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Letter to the Editor

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Adequate capture of spatial heterogeneity of Ki-67 proliferative index in meningiomas requires multiple tissue sections

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To the Editor:

While not a strict criterion for the grading of meningiomas according to the 2021 WHO Classification of Tumors of the Central Nervous System, the Ki-67 proliferative index provides insight into the proliferative capacity of meningiomas, has prognostic significance and is frequently requested by clinicians and reported by pathologists.[1] In meningioma reporting, Ki-67 proliferative index is typically provided as a single summary value or range (as in "2-3%" or "focally up to 10%"), but this is not standardized. Meningiomas, like other tumors, show substantial heterogeneity in their histologic and molecular features,[2] and regional variation in Ki-67 is frequently observed. The significance of this variation is not known, and typical reports do not capture it with sufficient precision to allow for detailed analysis of this phenomenon across a large number of cases. It is therefore desirable to know the degree of variation in Ki-67 proliferation fractions exhibited across meningiomas.

One hundred thirty-six sequential cases of meningioma resection for which Ki-67 immunohistochemistry had been performed on one or more blocks were retrieved from the Yale Pathology Department archives. Cases included CNS WHO grade 1 and grade 2 meningiomas; all were of meningothelial, fibrous, or transitional subtypes. For all cases, Ki-67 staining had been performed in a CLIA-certified clinical lab with standard clinical protocols using Ki67 antibody (CRM325 Biocare Medical) at a dilution ratio of 1:75. After de-identification, all slides were scanned on a Motic EasyScan Infinity 60 imaging system (Motic, Inc., Kowloon, Hong Kong) at 40× magnification, covering the whole profile of tissue on the slide at 0.26-μm/pixel resolution (Figure 1A). Whole slide images were analyzed using the QuPath[3] to perform the following operations for each image: estimate hematoxylin and DAB stain RGB vectors, detect tissue profile using threshold pixel classification, and positive cell detection (based on watershed algorithm applied to optical density in the hematoxylin and DAB channels). Images were then divided into 1 × 1 mm square tile regions and counts of positive and negative nuclei in each tile were exported for statistical analysis.

For every image, four 1 × 1 mm tiles were chosen for manual validation (Figure 1B). The tile in the image that was found to contain the highest number of positive nuclei by the automated detection algorithm and 3 more tiles selected at random. For each of these 4 tiles, the absolute number of positively staining nuclei was counted and an estimate of the number of negative cells was made (as a percentage relative to the automated count) by pathology trainees. Validation of the manual counts and estimations was made for 20% of the tiles by a neuropathologist. For each slide to be included in downstream analysis, the difference between automated and manual counts of positive nuclei was required to be <1% and the human assessment of error in automated count of negative nuclei was required to be <10% in all manually reviewed tiles. Among the 136 slides, 78 samples (57%) passed manual quality control criteria (Figure 1C). There was no association between QC failure and positive staining fraction. Among 58 slides that did not pass, the most frequent cause (35 slides) was artifactual staining (eg, edge artifact, high background, folding/crush artifact) that the digital segmentation interpreted as nuclear staining.

Among slides that passed quality control checks, correlation between maximum positive percentage identified in each image by computer detection and maximum value reported by a pathologist was $\rho = 0.4963049$ (Spearman' rank-order correlation). While a trend was observed that cases with higher reported values were found to have higher positive fractions

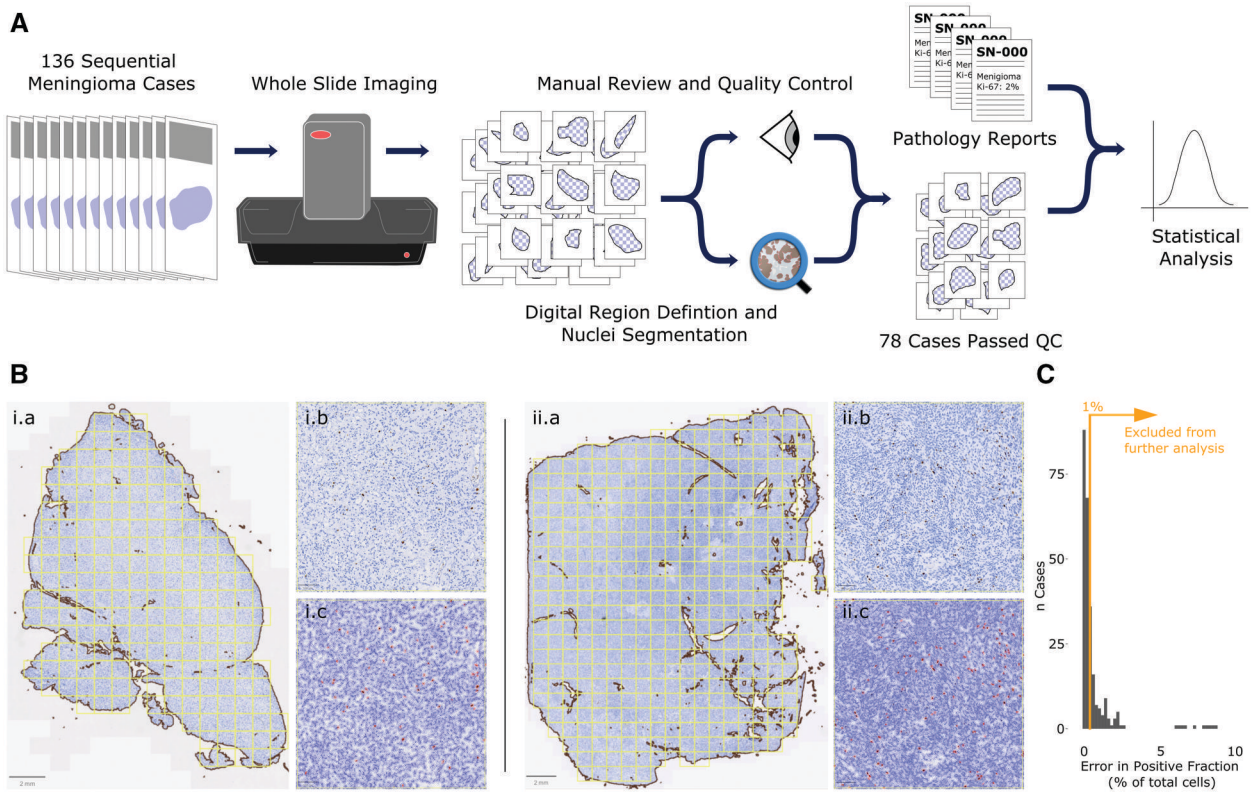

Figure 1. Diagram of process of slide selection, acquisition, and analysis. (A) One hundred thirty-six sequential cases were scanned for whole slide imaging and uploaded into QuPath for digital analysis. Quality control and manual counts were then performed. Seventy-eight cases passed quality control, which were then used for statistical analysis. (B) Example of 2 cases uploaded to QuPath with 1 mm^2 grid system (i.a and ii.a) with high power of a single grid (i.b and ii.b) and auto-detection of Ki-67-positive cells (i.c and ii.c). (C) Distribution of errors in digital estimate of positive staining fraction (versus human quantification ground truth) for all 136 cases.

by digital analysis, the digital quantification yielded lower maximum positive percentages on average (Figure 2A and B). Analysis of the spatial distribution of Ki-67-positive cells revealed substantial heterogeneity across meningioma samples. The excess kurtosis of the positive percentages in 1 × 1 mm tiles for each image was calculated to quantify the "tailedness" of the distribution relative to a normal distribution. The relationship between excess kurtosis and the mean positive percent of each case is illustrated in Figure 2C. This analysis revealed 2 distinct patterns: (1) A subset of cases, primarily lower-grade meningiomas, were characterized by rare "hotspots" of proliferation, as indicated by high excess kurtosis values. (2) Another subset, mostly higher-grade meningiomas, showed a broader elevation of proliferation throughout the tissue, as indicated by lower excess kurtosis values.

To assess the sampling requirements for capturing the full heterogeneity of Ki-67 staining, we fitted a generalized extreme value distribution to the positive percentage in every 1 × 1 mm tile for each image. The proportion of tissue needed to locate the 95th percentile proliferation index of each tumor is shown in Figure 2D. The majority of cases required a relatively low fraction of tissue to be sampled to reach this confidence level, suggesting that much of the tumor is similarly representative. However, some cases contained rare hotspots, indicating that high confidence in finding the highest region of proliferation would require more tissue than is typically present on a single slide. In fact, for 15 of 78 (19%) of cases, achieving 99% confidence that the highest proliferation density was captured would require review of 2 or more Ki-67-stained slides.

Although digital analysis of histology images has some limitations, such as its reliance on the accuracy and reliability of the algorithm used, it has been demonstrated to provide highly accurate and reproducible results.[4] Significantly, our findings demonstrate the wide variability in heterogeneity of proliferation fraction across tumors, of both low and intermediate grade, a feature that would be infeasible to capture through manual assessment. This heterogeneity compels caution in interpreting limited samples.

The high agreement between digital analysis and manual counts in quality-controlled slides aligns with previous studies showing good concordance between digital and manual Ki-67 quantification.[5,6] The largest source of error in our study was due to slide preparation and scanning artifacts, reflected in the substantial proportion of slides that failed quality control (43%). These preanalytical sources of error, while common to all digital pathology workflows,[7,8] are not a barrier to implementation in routine sign-out as long as strict quality control precedes image analysis, allowing for selection of appropriate slides for quantification. Detection algorithms may also suffer

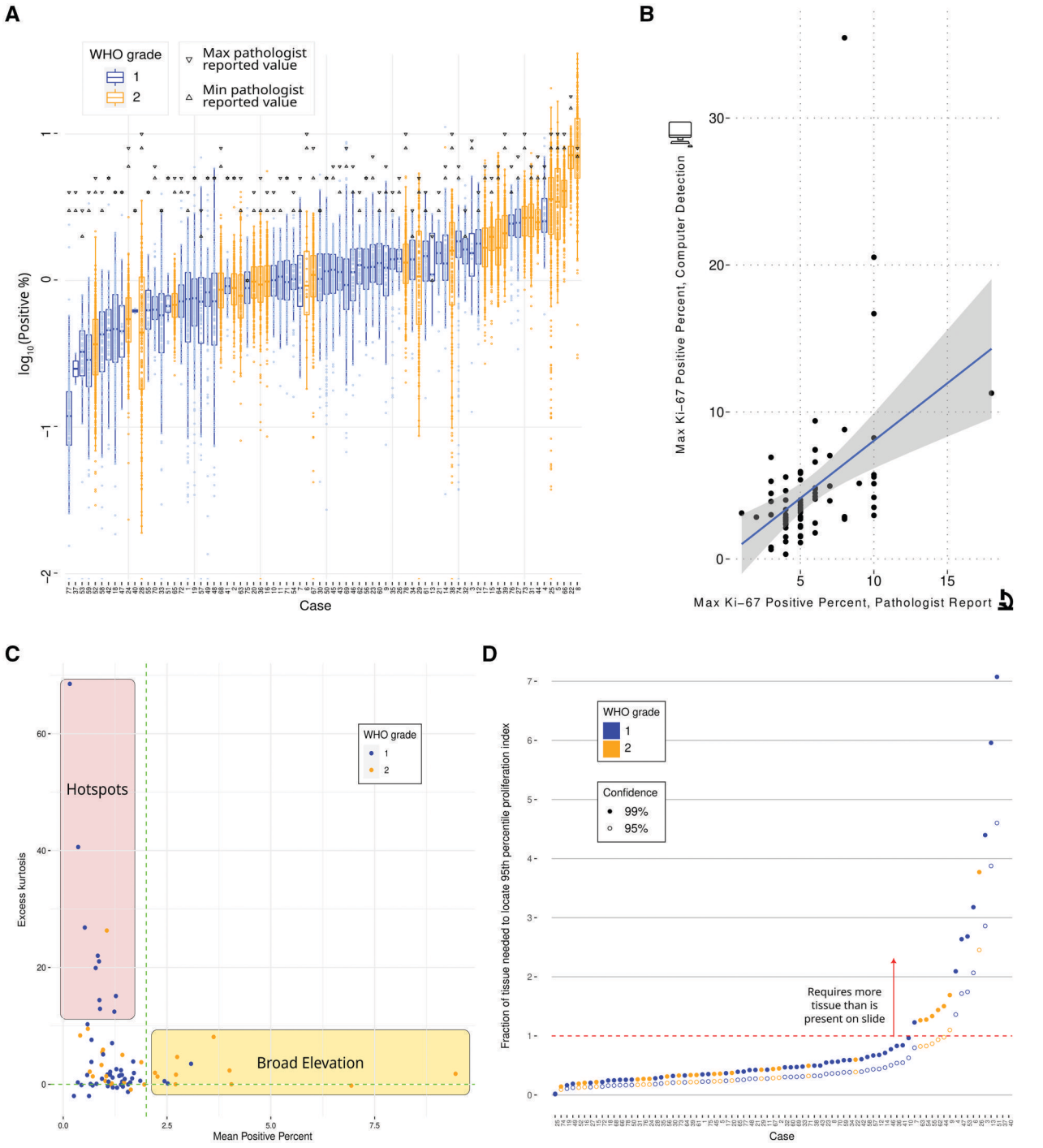

Figure 2. Statistical analysis of digital quantification and spatial heterogeneity of Ki-67 proliferation index. (A) Each dot represents $\log_{10}$ (positive fraction) for a single 1 mm^2 patch in the mage. Box-and-whisker plots represent the medians, upper and lower quartiles, and upper/lower quartiles plus/minus 1.5 × (interquartile range). (B) Correlation between maximum positive percentage identified in each image by computer detection and maximum value reported by a pathologist (Adjusted R-squared: 0.172. $F = 17$ on (1,76) DF; P value: 9.34e−05). (C) Excess kurtosis (the "tailedness" of a distribution relative to a normal distribution) versus mean positive percent of each case shows that a subset of cases (mostly lower grade) are characterized by rare "hotspots" of proliferation, while a different subset (mostly higher grade) shows a broader elevation of proliferation throughout the tissue. (D) Generalized extreme value (GEV) model fits show that the majority of cases have a low fraction of tissue needed to locate the 95th percentile proliferation index of the tumor (ie, much of tumor is similarly representative). Cases with values above 1 are more heterogeneous and obtaining high confidence that the highest region has been found requires more tissue than is present on a single slide.

from sources of systematic error, including the inability of algorithm to distinguish negative tumor nuclei from negative non-tumor nuclei (eg, blood vessels). This leads to overestimation of denominator (ie, underestimate of Ki-67 index), which may account for a component of the overall trend toward lower maximum values detected by image analysis compared with pathologist reported values (Figure 2B).

The moderate correlation ($\rho = 0.4963049$) between digitally quantified and pathologist-reported Ki-67 indices, along with the tendency for digital analysis to yield lower maximum values, warrants further investigation. This discrepancy could be due to several factors: (1) Systematic underestimation by digital analysis due to the inclusion of non-tumor nuclei in the denominator; (2) Differences in the area assessed by pathologists versus whole-slide digital analysis; and (3) Pathologist regression toward typically reported values. These findings underscore the need for standardized reporting practices and consideration of how digital tools might be integrated into clinical workflows.

Our analysis revealed significant heterogeneity in Ki-67 staining across meningioma samples, with distinct patterns observed in lower-grade versus higher-grade tumors. The presence of rare proliferative hotspots in some tumors, particularly lower-grade meningiomas, raises questions about the prognostic significance of these areas and the adequacy of current sampling practices. The finding that most cases require sampling of only a small fraction of tissue to capture the 95th percentile of proliferation is reassuring for current practices. However, the existence of cases requiring extensive sampling to confidently identify the highest proliferative regions suggests that some clinically significant information may be missed in routine assessments.

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CONFLICTS OF INTEREST

None declared.

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