Author's response to reviews

Title: In B-CLL, the codon 72 polymorphic variants of p53 are not related to drug resistance and disease prognosis

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Version: 2 Date: 8 July 2005

Author's response to reviews:

Dear Editors,

thank you for re-considering our revised manuscript. We wish to thank the reviewers for their positive comments which helped to improve the manuscript. We have added a detailed point-to-point reply and hope that all concerns are now addressed satisfactorily.

Sincerely

Prof. Dr. Peter Daniel

Reviewer Eric Solary

1- Did the polymorphism affect survival of patients that did not receive any previous treatment?

58 patients were treatment-naive. Of those, 36 carried Arg/Arg, 3 Pro/Pro and 19 Arg/Pro genotype. A subgroup analysis for survival estimates showed no significantly different result, i.e. no impact of the p53 codon 72 SNP (see new Fig. 2B).

2- Is there enough patients to determine whether the polymorphism affected the response to a given treatment such as fludarabine?

The patients were diagnosed to have B-CLL before 1998, mostly between 1991 and 1996. There are only 17 censored patients, those were followed a median of 98 months (i.e. 8.16 years). At this time, fludarabine was not yet considered a standard treatment for patients with B-CLL. The vast majority of patients was treated with alkylating agents. Concerning cell death induction by various cytotoxic stimuli, we have included, however, the ex vivo results from the DiSC assay, which was shown by Bosanquet at al. to correlate well with the in vivo results after fludarabine treatment (Br J Haematol 1999, 106: 71-77). There was, however, no impact of the codon 72 SNP on fludarabine induced cell death as determined by the LC90 doses in the ex vivo drug sensitivity test.

3- How was the mutational status of p53 determined and which mutations were found in 52 samples may be explained.

The p53 mutations in 22 samples were found by examination of exon 5-8 by SSCP-PCR and sequencing, as described (Sturm et al., Mutation of p53 and consecutive selective drug resistance in B-CLL occurs as a consequence of prior DNA-damaging chemotherapy. Cell Death Differ 2003, 10: 477-84.) The information was added in the methods section.

4- Where and how were collected the cells? Probably in peripheral blood but this may be indicated in the material and methods section.

Peripheral blood from 138 B-CLL patients was analysed for drug sensitivity to a panel of B-CLL drugs and
radiation using fresh cells. The same samples were analysed for mutations in the p53 DNA binding domain, using snap frozen cells from the same specimens. Of the 136 patients, 78 were pretreated with one to six drug regimens (mean number of pretreatments for these 80 patients +/- SEM: 1.93 +/- 1.005). Patients were staged according to Binet's classification and diagnosis of B-CLL was confirmed according to the NCI guidelines by morphology, a white cell count of >15 x 109 cells/l and cell markers including coexpression of CD5 and B-cell markers (CD19, CD20, and CD23) on leukemic cells. Only patients with high peripheral blood leukocyte count were used for this analysis (median WCC 120.8 /nl, range 20.7-1262.2 /nl). This information was added to the methods section.

Reviewer Massimiliano Bonafe

1- Authors should clarify the case of death of B-CLL patients. The relevance of this issue is due to the mean age of the patients enrolled in the study. Indeed, aged people are impinged upon by high mortality rate for causes unrelated to B-CLL, such as cardiovascular disease. In this regard,......p53 codon 72 impact...on myocardial ischemia... (Bonafe et al 2004)

It was not possible to clarify the cause of death in all 121 patients, because it is a retrospective study with a long follow up. Most of the patients died, however, of B-CLL related causes. Death from non B-CLL causes was coded as censored event and therefore does not affect the survival estimates following such a censored event. In addition, we believe that the patient number in this study is sufficient to compensate for some imbalances in co-morbidity.

We nevertheless read the article cited by the reviewer carefully and with great interest. Bonafe et al. describe an age related variance in apoptosis susceptibility for oxidative stress-induce apoptosis in fibroblasts and lymphocytes obtained from Arg/Arg or Pro/Pro patients and observed increased levels of Troponin and CK-MB after myocardial ischemia in old patients (66-99) with Arg/Arg compared to Pro/Pro carriers. We retested our data set and found no difference in distribution of Arg/Arg and Pro/Pro in two groups divided by the median in younger (< median of 63.18) and older ([greater than or equal to] median) patients. As expected the younger B-CLL patients show a better prognosis as compared to the older patients (p=0.055). This is also the case in the Arg/Arg and the Arg/Pro subgroups. The Pro/Pro subgroup with only n=9 is to small for such a dichotomy. We would like to thank the reviewer for this interesting hint.

2- Authors should specify the type cells which were employed....

We employed whole PBMC from high-counters". The percentage of contaminating normal" leukocytes is therefore very low. This information has been added to the methods section.

3- Authors should report data regarding chemotherapy to which the patients underwent....... We report a retrospective analysis. Of the 136 patients, 78 were pretreated with one to six drug regimens (mean number of pretreatments for these 80 patients +/- SEM: 1.93 +/- 1.005). Most patients received therapy with alkylating agents.

4- Authors should be cautious in estimating p53 codon 72 genotype frequency distribution in B-CLL.......whole blood leukocytes of these patients are predominantly neoplastic lymphocytes.....therefore, some Arg/Arg homozygotes could be in fact Arg hemizygotes.....

To clarify this point, we have added Concerning the p53 genotype of the B-CLL samples.....". We do not intend to estimate the patients p53 codon 72 genotype distribution in non-malignant tissue. The present data are all derived from neoplastic cells. Our data in a large cohort of 138 patients show that the homo- or heterozygosity for the 2 alleles has no significant impact on patient survival or response of B-CLL cells to chemotherapy or ionising irradiation.

5- Authors should avoid the concluding sentence in which they state that discrepancy among studies could be due to sample size. At variance they should underline that p53 codon 72 does not play any role in B-CLL neoplasms.

The conclusion was modified to include the statement by the reviewer.