PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Level of phosphohistone H3 among various types of human cancers
AUTHORS	Sun, Amy; Zhou, Wei; Lunceford, Jared; Strack, Peter; Dauffenbach, Lisa; Kerfoot, Christopher

VERSION 1 - REVIEW

REVIEWER	Rueymin Ray Lee Translational Medicine Leader Hoffmann La Roche, Inc. Nutley NJ 07110 No conflict of interest
REVIEW RETURNED	14-Apr-2012

GENERAL COMMENTS	This is a well executed study to determine and compare the phosphorylation of hostone Ser 10 and Ser28 by IHC in various tumor types. The authors successfully concluded that Ser10 staining was significantly higher than Ser28, and results are convincing. However, I wonder whether they can further improve the clinical significance of the conclusion by correlation with histology grades in part 1 (better sample size).

REVIEWER	Colman, Howard University of Utah, Huntsman Cancer Institute
REVIEW RETURNED	25-Apr-2012

RESULTS & CONCLUSIONS	While the data answer the question of whether IHC for PHH3 Ser10 or Ser28 demonstrate higher expression, this finding does not answer the underlying question of which marker is most sensitive and specific for the entity being assayed, namely cellular proliferation. So the results would be greatly strengthened by comparing the expression of these 2 markers with other measures of proliferation (e.g. Ki-67, mitoses) to determine which PHH3 marker is best.
GENERAL COMMENTS	Technically, the study appears to be sound. However, the main result reported is the relative expression levels of the the two PHH3 Ser10 or Ser28 markers relative to each other. However, no data is provided on the true sensitivity and specificity of either marker for measuring cellular proliferation. This would appear to be an important issue, particularly since the correlation between the Ser10 and Ser28 expression was relatively weak. Thus the manuscript

	would be greatly strengthened by inclusion of additional markers of cellular proliferation such as MIB-1 or mitoses counts to determine whether Ser10 or Ser28 staining is truly a better marker of this process.

REVIEWER	Christian L. Laboisse M.D. Professor of Pathology Head Department of Pathology Nantes University Hospital France
REVIEW RETURNED	04-May-2012

THE STUDY	This work is unfocused, based on a small series of samples. The rationale is unclear. The methodology is not adequately described. There is no attempt to correlate the findings to the determination of the mitotic index based on standard staining (HE). globally this work does not add significant information to the field.

VERSION 1 – AUTHOR RESPONSE

Reviewer #1: This is a well executed study to determine and compare the phosphorylation of histone Ser 10 and Ser28 by IHC in various tumor types. The authors successfully concluded that Ser10 staining was significantly higher than Ser28, and results are convincing. However, I wonder whether they can further improve the clinical significance of the conclusion by correlation with histology grades in part 1 (better sample size).

Response: We certainly agree that comparisons to grade would require a significant increase in the scope of this study. Although pHH3 is used in place of H&E mitotic counts for clinical purposes, I do not think the major focus of this manuscript is clinical implications. When lumping all cancers together (which is not appropriate) and sorting by grade, there is no significant difference.

Grade Number pHH3 (Ser10) Percent Positive
1 1 2.67%
2 7 2.75%
3 12 2.35%

Reviewer #2: While the data answer the question of whether IHC for pHH3 Ser10 or Ser28 demonstrate higher expression, this finding does not answer the underlying question of which marker is most sensitive and specific for the entity being assayed, namely cellular proliferation. So the results would be greatly strengthened by comparing the expression of these 2 markers with other measures of proliferation (e.g. Ki-67, mitoses) to determine which pHH3 marker is best.

Response: The relationship between Ki67 and pHH3 will not be linear, since Ki67 will label cells in G1 through M, while pHH3 is specific for M-phase. For example, Ki67 staining in xenografts is always near 100%, while pHH3 levels can vary dramatically dependent on the cell line and drug treatments. We have not performed a comparison with mitotic count in this sample set, but the pHH3 assays certainly do stain mitotic cells. In staining samples of tonsil, pHH3 staining is seen at levels approaching 5% in germinal center lymphocytes (area of proliferation), but many of these cells do not have the typical mitotic phenotype. All cells with a mitotic phenotype are stained. This indicates that the assays are staining cells in an earlier part of mitosis than can be detected by mitotic count of an

H&E stained slide.

Technically, the study appears to be sound. However, the main result reported is the relative expression levels of the two pHH3 Ser10 or Ser28 markers relative to each other. However, no data is provided on the true sensitivity and specificity of either marker for measuring cellular proliferation.

Response: We have added data and images of Untreated and Nocodazole-treated cell lines to address the sensitivity and specificity.

This would appear to be an important issue, particularly since the correlation between the Ser10 and Ser28 expression was relatively weak. Thus the manuscript would be greatly strengthened by inclusion of additional markers of cellular proliferation such as MIB-1 or mitoses counts to determine whether Ser10 or Ser28 staining is truly a better marker of this process.

Response: Please see response to Reviewer 2's first question. Further, the lack of linear relationship between Ki67 and pHH3, and a comparison of the two pHH3 antibodies were demonstrated in attached spreadsheet (in supplementary materials). Please note we do not have Ki67 staining data on all samples, just a subset.

Reviewer #3: This work is unfocused, based on a small series of samples. The rationale is unclear. The methodology is not adequately described.

Response: We have elaborated on the description of immunohistochemical methods.

There is no attempt to correlate the findings to the determination of the mitotic index based on standard staining (HE).

Response: Please see responses to Reviewer #2's question.

Globally this work does not add significant information to the field.

Response: We respect the reviewer's stand, but the authors believe this work will be a welcome addition to the existing knowledge.

VERSION 2 – REVIEW

REVIEWER	Colman, Howard University of Utah, Huntsman Cancer Institute
REVIEW RETURNED	20-Jun-2012

GENERAL COMMENTS	The authors determine that Ser10 expression is higher than Ser28. While this data answers the question of which IHC marker has higher expression, it does not answer the more important question of which marker is a better estimate of proliferation. In order to do this, the authors should include either correlation with other proliferation markers (e.g. mitoses, Ki-67) or patient outcome.

VERSION 2 – AUTHOR RESPONSE

“The authors determine that Ser10 expression is higher than Ser28. While this data answers the question of which IHC marker has higher expression, it does not answer the more important question of which marker is a better estimate of proliferation.”

Response: The purpose of this manuscript was to compare pHH3 Ser10 to Ser28, and we determined that Ser28 was less sensitive and results diverged. In addition, there is a well-known, well-published relationship between pHH3 (Ser10)-positive cell count and mitotic index.
So, analysis of pHH3 Ser10 expression would be the better estimate of proliferation.

“In order to do this, the authors should include either correlation with other proliferation markers (e.g. mitoses, Ki-67) or patient outcome.”

Response: Patient outcome is outside the scope of this particular study for it does not involve live patients. The authors agree that future studies should look at patient outcomes.

Since there is a well-known, well-published relationship between pHH3 (Ser10)-positive cell count and mitotic index, reinvestigating this is without merit since this relationship has been extensively described in the literature.

Also, the relationship between Ki67 and pHH3 will not be linear, since Ki67 will label cells in G1 through M, while pHH3 is specific for M-phase. For example, Ki67 staining in xenografts is always near 100%, while pHH3 levels can vary dramatically dependent on the cell line and drug treatments. We have not performed a comparison with mitotic count in this sample set, but the pHH3 assays certainly do stain mitotic cells. In staining samples of tonsil, pHH3 staining is seen at levels approaching 5% in germinal center lymphocytes (area of proliferation), but many of these cells do not have the typical mitotic phenotype. All cells with a mitotic phenotype are stained. This indicates that the assays are staining cells in an earlier part of mitosis than can be detected by mitotic count of an H&E stained slide.