Reviewer's report

**Title:** Prevalence and Spectrum of MLH1, MSH2, and MSH6 Pathogenic Germline Variants in Pakistani Colorectal Cancer Patients

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**Reviewer:** Mev Domínguez Valentin

**Reviewer's report:**

The manuscript entitled "Prevalence and Spectrum of MLH1, MSH2, and MSH6 Germline Mutations in Pakistani Colorectal Cancer (CRC) Patients" described the frequency and spectrum of pathogenic variants in MLH1, MSH2 and MSH6 gene in Pakistani HNPCC population. The identification of Lynch Syndrome patients is a major issue, because morbidity and mortality from CRC and extra-colonic cancers in these patients (and their relatives) can be decreased by early screening and intensive surveillance.

In this study, the selection criteria included a less stringent criteria of suspected-HNPCC including a family history of only two HNPCC-linked cancers.

**General comments**

- The term hereditary nonpolyposis colorectal cancer (HNPCC) was coined to distinguish familial aggregation of CRC from the polyposis phenotypes. The HNPCC subset of CRC families is heterogeneous and broadly consists of the 4% linked to LS (which may or may not fulfill the Amsterdam Criteria), <1% with a Lynch-like syndrome, and 2-4% classified as familial colorectal cancer type X (FCCTX).

- It would be more appropriate to use the term LS, when a defective mismatch repair (MMR) system due to the presence of pathogenic variants in at least one of the MMR genes (path_MLH1, path_MSH2, path_MSH6 and path_PMS2) or due to deletions of the 3' portion of the EPCAM gene is the cause.

- It would have been more appropriate to use the term "variant" throughout the manuscript, which is in line with the standards and guidelines for the interpretation of sequence variants [e.g. Richards et al, 2015]. The term "variant" should replace the term "mutation" or "polymorphism" with the following modifiers: (i) pathogenic, (ii) likely pathogenic, (iii) uncertain significance, (iv) likely benign, or (v) benign.

- Genetic analysis have not included PMS2 gene, which can be briefly mentioned.

**Specific comments**

- Introduction

  Include the general comments

- Methods
*Molecular analysis: The results from DHPLC, have been validated by another method?

*In silico analyses: VUS are relatively common, and complicate the interpretation of gene test results. There is a strong need to classify these VUS as either benign or deleterious. The use of bioinformatics tools combined with novel functional assays will be important for the interpretation of next generation sequencing analyzes of high numbers of genes, and the diagnostic assessment of cancer associated genetic variants

The links (Align_GVGD, SNAP, MaxEntScan, NNSPLICE) are not working.

* A section about the classification of the genetic variants found in the study (e.g. 5 tier-classification, etc) should be included.

- Results

* MSH2 germline mutations: Cannot rule out that the nonsense MSH2 variant is unique to the Pakistani population. Need to confirm/validate in a larger number of cases.

* Suggestion to change the headline MLH1, MSH2 and MSH6 sequence variants to Novel and VUS MMR variants. VUS need to be previously defined.

- Discussion

In general, the results presented in the present study highlight the challenge associated with using family history for detecting families with pathogenic MMR variant. There is a recommendation on the application of population-based screening protocols for all CRC and endometrial cancers diagnosed below age 70 using IHC of the MMR proteins. Nonetheless, patients with a young age of onset and/or a positive family history of LS-associated cancers without an identified pathogenic MMR variant, may suggest the involvement of pathogenic variants in as yet undiscovered genes.

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