Author's response to reviews

Title: DNA index determination with Automated Cellular Imaging System (ACIS) in Barrett's esophagus: Comparison with CAS 200

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Version: 3 Date: 13 May 2005

Author's response to reviews: see over
May 12, 2005

RE: MS: 1295492136604500 - DNA index determination with Automated Cellular Imaging System (ACIS) in Barrett's esophagus: Comparison with CAS 200

BMC Clinical Pathology

Dear Editors:

We are returning a revised version of our paper referenced above. We have carefully considered comments of the two reviewers. We found them to be thoughtful, constructive and useful. We have accepted all their suggestions and have revised our paper accordingly. In addition we have also added data on the reproducibility of the results of ACIS. Point-by-point response to the reviewer’s comments is also attached.

We also agree that it will fit well into the technique section.

I hope you find this paper suitable for publication.

Sincerely yours,

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Reviewer #1:

We thank Dr Sebo for his thorough, thoughtful constructive review. Our response is as follows:

Abstract:
1. We have rewritten as follows: “The ACIS was 25% more sensitive in detecting aneuploidy than CAS 200 with a mean DI of 18% higher than CAS 200 (p < 0.001; paired t test).
2. Rewritten as “In addition, the time required to perform DNA ploidy analysis on one slide is much less with the ACIS (30-40 min) than with the CAS 200 (40-70 min”).
3. Rewritten as: “Compared to CAS 200, the ACIS is more sensitive and less time consuming for DNA ploidy analysis in Barrett’s esophagus related lesions and adenocarcinomas.”

Background:
1. We have now defined DI.
2. Yes, switch is made.

Methods:
1. Yes, we corrected the typo (mitosis)
2. HGD definition and described it in 3 shorter sentences. 
3. Full terms rather than abbreviations have been used at the start of the sentences.
4. Done
5. This has been elaborated. ACIS may capture more Feulgen-stained DNA because of the better Fuelgen stain procedure.
6. Thicker tissue sections were used to eliminate underestimation of DI values due cutting off of the nuclei in the thinner sections and then apply a mathematical correction. ACIS uses a rigorous imaging strategy to eliminate overlapping nuclei. This has been clarified in the revised text.

One of the greatest challenges to quantitating DNA by image analysis is the occurrence of overlapping nuclei. There are two strategies that can be undertaken to overcome this, though both present a unique set of difficulties to surmount. One strategy is to cut thinner sections and thereby decrease the incidence of overlap. The downside of this approach, of course, is an increased frequency of cut nuclei. This is typically addressed by a tissue correction factor, which is in essence a right shift applied to the DI histogram designed to bring the values into an acceptable numerical range, as is seen for the CAS 200. In contrast to this, the second strategy that can be applied to nuclear overlap is to utilize the power of image analysis to consistently separate touching or moderately overlapping nuclei. Such a capability allows the use of thicker sections, with the result of a markedly decreased frequency of cut nuclei and consequent mitigation of the need for a mathematical shift in the DI histogram.

The ACIS utilizes the second approach, addressing touching/overlapping nuclei through a set of image processing algorithms known as: “Watershed Segmentation”. In brief, nuclei that touch or overlap are recognized through their size and other morphometric parameters. Nuclei are separated from each other through the segmentation process by insertion of a single pixel-wide boundary between them at the point of contact. The
analysis software provides five distinct cell separation profiles to allow optimal separation across a range of specimen morphologies.

The end result of this separation is individual nuclei for which DI values can be computed. This process is designed to work well for nuclei that touch or overlap to a modest degree; it should be obvious that that the individual DI values will diminish in accuracy as the degree of overlap becomes large. Due to this fact, the ACIS has two "fail-safes" built into the process that are designed to avoid analysis of excessively overlapped nuclei. Firstly, each of the cell separation profiles is designed to recognize and exclude nuclei that exhibit excessive overlap based on signature combinations of size, shape, color and morphometric filter descriptors. Secondly, in the event that an instance of markedly overlapping nuclei does pass through these filters, the user has the ability to delete such images from the dataset. In this fashion, sufficient numbers of nuclei can be obtained from thicker sections without the complication of cut cells and requirement for tissue correction factors.

**Results:**
1. The basic display format may contribute to the results. This has added in the revised text.
2. The filter threshold controlling the inclusion of stained nuclei is fixed and is set by the system.
3. Clearly, we could not analyze the DI values in the same exact cells. However, there was an excellent correlation between the results obtained by the two methods. We have also added data showing that there is very high degree of reproducibility of results between different observers using ACIS. This point is now discussed in a paragraph dealing with the potential limitations of the study.
4. We do not have precise data on the actual time required and moreover the data with the two instruments was obtained by different individuals. We have there deemphasized the time required part and had have also discussed it as a limitation of the study.

**Discussion:**
1. We like this suggestion and have added this concept at the end of the first paragraph in Discussion.
2. We have clarified that the performance of ACIS was compared with reference to well established CAS 200.
3. We agree that similar image cytometers from different manufacturers should yield similar results and they probably do if all parameters are identical. We have addressed this issue in the revised text.
4. ACIS provides specific instructions based on their internal analysis for yielding most optimal results. ACIS uses 7 um sections that was found to avoid underestimation of the DI values due to excessive cutting off of the nuclei with 5 um sections. No tissue correction factor is used. (see detailed discussion above).
5. As explained in the revised discussion, we were some what surprised by our results. Our purpose was to simply show that ACIS yield results which are similar to those of CAS 22. We have now discussed this point in more detail along with all possible causes for the observed differences.
Reviewer #2

We also thank Dr. Jean-Fracois Flejou for very helpful suggestions. We have modified the manuscript according your suggestions.

1. We have now more clearly emphasized the difference in staining techniques and tissue sections in the two methods.
2. Paragraph entitled aneuploidy analysis has been rewritten to clarify it.
3. The time required for analysis has been minimized as we do not have good data for it.
4. Typo have been corrected
5. You are right. We now added a sentence stating that only flow cytometry has been used in large series of patients with Barrett associated lesions (14) and that further studies with image cytometry are needed.