Response to: "Cytoplasmic dislocation of NPM1 and PU.1 in NPM1-mutated leukemia is obscured by paraformaldehyde fixation"

To the Editor:

Although we recognise that paraformaldehyde may influence the detection of NPM1 and PU.1 in immunofluorescence, the main goal of our study (Pianigiani *et al.*, 2020) was to test whether PU.1 localisation could be used to diagnose *NPM1*-mutated AML through immunohistochemistry in B5-fixed bone marrow biopsies. B5 fixation is routinely used in our laboratory to detect cytoplasmic localisation of NPM1 in acute myeloid leukaemia, based on Falini *et al.* (2005). It is unlikely that B5 fixation allows for correct detection of NPM1 but not that of PU.1.

The data provided by Gu *et al.* in this Letter and in previous work (2018) support the hypothesis that NPM1 directly binds PU.1, leading to PU.1 relocation from the nucleus to the cytoplasm. Given the heterozygous nature of *NPM1* mutations, a significant proportion of PU.1 would be expected to still be localised in the nuclei of *NPM1*-mutated cells (even accounting for the small amount of wild-type NPM1 dragged to the cytoplasm by the mutant protein). However, no PU.1 is detected in the nuclei of *NPM1*-mutated untreated cells in the vast majority of immunofluorescence and western blot experiments reported by Gu *et al.*, arguing for an overestimation of the actual amount of PU.1 in the cytoplasm. Also, all the experiments by Gu *et al.* have been performed using a polyclonal anti-PU.1 antibody whose production has been discontinued years ago, making it difficult to reproduce the data.

Concerning the nuclear/cytoplasmic fractionation, it is possible that in our experiments a proportion of PU.1 was not separated from the nuclear fraction. Considering the correct localisation of control proteins in our blot, and assuming, as claimed by Gu *et al.* (2018), that almost all PU.1 should be in the cytoplasm of *NPM1*-mutated cells, one would expect to find a visible proportion of PU.1 in the cytoplasm, even after an incomplete separation. However, we did not detect PU.1 at all in any of the cytoplasmic fractions, even after applying longer exposure times.

We would like to emphasize that we do not rule out that a proportion of PU.1 may be found in the cytoplasm of AML cells. However, our data indicate that PU.1 localisation studied by immunohistochemistry should not be used to diagnose *NPM1*-mutated AML. More experiments are necessary to establish the exact proportion of PU.1 localised to the cytoplasm of leukemic cells, and to define the contribution of cytoplasmic PU.1 to the development and maintenance of *NPM1*-mutated AML.

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