# To the editor:

## Challenging the adult T-cell leukemia/lymphoma (ATL) stem cell concept based on limiting dilution transplantation assay

In their article, Yamazaki et al[1] reported the identification of malignant stem cells in the Tax-transgenic (Tax-Tg) mouse model of adult T-cell leukemia/lymphoma (ATL). This statement was grounded partly on a limiting dilution transplantation assay (LDTA) reported in Table 2 of their paper,[1] aimed at estimating the frequency of cancer stem cells (CSCs) among splenic lymphomatous cells (SLCs). From this assay, the authors reported that one CSC existed in $10^4$ SLCs (0.01%, ie, 1 in 10 000), and this frequency estimate was found to be consistent with the frequency of the $CD38^-/CD71^-/CD117^+$ cells estimated by flow cytometry (0.03%, ie, 1 in 3333). Based on these cell frequencies, the authors claimed that $CD38^-/CD71^-/CD117^+$ cells can stand for the rare stem cell population among the whole SLC population, and this hypothesis was documented by the ability of sorted $CD38^-/CD71^-/CD117^+$ cells to regenerate the original lymphoma in transplanted animals in a single dose assay.

The rarity of cancer-initiating cells is usually considered as a major attribute favoring the cancer stem cell hypothesis.[2] This perception of cancer-initiating cells' rarity, and the related stem cell concept, mainly relate to the estimation of cancer-initiating cell frequencies by the "gold standard" limiting dilution transplantation assay in recipient animals.[3] However, the hypothesis of rarity of cancer-initiating cells is deeply impeded by the observation that LDTA practitioners, including Yamazaki et al,[1] usually do not use proper statistical analysis of LDTA data that may validate the reported cell frequency estimates. We are capable to demonstrate that appropriate statistical modeling of the LDTA performed with SLCs[1] invalidate this experiment and the related "stem" cell frequency estimate provided by the authors. Mathematical modeling of limiting dilution experiments of cells injected into recipient animals traditionally refers to the well-known single-hit Poisson model (SHPM), which posits that a single cell is necessary and sufficient to form a detectable tissue in the host.[3] In a previously published paper[4] we advised a statistical test aimed at estimating the fit of the SHPM to the data and based on a generalized linear modeling approach.[5] Briefly, a general linear model, termed $GLM_{loglog}$, can be written as

$$Y_i = \alpha + \beta X_i$$

with $Y_i = \ln[-\ln(\pi_i)]$ and $X_i = \ln(x_i)$, where $\pi_i$ is the proportion of recipient mice free of tumor, and $x_i$ is the cell dose, that is, the number of cells injected into each mouse at each group $i$ of cell dose. On elementary rearrangement[4] of the above equation, the $GLM_{loglog}$ reduces to the SHPM for the special case as the slope $\beta = 1$. Therefore, testing the null hypothesis $\beta = 1$ explores the SHPM hypothesis, and this can performed by a standard likelihood ratio test[5,6] and by a standard Wald test.[4] The results of our modeling study are presented in Table 1. The null hypothesis $\beta = 1$ is clearly rejected at a very significant level, indicating that the LDTA does not conform to the SHPM, and the consequence is that no frequency estimate can be rendered. The precise frequency of tumorigenic cells in the Tax-transgenic (Tax-Tg) model remains unknown, and the hypothesis that this frequency may be considerably higher than 0.01% must not be ruled out, ultimately challenging the stem cell concept.

**Table 1. Rejection of the SHPM hypothesis by fitting a generalized log-log linear regression model ($GLM_{loglog}$) to a limiting dilution transplantation assay (LDTA) performed with whole splenic lymphomatous cells (SLCs)**

| Characteristic | Definition |
|---|---|
| Cell population | SLCs |
| Deviance dispersion statistic* | 1.89 |
| β | 0.473 |
| SE(β) | 0.159 |
| **Null hypothesis, β = 1** | |
| *P(z)*† | 0.0009 |
| $P(\chi^2)$‡ | 0.0116 |
| SHPM hypothesis | Rejected |

*The deviance dispersion statistic can be considered as a summary measure of goodness of fit of the $GLM_{loglog}$ to the data and should be inferior to 1.5-2 when the model adequately fits the data.[5]

†*P(z)* associated to the Wald statistic Z.[4]

‡$P(\chi^2)$ associated to the standard likelihood ratio test used to compare nested models.[6] The $GLM_{loglog}$ holds, as demonstrated by the rejection of the null hypothesis $\beta = 0$ by both Wald and likelihood ratio test statistics with $P < .005$ (data not shown).

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## References

1. Yamazaki J, Mizukami T, Takizawa K, et al. Identification of cancer stem cells in a Tax-transgenic (Tax-Tg) mouse model of adult T-cell leukemia/lymphoma. *Blood.* 2009;114(13):2709-2720.
2. Rosen J, Jordan C. The increasing complexity of the cancer stem cell paradigm. *Science.* 2009;324(5935):1670-1673.
3. Porter HE, Berry RJ. The efficient design of transplantable tumour assays. *Br J Cancer.* 1963;17:583-595.
4. Bonnefoix T, Bonnefoix P, Verdiel P, Sotto JJ. Fitting limiting dilution experiments with generalized linear models results in a test of the single-hit Poisson assumption. *J Immunol Methods.* 1996;194(2):113-119.
5. Collett D. *Modelling Binary Data* (2nd ed). Boca Raton, FL: Chapman & Hall; 2003.
6. Hu Y, Smyth GK. ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods.* 2009;347(1-2):70-78.

# Response

## Estimating frequency of cancer stem cells in a mouse model of adult T-cell leukemia/lymphoma

Bonnefoix and Callanan, using a statistical analysis, have challenged our report on the frequency of cancer stem cells (CSCs) in a nonobese diabetic–severe combined immunodeficiency (NOD/SCID) mouse model of adult T-cell leukemia/lymphoma.[1] While we agree (as discussed below) that there are limitations in estimating frequency of CSCs in our model, we have clearly documented the existence of CSCs using xenografting of candidate CSCs into NOD/SCID mice. Specifically, we identified candidate CSCs in a side population (0.06%) that mostly overlapped with a minor population of $CD38^{-}CD71^{-}CD117^{+}$ cells (0.03%). Furthermore, we found that $10^2$ candidate CSCs could regenerate the original lymphoma when transplanted into the NOD/SCID mice. From the limiting dilution transplantation assay (LDTA) data in Table 2 (in our article), we estimated the frequency of candidate CSC to be around 0.01% on the basis of the observation that $10^4$ SLCs could regenerate the original tumor. However, we agree that we have used only limited transplantation data to determine this frequency and that more detailed studies would be required to accurately calculate this. In this regard it is important to note that the experimental conditions used would certainly influence frequency estimates. In our study, candidate CSCs could regenerate the original tumor only when analyzed at 60 days after transplantation, suggesting that the duration of analysis could also affect the LDTA data. In this respect it has already been shown that modification of xenotransplantation assays in NOD/SCID mice can also dramatically change the detection frequency of CSCs.[2]

We should also highlight that the cell population we identified by LDTA would likely include not only CSCs but also other tumorigenic cells, and the existence of such cells in addition to the candidate CSCs would also influence our estimates of CSC frequency.

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## References

1. Yamazaki J, Mizukami T, Takizawa K, et al. Identification of cancer stem cells in a Tax-transgenic (Tax-Tg) mouse model of adult T-cell leukemia/lymphoma. *Blood.* 2009;114(13):2709-2720.
2. Quintana E, Shackleton M, Sabel MS, et al. Efficient tumour formation by single human melanoma cells. *Nature.* 2008;456(7222):593-598.