Presence of unsampled Gleason pattern 4 can be predicted from characteristics of adjacent Gleason pattern 3

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Introduction

Prostate biopsies undergrade 25% to 50% of prostate cancers, delaying definitive treatment by up to 3 years.[1]
A cause of undergrading is the partial sampling inherent in biopsies: if a biopsy samples a low-grade (Gleason pattern 3) area but misses an adjacent high-grade (Gleason pattern 4 or 5) focus, the overall grade of the tumor will be underestimated. In other words, when faced with a prostate biopsy that appears to be Gleason 3+3=6, the pathologist may unknowingly be looking at a part of a higher-grade tumor that has been sampled only partially.
However, not all Gleason pattern 3 prostate cancers are equal: mounting evidence hints at the possibility that pure Gleason pattern 3 (pG3) is biologically distinct from Gleason pattern 3 associated with higher-grade prostate cancer (aG3).

In this study, we used immunohistochemistry and computer-aided image analysis to compare the expression of Ki67, cyclin D1, MYC, and p53 between foci of aG3 and pG3, in search of a reliable and cost-effective marker to distinguish them.
When a biopsy only shows Gleason pattern 3 prostate cancer (3+3=6), having such a marker would allow us to distinguish the true 3+3=6 from the false ones that only *appear* to be 3+3=6 because of a sampling error. This would dramatically improve the diagnostic performance of prostate biopsies and the management of early prostate cancer.

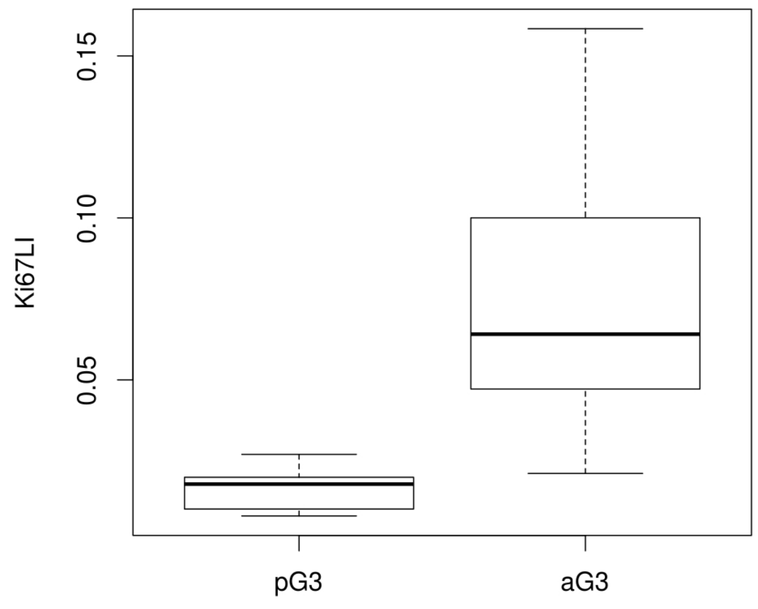
Results

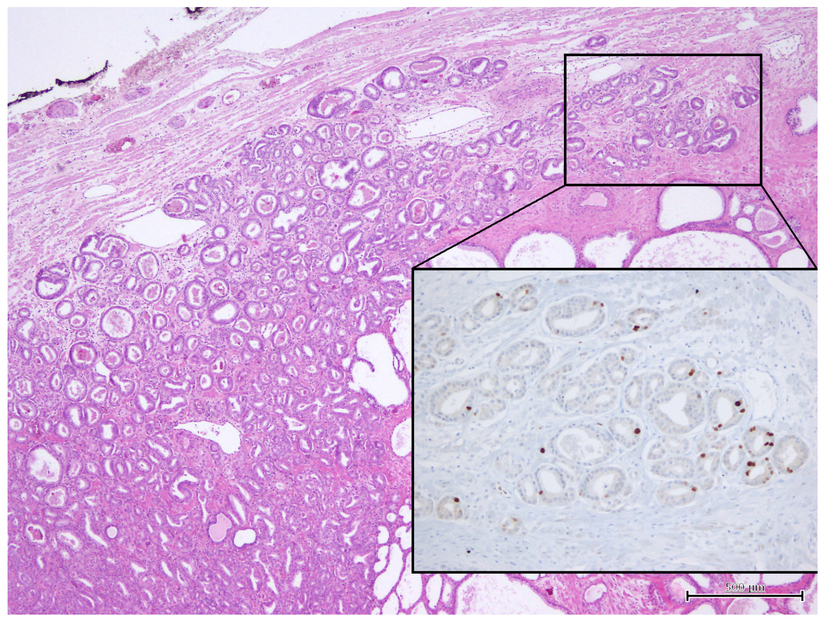
A total of 8 pG3 and 22 aG3 foci were included in the present study.

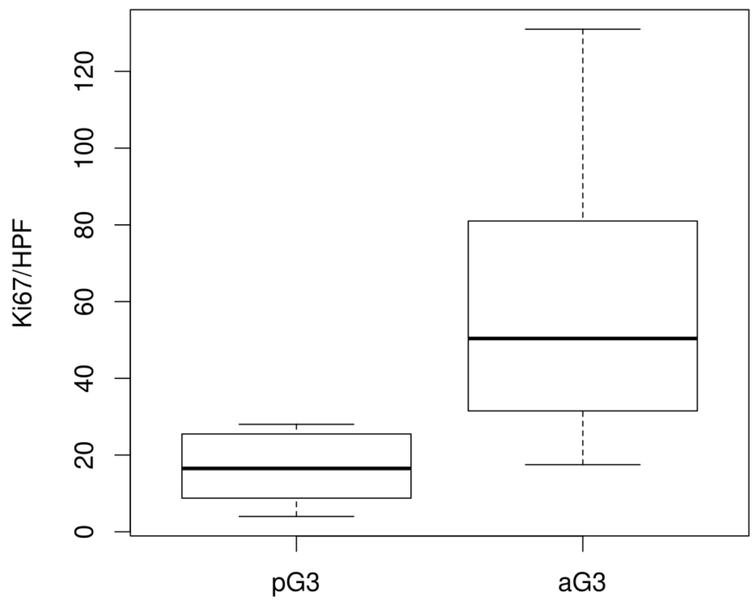
The average Ki67LI was 6.03% ± 4.20% (pG3: 1.63% ± 0.65%; aG3: 7.62% ± 3.77%). Ki67LI is significantly different between pG3 and aG3 ($p < 0.000001$).

The average Ki67/HPF was 48.6 ± 34.8 (pG3: 16.7 ± 9.61; aG3: 60.2 ± 33.4). The difference between pG3 and aG3 is statistically significant ($p < 0.00001$).

The expression of p53, Cyclin D1 and MYC did not significantly differ between pG3 and aG3.
Cyclin D1 positivity appeared to be significantly associated with higher Ki67/HPF ($p = 0.01$). An association with higher Ki67LI was also observed, but it did not reach the threshold for statistical significance ($p = 0.077$).







Materials and Methods

Our pathology database was searched for cases of prostatectomy or cystoprostatectomy with a diagnosis of prostatic adenocarcinoma.
The corresponding slides were retrieved from our archive and reviewed to search for:
- foci of pure Gleason 3 prostate cancer (pG3), consisting exclusively of Gleason pattern 3, without any adjacent pattern 4 or 5;
- foci of Gleason 3 adjacent to higher grade prostate cancer (aG3), where pattern 3 glands were spatially adjacent to Gleason 4 or 5 glands.

For each focus, the slide with the most Gleason pattern 3 was chosen and its relative formalin-fixed paraffin-embedded tissue block was retrieved to cut additional 3µm-thick sections for immunohistochemical analysis.

Immunohistochemistry

We used four antibodies: Ki67, Cyclin D1, p53, and MYC.
All immunohistochemical scoring was done for the Gleason 3 component of the focus: only cells belonging to a Gleason pattern 3 gland were considered. Stromal cells, benign glands, PIN and Gleason patterns 4 and 5 were ignored.

For each focus, Ki67 was evaluated using two indices: the percent of positive cells (labelling index, Ki67LI) and the number of positive cells per high-power field (Ki67/HPF, with 1 HPF = 0.36mm^2).

For each focus the regions with the highest density of positive cells were identified and captured in digital images (mean: 3.43 images per focus; each image represented 1 HPF).
Any nuclear DAB staining was considered positive.

The images were analyzed using QuPath (a free and open-source computer program for digital pathology). For each image, "Positive cell detection" was used to recognize each nucleus, and then a classifier was trained to distinguish positive, negative and stromal cells. The number of positive cells was also counted manually, on the same images, using the Multi-point tool in ImageJ. Each positive cell was clicked on in turn, while the software kept the tally and placed a marker on each counted cell to avoid double-counting.

The agreement between manual and computer-aided counting of positive cells was excellent, with an intraclass correlation coefficient of 0.946 (calculated with the *icc* function of the *irr* package in R).

Finally, for each focus the Ki67LI was calculated as total positive cells divided by total prostate cancer cells, and the Ki67/HPF was calculated as total positive cells divided by the number of images evaluated for that focus.

The other markers were evaluated by analyzing the slides directly under the microscope. A focus was considered positive for Cyclin D1 if at least 20% of its cells displayed any nuclear DAB staining, while p53-positivity required nuclear staining of moderate or high intensity in at least 20% of the cells. MYC was classified in three categories: absent/weak, moderate/strong cytoplasmic but not nuclear positivity in at least 20% of cells, and moderate/strong nuclear positivity (regardless of cytoplasmic positivity) in at least 20% of cells.

Statistical analysis

Statistical analysis was performed to evaluate the ability of each of the four markers to distinguish pG3 from aG3.
Fisher's exact test was used for binary variables (p53, MYC, Cyclin D1), while Student's t test was used for Ki67LI and Ki67/HPF.
All statistical analyses were performed using R version 3.5.1.
A p value lower than 0.05 was considered statistically significant for all tests.

Discussion and Conclusion

We studied the expression of Ki67, p53, Cyclin D1 and MYC in foci of pure Gleason pattern 3 prostate cancer (pG3) and compared them with those in Gleason pattern 3 associated with high-grade tumor (aG3), to find out whether one of these markers could be able to distinguish pG3 from aG3.

The Ki67 labelling index (Ki67LI) appears to be able to reliably distinguish pG3 from aG3 (pictured). Using 3.0% as a cutoff, Ki67LI is able to distinguish pG3 from aG3 with 95% sensitivity and 100% specificity. The number of Ki67-positive cells per high power field (Ki67/HPF) was also able to distinguish pG3 from aG3 (pictured) (using 30 as cutoff, the sensitivity was 86% and the specificity was 100%, whereas with 15 as cutoff, the sensitivity was 100% at the cost of only 50% specificity).

Our findings on Ki67LI are in line with those present in the literature.[2]
Moreover, it is possible that the worse prognosis of Gleason 3+3=6 prostate cancer with Ki67LI >10% observed by some authors is due to the fact that Gleason pattern 3 with high Ki67LI is actually aG3, and thus it is associated with (unsampled) Gleason pattern 4.[3]

It is interesting to note that the alterations characteristic of aG3 are not limited to the Gleason 3 glands directly adjacent to patterns 4 or 5, but they extend to the whole nodule of pattern 3.
In one of our cases we observed a focus of Gleason pattern 3 which on one side was adjacent to pattern 4, while on the other it extended for 5mm along the prostate margin. The region of this pattern 3 nodule furthest (>5mm) from Gleason pattern 4 still showed Ki67LI = 9.2% and Ki67/HPF = 60 (pictured).

The absence of statistically significant differences in the expression of Cyclin D1, MYC and p53 between pG3 and aG3 is in line with the literature, as is our finding regarding the differences in Ki67 expression between Cyclin D1-positive and -negative cases of prostate cancer.

Calculating the Ki67LI is relatively fast using appropriate software. However, it does require taking digital pictures and then analyzing them, which can quickly become a burden in a regular hospital without experience in digital pathology. For this reason, we demonstrated that an index as simple as the number of Ki67-positive cells per high power field can be used as a first-line test: cases with Ki67/HPF <15 are very likely to be pG3 ("true" 3+3=6), whereas cases with Ki67/HPF >30 are very likely to be aG3 ("false" 3+3=6, with unsampled pattern 4 or 5). Only the grey area in between would require the calculation of the Ki67LI.

To sum up, this proof-of-concept study confirms that pG3 and aG3 are two distinct entities, and is the first to our knowledge to translate this concept to the clinical practice by identifying an affordable and straightforward method to distinguish these two entities. Such method is able to predict which patients with a Gleason 3+3=6 prostate biopsy are truly 3+3=6 (pG3) and which *appear* to be 3+3=6 due to a sampling error, but actually harbor a prostate cancer of Gleason score at least equal to 7, and thus would likely benefit from early radical treatment more than from active surveillance. Using Ki67/HPF and Ki67LI it is possible to distinguish these patients with >95% specificity and sensitivity.

Should our observations be validated by future studies, the diagnostic performance of prostate biopsies would noticeably improve. Fewer patients with unsampled high-grade prostate cancer would be erroneously enrolled in active surveillance protocols. So not only would they not undergo the additional biopsies that within 2-3 years would eventually sample the Gleason pattern 4, but also their radical surgical treatment would not be delayed so much.

References

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