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ORIGINAL ARTICLE

# Loss of H3K27me3 in WHO grade 3 meningioma

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**Abstract**

Immunohistochemical quantification of H3K27me3 was reported to distinguish meningioma patients with an unfavorable prognosis but is not yet established as a prognostic biomarker within WHO grade 3 meningiomas. We studied H3K27me3 loss in a series of biopsies from primary and secondary malignant meningioma to validate its prognostic performance and describe if loss of H3K27me3 occurs during malignant transformation. Two observers quantified H3K27me3 status as "complete loss", < 50% and > 50% stained cells in 110 tumor samples from a population-based consecutive cohort of 40 WHO grade 3 meningioma patients. We found no difference in overall survival (OS) in patients with > 50% H3K27me3 retention compared to < 50% in the cohort of patients with WHO grade 3 meningioma (Wald test $p=0.5$). H3K27me3 staining showed heterogeneity in full section tumor slides while staining of the Barr body and peri-necrotic cells complicated quantification further. H3K27me3 expression differed without a discernible pattern between biopsies from repeated surgeries of meningioma recurrences. In conclusion, our results were not compatible with a systematic pattern of immunohistochemical H3K27me3 loss being associated with OS or malignant transformation of meningiomas and did not support H3K27me3 loss as a useful immunohistochemical biomarker within grade 3 meningiomas due to staining-specific challenges in quantification.

**Keywords** Meningioma · H3K27me3 · Heterogeneity

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## Introduction

Meningiomas account for approximately 37% of all primary intracranial tumors [1]. They are classified as WHO grade 1–3 tumors according to the 2016 and 2021 WHO grading systems based on histopathological and molecular features [2–4]. Most meningiomas are classified as slow-growing and indolent WHO grade 1 tumors [1], but a subgroup of benign meningiomas either undergo malignant transformation to WHO grade 3 meningioma (secondary malignant meningiomas) or are diagnosed as malignant meningiomas at first surgery (primary malignant meningiomas) and associated with a dismal prognosis [5]. The 2016 WHO grading system was based on histological criteria and allocated 1–3% of all meningiomas to grade 3, while the recent update also includes molecular criteria and probably increases the proportion of grade 3 meningiomas since genetic mutations that lead to a molecularly defined WHO grade 3 diagnosis are present in 5–15% of all meningiomas [6, 7]. Despite access to molecular prognostic markers, aggressive phenotypes cannot be reliably diagnosed [8].

Histone methylation is a key mechanism for regulation of gene-expression and instrumental for regulation of differentiation and growth control. Regulation of H3K27me3 (histone H3 lysine 27 trimethylation) is a critical regulatory mechanism for differentiation states of normal cells and aberrant methylation or demethylation can lead to malignancy [9–13]. In brain tumors, H3K27 mutation is oncogenic in pediatric gliomas and a hallmark for diffuse midline gliomas [14, 15]. Trimethylation at lysine 27 on histone H3 (H3K27me3) mediates gene silencing by the histone methyltransferase Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2), a putative oncogene [16]. Upregulation is associated with neoplasia [16, 17]. In CNS-related tumors, loss of H3K27me3 has also been suggested to be a diagnostic marker for malignant peripheral nerve cell tumors [11, 18] and pediatric ependymomas [19].

Subsequently, loss of H3K27me3 [9] was investigated to fulfil the need of additional biomarkers of an aggressive meningioma phenotype [20–22]. Initial studies suggested a role for H3K27me3 as a robust biomarker and also claimed refined individual prognostication among patients with WHO grade 3 meningiomas [23]. However, findings of H3K27me3 as a prognostic biomarker in meningiomas [21, 23, 24] were contradictory and traceability of data was limited; one study found prognostic value of semiquantitative assessment of H3K27me3 if divided into three categories of 0%, 0–50% and > 50% loss within WHO grade 3 [23] while others did not [21, 25, 26]. A recent report on a limited number of WHO grade 3 patients ($n=4$) suggested that loss occurred in association with tumor recurrence unless H3K27me3 was lost as a sign of an aggressive phenotype already in the primary meningioma [27]. A H3K27 mutation in midline gliomas suggests specificity and causality, while the inconsistent associations between phenotype and H3K27me3 loss in meningioma could be an epiphenomenon resulting from non-specific hypomethylation associated with neoplasia and thereby problematic as biomarkers or clues to pathogenesis.

There is a need to (1) analyze histological patterns of H3K27me3 immunohistochemistry, (2) validate previous findings of H3K27me3 loss on consecutive population-based meningioma tissue, and (3) to investigate how methylation status changes during malignant transformation from grade 1 and 2 to secondary grade 3 meningioma. We have gathered a unique material of 40 consecutive grade 3 patients to analyze whether: (1) H3K27me3 is readily quantifiable with immunohistochemistry, (2) the methylation status changes predictably during malignant transformation and recurrences (3) there is an association between overall survival (OS) and methylation status in a population-based cohort of grade 3 meningiomas. We also screened malignant meningiomas for the H3K27 mutation.

## Materials and methods

### Cohort and clinical parameters

We recently described the TERT promoter mutational status in the current population-based cohort of 20 primary and 20 secondary WHO grade 3 meningiomas defined per WHO 2016 criteria in patients who were operated at the Department of Neurosurgery at Rigshospitalet between 2000–2018 [28]. Clinical data included age, sex, performance status pre-meningioma diagnosis and extent of resection (Simpson grade). Linkage provided by the Danish social security number enabled us to localize all archived formalin-fixed paraffin embedded (FFPE) tumor specimens for each patient in this malignant cohort (MC, $n=40$ patients). Each of the forty patients underwent 1–10 operations for the original meningioma and recurrences at the of site of the original tumor, in total 119 operations. Full section tissue slides were available for analysis from 110 of 122 operations (90%). Of the 110 samples, 15 were from WHO grade 1, 15 grade 2 and 80 grade 3 meningiomas. Meningiomas histologically defined as grade 1 or 2 from a patient, that had a later recurrence classified as a WHO 2016 grade 3 ($n=20$ patients) were defined as "premalignant". Adequate premalignant tissue samples were only available from 17 of the 20 patients ($n=30$ meningiomas). All full section tissue slides (hematoxylin–eosin staining) were reviewed by a senior neuropathologist to fulfill the 2016 WHO classification, and none were reclassified compared to original diagnosis.

Controls were included to evaluate immunohistochemical staining in relation to tissue age and to compare benign meningiomas to the premalignant samples in our cohort of malignant meningiomas. The controls comprised new FFPE material from 33 patients with meningiomas diagnosed as WHO grade 1 ($n=21$) and WHO grade 2 ($n=12$) graded according to the WHO 2016 criteria and who underwent surgery at the Department of Neurosurgery in 2020 (Table 1).

### Immunohistochemistry

Immunohistochemistry was performed on 4-µm-thick formalin-fixed paraffin-embedded (FFPE) tumor sections. For H3K27me3 staining, the tumor sections were pre-treated

**Table 1** Overview of cohorts and samples

| Tumors and WHO grade | 1 | 2 | 3 | Total |
|---|---|---|---|---|
| Malignant cohort, MC (40 patients, gathered 2000–2020)[1] | 15 | 15 | 80 | 110 |
| Controls (33 patients, gathered in 2020) | 21 | 12 | 0 | 33 |

[1]Patients in the malignant cohort have 1–10 recurrent tumors

at 97 °C for 30 min and incubated with a wash buffer for 40 min (BenchMark ULTRA systems, Roche Diagnostics, Rotkreuz, Switzerland). We subsequently incubated the tumor sections with the primary antibody (TRIMh, Lys27 clone, CellSignaling, cat.no: 9733S, clone C36B11, Danvers, MA, USA) for 20 min at a 1:200 dilution on an automated Dako Omnis stainer (Agilent, Santa Clara, USA). Vessels were used as internal positive control and as external positive control we used human colon, tonsil, and liver tissue. For histone H3 mutation staining (H3K27 mutation), we incubated the tumor sections from the MC with the primary antibody (Histon3, Abcam, cat.no. ab190631, clone EPR18340, Cambridge, UK) for 32 min at a 1:1000 dilution on an automated stainer (Benchmark ULTRA). All tumor sections were counterstained with hematoxylin following standard protocol.

### Histological examination and quantification of H3K27me3 staining

The first WHO grade 3 diagnosis was based on frank anaplasia in 12/40 patients (30%), more than 20 mitotic figures per high power field in 20 patients (50%) and rhabdoid or papillary morphology in 8 patients (20%). Of the 8 patients with dominant rhabdoid or papillary components, 4 had concomitant anaplasia (2 rhabdoid and 2 papillary). Of these 4 patients without concomitant anaplasia, 2 later had grade 3 recurrences with anaplasia and elevated mitotic index. Of 80 WHO grade 3 tumors, 61 were anaplastic, 11 rhabdoid and 8 papillary. Two observers blinded to sample identity, previous diagnoses, and clinical information (ADM, PhD fellow, and CBB, specialist registrar in pathology) independently quantified H3K27me3 staining under light microscopy of each sample. Staining was quantified in three categories: 1: Completely negative, 2: < 50% stained cells, 3: > 50% stained cells. Each observer noted staining of vessels (yes/no), staining of perinecrotic cells (if present, yes/no) and intracellular staining of the inactivated X-chromosome (Barr body) (yes/no). Following quantification, discrepancies were reviewed by both observers and consensus was reached. Selected cases were scanned with the Hamamatsu NanoZoomer for documentation and viewed with the software NDP.viewer (Hamamatsu Photonics, Hamamatsu City, Japan).

### Statistical analysis

For assessment of inter-rater reliability of H3K27me3 quantification between the two observers, we used (1) the weighted Kappa for the three H3K27me3 staining categories and (2) Cohen's Kappa for complete loss (category 1 vs. 2 and 3).

Further analyses were applied to the consensus dataset. For investigation of differences in staining between old and new FFPE blocks, we compared H3K27me3 staining quantification in grade 1/2 tumors from the controls ($n = 33$ patients) versus the first premalignant grade 1/2 meningiomas from patients with secondary grade 3 meningiomas ($n = 17$ patients) using Fisher's exact test. For differences in anaplastic and non-anaplastic tumors' loss of H3K27me3 we used Fisher's exact test (8 non-anaplastic vs. 32 anaplastic). One WHO grade 3 meningioma per patient was investigated (the first in cases with consecutive recurrences).

For investigation of H3K27me3 status associated to overall survival (OS) within grade 3 patients, we defined OS as time from first WHO grade 3 surgery to death. We applied Cox proportional hazards regression when investigating the effect of > 50% H3K27me3 retention vs. < 50% retention or completely negative staining of the first WHO grade 3 meningioma on death. Age at diagnosis was included as a continuous covariate. We found valid assumptions for the Cox regression when testing proportionality using the Schoenfeld residuals. We depicted overall survival for the two groups using Kaplan–Meier curves and tested the difference with a log-rank test. Moreover, we applied a sensitivity analysis to evaluate the potential benefit of augmented multivariate regression analyses. Here, we assessed and compared four separate regression models and their ability to predict death after 3 years. In addition to H3K27me3, each of the four models comprised (1) age, (2) secondary/primary status, (3) Gross total resection (GTR, Simpson grade I–III) vs. subtotal resection (STR, Simpson grade IV–V), and (4) Performance status pre-meningioma diagnosis dichotomized as > 70 on the Karnofsky Performance Status Scale. We used a leave-one-out bootstrap based on 100 random subsamples each comprising the 40 patients randomly drawn with replacement. Prognostic performance of the individual models was averaged based on the 100 subsamples and assessed using the area under the receiver operating characteristics curve (AUC) and the Brier score. The model performing best, was included.

We considered $P < 0.05$ as significant. For computing, we used R version 4.0.2 [29].

## Results

### Moderate inter-observer reliability and pitfalls in staining quantification

For H3K27me3 quantification, weighted Kappa for the two observers was 0.58 (95% CI 0.48–0.68) and showed moderate inter-observer reliability in placing samples in the three H3K27me3 categories (completely negative, < 50% loss, > 50% loss). Cohen's Kappa for classifying complete

loss between the two observers was 0.65 (95% 0.46–0.84). Figure 1 shows examples of staining quantification. Discrepancy amongst the two observers was found in (1) large full section tumor slides (2) samples with intratumoral heterogeneity (3) perinecrotic staining and (4) samples with staining of inactivated X chromosome in female patients (Fig. 2). External positive controls (colon, tonsil, and liver) showed homogenous staining and retention of H3K27me3 in all cases.

## Artefacts, diagnostically irrelevant staining, and impact of tissue age

Artefacts were limited to occasional air bubbles which were easily identified. Specific H3K27me3 staining of intratumoral vessels was detected in 135/143 (95%) of samples (Table 2). In nine of the cases with no staining of vessels, four cases had faint staining of tumor cells. 70 of the tumor samples came from male patients and staining of the inactivated X chromosome (staining of the Barr body) was observed in 0% of these. In samples from female patients ($n=73$), dotting of Barr bodies was observed in 44 samples (60%). We compared loss of H3K27me3 in premalignant samples (first MC grade 1–2, $n=17$) to cases from controls (grade 1–2, $n=33$) and found no difference between the cohorts' distribution of staining quantification (Fisher's exact test, $p=1$).

## H3K27me3 status and association to progression free survival within WHO grade 3

Cox proportional hazards regression analysis did not show a significant effect of H3K27me3 loss/retention status on overall survival from the first WHO grade 3 meningioma (Wald test $p=0.3$). The 30 patients with loss of H3K27me3 (completely negative = 5 and < 50% H3K27me3 positive cells = 25) had a hazard ratio of 0.64 (CI 95% 0.26–1.54) compared to the 10 patients with retention (> 50% H3K27me3 positive cells) when adjusting for age. Figure 3 shows the Kaplan Meier curves for the patients with loss and retention of H3K27me3.

## Sensitivity analysis

Inclusion of secondary/primary status, extent of resection, and performance status apparently impaired the regression model (Fig. 4). The plots, AUC and Brier scores strongly indicated that the model with the best prognostic performance was the model which included only H3K27me3 status and age as a covariate.

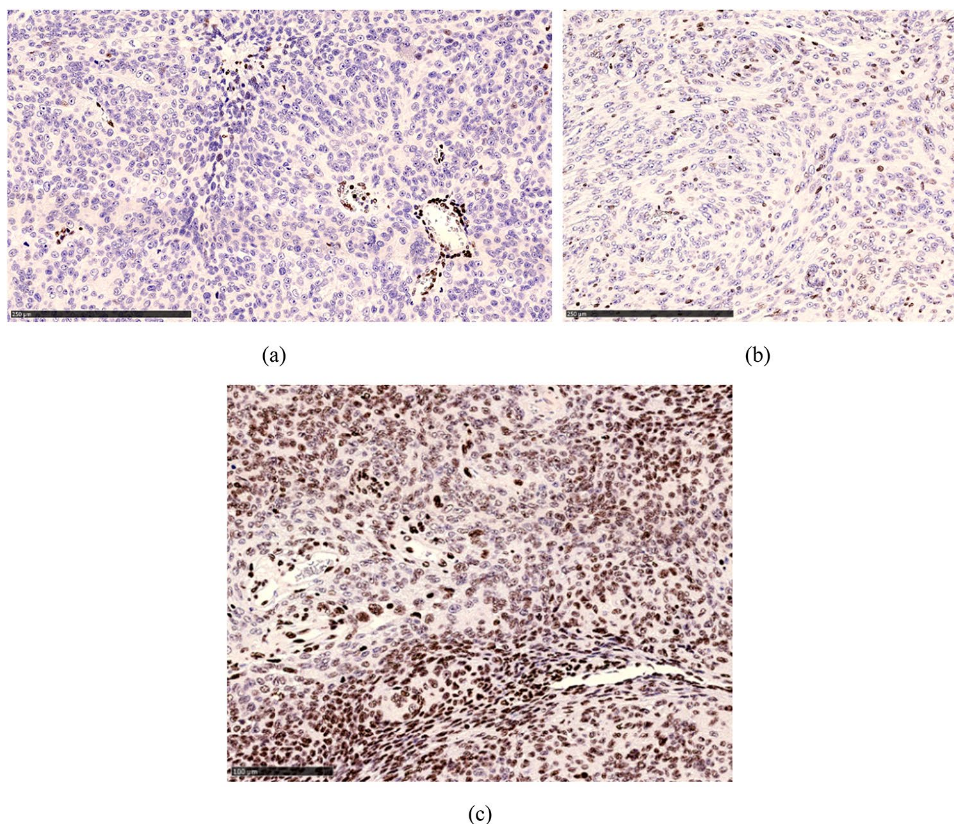


**Fig. 1** Examples of staining quantification of H3K27me3 in meningiomas **a** Completely negative tumor sample with retention of H3K27me3 in intratumoral vessels. Scale bar 250 µm, × 200 magnification. **b** Tumor sample placed in the < 50% H3K27me3 positive cells category. Scale bar 250 µm, × 200 magnification. **c** Sample with > 50% retention of H3K27me3 in tumoral cells category. Scale bar 100 µm, × 250 magnification

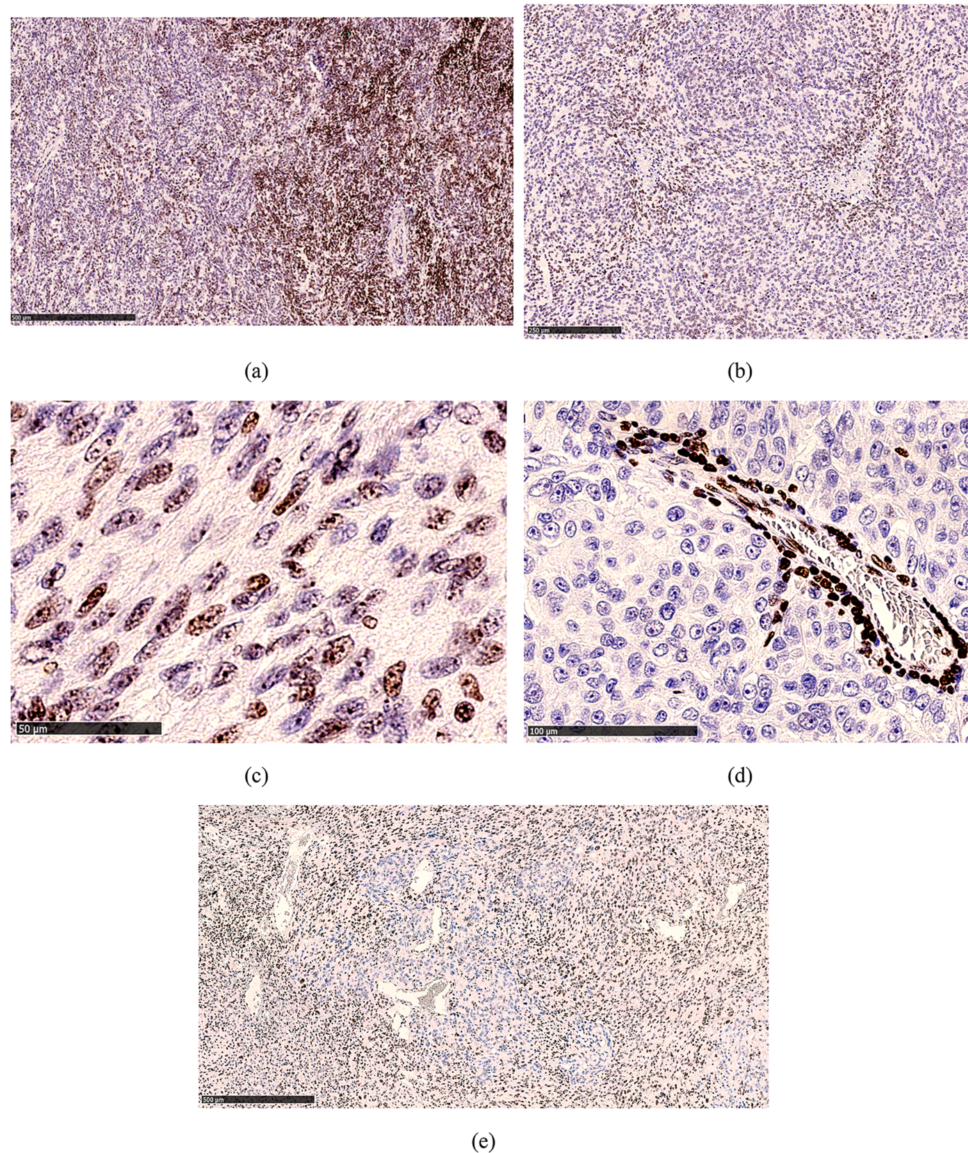

**Fig. 2** Features and pitfalls in staining quantification of H3K27me3 in meningiomas. **a** Large, heterogeneous tumor slides as this make placement in the > / < 50% H3K27me3 positive cells staining category difficult. Scale bar 500 µm, × 80 magnification. **b** Staining of perinecrotic cells in a WHO grade 3 sample. Staining of perinecrotic cells vary in intensity. In borderline cases multiple necrotic areas can make quantification difficult. Scale bar 250 µm, × 100 magnification. **c** Detail of inactivated X-chromosome staining in a female patient (Barr body staining); this feature can make staining quantification difficult in borderline cases. Scale bar 50 µm, × 500 magnification. **d** Detail of strong staining of intratumoral vessels in an otherwise completely negative case. Vessels too exhibit varying intensity of H3K27me3 staining. Scale bar 100 µm, × 400 magnification. **e** Example of case with loss of H3K27me3 in an area of otherwise > 50% H3K27me3 retention. Scale bar 500 µm, × 70 magnification

**Table 2** Overview of staining quantification divided on malignant cohort (MC) and new cohort (NC) and WHO grades

| | | NC 1/2 $N=33$ | MC 1/2 $N=30$ | MC 3 $N=80$ | MC Anaplastic $N=61$ | MC Rhabdoid and papillary $N=19$ | All $N=143$ |
|---|---|---|---|---|---|---|---|
| H3K27me3 staining quantification (%) | Complete loss | 2 (6) | 2 (6.7) | 15 (18.8) | 9 (14.7) | 6 (31.6) | 19 (13.3) |
| | < 50% | 14 (42) | 11 (26.7) | 45 (56.2) | 34 (55.7) | 11 (57.9) | 70 (49) |
| | > 50% | 17 (52) | 17 (56.7) | 20 (25) | 18 (29.5) | 2 (10.5) | 54 (37.7) |
| Vessels stained (%) | Yes | 32 (97) | 28 (93) | 74 (92.5) | 57 (93.4) | 17 (89.5) | 134 (94) |
| | No | 1 (3) | 2 (7) | 6 (7.5) | 4 (6.6) | 2 (10.5) | 9 (6) |
| Staining of Barr body ("dotting") (%) | Yes | 15 (45) | 4 (13.3) | 25 (31.3) | 20 (32.8) | 5 (26.3) | 44 (30.7) |
| | No | 18 (55) | 26 (86.7) | 55 (68.7) | 41 (67.2) | 14 (73.7) | 99 (69.2) |
| Perinecrotic cells stained (%)[1] | Yes | 3 (9) | 3 (10) | 19 (23.8) | 15 (24.6) | 4 (21.1) | 25 (17.5) |
| | No | 30 (91) | 27 (90) | 61 (76.2) | 46 (75.4) | 15 (78.9) | 118 (82.5) |

[1] All perinecrotic cells were stained, with varying intensity

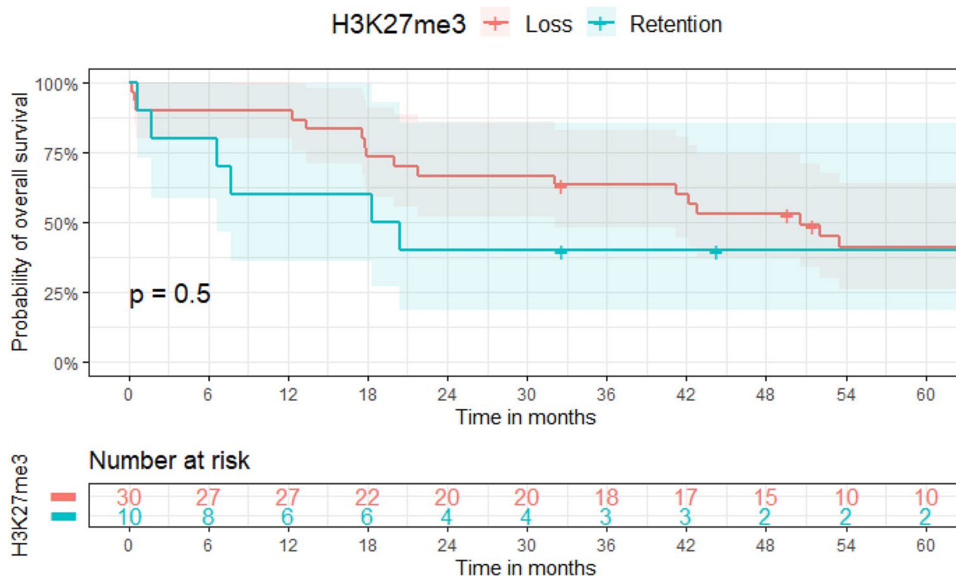


**Fig. 3** Kaplan Meier curves for the H3K27me3 status in the malignant cohort. Retention denotes the 10 patients with > 50% H3K27me3 positive cells in their first WHO grade 3 tumor. Log rank test *p* value = 0.5

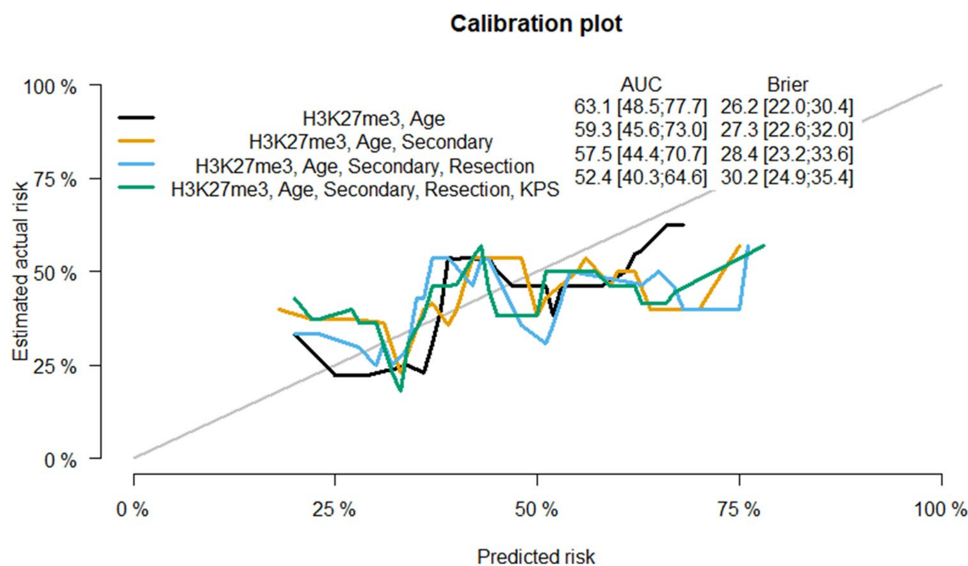


**Fig. 4** Calibration plot which describes the agreement between observed frequency of death and predicted risk of death at 3 years for four different multivariate models. The diagonal line indicates perfect calibration. A higher AUC score and a lower Brier score indicates a better model. *Secondary* denotes secondary or primary malignant meningioma. *Resection* denotes gross total resection (Simpson grade I–III) and subtotal resection (Simpson grade IV–V). *KPS* denotes pre-meningioma diagnosis performance status quantified on the Karnofsky Performance Scale and dichotomized to > 70. The AUC (area under the receiver operating characteristics curve) and Brier Score indicate that the best model for predicting death at 3 years is the one which includes only H3K27me3 status and age as a covariate

## Loss of H3K27me3 in WHO grade 3 meningioma

Figure 5 shows patients with multiple meningiomas in the MC ($n = 25$), the order of the tumor recurrences and the H3K27me3 staining category. Of the 25 patients, 9 had a constant H3K27me3 status across recurrences (7 of these in the < 50% category). In 11 patients, the last WHO grade 3 tumor had loss of H3K27me3 compared to previous tumors and in 5 patients the last WHO grade 3 tumor had gain of H3K27me3 expression compared to previous tumor(s). Comparison between anaplastic and non-anaplastic meningiomas did not imply a difference between staining quantification in these two groups (Fisher's exact $p = 0.44$). No tumor samples among primary and secondary malignant or premalignant samples stained positive for the H3K27 mutation.

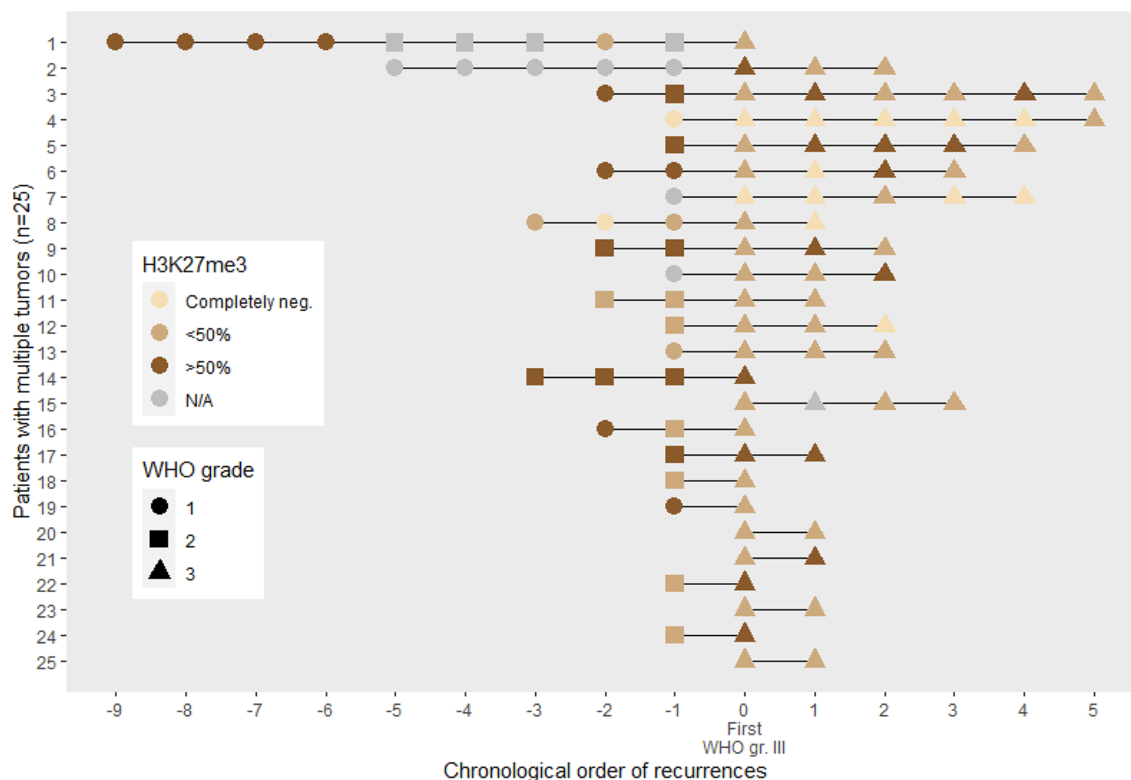


**Fig. 5** Temporal development in H3K27me3 status within patients with multiple tumors in the malignant cohort. Each line represents a patient, and colored shapes are tumors with grade (shape) and H3K27me3 status (color). Tumors not available for analysis (92% were available in the entire material) are also denoted for each patient with gray. The *y* axis lists patients with multiple meningiomas ordered by number of meningiomas. The *x* axis denotes the chronological order of recurrences. We see loss of H3K27me3 in 11/25 patients, gain in 5/25 and unchanged status in 9/25. One patient showed all staining categories across tumor recurrences (6th from the top)

## Discussion

We analyzed loss of H3K27me3 as a potential biomarker for aggressive phenotypes and could neither confirm the immunohistochemical marker as a robust, readily quantifiable marker during malignant transformation, nor find an association between methylation status and overall survival. Moreover, the H3K27 mutation was not detectable in our cohort of grade 3 meningiomas.

### Overall survival

We did not find an association between H3K27me3 status and overall survival in our cohort. We did not analyze loss of H3K27me3 as a prognostic marker across all meningioma grades to confirm previous reports [22, 24] and only analyzed loss of H3K27me3 as a marker for phenotypes among grade 3 meningiomas. We could not corroborate prognostic impact of loss of > 50% of H3K27me3 in our WHO grade 3 cohort as described by Gauchotte et al. [26]. We applied the same dichotomization between > 50% vs. < 50% stained cells and found no statistically significant difference between the two groups. Moreover, our sensitivity analysis did not indicate an influence of performance status, extent of resection or differentiation between primary and secondary tumors on our conclusion. Unexpectedly, the hazard ratio for OS favored loss of methylation (0.64, CI 95% 0.26–1.54) in the group of 30 patients with loss (< 50% stained cells) compared to 10 patients in the with retention (> 50% stained cells). Several factors can explain differences between our findings and previous literature. Our findings might be due to low statistical power and methodological differences which include the definition of ‘loss of H3K27me3’ that could compromise conclusions. Our cohort included rhabdoid and papillary subtypes and thereby differed from the selected population of anaplastic tumors studied by Gauchotte et al. This difference of histological subtypes is probably not important since our material contained only four purely rhabdoid/papillary meningiomas and four with concomitant anaplasia. Moreover, we found no difference in the distribution of staining categories between anaplastic and non-anaplastic tumors.

## Reproducibility

There were issues that could fundamentally affect reproducibility. We found extensive heterogeneity of H3K27me3 within samples, across serial samples and cellular/subcellular localization of H3K27me3 staining. Technical reasons for heterogeneity include definition, quantification, and reproducibility of immunohistochemically defined loss of methylation, which can comprise fundamental sources of error. We evaluated a control cohort and found that tissue age did not seem to affect staining patterns, while other factors could severely affect reproducibility. Previous studies employ semi-quantitative methodologies but differ in the definition of 'loss' of H3K27me3. Our study and a previous study investigating WHO grade 3 meningioma [23] classified 'loss' as < 50% cells stained positive for H3K27me3. However, other studies classified loss as a tumor completely negative for H3K27me3 positive cells [21, 22]. Meningiomas with < 50% would be designated as 'retained' or with ambiguous staining pattern as described by Katz et al. [22] (13 cases with ambiguous patterns out of 232 assessed cases). In our material, a large proportion of meningiomas showed < 50% stained cells without being completely negative, and these were grouped with complete loss in the survival analyses. Table 3 shows an overview of previous studies investigating H3K27me3 loss in meningioma, and interobserver agreement was consistently low across studies. We also found insufficient inter-observer reliability between our two observers (weighted Kappa = 0.58) when classifying cases as "complete loss", < 50% retention and > 50% retention of H3K27me3.

Quantification was also complicated by H3K27me3 antibody specificity, as we observed staining of inactive X chromosome in female cases [30] and staining of perinecrotic cells in cases with necrotic areas [31]. Quantification is particularly problematic around 50% positive cells, where small differences determine the category. Interobserver agreement and reproducibility require homogenous staining patterns, which were rare. A reliable method for reliable and quantitative measurements of immunohistochemical H3K27me3 expression is needed. Only complete loss of trimethylation and completely retained trimethylation could be unambiguously described, while most samples showed variable patterns of loss of trimethylation. External positive controls showed technical success of the staining in all cases. In contrast, internal controls should be questioned. Intra-tumoral vessels were used as internal positive controls in previous studies [23, 26]. In four cases with positive external controls, we found faint but true staining of the tumor cells but no staining in endothelial cells in intra-tumoral vessels. Taken together, reproducibility is probably too low for prognostic applications within WHO grade 3 meningioma based on our material. Factors such as staining of Barr bodies, staining of intra-tumoral vessels and necroses comprised potential sources of error that can be considered by a human observer, but largely preclude automatic quantification.

In addition to methodological challenges concerning reproducibility, spatial variation within samples (Fig. 2a,

**Table 3** Overview of previous meningioma cohorts investigating H3K27me3 staining

| | | Katz et al. [22] | Gauchotte et al. [23] | Behling et al. [24] | Nassiri et al. [21] | Jung et al. [25] | Maier et al. |
|---|---|---|---|---|---|---|---|
| Meningiomas, $n$ | | 232 | 47 | 1268 | 151 | 141 | 143 |
| Inter-observer reliability | | NA | $K = 0.64$ | $K = 0.73$ | $K = 0.88$ | NA | $K = 0.65$ |
| 2016 WHO grade of tumours | 1 | 49 | - | 1001 | 48 | 0 | 36 |
| | 2 | 155 | - | 250 | 74 | 115 | 27 |
| | 3 | 28 | 47 | 17 | 29 | 26 | 80 |
| Loss of H3K27me3 (%) | 1 | 2.0 | - | 3.1 | 4.2 | - | 11 |
| | 2 | 11.6 | - | 10.4 | 12.2 | 29.6 | 0 |
| | 3 | 21.4 | 21.3[1] | 17.7 | 27.6 | 53.8 | 18.8[1] |
| Ambiguous cases (< 50% positive cells) | | 13 | 10 | NA | 11 | 48 | 70 |
| Dichotomization of ambiguous cases | | Classified as 'retained' | Classified as loss | NA | Classified as 'retained' | Classified as loss (< 45%) | Classified as loss |
| Prognostic impact | | Grade 1/2 | Within grade 3 | Grade 1/2/3 | Grade 1/2 | Grade 2 | No |

[1]Loss was here classified as < 50% H3K27me3 positive cells in contrast to 'complete loss' with no positive cells

[2]Loss was here classified as < 45% H3K27me3 positive cells

All studies used the C36B11 antibody clone

Fig. 2e) and temporal variation between serial samples, was detected (Fig. 5). Spatial heterogeneity provides for variation of results depending on where biopsies and slides were obtained, and which part of a tumor was evaluated.

Finally, our investigation of H3K27me3 loss across serial recurrences (Fig. 5) showed temporal variability with frequent status changes across recurrences. Most patients ($n = 11$, 44%) had an end-point tumor with relative loss of H3K27me3, nine (36%) patients retained a stable status through recurrences while five (20%) patients had a relative gain of H3K27me3 methylation. We did thus not detect a consistent pattern of loss, as recently claimed in a report of four patients [31], or gain of methylation during malignant transformation. Methylation status was variable and unpredictable and could be an epiphenomenon associated with a general tendency to aberrant methylation in more aggressive tumors [13] but with limited robustness as a biomarker. Another biological explanation could be that subclones with or without H3K27me3 loss co-exist and that different subclones dominated in different recurrent tumors. Taken together, the lack of a detectable statistical association to overall survival, invalidates immunohistochemical loss of H3K27me3 as a prognostic biomarker for grade 3 meningiomas. Our findings agree with Jung et al. [19] and extend their analyses by showing that the marker varies inconsistently across recurrences.

### H3K27 mutation

As none of the investigated meningiomas had the H3K27 mutation in the immunohistochemical analysis, it is highly unlikely that the mutational status impacts trimethylation of H3K27 in meningiomas, as it does in pediatric gliomas [32], but sequencing analyses must be done to confirm this in meningioma.

## Conclusions

We investigated the immunohistochemical expression of (loss of) H3K27me3 in a population-based cohort of 40 malignant meningioma patients. We did not address the population of *all* meningiomas, just grade 3 meningiomas. We could not corroborate previous claims of H3K27me3 as a robust, reproducible immunohistochemical prognostic marker with an association to overall survival among WHO grade 3 meningiomas. Our study highlights pitfalls in H3K27me3 staining and temporal heterogeneity across serial recurrences. The findings may not be surprising, but it is necessary to make empirical data available in published literature.

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**Data availability statement** The data presented in this study are available on request from the corresponding author.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Institutional review board statement** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Danish Ethics Committee (number H-6–2014-010).

**Informed consent statement** Patient consent was waived since the study does not involve any further health risks or cause the patients any further harm and obtaining informed consent was estimated as disproportionately difficult.

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