Author's response to reviews

Title: Genotyping Panel for Assessing Response to Cancer Chemotherapy

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Author's response to reviews: see over
Dear Dr. Edmunds

Thank you for forwarding the comments of the reviewers on our manuscript, entitled “Genotyping Panel for Assessing Cancer Risk and Response to Chemotherapy,” by Dai et al. (2666304591667989). We are grateful to the reviewers for their valuable comments and suggestions, and have revised the manuscript accordingly. Specific responses are explained in detail below.

Response to reviewer Dr. Federico Innocenti:

1) The main purpose of this study is not entirely clear. It seems that several are the aims of this study, including 1) to create a platform suitable to genotype several variants, 2) to select variants that play a role in cancer pharmacogenetics and cancer risk from existing published data, 3) to identify variants that are associated with cancer risk (as a pilot study, as stated at page 20), or a combination of 1), 2), 3). The aim(s) should be clearly specified.

We have added the following sentence to the abstract, “The purpose of the current study is to establish a flexible, cost-effective, high-throughput genotyping platform for candidate genes involved in chemoresistance and –sensitivity, and treatment outcomes.” Many of the genes involved in cancer biology are also likely candidates for affecting treatment outcomes. This is the main purpose of the genotyping panels.

2) The inclusion of the genotyping data from 90 colorectal cancer patients and 39 phase I patients does not seem to provide useful information, for several reasons. First, this study is not designed to identify cancer risk genes, because is not prospectively designed, there is not a matched control arm and there is no control for variables of patient characteristics and environmental factors (diet, smoking etc.), which have not been reported in this study. Hence, the comparison of allele frequencies from SNPs reported in the CEPH HapMap (and other databases) should be removed from this manuscript.

We agree with the referee that the use of historical controls is problematic because population stratification can be inadvertently introduced. However, this is difficult to avoid unless a suitable control sample has been carefully assembled, but this is not the case. Because our genotyping panels are geared towards assessing treatment outcomes, we anticipate that response/no response/toxicity will be the clinical outcomes to be assessed. The number of subjects here is too small to achieve this purpose; rather we wanted to establish the genotyping platform with an initial cohort upon which we can build further studies. To compare genotypes to controls, we have nevertheless used the CEPH collection as appropriate as a first step, to guide further exploration. We clearly state that any association results with this historical control need to be further validated.
We expect that in the future more and more historical control collections will become available that can be used in this sense. We prefer to leave the results in the manuscript, but would be prepared to remove them if this is the editorial decision. We have modified the text to clarify our interpretation further, as follows on page 19.

“Since we did not have access to a control population specifically designed to match the colon cancer patients in this study, we compared genotype frequencies to historical controls, as provided by HapMap and further databases. While this approach incurs the risk of population stratification, it nevertheless provides a first pass at identifying potential risk factors, in particular if more than one database supports potential association with colon cancer.”

In addition, the genotyping information from 39 phase I patients treated with flavopiridol does not add useful information if the genotype-phenotypic association data will be published in a different publication (as stated at page 19). Instead of germline DNA collected from cancer patients, this platform should have been validated using unrelated HapMap samples. In this study, the use of germline samples from cancer patients serves purely to the purpose of determining the success rate of genotyping.

The reviewer is correct. “In this study, the use of germline samples from cancer patients serves purely to the purpose of determining the success rate of genotyping.”

The purpose of the report is indeed to establish the genotyping platform and to provide a first cohort with genotyping results that we hope to expand in the future for biological and pharmacological studies. The flavopiridol genotyping study did yield several interesting associations with pharmacokinetic parameters; however, these can only be published once the inferred mechanisms have been validated by in vitro studies. While the SNPlex platform has already been validated by Applied Biosystem (see reference #18) to have high precision, our goal was to test this approach for the relevant SNP panels used here. We modified the following sentence on page 6 to include the information. “To establish a flexible, cost-effective, high-throughput genotyping method, we adapted SNPlex genotyping established and systemically validated by Applied Biosystems to have high precision [18, 19].”

To test whether our panels and lab environment can produce similar results, we have selected three SNPs and confirmed the results by SNaPshot assay. Our results indicate the same precision rate as reported by Applied Biosystems. Using germline DNA samples from CLL patients and from colorectal cancer patients validated the applicability of our SNPlex panels to clinical samples.

3). Hardy-Weinberg equilibrium should be analyzed for each SNP.

Hardy-Weinberg equilibrium may not be accurately assessed by the small sample size as in this study. Nevertheless, we have analyzed each SNP for Hardy-Weinberg equilibrium, and found over 90% of SNPs do not deviate from Hardy-Weinberg’s distribution. The results have been added to page 19.
Were positive control samples used for the alleles not included in the SNPlex?

The authors are not sure what “positive control samples used for the alleles not included in the SNPlex” means. We did run both positive and no DNA template controls for the SNPlex. The following sentence on page 10 was modified. “SNPlex genotyping was carried out according to the manufacturer’s suggested protocol with slight modifications to accommodate a manual procedure using 96-well plate (90 testing DNAs plus positive and no DNA template controls, and 4 wells for allelic ladders).”

4). In addition to the criteria used by the authors, a htSNP selection from the HapMap data should have been used from the 3 ethnic groups (taking into account potential overlaps among SNPs in different ethnicity). The selection criteria in this paper do not utilize a haplotype-based approach, and this should be highlighted as a limitation of the study in the discussion. A combined approach of htSNPs and individual variants that are shown to be functional (at in vitro and/or clinical level) and not tagged by the htSNPs would accurately capture all important information from these genes.

We have added a statement to this effect on page 8. “The genotyping panels have not been geared primarily to cover all main haplotypes for each gene, but rather to focus on functional SNPs as much as they are known. The purpose therefore is not primarily the discovery of new functional polymorphisms, but rather the assessment of the clinical impact of known ones. We anticipate that in the future we will be able to focus the genotyping panels even more on known functional SNPs, in an effort to develop clinically relevant biomarker panels. The approach takes into consideration that new candidate genes and polymorphisms continue to emerge [20] that need to be flexibly included in the genotyping panels.”

5). For the selection of candidate SNPs, more information should be provided. For example, did the authors only choose studies that were independently replicated?

Added to page 8. “We chose candidate SNPs that for the most part have been implicated in cancer biology and chemotherapy in more than one study.”

Why the authors did not take advantage of the candidate genes from genome-wide studies of cancer risk?

We think that the evidence for such genes was insufficient at this point to include with our panels and could be easily added to our panels if necessary since the panels are flexible and expandable.

Concerning the SNPs selected from dbSNP, were they validated?
Added to page 9. “SNPs from dbSNP were frequent and fully validated by different research projects, such as HapMap project [22] and the NCI SNP500Cancer project [23].”

**Which transporter databases were used (PharmGKB is not a database for transporters)?**

We did not systematically cover all membrane transporters but only those already implicated in chemotherapy response. We added the following website to the manuscript on page 9, the human membrane transporter database [24]. PharmGKB dose contain the relevant SNPs for this purpose, in addition to the extant literature.

**How redundant SNPs (due the LD) were excluded before the inclusion in the final list?**

Added to page 9. “SNPs in high LD (>0.7) with another SNP already in the panel were generally excluded, although in some case we added such SNPs for the assays design, to assure that either one was represented in the panel design.”

6) The data in Fig. 3 and the discussion from page 22 to 26 are suitable for a nice review paper, but not for the purpose of this publication.

We agree with this statement and have discussed the issue before the manuscript’s submission. Since the manuscript include a large number of genes and SNPs, and the purpose is to generate a clinically useful genotyping panel, we feel that this discussion is useful for readers that are not familiar with pharmacogenetics/pharmacogenomics. We provide background information for some well studied SNPs detected using the methods established in the current study. We are willing to remove the information if the editor so decides, but prefer to leave this survey in the Discussion.

**Response to reviewer Dr. Michael Gottesman:**

1. Supplemental table 1: Numerous SNPs failed to be detected by the SNPlex system for some technical reasons partially explained in Materials and Methods, in the SNPlex pools and reagents section. While some of the results obtained from the SNPlex platform were validated using other methods such as SNaPshot or PCR, few SNPs that failed to be detected by the adapted SNPlex method were studied using these methods. What is the reason for this choice? Is it related to a technical issue or their clinical irrelevance, which was previously demonstrated? Is their detection possible using these additional approaches? To be clear, it should also be mentioned in the table what SNPs that appear in bold type can be detected by alternative methods and which methods.
In some of the cases where one SNP failed, we had included additional SNPs in high LD as surrogates. In other cases we did not establish alternative methods as the genotyping panels were not designed to be comprehensive; alternative methods were established only where a SNP had known functional relevance. CYP2D6 is an example that failed SNPlex design because of genomic reasons (high homology to other genes and pseudogenes). The information for “SNPs that appear in bold type can be detected by alternative methods” has been added to the Supplemental Table 1. In addition, please see explanation in comment No. 2.

2. There is a discrepancy between the Materials and Methods section and the Results that brings confusion. In the M&M, the authors report that they used the SNaPshot approach to detect 3 SNPs analyzed using their SNPlex method. The authors should also mention that they used this alternative method and others as well to detect polymorphisms that failed to be detected by their platform.

On page 16, after the following sentence in the original manuscript, “Any polymorphisms that could not be included with the SNPlex panels were omitted, or if thought to be critical, targeted by alternative methods.” we added the following information, “For example, a majority of the SNPs that are not suitable for SNPlex genotyping can be genotyped by multiplexed SNaPshot assay (see multiplexed SNaPshot for CYP2D6 in this manuscript as an example). Similarly, small insertions/deletions and repeats can be amplified by PCR and the variants determined by PCR product size difference based on gel electrophoresis or capillary electrophoresis using fluorescent-labeled primers (see UGT1A1 promoter dinucleotide repeat polymorphism in this manuscript). Based on the sequence information and literature search, possible alternative methods for detection of these genetic variants are listed in Supplemental Table 1.”

3. The selection of genes was careful and well established. However, the number of selected uptake transporters, important actors in drug response, is extremely low. Is this choice related to the current lack of knowledge or scientifically unrelated?

We did not systematically cover all membrane transporters, but only these already implicated in cancer chemotherapy response. Because of our genotyping platform is flexible and expandable, new SNPs found to be important later on can be easily added to the panels.

We have added the following information to Materials and Methods section on page 8. “The main focus of the current study was to include known functional polymorphisms candidate genes based on available literature. The genotyping panels have not been geared primarily to cover all main haplotypes for each gene, but rather to focus on functional SNPs as much as they are known. The purpose therefore is not primarily the discovery of new functional polymorphisms, but rather the assessment of the clinical impact of known ones. We anticipate that in the future we will be able to focus the genotyping panels even more on known functional SNPs, in an effort to develop
clinically relevant biomarker panels. The approach takes into consideration that new candidate genes and polymorphisms continue to emerge [20] that need to be flexibly included in the genotyping panels.”

We would like to thank the reviewers for their thoughtful comments. None of those comments seem to have reflected doubts about the basic significance or validity of the study, but they have helped us substantially to strengthen the manuscript. We hope that you will now consider the paper fully acceptable for publication in Journal of BMC Medical Genomics. We look forward to hearing your decision.

Sincerely,

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