Author's response to reviews

Title: Breakpoint characterization of a novel large intragenic deletion of MUTYH detected in a MAP patient: Case report

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

Thank you very much for your message, and for sending us the positive criticisms raised by the referees of our manuscript “Breakpoint characterization of a novel large intragenic deletion of MUTYH detected in a MAP patient: Case report” (MS: 1029321632571047). The new version of the manuscript was fully revised, and hopefully will meet your expectations after the clarification of some aspects and the incorporation of all points raised by the referees.

In this letter you will find a point-by-point reply to all reviewers questions, as well as the paragraphs that have been modified in the new version, which has been uploaded with 'tracked changes' of all the corrections, following the instructions of BMC Medical Genetics.

On behalf of my colleagues, I want to thank you and hope that the current version is now suitable for publication in the BMC Medical Genetics.

We appreciate your attention and time.

Sincerely yours,

[Signature]

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Reviewer 1 – Dr. ASTRID OUT:

1. In order to find more patients with the large MUTYH deletion, the authors have screened a relatively small group of MMR gene mutation negative Lynch syndrome patients and controls, without detecting the MUTYH deletion. Overall, MUTYH mutations are rare in Lynch syndrome patients, supporting the fact that the deletion was not found in the group of 50 patients (see e.g. Riegert-Johnson et al. 2007, Stormorken et al. 2006). It would be more interesting to screen a large group of (APC-mutation negative) polyposis patients. The reviewer suggests discussing this in the paper. How many polyposis patients were screened before this deletion was found in patient FAP15?

We thank Dr. Out for the careful analysis of our manuscript. We agree that MUTYH mutations are rare in Lynch syndrome patients. However, MAP has a highly variable clinical picture and descriptions of MAP patients presenting a phenotypic overlap with Lynch syndrome have been made. This becomes even more important in our sample, since our clinically suspected Lynch patients fulfilled not only the Amsterdam I/II but also the Bethesda criteria, which includes patients diagnosed with colorectal cancer under the age of 50 years without family history. In addition, we have also screened for the presence of this MUTYH deletion in other polyposis patients: three APC/MUTYH-mutation negative and 18 APC/MUTYH-mutation positive. In the second paragraph of the case presentation section (page 3) of the revised manuscript we have included these polyposis patients and have better defined our Lynch syndrome group.

2a. The authors explain a plausible mechanism by which the mutation might have occurred. However, the question remains when it has occurred. It might be a new variant, but it seems not unlikely that the homozygous variant in this patient (without consanguineous parents), results from inheriting two alleles of a founder mutation in the population. Another possibility could be a recombination event in the zygote, resulting in the biallelic state. Unfortunately no DNA analysis was possible for the parents and the affected sibling. It might be interesting to discuss these possibilities in the paper.

2b. It has been shown that the MUTYH gene variation spectrum differs among ethnicities. Until now most described MUTYH associated polyposis patients are of European descent, in which this deletion has not yet been found. The described variant might be a founder variant in the Brazilian population, perhaps in a subpopulation of non-European descent. This might mean that this variant also causes polyposis in descendants of the same population in other areas of the world. The primers used for MUTYH mutation screening in the Netherlands should have detected this deletion in homozygous as well as heterozygous state, but until now the mutation has not been found there (by using sequencing amplicons for exons 1, 2, 3-5, 6-8, 9-11, 12-13, 14, 15 and 16, as described by Nielsen et al. 2005). Incompletely screened polyposis patient groups might be interesting to be tested for the large deletion.
We have included this important discussion points suggested by the referee in the last sentence of paragraph 4th of the “Identification and characterization of the deletion” section (page 5). In this discussion, we take into consideration a recently published manuscript that describes this same deletion in a European patient (ROULEAU et al. 2011) and we discuss the possible origins and implications of this mutation as follows: “One possible explanation for the homozygous occurrence of the deletion in this patient, without consanguineous parents, is the inheritance of two alleles of a founder mutation in the population. The proband’s great-grandparents were Portuguese, therefore she had Caucasian ancestry. In this sense, very recently this same deletion was found in a European MAP patient in compound heterozygosity with the common MUTYH Caucasian mutation p.Gly396Asp [10]. The description of the same rearrangement in patients from different geographic regions is a very exciting discovery and additional studies to uncover the origin and frequency of this mutation would be of great value. Another potential explanation for the homozygosity is the occurrence of a recombination event in the zygote, resulting in a biallelic state, though the existence of an affected sibling does not support this hypothesis.”

**Minor Essential Revisions: (small adaptations to the text)**

All following “minor essential revisions” (small adaptations to the text and spelling errors) from 1-10 suggested by referee 1 were accepted and the corrections were performed at the corresponding paragraph.

1. **Background, First paragraph:**
   1a: write “MutY” instead of “Mut Y”? Corrected as requested.
   1b: write “G:C to T:A transversions” instead of “G: C to T:A transversion”? Corrected as requested.

2. **Background, Second paragraph:** The last sentence “Mutations … APC 30% [7].” might suggest that MUTYH accounts for a larger proportion of polyposis patients than APC.

   This statement was rephrased to: “Mutations in MUTYH account for approximately 40% of patients with 10-100 colorectal adenomas (attenuated FAP patients) and positive familial history, a proportion slightly higher than that of APC mutations in these patients (30%) [7].”

3. **Background, Third paragraph:**
   3a: “most pathogenic variants are missense … truncating mutations.” Suggestion to change this part of the sentence to e.g.: “most pathogenic variants are missense variants and only a minority consists of splice site and truncating variants.”

   This statement was rephrased as suggested by the referee.
   3b: Although frequently called hotspots, p.Tyr179Cys and p.Gly396Asp are very likely to be founder variants (see e.g. ref 5 in the paper: Nielsen et al. 2010).

   This paragraph was changed to: “The two hotspot mutations p.Tyr179Cys (exon 7) and p.Gly396Asp (exon 13) are prevalent in populations of European origin,
probably due to a founder effect, and account for approximately 80% of all reported mutant alleles [5,8]."

4. Case presentation, First paragraph: Starts with “A Brazilian female patient...” but later mentions “the patient stated that he...”. Corrected to “the patient stated that she...”

5. Identification and characterization of the deletion, First paragraph: “Eleven primers pairs” should be “Eleven primer pairs”. Corrected as requested.

6. Identification and characterization of the deletion, Second paragraph: Why is “constitutive” in Italic? Corrected to “constitutive”.

7. Identification and characterization of the deletion, Third paragraph: “the deletion at 5’ of...” change into “the deletion at the 5’ end of...”? Corrected as requested.

8. Identification and characterization of the deletion, Fifth paragraph: “repetitive element is present on...” change into “repetitive element is present at” or “repetitive element is present near”? Corrected to “repetitive element is present at”.

9. Identification and characterization of the deletion, Sixth paragraph: 9a: adapt the mutation nomenclature in accordance with the LOVD submission, c.348+33_*64+146del4285insTA or c.348+33_*64+146delinsTA. Corrected to c.348+33_*64+146del4285insTA. 9b: instead of “as a 2-nt” better use “as a 2-bp”? Corrected as requested.

10. Identification and characterization of the deletion, Seventh paragraph: mention that the AluY insertion is a polymorphic insertion? Corrected to “...a recent study has shown that a polymorphic AluY insertion in intron 15 when in a homozygous state..”.

Reviewer 2 – Dr. ROSSELLA TRICARICO:

Abstract
1. The statement “Most MUTYH pathogenic variants are missense mutations, and currently, no gross genomic deletions have been described. Case Presentation: We have identified the first large deletion in the MUTYH gene: a >4.2 kb deletion encompassing exons 4-16...” is not correct because the c.348+33_*64+146del4285 deletion has already been identified in a MAP patient (Rouleau et al., Clin Genet. 2011Sep;80(3):301-3). This data should be presented, and the implications of finding the same rearrangement in (presumably) different ethnic groups should be discussed.

We thank Dr. Tricarico for carefully evaluating our manuscript.

During the revision period of our manuscript, we were very surprised by the publication of Rouleau et al (Clin Genet. 2011Sep;80(3):301-3), first published online on August 5th. The referred manuscript reported for the first time the large MUTYH deletion c.[348+33_*64+146delinsTA]. This mutation is the same variant described in our manuscript. Our group has submitted this deletion to the LOVD mutation
database, where it has been publicly available since May 12\textsuperscript{th} (http://chromium.liacs.nl/LOVD2/colon_cancer/variants.php?select_db=MUTYH\&action=view&view=1012041\%2C0002450\%2C10). We have now updated two sentences of our manuscript to include the description and discuss this freshly finding, as follows:

“During the composition of this manuscript, an independent study performed by a French group identified this same rearrangement in one of their polyposis patients [10]. This deletion is the first report of a large intragenic $MUTYH$ rearrangement.” – Fourth paragraph, page 3.

“In this sense, very recently this same deletion was found in a European MAP patient in compound heterozygosity with the common $MUTYH$ Caucasian mutation p.Gly396Asp [10].” – Second paragraph, page 5.

\textbf{Background:}

1. The statement in the second paragraph “Most biallelic $MUTYH$ mutation carriers have between ten and several hundred polyps, usually with later onset compared to FAP patients” is incomplete because the clinical presentation of MAP also includes a number of patients with early-onset CRC and none or few polyps (>10) (Nielsen et al., Crit Rev Oncol Hematol 2010, 1-16.). Please refer to this data.

The requested information was included in the second paragraph of the background section and the corrected version is the following: “Most biallelic $MUTYH$ mutation carriers have between ten and several hundred polyps, usually with later onset compared to FAP patients [1,6]. Also, a number of MAP patients with CRC and no polyps have been reported [5].”

2. Please refer to Rouleau et al., Clin Genet. 2011Sep;80(3):301-3 (Fourth paragraph, last sentence).

We have rephrased our last paragraph of the background to include the recently published manuscript, as follows:

Page 3: “Here, we report a case study in which we have characterized a large $MUTYH$ deletion in a MAP patient. During the composition of this manuscript, an independent study performed by a French group identified this same rearrangement in one of their polyposis patients [10]. This deletion is the first report of a large intragenic $MUTYH$ rearrangement. We have identified this novel >4.2 kb deletion and refined the breakpoints to the base pair level. Based on the analysis of the sequences at breakpoints, we suggest a possible mechanism of origin for this alteration. Presence of this deletion was also analyzed in familial colorectal patients and a control group.”

\textbf{Case Presentation:}

1. The case report should contain a detailed description of the patient including more detailed information on ethnic origin. More detailed information should also be provided on clinical and family history, tumour histology, presence of extracolonic manifestations, any other tests that were carried out (e.g. APC screening), phenotype
We have extended the description of ethnic origin and phenotype of the patient and included the family pedigree as figure 1. The numbers of the other 2 figures of the manuscript were updated accordingly. The reviewed version of “Case presentation” (Pages 3 and 4) is the following:

“A Brazilian female patient (FAP15) from the Hereditary Colorectal Cancer Registry of Hospital AC Camargo (São Paulo, Brazil), who was clinically suspected for MAP, was screened for mutations in the *MUTYH* gene by direct sequencing. The age at onset of the attenuated polyposis (approximately 40 polyps) was 42 years and of the rectal cancer was 44 years. No extracolonic manifestations were observed in this patient. Family history was accessed through personal report. The patient stated that she had unaffected deceased parents, four unscreened siblings (without colonoscopy) and one affected sister from whom biological material was unavailable due to residence distance (figure 1). The affected sister presented with attenuated polyposis at the age of 44 years. The proband had a Caucasian ancestry, since her great-grandparents were Portuguese.”

A legend for the incorporated Figure 1 was included: “Family tree of patient FAP15. The proband is indicated by the arrow. The proband’s age at onset of the polyposis (42 years) and of the rectal cancer (44 years) are indicated. The patient presented unaffected deceased parents, four unscreened (without colonoscopy) siblings and one affected sister (figure 1). The affected sister presented with polyposis at the age of 44 years. The Caucasian patient stated having Portuguese great-grandparents and no report of inbreeding in her family.”

As the patient presented attenuated polyposis, we started the mutation screening by the *MUTYH* gene, hence *APC* screening was not performed in this patient.

2. The geographic/ethnic origin of the controls and of the 51 familial CRC patients should be the same as that of the proband, and should be specified.

All analyzed individuals in our study were Brazilian and this was clarified at the second paragraph of the case presentation section. Furthermore, during the revising period of the manuscript, we were able to screen 95 additional control individuals for this deletion and this final number of controls (111) was updated in the manuscript: “In addition, the presence of this *MUTYH* deletion was screened through PCR in other 183 Brazilian individuals: three *APC/MUTYH*-mutation negative and 18 *APC/MUTYH*-mutation positive polyposis patients; 51 clinically suspected Lynch syndrome patients (fulfilling Amsterdam II or Bethesda criteria), who were non-carriers of germline mutations in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2*; and 111 healthy controls” (second paragraph, page 4).

Identification and characterization of the deletion

1. Synthetic information about the primers set and condition used for the junction fragment sequencing analysis should be provided.
As requested, we included the sequence of the used primers and PCR conditions to amplify the junction fragment. This paragraph was rephrased to “Using an exon 2 forward primer (5’-GGTGAGAAGGGAGATTAG-3’) and reverse primers designed on the position of the A_14_P118536 undeleted probe and 600 bp downstream (primers 16R2: 5’-CTTCAGGTCTTCACCAAGTCC-3’ and 16R1: 5’-CTTCTCTGTGCCCTCTTC-3’, respectively), we successfully amplified the junction fragments for this deletion. As the primer 16R1 was located at an undeleted region and closest to the breakpoint, further analyses were performed only with this primer. The PCR reaction of the control DNA pool failed to amplify any detectable PCR product (theoretically 5889 bp in length), whereas PCR products of ≈ 1600 bp were detected using genomic DNA from the patient (Fig. 2E). Briefly, the PCR conditions consisted of 25 ng of genomic DNA, 0.3 µmol of each primer and 1X Platinum® PCR SuperMix High Fidelity (Invitrogen) in a 20 uL reaction. Cycling conditions entailed a preincubation at 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 62°C and extension at 72°C for 2 minutes.”

2. Please specify if a region of another gene is deleted downstream of the 3’end of the MUTYH gene.

The following statement was included at the end of the third paragraph (page 5) of this section: “No region of the adjacent HPDL gene is deleted downstream of the 3’ end of MUTYH.”

3. The authors should adhere to the Human Genome Variation Society (http://www.hgvs.org/rec.html) recommendations for the description of genomic deletions. These are some points to consider in this regard:

- The HGVS gene nomenclature is in italic character. Please check the name of the genes in abstract, background, first sentence and in case presentation, second paragraph, second and third sentence - The sequence deletion at genomic level as described as chrX:g.32,218,983_32,984,039del and using “del” after an indication of the first and last nucleotide(s) deleted. In addition, a combination of a deletion and insertion at the same site is described using the format c.112_117delinsTG. These designations should be used in the description at genomic and cDNA level of the variant “chr1: 45794768_45799052del4285” and “c.348+33_*64+146del4285” identified in the proband.
- The description of the homozygous changes in a recessive disease are the following: c.[change allele 1];[change allele 2] (last HGVS update August 4, 2011). It should be used in the genotype description of the proband (sixth paragraph, fourth sentence). Please, note that the chromosome build used should always be mentioned (e.g. NCBI Build 36.1 or UCSC Feb. 2009 (GRCh37/hg19) assembly). It should be added to Hg19 in Identification and characterization of the deletion, third paragraph, fourth sentence and Legends, figure 1, last sentence.

Regarding these considerations, the names of all cited genes were formatted in italic in the abstract, background (first sentence) and in case presentation (second paragraph). Also, the mutation nomenclature was checked and changed according to HGVS recommendations to chr1:g.45,794,768_45,799,052del4285insTA,
c.348+33_*64+146del4285insTA, third paragraph (page 5) of “Identification and characterization of the deletion” section. The genotype of the proband was changed to c.[348+33_*64+146del4285insTA];[348+33_*64+146del4285insTA] (sixth paragraph, fourth sentence). The correct description of the chromosome build was introduced in both, the cited paragraph and the legend.

4. In the identification and characterization of the deletion, eight paragraph, eleventh sentence, some references should be specified.

We have included the reference 22 (Batzer and Deininger, 2002) for this sentence.

5. The c.348+33_*64+146del deletion has already been identified in a MAP patient by qPCR-HRM and aCGH (Rouleau et al., Clin Genet. 2011Sep;80(3):301-3). This reference should be included and discussed.

This reference was included and discussed in two paragraphs: background fourth paragraph and Identification and characterization of the deletion fourth paragraph.

Legends
1. Figure 1, section G: The description is not clear. Please clarify and indicate what the light gray text indicates.

The legend to the previously Figure 1, currently Figure 2 was re-written as shown below:
“G: Nucleotide sequence around the deletion breakpoints and the deletion junction. Red larger nucleotides are the non-template insertion (filler DNA). Light gray nucleotides indicate the deleted sequence. Shaded nucleotides represent shared sequences between the breakpoints. Rearrangement-promoting elements GAG/GCS* are shown underlined. The gray arrow beneath the intergenic deleted sequence shows the location of the AluJr element.”

Figures
1. Figure 1, section D: Please indicate the location of the 16R2 primer.

For characterization of the breakpoint, we have design a reverse primer on the position of the A_14_P118536 undeleted (16R2) and a second reverse primer 600pb upstream this region, that we wasn’t aware if it would be in a deleted or undeleted region. After we observed that the primer 16R1 was located at an undeleted region and as it was closest to the breakpoint, we decided to use only with this primer for further analyses, since it gave a smaller amplicon. For this reason, the location of the primer 16R2 is not represented in Figure 1-D (now 2-D). We have clarified this issue better in the third paragraph of the section Identification and characterization of the deletion, as follows:
“Using an exon 2 forward primer (5'-GGTGAGAGGGAGGATAG-3’) and reverse primers designed on the position of the A_14_P118536 undeleted probe and 600 bp upstream (primers 16R2: 5'-CTTCAGGCCTTCCACCAAGTCC-3’ and 16R1: 5'-CTTCTCCTGCTGCTCCTCTC-3’, respectively), we successfully amplified the junction fragments for this deletion. After confirming that the primer 16R1 was located at an undeleted region and as it was closest to the breakpoint, further analyses were performed only with this primer.”

In addition, the following language corrections should be introduced:
- Background, First paragraph, third sentence: “Mut Y” should be replaced with “mutY”
- Background, Third paragraph, sixth sentence: “~” should be replaced with “approximately”
- Identification and characterization of the deletion, eighth paragraph, thirteenth sentence: “8-oxyG” should be replaced with “8-oxoG”
- Figure legends, figure 2, second sentence: “USCS” should be replaced with “UCSC”

All language mistakes appointed by Dr. Tricarico were corrected at the corresponding paragraphs.

After the introduction of two new references (Batzer and Deininger, 2002 and Rouleau et al, 2011) all the references were reorganized accordingly.