Reviewer's report

Title: Concomitant heterochromatinisation and down-regulation of gene expression unveils epigenetic silencing of RELB in an aggressive subset of chronic lymphocytic leukemia in males

Version: 2 Date: 18 February 2010

Reviewer: Curt Balch

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This manuscript describes findings of a high level of heterochromatinization in apoptosis-resistant chronic lymphocytic leukemia (CLL) cells from male patients, but not in CLL cells from female patients. Microarray analyses demonstrated 17 genes commonly dysregulated in apoptosis-resistant CLL cells from males and females; one of these, RELB, was downregulated in male resistant CLL cells but upregulated in resistant CLL cells from females. Strengths of the study are the study of a devastating malignancy (CLL), identification of resistance-correlated genes (including possible gender-related differences), and the interesting finding of increased heterochromatinization in male resistant CLL cells. Weaknesses include inadequate integration (and interpretation/speculation) of the results (as they relate to chemoresistance), how the findings might be eventually translated to clinical applications, and a lack of discussion clarifying results that may deviate from the original hypothesis.

Major Compulsory Revisions

1. Apoptosis-resistant CLL cells were selected by 24-hour exposure to 10 Gy of 137Cs, which was previously demonstrated to elicit gene misexpression similar to fludarabine. However, this important control (gene signature similarity) should also be mentioned in the current study. Did the authors of the current study also observe double-strand breaks (and alterations in the corresponding DNA damage response pathways), similar to that previous study?

2. Why (apparently) was a different Affymetrix GeneChip used for the male CLL samples (Set A-B for male samples vs. 2.0 Plus female samples)? Also, the discussion of the microarray results is quite confusing, with the male samples apparently assessed for expression of 472 genes, and the female samples assessed for 803. This seems inconsistent with the Affymetrix U133 array, which contains over 33,000 genes. Was only a subpanel of the total array used for the male vs. female CLL cell comparisons? This should be clarified.

3. The region of the RELB locus that was bisulfite sequenced was a 428-bp region comprising the promoter (from -259 bp) and first exon (to +169 bp), while the three promoter subregions assessed by anti-H3K9me3 ChIP were -333 to -391, -529 to -650, and -1117 to -1191. Thus, it would not appear that the sequences analyzed for H3K9 methylation overlapped with the region analyzed for 5-methyl-dC; nonetheless, the authors assert that RELB transcriptional
silencing is due to histone H3K9 methylation, and not deoxycytosine methylation. Unless the authors have additional data or some justification for a lack of 5-me-dC in those three subregions, that specific conclusion is not supported and should be removed.

4. The authors should speculate a possible mechanism for heterochromatinization specifically in male CLL cells, as compared to female CLL cells.

5. Despite the finding of gender-specific silencing of RELB, the authors do not speculate on a possible role for canonical or noncanonical NF-κB signaling in sensitivity to double strand DNA breakage (e.g., contribution to apoptotic capacity, etc.), and why loss of RELB might result in resistance. Those possible interpretations, including their specificity to male CLL cells, should be included in the discussion. The converse finding, of elevated RELB levels in female resistant CLL cells, should also be discussed.

6. If the authors speculate a loss of PAX5 or STAT1 tranactivation (due to H3K9me3 or 5-methyl-C) as contributory to RELB silencing, they should demonstrate a loss of binding of these transcription factors (TFs) in the resistant cells, or at a minimum, discuss the possible roles of these TFs in signaling cascades induced by double strand DNA breakage.

7. The authors frequently discuss CLL resistance as due to decreased susceptibility to DNA damage-induced apoptosis. However, of the 17 genes found commonly dysregulated in the resistant CLL male and female samples, how many were members of that specific cascade? Gene set enrichment, and/or pathway determinations, should be provided for those particular genes (in addition to chromosome location, etc.), and how these may contribute to apoptosis resistance.

Minor Essential Revisions

1. The perinuclear distribution of the 5-methyl-C labeling in healthy donors, as compared to CLL patient samples, should be explained to the reader.

2. The nomenclature for trimethylated histone H3 lysine 9 (H3K9me3, 3metH3K9) should be consistent.

Discretionary Revisions

1. The pertinence of the IgVH mutations, with respect to the current study, should be contemplated by the authors and modified or eliminated, if deemed unrelated to the manuscript.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:

I declare that I have no competing interests.