Reviewer's report

Title: Promoter methylation and large intragenic rearrangements of DPYD are not predictive of severe toxicity to 5-fluorouracil-based chemotherapy in gastrointestinal cancer

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Reviewer: Linda JW Bosch

Reviewer's report:

In this manuscript, Savva-Bordelo and collaborators investigated whether DNA promoter methylation and large intragenic rearrangements of the DPYD gene contribute to the development of severe 5-FU toxicity in patients with gastrointestinal cancer. In addition, they analyzed immunoexpression of DPD and TYMS in tumors from 29 of those patients.

The authors found no promoter methylation or large intragenic rearrangements in DPYD in a series of 45 patients, indicating that those mechanisms do not play a role in DPD deficiency in those patients. Although a relevant research question, some other studies have already reported the lack of these mechanisms in DPD deficiency. Nevertheless, as for the DNA methylation analysis, contradicting results have been published and the number of patients analyzed is largest in this manuscript. Therefore, in my opinion, these results are still relevant to report. However, I do have some major and minor comments to the manuscript.

Major Compulsory Revisions:

- Title: it is too strong to state that promoter methylation and large intragenic rearrangements of DPYD are not predictive of severe toxicity to 5-FU, because the authors did not find these aberrations and therefore they could not proof that when present, it would not be predictive. It is more accurate to stress the absence of these mechanisms in patients with severe 5-FU toxicity.

- Although the methods are appropriate to answer the research questions, I lack relevant details on the descriptions. For example, for the MLPA analysis, it would be very helpful to elaborate on the composition of the probe-mix (probes present for all of the 23 exons of DPYD), to better understand how this method is used to measure intragenic rearrangements. Also the analysis of the data is missing (e.g. which cut-offs are used to determine the presence of a deletion or duplication?). Furthermore, I would like to see more details on the MSP analysis (PCR program, annealing temperature) and on the immunostainings (which protocol, dilution of antibodies, staining controls)

- Introduction/Study design: The research question for the immunoexpression of DPD and TYMS in tumor tissues is not clear to me in the context of this manuscript. The main aim of the manuscript is to find a molecular explanation for the development of severe 5-FU toxicity. Expression of these proteins in the
tumor itself would be of relevance to predict response of the tumor to the drug, rather than to predict development of toxicity of the patient. However, clinical data on tumor response is missing and therefore no conclusions can be made from this data. For me, this data is therefore redundant. Also, I don’t see the additive value of the correlation to the standard clinical parameters given in table 2.

- Discussion: the authors say that they are the first to analyze DPYD methylation using a quantitative MSP approach, which is more specific and sensitive than conventional methylation-specific PCR. I agree that a quantitative MSP approach can be more specific than conventional MSP, however, only when the primers are well designed and when a probe is used to detect the amplified PCR product (The probe adds extra specificity by sequence-specific binding). In the present manuscript, SYBRgreen is used to detect amplified PCR products, which in essence is as specific as ethidium-bromide used to detect the product on gel. Please delete this sentence from the discussion.

-Discussion, fourth alinea: the authors state that “genetic screening of DPYD mutations in GI cancer patients is only cost-effective when this procedure is restricted to patients which develop toxicity following the first administration of 5-FU, leading to a change in chemotherapy regimen if a mutation is detected.” Why would we want to screen for a mutation that is predictive for toxicity, when toxicity is already measured? Don’t we want to change the chemotherapy regimen in any case of toxicity, also in the absence of a DPYD mutation is not found? What is then the purpose of screening?

Minor Essential Revisions:
- Please, change colo-rectal to colorectal.
- Material and Methods, section 2.2: add the number of patients that have esophageal, gastric or colorectal cancer or refer to table 1.
- Material and Methods, section 2.3: remove ‘[’ before “from American Type Tissue Collection
- Results, section 3.2, starting from “Importantly, …” The text is hard to follow for the reader. e.g. the flow of tested samples: 45 samples tested, 2 confirmed diagnostics, 1 not, “the remaining 42…”, “the remaining 39…”, “(MPLA analysis was not performed in 3/42 …)”. Please, start first with the broad findings, then go to details.
- Results, section 3.3: “DPD immunoexpression was normal in the corresponding tumor and paired morphologically normal epithelium”. What is ‘normal’ expression? As indicated in the Introduction, expression levels of DPD and TYMS vary among individuals, thus ‘normal’ expression does not exist. Please, rephrase.
- Discussion, third alinea: “This mutation was found in two patients (one previously reported) and all of them developed severe toxicity following 5-FU administration.” Change “all of them” to “both”
- Table 1: change "19, 42.2" to "9 (42.2)"
- Table 3: As said in the Material and Methods, 3+ scoring was also included in the analysis, but none of the tissue scored 3+. For completeness, please add this in the legend.

- Please, adapt p-values to one significant decimal

- Legend to Figure 2: change “…ratios determined using …” to “ratios were determined using …”

- Figure 2: The indicated threshold of 0.0155 is not called “Ct”, but just the threshold. Please, adapt.

- Figure 2: In the legend, “A” and “B” are indicated as amplification plots for RKO and serial dilution of fully methylated DNA, respectively, but swapped in the figure. Please, adapt.


Discretionary Revisions

- Introduction: “…, whereas inhibition of TYMS…, becoming therefore less toxic” is difficult to follow. Please rephrase.

- Abstract: “In this study …from 29 of those patients”. The sentence is too long and not nice to read due to the brackets. I would create two sentences and leave out the brackets.

- The terms “high-grade toxicity” and “severe toxicity” are both used in the manuscript. If the authors mean the same with these terms, it would be nicer to just use one of them.

Level of interest: An article of limited interest

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’