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: Zdenek Kleibl

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This study primarily aims at characterization of DPYD promoter methylation and LGRs in DPYD gene in 45 cancer patients suffering from high toxicity following 5FU treatment. The secondary involved immunoexpression of DPD and TYMS in paired tumor/normal tissue of subgroup of analyzed patients (29 individuals). Characterization of (epi)genetic factors contributing to the development of 5FU toxicity belongs to the major concern in current clinical oncology research and despite the numerous articles published on this topic, the unambiguous evidence supporting or rejecting the routine use of genetic tests of DPYD gene (perhaps except to the analysis of IVS14+1G>A) is the task for the future. In general, the manuscript adds some valuable data to this growing evidence.

Major Compulsory Revisions:

1) Patient samples.

There are several unclarities/inconsistencies regarding to the samples analyzed population:

The method of analysis of IVS14+1G>A is not specified in Methods section. The notices could be found only in paragraph 3.2. (“…previously been detected in routine analysis; …previously identified through direct sequencing.”) and in Discussion (p11; “All patients enrolled in this study were screened for DPYD exon 14 mutations by sequencing…”). Is this “routine” analysis of exon 14 included in institutional or national guidelines?

The characteristics of patients and their toxicities (incl. Tab.1) based on which they were enrolled into the study could be included in Methods instead of Results.

The sequencing of exon 14 prior MLPA analysis is dispensable as the MLPA DPYD kit contains the specific probe for IVS14+1G>A and the effect of 1845G>T (E615D) is highly questionable (in HGMD dbf is considered to be probably a neutral alteration). Moreover, the authors did not correlate the mutation status with toxicity or results of immunohistochemistry. What was the toxicity of patients with IVS14+1G>A (and 1845G>T) compared to the other toxicities?

2) qPCR analysis for methylation quantification:

The procedure is described insufficiently. The authors stated (p6; paragraph
2.5.): “The chemically modified DNA from the RKO cell line, peripheral blood and microdissected tumor tissue was amplified through quantitative methylation-specific PCR (QMSP)[24]”. The citation 24 (Olek et al) refers about the use of modified bisulphite treatment of analyzed DNA prior the standard PCR analysis, not about qPCR. The amount of isolated/modified DNA, especially in case of microdissected samples, should be also described. Do authors really used bisulphite modification on agarose beads?

Alongside the primer sequences for methylation-specific qPCR analysis of DPYD promoter the positions of primers in gene, length or PCR product, and the coverage of DPYD promoter should be noted.

The conditions for qPCR on ABI7500 are not described in MS. Also, there is no description of which serial dilutions (2x, 10x, other) were used for calibrations. Moreover, the Fig 2 does not illustrate the result of methylation specific qPCR. I guess, that “A” represent the serial dilutions and “B” methylated promoter in RKO cells (Fig. 2 and Legend p16)? The Fig2 shows the optimization procedure without any analyzed sample of patients and, therefore, it should be redesigned and could be transferred to the supplementary data for illustrative purposes only optimally together with some representative sequencing chromatograms of amplicons from methylated/unmethylated DNA.

The quantification protocol calculating the relative quantification using ##Ct using ACTB should be also specified; at least with given amplification efficiencies of both ACTB and DPYD PCRs. Instead of useless Fig 2, some data (e.g. table) representing the results of qPCR analysis in patients should be presented, especially when authors themselves wrote (p.7): “The ratio thus generated, which constitutes an index of the percentage of input copies of DNA that are fully methylated at the primer site, was then multiplied by 1000 for easier tabulation (methylation level =target gene/reference gene × 1000)”

Finally, the use of qPCR approach for analysis of DPYD promoter methylation that is not present in any of analyzed cancer patient seems to me disputable and without significant improvement compared to the classical end-point methylation-specific PCR. Based on presented (negative) qPCR results (that are not shown for patients in the MS) the statement: “… our study …was also the first to use a quantitative methylation-specific PCR approach, which is more specific and sensitive than conventional methylation-specific PCR.” seems to me too ambitious. How authors assessed specificity and sensitivity of methylation specific qPCR analysis?

3) Immunohistochemistry:

In general, I agree that DPD and TYMS expression could be potentially used as predictive factor for 5FU efficacy or prognostic factor in 5FU treated patients, however, I found no evidence for the introductory sentence (Introduction, p.3): “The expression levels of DPD and TYMS … may be related to different toxicity profiles”. This statement that intratumoral expression is related to site-related toxicity is also not supported by referenced papers (in Discussion p12):“Indeed, lower DPD and TYMS expression have been associated with higher toxicity[3, 11]” How the expression of DPD/TYMS in GI cancer cells and surrounding tissue
could influence e.g. hematological toxicity or hand-foot syndrome according to the authors? Moreover, despite performed immunohistochemical analysis in 29 patients (or 30? – p.7?), this hypothesis is not commented in the results/discussion. Why authors correlated the immunohistochemical data only with clinic-pathological variables (Tab. 2) but no with site-related toxicities?

The characteristics (tumor, toxicity, gender,...) of 29 patients analyzed by immunohistochemistry should be included. What was the difference in DPD or TYMS expression in normal epithelium from different patients and different GI tissues?

Similarly to the above mentioned Fig 2, I suppose that the information value of Fig 4 is low and this figure could be listed in Supplementary material showing the evaluation of immunohistochemical analysis. Instead of this, the data representing the results of immunohistochemical analysis of DPD and TYMS classified according to the scoring system (0-3+) described in Method section (p. 8) are highly desirable and must be presented. If the authors stated that “immunoexpression was decreased in XY%...” it is not evident whether this decrease was scored as 0 or 1+. The authors should also comment that any patient exerted increased expression of DPD and TYMS. What was the proportion of patients showing reduced DPD expression together with TYMS expression?

Minor Essential Revisions
1) Abstract: Decreased immunoexpression of DPD and TYMS was found in 59% and 11% of the carcinomas, respectively. (should be 59% and 37% - according to the Results).
2) Paragraph in 3.2 following second sentence would be better.
3) The paragraph 3.2. should be revised to be clear in which/how many patients were analyzed by MLPA.

Taken together, I assume the despite its value, the manuscript should be carefully revised. The key endpoints are negative results of LGRs performed by MLPA and lack of DPYD promoter methylation assessed by methylation-specific PCR. Despite this findings were already published, the data are rare and a new data from independent studies are desirable. Therefore, this part of manuscript could be considered as a part that is of scientific value and could contribute to the growing evidence necessary for future evaluation of different strategies for toxicity prediction based on DPYD/DPD analysis in 5FU-treated patients in clinical settings. If the authors will not provide clear rationale for their immunohistochemical analyses, together with correct demonstration of immunohistochemical results and their evaluation with different toxicities, I assume that the data should not be presented in this the manuscript.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interests.