defined and subject to a high inter-observer bias, indicating the need for more reliable biomarkers\textsuperscript{5,7}.

For various other CNS tumors, molecular profiling has identified distinct subtypes with characteristic aberrations. Many of these correlate with prognosis or provide targets for treatment, and therefore support clinical decision making, e.g. epigenetic subgroups in medulloblastoma\textsuperscript{8-10} and ependymoma\textsuperscript{11}, or isocitrate dehydrogenase (IDH) status in diffuse glioma\textsuperscript{12-14}. Recent studies identified telomerase reverse transcriptase (TERT) promoter mutations in a small subset of meningiomas to be associated with higher risk of recurrence and shorter time to progression\textsuperscript{15,16}, and four large exome-sequencing efforts focusing on WHO grade I meningiomas have identified recurrently mutated genes beyond the long-known association with NF2\textsuperscript{17-20}. Yet, these findings cover only a fraction of meningiomas and have not all been adequately thoroughly tested for their prognostic relevance. In this study, we aimed at a comprehensive characterization of the entire molecular genetic landscape of meningioma in order to identify biologically and clinically relevant subgroups that able to refine the current classification scheme.
Results

**DNA methylation analysis identifies six distinct methylation classes of meningioma**

We generated genome-wide DNA methylation profiles from a discovery cohort of 497 meningiomas (Suppl. Fig. 1) along with 309 samples of other extra-axial skull tumors that histologically mimic meningioma variants, including solitary fibrous tumor/hemangiopericytoma, schwannoma, malignant peripheral nerve sheath tumors, chordoma, chondrosarcoma, fibrous dysplasia, and hemangioblastoma. Despite sharing mesodermal origin, unsupervised clustering of DNA methylation data clearly segregated all meningiomas from these other skull tumors (Suppl. Fig 2). Unsupervised clustering of meningiomas alone revealed two major epigenetic groups (Groups A and B, Fig. 1A), with both groups further subdividing into four and two subgroups, respectively, termed “methylation classes” (MCs). Based on further molecular and clinical characteristics outlined below, the four MCs of Group A were designated MC benign 1 through 3 (MC ben-1, ben-2, ben-3) and MC intermediate A (MC int-A). The two MCs of Group B MC intermediate B (MC int-B), and malignant (MC mal).

There was an enrichment of grade I tumors among MC ben-1, MC ben-2, and MC ben-3, and an enrichment of WHO grade III tumors in MC mal, while WHO grade II tumors where scattered across all MCs. Analysis of 75 primary and matched recurrent tumors from 37 patients showed that association with Group A or B was stable upon recurrence (Fig. 1B), supporting further assessment of methylation profiling for diagnostic and prognostic implications.

**MC predict clinical course with higher accuracy than WHO grading**

The wide spectrum of clinical behavior among WHO grade I and II meningiomas points towards the limited prognostic power of the current classification, particularly at the border between grade I and II. As a result, deciding on radiotherapy based on the current grading is heavily debated \(^5\). Thus, we correlated meningioma MCs with progression-free survival (PFS) to evaluate their potential for predicting outcome compared to WHO grading (Fig. 2A, B). We further combined MCs exhibiting virtually identical benign (MC ben-1, MC ben-2, MC ben-3) or intermediate (MC int-A, MC int-B) outcome into combined MCs (Fig. 2C). Classification by individual and combined MCs demonstrates more precise prognostication than by WHO grading (Fig. 2D, Brier prediction test, \(p<0.01\)). These findings were confirmed in 140 meningiomas from an independent validation cohort (Suppl. Fig 3A, B).

We next focused on the predictive power of MCs within WHO grades and, particularly, patients divergently diagnosed by WHO grading and DNA methylation-based classification. Patients with WHO grade I meningiomas molecularly assigned to an intermediate MC experienced a less favorable clinical course than patients with WHO grade I meningiomas diagnosed solely based on histology. In fact, there
outcome was indistinguishable from that of patients with WHO grade II meningiomas (Fig. 3A). Likewise, patients with WHO grade II meningiomas molecularly assigned to a benign MC had a better outcome than the average outcome of patients with WHO grade II meningiomas. Consequently, stratification for MC is of higher value for prediction of PFS than WHO grading. Within the combined MCs, WHO grading confers limited additional information (Fig. 3B, Suppl. Table 2). However, combined MCs delineate subgroups with significantly distinct prognosis within all WHO grades (Fig. 3C), demonstrating the benefit of MC-based grading for patients and the potential to significantly reduce under- or overtreatment.

Methylation classes are associated with distinct driver mutations and copy-number-alterations

We next sequenced 304 meningiomas with sufficient material available using a custom hybrid-capture next-generation sequencing (NGS) panel dedicated to 40 genes previously reported to be mutant in meningioma (Suppl. Table 1), based on our recently established custom NGS approach for routine brain tumor diagnostics. Known recurrent mutations (most frequently NF2, followed by TRAF7 and AKT1) were significantly enriched in certain MCs (Suppl. Table 3, Fig. 4). Within Group A, NF2 mutations were observed in 63 % of MC ben-1 tumors (accumulation of parameter in this MC p < 0·0001, Fisher’s exact test). MC ben-2 contained the vast majority of meningiomas carrying AKT1 (33 % in this subgroup; p < 0·0001), SMO (7 %; p = 0·0002), KLF4 (15 %; p < 0·0001), and TRAF7 (49 %; p < 0·0001) mutations and rarely NF2 mutations. Only one AKT1 and five KLF4 mutations were detected outside this MC. MC ben-3 exhibited NF2 mutations in 32 % and PIK3CA mutations in 11 % of tumors, representing the majority (5/7, 71 %) of PIK3CA mutations in the cohort. MC int-A carried NF2 mutations in 53 %. Within Group B, MC int-B tumors harbored NF2 mutations in 35 % and MC mal in 31 %. SUFU mutations were confined to Group B, with 5 % of MC int-B and 6 % MC mal tumors being mutated. Four out of five TERT promoter mutations mapped to the meningiomas in Group B (p = 0·005 Fisher’s exact test).

Annotation of copy-number-variations (CNV) revealed that MCs are associated with distinct cytogenetic aberrations (Fig. 4A, B): MC ben-1 was associated with deletions of 22q (95 %) but otherwise virtually no CNV. MC ben-2 presented with absence of recurrent CNVs. Typical for MC ben-3 were multiple chromosomal gains most frequently affecting chromosome 5 (47 %). MC int-A frequently exhibited losses on 1p (70 %) and 22q (84 %). In Group B, MC int-B frequently exhibited losses on 1p (89 %), 10 and 22 (89 %), all features also shared with MC malignant. However, in MC mal, a higher frequency of CDKN2A deletion occurred (70 %).

Representative cases with sufficient material available of all MCs underwent RNA-sequencing in order to identify differentially upregulated genes and activated pathways (Suppl. Fig. 4). (In preparation...)
Methylation classes and WHO subtypes, localization, and gender

Examining the distribution of histological subtypes across MCs revealed which histological subtypes comprise the MCs and, conversely, to which MC the samples of a respective subtype are assigned (Fig. 5). The rare lymphoplasmacytoid-rich meningiomas (WHO grade I) was not assessed due to the overwhelming dominance of constitutional (non-tumor) DNA in these samples. In general, two patterns were observed: Either a given MC was strongly associated with a small set of or even single subtypes, or samples of a MC or subtype, respectively, being widely spread across all variants. MC ben-1 comprised the majority of fibroblastic meningiomas and is also enriched for psammomatous meningioma. Fibroblastic meningiomas frequently harbor calcifications called psammoma bodies, and a high abundance of these calcifications defines psammomatous meningioma. The overexpression of SERPINF1 (Suppl. Fig. 4), which has been implicated in osteogenesis and calcification, in MC ben-1 might contribute to this histologically detected phenomenon. MC ben-2 was highly enriched for meningothelial meningiomas, and contained the vast majority of secretory meningiomas. MC ben-3 harboured cases from several subtypes but was particularly enriched for angiomatous meningiomas. This is in line with the overexpression of vessel-associated markers in samples of this MC (Suppl. Fig. 4). Transitional meningioma, a hybrid of meningothelial and fibroblastic histology, dissolved into several MCs, along with the samples of the rare microcystic, clear cell, chordoid, metaplastic and psammomatous subtype.

The two intermediate MCs were predominantly constituted of atypical meningiomas. However, a considerable fraction of atypical meningioma (n=31) fell into MC ben-1. These observations majorly contribute to the higher prognostic power of MC class over histology. Anaplastic meningiomas predominantly mapped to MC malignant. Of note, the six rhabdoid/papillary meningiomas, by definition WHO grade III, all ended up in one of the MC benign or intermediate meningioma groups. However, the number was too low to assess the statistical relevance of WHO grading and MC classification individually for rhabdoid/papillary meningioma.

Transitional meningioma WHO grade I was much more frequently assigned to an intermediate MC than fibroblastic or meningothelial meningioma, mostly to MC int-A. Atypical meningiomas assigned to a benign MC accumulated in MC ben-1.

The most frequent localizations for all subgroups were the frontal and central convexity, except for MC ben-2 (Fig 6). For the latter, basal localization was common, in line with the high occurrence of AKT1 and SMO mutations in this MC, which are known to be enriched in this localization. Interestingly, all MC mal cases were located along the convexity. In contrast, none of the basal tumors was allotted to MC mal, including four intraventricular and ten spinal meningiomas that all localized to intermediate or benign MCs. Gender and age distribution was equal throughout all
MCs. With the exception of a predominance of male patient in MC mal, while all other MCs mainly comprised female patients (Fig 6).
Discussion

The 15 subtypes of meningioma included in the current WHO classification have evolved over decades. The major aim of introducing including this variety of subgroups was to cover the whole histological spectrum of meningioma and to avoid misclassification. For example, meningeal tumors with chordoid or rhabdoid cytology may initially raise suspicion of a chordoma or rhabdoid tumor rather than but not point towards a meningioma. Therefore, particular subtypes with these ambiguous features were introduced into the classification in order to draw attention to the morphologic diversity of highlight the existence of these cytological differentiations in meningiomas. In addition, some cytological features have been reported to be associated with distinct outcomes. Although oftenthis was based on small series, it prompted the allocation of distinct WHO grades to specific meningioma subtypes. However, this approach has been increasingly questioned due to suboptimal inter-observer reproducibility, most recently reported in a large Radiation Therapy Oncology Group (RTOG) meningioma trial in which the authors expressed the urgent need for more objective molecular markers.

This resulted in an overall critical view of clinicians with respect to the current meningioma WHO classification and grading, which has been reiterated in the most recent published European Association of Neuro-oncology (EANO) guideline for the diagnosis and treatment of meningiomas. Accordingly, revisiting meningioma diagnostics based on epigenetic profiling by defining MCs with enhanced predictive power will greatly improve the acceptance of meningioma classification and facilitate more successfully guide clinical decisions regarding postoperative treatment. An overview of the molecular and clinical hallmarks of the six meningioma MCs is given in Figure 6.

Distinct methylation profiles suggest different development

Beyond the identification of clinically relevant groups and the basis for a novel classification, our dataset might give provide insight into the development of meningiomas. This has previously been shown for other entities such as the four variants of medulloblastoma, distinguishable by their DNA methylation patterns, which were shown to be determined by different precursor cell populations, and exhibit very different clinical characteristics and therapy needs. Our data indicate that the spectrum of meningiomas is divided into two major epigenetically highly distinct Groups (A and B, Fig. 1). This strong separation suggests either the existence of distinct cells of origin or an underlying event with a major impact on genome-wide DNA methylation. The distinctive very different DNA methylation profiles of Groups A and B, despite the shared occurrence of NF2 mutations, might suggest that meningiomas arise from two different precursor cell populations. Based on our own and published high-throughput sequencing data, there is no evidence for the existence of a single mutation being solely responsible for the separation of these two groups. However, we cannot fully exclude the
existence of alterations not readily detectable by these approaches, such as translocations or fusions, causing the responsible changes in the methylome. Moreover, the fact that patients with meningiomas clustering in Group A share a predominantly benign, with a small proportion exhibiting a semi-aggressive clinical course, and that patients with meningiomas of Group B follow a semi-aggressive to malignant clinical course, may further argue towards for a distinct cell of origin with different intrinsic propensities for malignant transformation. However, analyses dissecting the full regulatory background of the tumor cells in comparison to arachnoidal cells, e.g. by ChIP-Sequencing, are needed to fully elucidate this.

Methylation-based versus WHO subgrouping versus other molecular markers

Extensive whole exome or -genome sequencing has provided a large body of information on the mutational landscape of meningioma^17-20. Four distinct meningioma mutational subgroups have been proposed, defined by mutations either in NF2, TRAF7, the hedgehog pathway, or POLR2A^18. However, such a model of meningioma development based on mutational analysis alone currently does not currently satisfy the clinical need for distinction between patients in need of adjuvant treatment or not. A major drawback is the lack of risk stratification among NF2-mutant cases that which can present with any clinical course. While the strong association of AKT1, TRAF7/KLF4, or SMO mutations with benign, or TERT promoter mutations with unfavorable course may allow for mutation-based risk assessment in these subgroups, the current inability to stratify NF2-mutated meningiomas for other mutational events associated with clinical outcome is a major obstacle for a classification and grading system based on mutational profiling alone.

Similarly, strong limitations apply to approaches based on copy-number-profiles: They leverage the accumulation of aberrations during progression but are not capable of predicting the clinical behavior upfront. The current dataset attributes the highest prognostic power to methylome-based subgrouping, which proves to be superior to WHO classification (Fig. 2, 3). However, with an exclusively mutation-based subgrouping for classification of the full spectrum of meningioma is not yet in place.

An integrated diagnosis for meningioma evaluation

The WHO 2016 revision of the classification of CNS tumors supports the concept of an integrated diagnosis. It relies on a multilayered approach combining data from histology, molecular genetic analyses, and clinical findings^4,27,28. Adopting this WHO approach to the diagnosis of meningioma, the morphological layer corresponds to the current diagnostic standard, i.e. diagnosing the 15 WHO meningioma subtypes and grading according to the morphological scheme. In absence of
molecular analyses the morphological diagnosis should be suffixed with NOS (not otherwise specified), as agreed for parenchymal brain tumors without molecular workup⁴. The molecular diagnostic layer contains either DNA methylation or mutation analyses or both. With methylation analysis performed, one of the six MCs can be diagnosed. Mutational data may only enable inferring the MC for a subset within the MC ben-2, e.g. for AKT1 mutant cases, but not in each instance. If the data allow diagnosis of a MC, this results in a significantly improved more powerful prediction of the clinical course. This corresponds to the current approach for other entities, e.g. ependymoma and medulloblastoma, in that which methylation data has proven to be more relevant than histological grading⁴,⁵. Based on the data presented here, the integrated diagnosis of meningioma will not only highlight identify the prognostically relevant MC with regard to prognosis but also provide in addition refer to the morphological subtype identified on histological examination. Collectively, the accompanying dataset and proposed accompanying classification scheme proposed here advances meningioma diagnostics from the traditional histopathological approach histology into an integrated profiling with higher accuracy of risk assessment for individual patients.

**Author contributions**

FS and AvD conceived the project, coordinated data generation, and wrote the manuscript with input from all co-authors. FS, D Schrimpf, D Stichel, DTWJ, SS, D Sturm, M Sill, VH, LC and SMP designed methylation experiments and analyzed DNA methylation data. FS, D Schrimpf, D Stichel, LC and KO analyzed RNA sequencing data. FS, D Schrimpf and TH analyzed survival data. FS, D Schrimpf, D Stichel, SS, DR, CK, DC, KH, AK, AW analyzed DNA sequencing data. M Schick and MBH performed array experiments. FS, DR, CK, DC, KH, AK, AW, PB, K Kurian, AFO, CM, AK, JS, EJR, VPC, WP, MM, and AvD performed histological evaluation. HGW, ASB, PB, HE, K Kurian, AFO, CM, CJ, MSR, RK, M Simon, AB, KL, AK, JS, VPC, SB, M Platten, DH, AU, WP, WW, MM, M Preusser, CHM, and MW collected and interpreted clinical data and/or compiled respective tissue collections.
Materials and Methods

Samples

Samples with clinical data were retrospectively collected from the Dept. of Neuropathology Heidelberg, Germany (local and referral cases), Dept. of Neurosurgery Heidelberg and the FORAMEN network, the Dept. of Neurology and Neuropathology, Zürich, Switzerland, and the Neurological Institute (Edinger Institute) Frankfurt/Main, Germany. Additional samples without survival annotation were included from the Dept. of Neuropathology Berlin, Bonn, Magdeburg, Münster, Tübingen (all Germany), and Bristol (UK). The validation cohort was provided by the Medical University of Vienna.

Methylation analysis, copy-number analysis

Illumina 450k Human BeadChip (discovery cohort) and 850k EPIC (validation cohort) analysis were performed as previously described (ref). Unsupervised clustering for the discovery and validation cohort was performed based on XYZ (Euclidian ward SD 0.2). Copy-number aberrations were inferred from methylation array data (Ref VH).

Copy number analysis in MCs

Damian

Panel and RNA sequencing

Panel sequencing for genes reported to be mutant in meningioma (Suppl Table 2) was performed applying a custom hybrid-capture approach (Agilent) as described before (Ref). RNA libraries were generated with TruSeq RNA Access (Illumina). Sequencing was performed on a NextSeq 500 (Illumina).

Expression analysis based on RNA-seq data

Konstantin

Statistical analysis of clinical parameters

Distribution of survival times was estimated by the method of Kaplan and Meier and compared between groups with the log-rank test. Hazard ratios including 95% confidence intervals based on Cox regression models were calculated. For the multivariable Cox regression model, imputations of missing covariate values was done applying the multivariate imputations using chained equations (mice) algorithm with 100 imputation runs. Hazard ratio for age is given per 10 year increment. Prediction error curves based on the Brier score were computed. Integrated Brier score was tested between risk stratifications using 1000 bootstrap samples. P-values below 0.05 were considered statistically significant. Analyses were performed with statistical software R 3.3. Details and references are given in the supplemental information.
Figure 1 Unsupervised clustering of methylation data of 497 meningioma samples (A). Unsupervised clustering of matched primary and recurrent samples (matched primary/recurrent samples of identical patient identified by arrows) combined with reference samples from group A and B shows that no shift between groups occurs upon recurrence (B).
Figure 2 Progression free survival (PFS) of 228 case with clinical data stratified for WHO grade (A), methylation class (B), combined methylation classes (C). Brier prediction plot calculated for the models A-C (D, WHO vs combined MCs p=0.0138, 0.0096, 0.0062 for 5, 10 and 12 years, respectively).
Figure 3 Comparison of WHO grading and methylation-based risk prediction: WHO grade I cases allotted to an intermediate methylation class show PFS similar to the average grade II tumors. In turn, WHO grade II cases assigned to a benign methylation class have longer PFS than the average WHO grade II cases (A). Hazard ratio (including 95% confidence intervals) forest plot for WHO grading, overall and stratified for combined methylation classes (B). Hazard ratio forest plot for combined methylation classes, overall and stratified for WHO grading (C). While sub-stratification for WHO grade among MCs is of limited additional value (B), MCs stratify for distinct PFS within WHO grades (C).
Figure 4 Distribution of mutations across sample that underwent panel-sequencing (304) stratified for MCs (A). Copy number variations across all samples that underwent 450k analysis (497) the MCs (B).
Figure 5 Association of histological subtypes and MCs
Figure 6 Schematic overview over the six identified MCs and their molecular and clinical characteristics.
Validation cohort stratified for WHO grade (A) and combined methylation classes (B).
Most differentially expressed genes in the six MCs (A) and ClueGo based on KEGG source data for MC ben-2 (B) (further MCs following)
Supplementary Table 1

In preparation

Supplementary Tables 2

Multivariable analysis

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<tr>
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<td>1.04</td>
<td>[0.58, 1.87]</td>
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<td>1.31</td>
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<td>[0.63, 3.37]</td>
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<td>[0.67, 2.44]</td>
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<tr>
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<td>0.52</td>
<td>1.69</td>
<td>[0.73, 3.91]</td>
<td>0.22</td>
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<tr>
<td>other</td>
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<td>1.09</td>
<td>[0.23, 5.24]</td>
<td>0.91</td>
</tr>
<tr>
<td>posterior fossa</td>
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<td>0.96</td>
<td>[0.34, 2.69]</td>
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<td>ClinSubgroup intermediate</td>
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<td>6.29</td>
<td>[3.48, 11.39]</td>
<td>&lt; 0.0001</td>
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<tr>
<td>ClinSubgroup malignant</td>
<td>3.41</td>
<td>30.38</td>
<td>[13.49, 68.38]</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>center HD</td>
<td>-0.42</td>
<td>0.66</td>
<td>[0.34, 1.29]</td>
<td>0.22</td>
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<tr>
<td>center Zurich</td>
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<td>[0.46, 1.99]</td>
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### Supplementary Table 3

**Distribution of Mutations across MCs**

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<th>PIK3CA</th>
<th>AKT1</th>
<th>KLF4</th>
<th>TRAF7</th>
<th>NF2</th>
<th>SMO</th>
<th>SUFU</th>
<th>TERT</th>
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<td>Ben-2</td>
<td>1 1%</td>
<td>24 32%</td>
<td>11 15%</td>
<td>36 49%</td>
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<td>0 0%</td>
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<td>67</td>
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<tr>
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<td>5 11%</td>
<td>5 11%</td>
<td>15 32%</td>
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<tr>
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<td>0 0%</td>
<td>2 6%</td>
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References


