A Prospective, Multi-Institutional Diagnostic Trial to Determine Pathologist Accuracy in Estimation of Percentage of Malignant Cells

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Context.—The fraction of malignant cells in tumor tissue submitted for tests of genetic alterations is a critical variable in testing accuracy. That fraction is currently determined by pathologist visual estimation of the percentage of malignant cells. Inaccuracy could lead to a false-negative test result.

Objective.—To describe a prospective, multi-institutional study to determine pathologist estimation accuracy.

Design.—Ten ×20 magnification images of hematoxylin–eosin–stained colon tissue specimens were sent as an educational component of the College of American Pathologists KRAS-B 2011 Survey. Data from 194 labs were analyzed and compared to a criterion standard with comprehensive manual nuclear counts.

Results.—Survey responses indicated low interlaboratory precision of pathologist estimation, but mean estimates were fairly accurate. A total of 5 of the 10 cases assessed showed more than 10% of respondents overestimating in a manner that could lead to false-negative test results.

Conclusions.—The significance of estimation errors resulting in molecular testing failures with implications for patient care is unknown, but the current study suggests false-negative test results may occur.


Successful molecular oncology testing depends on accurate assessment of the percentage of malignant cells in the analyzed tissue specimen. Although sensitivity is highly variable, depending on the individual technical approach, accurate determination of neoplastic cellularity is critical for achieving reliable results.1–4 Some assays, like Sanger or some next-generation sequencing methods, may require that malignant cells represent between 25% and 40% of the total cellular content. An overestimation of the percentage of malignant cells could lead to false-negative results if the true percentage of malignant cells in the specimen fell below the particular assay’s analytic sensitivity, and could subsequently lead to incorrect selection of therapy.1,3,5–8 Estimation of percent neoplastic cellularity can also affect care in other diagnostic settings, including assessment of copy number variation, chromosomal translocation, determination of residual cancer burden after neoadjuvant therapy,9–12 and other genomic aberrations that can be affected by contaminating normal DNA.13–16

Few methods exist for determining the percentage of malignant nuclei.1 Counting the total nuclei in a tissue section is extremely time consuming and not practical for clinical use. Usually, a pathologist reviews the tissue section to be analyzed and roughly estimates the percentage of malignant nuclei.1,2,5,6 Previous studies have evaluated pathologist agreement when estimating percentage of malignant cells (concordance) and have illustrated the difficulty in using visual assessment to quantify absolute numbers of cells.2,5,17,18 These studies were relatively limited and included small numbers of cases, and in particular small numbers of participants. Therefore, we designed a statistically powered study to evaluate pathologist accuracy in estimating percentage of malignant cells as part of the College of American Pathologists (CAP) KRAS-B 2011 Survey. Here, we report the results of this prospective, multi-institutional diagnostic trial.

MATERIALS AND METHODS

Ten ×20 magnification images of colon adenocarcinoma specimens stained with hematoxylin–eosin (Figure 1) were selected by the CAP Molecular Oncology Committee to represent a range of specimen appearances encountered in routine diagnostic practice,
Figure 1. The complete set of 10 images distributed as part of the College of American Pathologists (CAP) KRAS-B 2011 Survey for the Photo Challenge. A through J. The 10 images are presented in order, each representing a random field from a random colon cancer specimen (hematoxylin-eosin, original magnifications ×20).
and the images were distributed as an educational component of the KRAS-B 2011 Survey (a survey that the CAP offers for clinical laboratories to assess their proficiency at detection of mutations in KRAS). Included in the survey were carefully detailed instructions as well as an example of how lab personnel should calculate the percentage of malignant cells. The instructions included a brief description regarding the importance of accurate determination of the percentage, and participating laboratories were asked to identify who was responsible for determining the percentage of malignant cells and the method used for achieving that number. Data from 194 labs were received and analyzed for accuracy through comparison of estimated percentages to a criterion standard achieved by counting all malignant and nonmalignant nuclei in the survey images and calculating the percentage of malignant cells. The criterion standard for the correct percentage of malignant cells in each image was determined by subdividing the images into 9 parts for better detail resolution and marking each nucleus with a red or green dot corresponding to neoplastic and nonneoplastic cells, respectively, using Photoshop (Adobe, San Jose, California). The number of red and green dots was counted for each part of each image and totaled. A sample image, one of its subdivided parts, and the same part with marked nuclei are shown (Figure 2, A through C). All dotting and counting was done by technicians (H.V. and K.L.) in the Rimm lab, and designsations of benign versus malignant nuclei were reviewed by
a pathologist (D.L.R.). We believe this approach accurately represents a criterion standard for evaluating pathologist estimation.

**RESULTS**

None of the institutions surveyed in this study reported counting the total nuclei in a tissue section to obtain the percentage of tumor cells in the sample. When asked to describe their method for determining the percentage of malignant cells in a tissue section, the vast majority of laboratories reported using pathologist estimation. Survey results indicated a high level of interlaboratory variation and the presence of a wide range of estimated percentages on multiple study images. The mean estimate for 8 of 10 images was within 10% of the criterion standard, suggesting that, on average, pathologists perform well. For example, image KRAS-90 (Figure 2, D) contained the highest percentage of malignant cells (66.2%), and the mean estimate was 64.4% malignant cells. On the other end of the scale, KRAS-91 (Figure 2, E) had the lowest percentage of malignant cells (6.6%), and the mean estimate was within 1% of the count. Other examples suggested less accurate pathologist estimates, with the potential to impact patient care. For

![Image of histograms and graphs showing the estimation of percentage of malignant cells across different images](image-url)
example, KRAS-89 (Figure 2, F) yielded the most discordant responses, ranging from 10% to 95% malignant cells and with the mean estimate of percentage of malignant cells differing by 24.4% from the criterion standard.

The frequency distribution of all estimated percentages for each image is shown in Figure 3, A through J. The range of responses indicated a low level of interlaboratory precision in estimating the percentage of malignant cells. The mean estimate for each image in the survey was also plotted versus the criterion standard (Figure 3, K). Linear regression showed a statistically significant correlation between the mean estimate and criterion standard ($R^2 = 0.8236; P < .001)$. The greatest variation occurred in images containing higher percentages of malignant cells (>40%).

Patient care could be affected by pathologist overestimation of malignant cells, which could generate a false-negative test result for a mutation. Based on the generally accepted ranges of analytic sensitivity for various diagnostic assays, we categorized overestimation errors of greater than 20% as having the potential to change patient outcome because of false-negative DNA testing results (although this figure is highly dependent on the analytic sensitivity of the assay selected by each laboratory). For each image, we graphed the percent of survey responders that overestimated the percentage of malignant cells by greater than 20% (Figure 3, L). Although some cases showed low rates of overestimation, others were more problematic. The most discrepant image was KRAS-89, in which 57% of survey participants overestimated the percentage of malignant cells by greater than 20%.

**COMMENT**

With respect to mean pathologist estimation, the data indicate that pathologists more accurately estimate the percentage of malignant nuclei in cases containing low amounts of tumor cells. Based on our linear regression plot of mean pathologist estimates versus the counted malignant cellularity (Figure 3, K), that accuracy seems to decrease as a function of increasing tumor cell content, suggesting overestimation affecting patient care may be less likely. Pathologist concordance, however, remains low for all study images except KRAS-91 and appears to be unrelated to malignant cellularity. Other specimen factors, such as staining intensity or section thickness, may also affect pathologist estimation accuracy, although these factors were unable to be assessed in this study. The study also suggests that pathologists are more likely to overestimate than underestimate the percentage of malignant cells. This is of some concern, because overestimation is more likely to affect patient care than underestimation. We averaged each pathologist’s error across 10 images and found that 5.4% of survey participants overestimated by more than 20% on average. This is particularly concerning because overestimation errors greater than 20% are most likely to affect molecular testing results because of insufficient malignant DNA.

Although the results of this study were powered based on the number of participants and pilot data on estimation of percent neoplastic cellularity, they are limited by inclusion of only 10 representative images for review and the exclusive use of ×20 images for evaluation of malignant cellularity, rather than whole-slide examination at multiple magnifications, as is done in routine clinical practice. Finally, this study does not address how errors in the estimation of percentage of malignant cells affect patient outcome. Nonetheless, the data demonstrate that most pathologists adequately estimate the percentage of malignant cells. However, they also illustrate the potential for a false-negative rate of at least 5%. As molecular testing continues to become a routine part of pathologic evaluation, pathologists should be conservative in their estimations of the percentage of malignant cells and consider the importance of tissue macrospecimen or microdissection to increase malignant cellularity. In addition, pathologists should have an understanding of the analytic sensitivity of the assay selected by their lab (Sanger sequencing, pyrosequencing, real-time polymerase chain reaction, or next-generation sequencing). Finally, new automated objective methods should be sought that can quickly and accurately determine the percentage of malignant cells.

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