Author's response to reviews

Title: Novel SYBR-based duplex qPCR for the detection of gene dosage: Detection of an APC large deletion in a Familial adenomatous polyposis patient with unusual phenotype.

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Version: 3 Date: 21 May 2012

Author's response to reviews: see over
May 21th, 2012

Prof Maurizio Genuardi
Editor - BMC Medical Genetics

Ref: MS: 1628038326670125

Dear Dr. Genuardi

Thank you for your message, and for sending us the concerns of the referees about our manuscript “Novel SYBR-based duplex qPCR for the detection of gene dosage: Detection of an APC large deletion in a Familial adenomatous polyposis patient with unusual phenotype” (MS: 1628038326670125). The new version of the manuscript was fully revised addressing all points raised by the referees. Paragraphs that were subjected to major modifications appear underlined in the revised manuscript.

The Associate Editor’s comments that we “should specify that the method used in this study for the APC gene can only detect copy number alterations if these involve the exons corresponding to the probes used, so that partial deletions not spanning these parts of the gene would escape detection” was addressed in the Discussion, sixth paragraph (page 10): “Another important point to consider is that, similar to other amplicon-based gene dosage approaches, the duplex qPCR only assesses the copy number of the amplified region (90-220 bp), meaning that primers for each exon should be designed to screen the entire coding-sequence of a gene”. Also, the misspelling error pointed out in the abstract - results section, the first word, ”Though”, was corrected to ”Through”.

In addition, it was observed by the editorial office that the quality of the written English needed to be improved and that particular attention to the abstract was necessary. Prior to submitting the revised version of our manuscript to BMC Medical Genetics, we submitted it to a professional editing service - the “American Journal Experts”, and we hope the manuscript now meets the required quality of written English.

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

We appreciate your attention and time.

Sincerely yours,

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Reviewer 1 – Dr. ROSSELLA TRICARICO:

MAJOR ESSENTIAL REVISIONS:

1. Background, second paragraph, first sentence: The authors state that the main aim of the study is to characterize the genomic alteration in a FAP patient. However, the title and discussion deal mostly with technical aspects, that are not fully novel, while the characterization of the genomic alteration is marginally discussed.

   We thank Dr. Tricarico for the careful analysis of our manuscript. We agree that the aim of the study was not coherent with the title and most of the manuscript discussion. Therefore, we have revised that paragraph and the novel version stands as follows (Background, last paragraph, page 3): “The aim of this study was to develop a novel gene dosage method using SYBR Green to confirm a genomic alteration in a FAP patient. The technique is based on the coamplification, by duplex PCR, of the target and reference genes and the comparison of the peaks of the melting curve (PMc) ratio between patient and controls. This method was successfully validated and applied to identify a large genomic deletion encompassing the APC and 19 additional genes. Thus, this paper presents the detailed description of this novel gene dosage method, as well as evaluates the genotype-phenotype correlation of the APC-deleted patient”.

2. Methods: Somatic mosaicism has been described in some FAP patients (Aretz et al., 2007). It would be useful to provide information about the sensitivity (i.e. set up a serial dilution experiments) and the specificity of the method.

   In the light of this important point mentioned by the Reviewer, we performed a new experiment to investigate the sensitivity of this methodology. The description of this assay was included in Methods - SYBR-based duplex qPCR, last paragraph, page 6: “After confirming the APC deletion in this patient, the detection sensitivity of this method was assessed by mixing five different DNA proportions of the APC-deleted patient (P) and a healthy control (C). We used 18 ng of DNA in each reaction as follows: 1 P : 0 C (18 ng P : 0 ng C); 3 P : 1 C (13.5 ng P : 3.5 ng C); 1 P : 1 C (9 ng P : 9 ng C); 1 P : 3 C (3.5 ng P : 13.5 ng C); and 0 P : 1 C (0 ng P : 18 ng C). All mixtures were submitted to a duplex qPCR for the APC exon 15 as the target gene and GAPDH as the reference gene. Experiments were performed in duplicate and data obtained from the sample containing only DNA from the wild-type control was used to normalize the PMc ratios.”

   A novel figure was added to the manuscript (figure 3A and B) in order to present the results obtained from this assay. Also a paragraph was added to the Results (last paragraph, page 8) and Discussion (last paragraph, page 10) with the purpose of describing this novel data.

3. Discussion, third paragraph, eighth sentence: As reported in the abstract “This paper presents the description and validation of this novel gene dosage method, as well as discusses the phenotype of patients presenting with large genomic deletions encompassing the APC gene”. The discussion of the phenotype should be expanded
by adding information on developmental milestones and current performance. Has a
dysmorphologic evaluation been performed?

We have expanded the description of the patient’s phenotype regarding clinical
features (see Methods – first paragraph, page 3). Examination performed by the
gastroenterologist and genetic counselor did not identify any dysmorphic features or
severe mental impairment. Nevertheless, assessment of the developmental milestones
and dysmorphologic evaluation were not performed in this initial medical
appointment. We have tried to re-evaluate the index patient in order to perform more
comprehensive tests; however the patient is not being followed in this hospital any
longer. What we can infer from the medical records and clinical evaluation performed
by the responsible physician is that either major physical or mental impairment were
present in this patient, since he was a 40 years old man that have concluded high
school education at a regular school and lived abroad working as an electrical
technician for several years.

MINOR ESSENTIAL REVISIONS:

All “minor essential revisions” suggested by Referee 1 were accepted and the
corrections were performed at the corresponding paragraph.

Abstract
1. Methods, last sentence: “The reliability of the herein described qPCR method was
   validated for additional genes (HPRT1, ATM, PTEN and BRCA1)”: is not clear why
   (HPRT1, ATM, PTEN and BRCA1) are considered additional genes, since the test has
   been set up for all genes listed within brackets.
   Corrected to (page 2): “The reliability of the herein described qPCR method was
   validated for several genes (APC, HPRT1, ATM, PTEN and BRCA1)”.

Background
1. First paragraph, sixth sentence: a link to the LOVD website should be included.
   The link was included as requested (page 3).

2. First paragraph, third sentence: references 1 and 2 should be replaced with more
   recent ones.
   It was corrected as requested. Reference 1 - Gardner et al (1980) was replaced
   by Lipton & Tomlinson (2006); reference 2 – Leppert (1987) was replaced by Half et al

Methods
1. Samples, first paragraph, fourth sentence: Since the title refers to a case with
   unusual FAP phenotype, a more detailed description of the patient should be given.
   Please move here from results (fourth paragraph), discussion (second paragraph) and
   supplementary figure 1 legend, information about the clinical and family history of
   the patient. If available, provide more detailed data including the age at diagnosis of
   the polyps (are polyps and rectal cancer synchronous or metacronous?), the tumour
histology, the description of any treatment or intervention, the clinical history of all affected index patient’s relatives.

This first paragraph of Methods (page 3 and 4) was rephrased to: “The index patient (FAP02) was a Brazilian male with clinically suspected FAP identified in the Hereditary Colorectal Cancer Registry of AC Camargo Hospital (São Paulo, Brazil). His family history was accessed through the index patient report: his paternal grandmother, his father and one paternal uncle were affected with polyps and CRC at unknown ages, and one sister presented with polyps/CRC at the age of 44 that progressed to liver metastasis. The affected relatives were deceased; therefore, no biological material was available for mutation screening. One unaffected sister and one unaffected niece were available for genetic testing (Additional file 1, supplementary figure 1). The index patient was diagnosed with colorectal cancer (T1N0M0) at the age of 40, and harbored more than 100 synchronous adenomatous colorectal polyps, including duodenal and gastric polyps; no signs of other extracolonic manifestations commonly associated with FAP were observed. The patient was referred for total proctocolectomy with ileoanal pouch anastomosis. Examinations performed by a gastroenterologist and a genetic counselor did not identified any dysmorphic features or severe mental impairment. The patient completed his high school education at a regular school and lived abroad working as an electrical technician for several years”.

Since the index patient was the only affected member treated at AC Camargo Hospital, information of the other affected members was restricted to what was reported in the genetic counseling interview.

2. Samples, first paragraph, fourth sentence: Please move here information about healthy and mutated tested controls from “SYBR-based duplex PCR” section, fourth paragraph. In addition, describe also here the deletions and duplications of mutated controls used.

It was corrected as requested (page 4).

3. Samples, first paragraph, last sentence: Provide information about the initial concentration of samples used to set up and perform the experiments. The novel gene dosage method developed is a quantitative test, thus it should be specified that all samples (controls and patients) had the same initial concentration.

The following sentence was included in Samples, last paragraph, page 4: “The DNA concentration was verified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific – Waltham, MA, USA) and DNA samples were diluted to a concentration of 25 ng/µL. Immediately before the qPCR assay, the concentration of DNA samples was reassessed and they were diluted to a 3.6 ng/µL solution, from which 5 µL was used in each qPCR reaction (a total of 18 ng)”.

4. SYBR-based duplex PCR. It would be better to describe the set up experiments for HPRT1 and validation experiments for ATM, PTEN and BRCA1 before performing experiments for APC.

It was corrected as requested (page 5).

5. SYBR-based duplex PCR, first paragraph, last sentence: Are also healthy controls tested in duplicate?
Healthy controls were also tested in duplicate, and this information was included in SYBR-based duplex PCR, sixth paragraph, page 6.

6. SYBR-based duplex PCR, last paragraph: Please specify that male and female controls are used to validate the novel test and to identify the value ranges corresponding to wild-type, hemideleted and hemiduplicated samples.

The following phrase was included in SYBR-based duplex PCR, sixth paragraph, page 5: “Ratios between 0.9 and 1.1 were considered normal, ratios between 0.6 and 0.7 indicated deletions of one allele, and ratios above 1.4 indicated amplifications; ratios outside these intervals were considered inconclusive and repeated. Values were determined empirically based on the X chromosome dosage assay, target genes validation tests and sensibility assay described below. All the assays included three healthy controls and were performed in duplicate for patients and controls”.

7. aCGH experiments and data analysis, eight sentence: Please, include information about ADM-2 software (i.e. manufacturer etc).

The ADM-2 is a statistical algorithm available inside the Genomic Workbench software (Agilent Technologies - Santa Clara, USA). That information was included in the manuscript – Methods, aCGH experiments and data analysis, first paragraph, page 6.

Results

1. Results of set up and validation experiments should be described before APC experiments.

It was corrected as requested – Results, second and third paragraph, page 7.

2. First paragraph, first sentence: Healthy controls should be tested to verify the frequency of the novel missense variant.

As suggested by the referee, we screened 95 healthy controls for this missense variant, and the missense variant was not detected. This information was included in Methods, second paragraph, page 4 and Discussion, first paragraph, page 8.

3. First paragraph, third sentence: Please include link to of LOVD, dbSNP and 1000Genomes websites.

The suggested links were included as requested (page 7).

4. Second paragraph, third sentence: “The duplex qPCR showed PMc ratios of 0.67 and 0.63 for APC exons 2 and 15 respectively (figure 1B), revealing a haploidy for these APC exons in the FAP patient’s sample”. It is necessary to provide here information on the range of normal, deleted and duplicated PMc ratios (with the percentage of error) and validation of the test before asserting the presence of APC haploidy.

The following phrase was included in Methods, SYBR-based duplex PCR, sixth paragraph, page 5: “Ratios between 0.9 and 1.1 were considered normal, ratios between 0.6 and 0.7 indicated deletions of one allele, and ratios above 1.4 indicated amplifications; ratios outside these intervals were considered inconclusive and
repeated. Values were determined empirically based on the X chromosome dosage assay, target genes validation tests and sensibility assay described below.

5. Please report the APC sequence deletion at the genomic level as recommended by HGVS (http://www.hgvs.org/rec.html).

The corrected description of the deletion was included in Results, fourth paragraph, page 8: chr5:g.(107,755,923_107,818,559)_(113,079,145_113,113,875)del (UCSC Feb. 2009 – GRCh37/hg19).

Discussion
1. Second paragraph, last sentence: See comment above on healthy controls.

The following phrase was included in Discussion, first paragraph, page 8: “We examined the presence of this undescribed missense variant in 95 healthy controls, and the alteration was not detected in any control individual”.

2. Third paragraph, fourth sentence: Please, include link to Decipher Database website.

The link was included as requested (page 8).

3. Third paragraph, last sentence: The statement “In our study, the identified gross deletion encompasses the entire APC gene and 19 additional genes and is likely to have been present in this family for at least three generations, with a quite unusual phenotype of absence of mental impairment and dysmorphic features” should be followed by discussion (i.e. influences of modifier genes or endogenous or exogeneous factors etc). Further reports of 5q deletion encompassing APC should be discussed (Lindgren et al., 1992, Raedle et al. 2001). Please, refer to this data.

These references were included and discussed in two paragraphs (second, and third) of the Discussion section (page 8 and 9).

Legends
1. Supplementary Figure 1, It is not clear if subject II:1 has been tested and his genotype. Please, clarify. In addition, indicate what the symbol “?” means.

To clarify these questions, the legend of Supplementary Figure 1 was updated to: “Family pedigree of patient FAP02. The arrow indicates the index patient. All affected individuals presented both polyposis and colorectal cancer. The patient presented four affected deceased relatives: grandmother, uncle, father and one sister. One unaffected sister (III:4) and one unaffected niece (IV:1) were tested and neither the deletion nor the missense variant were detected. Genetic testing of the unaffected individual II:1 could not be performed. The symbol (?) indicates unknown cause of death in individuals II:3 and II:4”.

2. Supplementary Figure 2: “Supplementary figure 2A” should be “A”. The same correction should be done for “Supplementary figure 2B, 2C, 2E and 2F.”
The supplementary figure 2 was removed from the manuscript and the results from this validation assay appear now in **Results**, third paragraph, page 7:

**Figures**

1. **Figure 1B:** The peak heights of the melting curves of all genes tested (figure 2B and supplementary figure 2) show a reduction or increasing of target gene’s peak height for exon deletion or duplication, respectively. Please detail the melting curve peak heights observed in Figure 1B: why is reduction of APC peak heights not observed in patient FAP02?

   The melting peak heights of each gene represent the amount of amplicons generated for that gene in the end of the qPCR reaction. Several factors are responsible for determining the amplification yield of each target, e.g. size of the amplicon, primers efficiency, GC content, and others. Due to these factors, in a duplex PCR, one of the genes can be expected to be more efficiently amplified than the other, and for that reason one gene produced a higher melting peak than the other.

   Therefore, in order to determine the gene dosage with this new assay, we evaluated the ratio between target and reference genes of the patient in comparison with the ratio of a normal control. In the figure 1B submitted in the first version of our manuscript (previous figure 1B), although an “inversion” of the peaks heights is not observed as it is for other tested genes, the APC/GAPDH ratio is reduced in the patient (1.12) when compared to the control (1.77), resulting in a normalized ratio of 0.63 in the patient – which indicated the deletion. However, to avoid misunderstanding, we performed an additional duplex qPCR experiment using a different primer pair for the APC exon 15 that showed amplification efficiency similar to the GAPDH gene. The figure 1B now presents this result (updated figure 1B), depicting the “inversion” of the peaks heights.

2. **Supplementary Figure 1, section D:** Please add the minus symbol near subject III:4, and replace the plus symbol with minus near subject IV:1.

   Minus and plus symbols that appeared in this figure represented if individuals had been subjected to colonoscopy examination at AC Camargo Hospital or not. In order to avoid confusion with the disease status of the family members, this information was removed from the figure in the new version of the manuscript.
DISCRETIONARY REVISIONS

1. The paper would benefit from the following corrections in language:
- Background, fourth sentence and Methods, second paragraph, first sentence: “NM_000038” should be replaced with the current accession number “NM_000038.5”
- Results, first paragraph, fourth sentence: “lost” should be replaced with “loss”
- Results, third paragraph, fifth sentence and supplementary figure 2, last sentence: “Hg18” should be replaced with the current chromosome build (e.g. NCBI Build 36.1 or UCSC Feb. 2009 (GRCh37/hg19) assembly)
- Figure Captions, Figure 1; eight sentence: “hg18, Build36” should be replaced with the current chromosome build (e.g. NCBI Build 36.1 or UCSC Feb. 2009 (GRCh37/hg19) assembly)
- Supplementary Figure 1, first sentence: “family tree” should be replaced with “Pedigree”

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

Reviewer 2 – Dr. José Luis Soto

Major Compulsory Revisions
1. This assay requires that the reference and testing amplicons have significant differences between the melting temperatures to be analyzed in duplex PCR. This requirement should be noted in the discussion section with a recommendation in the minimum difference in the amplicon melting temperatures.

We thank Dr. Soto for carefully evaluating our manuscript.
We have now updated two sentences of our manuscript to include the description and discussion of this point suggested by Dr. Soto. In the Methods, SYBR-based duplex qPCR, first paragraph, page 5 we have included the following statement: “Two main principles were followed for designing the duplex qPCR primers, as follows:
1) The total length of the amplicon (including the primers) should be between 90 and 220 bases.
2) Amplicons for the target and reference genes should have a minimum of 5 °C of difference between their melting temperatures (Tm), such that a clear individualization of target and reference gene melting peaks can be achieved”.
In the Discussion, seventh paragraph, page 9 and 10 we have included: “It is important to notice that this method requires that target and reference gene amplicons display a minimum difference between the melting temperatures, such that a clear individualization of the target and the reference gene melting peaks can be achieved. In our experience, this minimum difference was ≥5°C”.
2. To validate this method for diagnostic porpoises, higher demanding analytical studies should be address. The authors may offer this assay as an alternative method to screen for gene dosage changes, or as a confirmatory analysis for previously detected alterations by other -not for diagnostic use- techniques. The analytic validity of this approach should be discussed in these terms.

Regarding this consideration, we have included in Discussion, last paragraph, page 9: “The novel assay presented here accurately detected losses and gains of one copy of the target sequence in all analyses (including mosaics of down to 25% of heterozygous mutated cells). Therefore, this methodology represents a reliable and flexible alternative both for screening gene dosage changes and as a validation assay for previously detected alterations. Nevertheless, validation of this technique for diagnostic purposes demands additional analytical studies in larger cohorts and for different genes”.

Minor Essential Revisions

1. Please review the Supplementary Figure 1: Family tree of patient FAP02. There are some incoherencies between the figure and its legend: Unaffected niece (IV:1) is marked with a plus symbol in the figure (instead of a minus symbol). There is not minus symbol in the unaffected sister (III:4). Unaffected aunt (II:1) is marked with a minus symbol.

Minus and plus symbols that appeared in this figure indicated if colonoscopy examination had been performed at AC Camargo Hospital or not. In order to avoid confusion with the disease status of the family members, this information was removed from the figure in the new version of the manuscript. The legend of Supplementary Figure 1 was updated to: “Family pedigree of patient FAP02. The arrow indicates the index patient. All affected individuals presented both polyposis and colorectal cancer. The patient presented four affected deceased relatives: grandmother, uncle, father and one sister. One unaffected sister (III:4) and one unaffected niece (IV:1) were tested and neither the deletion nor the missense variant were detected. Genetic testing of the unaffected individual II:1 could not be performed. The symbol (?) indicates unknown cause of death in individuals II:3 and II:4”.