

## Identification of Germline Mutations in Genes Involved in Classic FAP in Patients from Northern Brazil

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**Abstract.** *Background:* Colorectal cancer is a common cancer worldwide, with 5-10% of cases being hereditary. Familial adenomatous polyposis syndrome (FAP) is caused by germline mutations in the APC gene or rarely in the MUTYH gene. *Patients and Methods:* This work did not identify germline mutations in the MUTYH, NTHL1, POLD1 and POLE genes in 15 individuals belonging to five families with classic FAP, who had the mutation in the APC gene confirmed in a previous study. Our results support mutations in the APC gene as the main genetic contribution of classical FAP with severe phenotype. In the family that had the most aggressive form of the disease, we performed an array-based Comparative Genomic Hybridization analysis and identified the germinal loss of an allele of the NOTCH2 and BMPR2 genes in the mother (proband) and daughter. In order to validate the involvement of these genes in the other four families of this study, we analyzed the DNA copy number variation in the peripheral blood of the 15 participants.

*Results:* FAP is a syndrome with considerable genetic and phenotypic heterogeneity and this phenomenon may explain the presence of secondary genetic alterations, such as the allelic loss of NOTCH2 and BMPR2 genes, found only in one family in this study. The CNV analysis confirmed that only the two members of the FAP2 family (patient 02H and 02F) had a deletion of these two genes, as the aCGH methodology had found. The other study participants did not show allelic loss for these two genes. *Conclusion:* Validation in a larger number of families could confirm the presence of these new genetic alterations in classic FAP and improve understanding of the different types of aggressiveness of the disease.

Colorectal cancer is a common cancer worldwide, with 5-10% of cases being hereditary. Familial adenomatous polyposis syndrome (FAP) is caused by germline mutations in the APC gene or rarely in the MUTYH gene. The NTHL1, POLD1 and POLE genes have also been reported in previously unexplained FAP cases (1). Many of the phenotypes of cancer syndromes are overlapping; additionally, the sensitivity of using only clinical criteria is limited, and for this reason a broader genetic analysis is necessary (2).

According to the number of polyps and the age of disease onset, the phenotype of patients with mutations in the APC gene can be classified as classic FAP (more than 100 polyps and early onset of disease) or attenuated FAP (less of 100 polyps and late onset of disease) (3-5).

FAP is caused by monoallelic mutations in the APC gene, but in up to 10% of cases, in which the APC mutation is not identified, there is a biallelic germline mutation in the MUTYH gene. Unlike classic FAP, MUTYH-associated polyposis is attenuated and has a lower polyp load, which rarely exceeds 100 (6, 7). In a small proportion of cases, when genetic analysis of APC and MUTYH does not identify

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a responsible mutation, FAP has its etiology associated with autosomal recessive polyposis associated with the *NTHL1* gene and autosomal dominant polyposis syndrome due to mutations in the *POLD1* and *POLE* genes (8-10).

The present work aims to identify germline mutations in the *MUTYH*, *NTHL1*, *POLD1* and *POLE* genes in 15 individuals belonging to five families with classic FAP, who had the mutation in the APC gene confirmed (11). Additionally, we performed an array-based comparative genomic hybridization (aCGH) analysis in one of the five families in this study and identified germline alterations in the DNA copy number of the *NOTCH2* and *BMPR2* genes.

To validate the involvement of these genes in colorectal carcinogenesis, we analyzed the DNA copy number (CNV) variation in the peripheral blood of all participants in this study.

## Patients and Methods

**Ethics committee.** The study was approved by the Research Ethics Committee of the João de Barros Barreto University Hospital (Belém, Pará, Brazil; approval number: 274/12). All analyzed patients or their guardians signed a consent form, guaranteeing that the use of biological material and participation in the study and all methods were carried out in accordance with the Helsinki guidelines and regulations.

**Patients.** This study analyzed 15 patients belonging to 5 different families, who had confirmed germline mutations in the APC gene (11). All patients reside in the State of Pará in Brazil and were treated at the Colon Proctology Outpatient Clinic of the João de Barros Barreto University Hospital (Belém, Pará, Brazil). Peripheral blood samples were collected for analysis. In addition to patients with a histopathological diagnosis of FAP, family members who did not develop the disease were also tested for possible identification of mutations and for genetic counseling (Table I).

**DNA extraction.** Peripheral blood samples from patients and family members were collected in EDTA tubes. Genomic DNA was extracted using the QIAamp DNA Blood Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions.

**Sanger sequencing method.** Sanger sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit. For germline mutation analysis, the complete coding sequence of the *APC*, *MUTYH* and *NTHL1* genes and the exonuclease domain of the *POLD1* gene (exons 6–13) and the *POLE* gene (exons 9-14) were amplified by Polymerase Chain Reaction (PCR). Primer sequences and annealing temperatures for PCR used were those described by Khan *et al.* (1). PCR products were purified with ExoSAP-IT (Affymetrix®, Santa Clara, CA, USA) and sequenced using the ABI 3730 DNA sequencer (Applied Biosystems®, Foster City, CA, USA). Bidirectional sequencing was performed on all samples. The chromatogram of the Sanger sequencing results was analyzed using the Sequencing Analysis v. 5.2 software (Applied Biosystems®).

**aCGH and PCR for CNV evaluation.** We performed high-density comparative genomic hybridization analyses to assess the genome of all 3 patients in the FAP2 family. Analyses were performed to identify CNV in the complete genome of patients with FAP. The

system used was the Affymetrix CytoScan HD Array (Affymetrix), which comprises a total of 1.9 million probes for detection of CNV. Chromosome Analysis Suite version 1.2.1 (Affymetrix®) was used for microarray analysis.

The CNV of *NOTCH2* (Hs01519239\_cn) and *BMPR2* (Hs04642658\_cn) genes was evaluated using the TaqMan® CNV assays (Thermo Fisher Scientific®, Waltham MA, USA). Gene assays were run concurrently with the reference TaqMan® CopyNumber RNase P assay (Hs00468130\_cn) (Thermo Fisher Scientific®) for internal control. Reactions were performed in triplicate with gDNA using ABI 7500 Fast Real-Time PCR thermocycler (Applied Biosystems®). Sex-matched human genomic DNA (Promega®, Madison, WI, USA) samples obtained commercially were used as calibrators to estimate the copy number of the target gene. The analysis of relative quantification was performed using CopyCaller™ software (Thermo Fisher Scientific®).

## Results

Through interviews with family members and searching through medical records, it was possible to build family pedigrees for three of the five families included in the study - FAP1, FAP2 and FAP3 - which, coincidentally, are the families with the highest number of individuals affected by FAP in subsequent generations [Figure 1, adapted from Moreira-Nunes *et al.*, (11)]. Pedigree analysis of the remaining two families was not possible due to the patients' lack of information about their relatives.

In the previous study we reported that all 15 patients from the five families with FAP had frameshift germline mutations in the APC gene (c.3183\_3187delACAAA, c.3927\_3931delAAAGA, c.3921\_3925delAAAAG, c.3956delC), which gave the severe/aggressive phenotype to the aggressive phenotype syndrome (11). In addition to patients with a histological diagnosis of FAP, relatives without symptoms of the disease had mutations in the APC gene (Table I). In this study, the 15 individuals from the 5 families with FAP did not show pathological mutations for the *MUTYH*, *NTHL1*, *POLD1* and *POLE* genes.

The aCGH technique was performed only for the FAP2 family, since, among the families analyzed, it had the aggressive form of the disease (Table II). The approach used in the present work aims to verify if the alterations found in the FAP2 family, may be present in the other four families participating in this study. The FAP2 family was genomically analyzed using peripheral blood from three members, father, mother (proband) and daughter; the fourth member (the grandfather) was also a carrier of FAP but died before the beginning of this study (Figure 1). Only the mother and daughter of the FAP2 family (patients 02H and 02F) revealed DNA loss in chromosome arms 1p13-p11 (*NOTCH2* gene) and 2q33.1-q33.2 (*BMPR2* gene). The *APC* (located in the chromosome region 5q21), *MUTYH* (1p34.1), *NTHL1* (16p13.3), *POLD1* (19q13.33) and *POLE* (12q24.33) did not show quantitative alterations in the DNA.

Table I. Clinical features of patients analyzed in this study.

| Patients        | Sex    | Age at diagnosis | Histopathology | Mutations in the APC gene*#        | CNV NOTCH | CNV BMP2 |
|-----------------|--------|------------------|----------------|------------------------------------|-----------|----------|
| Family 1 (FAP1) |        |                  |                |                                    |           |          |
| 01A             | Female | 23               | FAP            | c.3956delC<br>c.3183_3187delACAAA  | 2         | 2        |
| 01B             | Female | 25               | FAP            | c.3956delC<br>c.3183_3187delACAAA  | 2         | 2        |
| 01C             | Female | 18               | FAP            | c.3956delC<br>c.3183_3187delACAAA  | 2         | 2        |
| 01D             | Male   | 14               | FAP            | c.3956delC<br>c.3183_3187delACAAA  | 2         | 2        |
| 01E             | Male   | 17               | FAP            | c.3956delC<br>c.3183_3187delACAAA  | 2         | 2        |
| Family 2 (FAP2) |        |                  |                |                                    |           |          |
| 02F             | Female | 40               | FAP            | c.3956delC<br>c.3927_3931delAAAAGA | 1         | 1        |
| 02G             | Male   | +                | FAP            | c.3956delC<br>c.3927_3931delAAAAGA | 2         | 2        |
| 02H             | Female | 15               | FAP            | c.3956delC<br>c.3927_3931delAAAAGA | 1         | 1        |
| 02I             | Male   | Not applicable   | -              | c.3956delC<br>c.3927_3931delAAAAGA | 2         | 2        |
| Family 3 (FAP3) |        |                  |                |                                    |           |          |
| 03J             | Male   | 30               | FAP            | c.3956delC                         | 2         | 2        |
| 03H             | Female | Not applicable   | -              | c.3956delC                         | 2         | 2        |
| 03L             | Female | Not applicable   | -              | c.3956delC                         | 2         | 2        |
| 03M             | Female | Not applicable   | -              | c.3956delC                         | 2         | 2        |
| Family 4 (FAP4) |        |                  |                |                                    |           |          |
| 04N             | Male   | 40               | FAP            | c.3956delC                         | 2         | 2        |
| Family 5 (FAP5) |        |                  |                |                                    |           |          |
| 05O             | Male   | 25               | FAP            | c.3956delC<br>c.3921_3925delAAAAG  | 2         | 2        |

FAP: Familial adenomatous polyposis; +: Unknown; Not applicable – patients without disease. \*Previously reported in Moreira-Nunes *et al.* 2015 (11). #The presence of mutations in the MUTYH, NTHL1, POLD1 and POLE genes was not detected.

The CNV detected by real-time PCR was used, in all 15 patients belonging to the five families that participated in this study, to verify the possibility that other FAP patients had allelic loss of the *BMP2* and *NOTCH2* genes. The CNV analysis confirmed that only the two members of the FAP2 family (patient 02H and 02F) had a deletion of these two genes, as the aCGH methodology had found. The other study participants did not show allelic loss for these two genes.

## Discussion

Tumorigenesis of colorectal carcinoma is a multi-step process, starting from mutations that cause loss of function of tumor suppressor genes, such as *APC*. Although other genes have been shown to be involved in this process, there are still

unrecognized molecular events, which when elucidated can help to improve the treatment of the disease (12-14).

By aCGH analysis, the *APC* gene did not show quantitative DNA alterations in the five families studied, although large *APC* deletions are a common cause of FAP (15). *APC* sequencing of family members participating in this study also demonstrated the absence of large deletions, the main change being the presence of frameshift c.3956delC mutation (11), which confers the aggressive phenotype on family members (16).

In *The Human Gene Mutation Database* there are approximately 1800 pathogenic gene mutations. Currently, prevention treatment remains the most important strategy for the clinical management of patients with FAP. Gastrointestinal endoscopy monitoring and assessment of the risk of disease progression are the mainstays of choosing local endoscopic

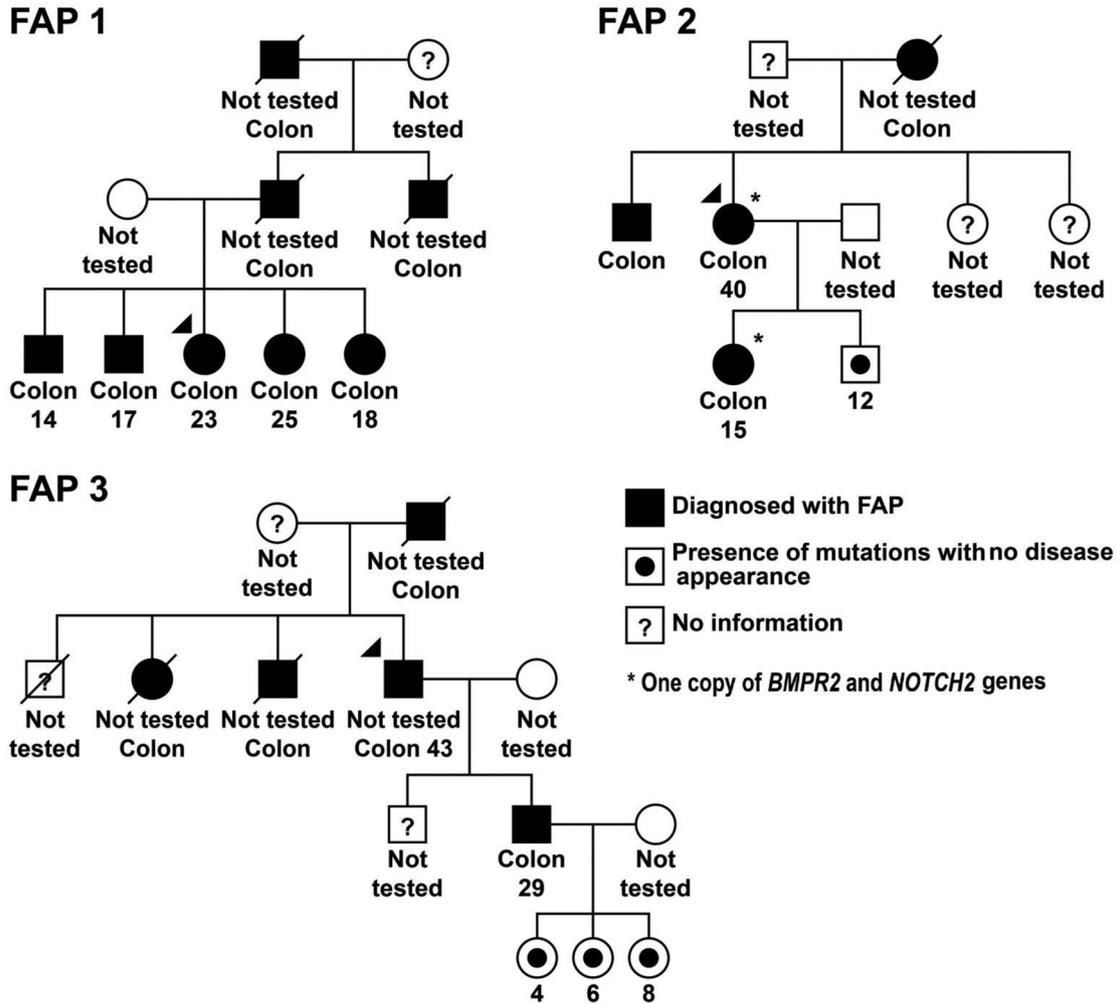


Figure 1. Pedigrees of families with FAP. (A): FAP1 - Family with eight affected members, presence of mutations in the APC gene, low expression of mRNAs and deletion of an allele of the *BMPR2* and *NOTCH2* genes; (B): FAP2 - family with four affected members and presence of APC mutations; (C): FAP3 - a family with five members affected with APC mutations and three members without onset of disease, but with presence of APC mutations. The numbers under the symbols represent age at diagnosis. Solid symbols represent affected limbs with confirmed diagnoses of adenomatous polyposis. Left upper arrows indicate the probands. In all affected limbs with mutations in the APC gene, mutations in the *MUTYH*, *NTHL1*, *POLD1* and *POLE* genes were not detected (adapted from Moreira-Nunes et al., 2015) (11).

Table II. Summary of the genomic alterations found in one member of the FAP2 family (02H).

| ++CR | Band (start) | Band (end) | Genomic Alteration | Copies | Localization (CR: start) | Localization (CR: end) | Size (kbp) | Gene          |
|------|--------------|------------|--------------------|--------|--------------------------|------------------------|------------|---------------|
| 1    | p12          | p12        | Loss/deletion      | 1      | 120.558.305              | 120.568.322            | 10         | <i>NOTCH2</i> |
| 2    | q33.1        | q33.2      | Loss/deletion      | 1      | 203.295.762              | 203.309.483            | 14         | <i>BMPR2</i>  |

+CR: Chromosome.

treatment or preventive radical resection of the stomach and colon (17). Between 20% and 30% of individuals diagnosed with FAP do not carry a pathogenic variant in the APC gene and mutations in the *MUTYH*, *NTHL1*, *POLD1* and *POLE*

genes may be related to the disease etiology. Patients with FAP can carry pathogenic variants in more than one gene at the same time and for this reason the sequencing of the five genes mentioned above is necessary (18).

In the present study, the 15 individuals from the 5 families with FAP did not show mutations that cause DNA repair deficiency by base excision (in the *MUTYH* and/or *NTHL1* genes) and/or inactivation of polymerase revision (in the domain of exonuclease genes *POLE* and/or *POLD1*). Mutations in other target genes, including age-related genes, could also play an important role in colorectal cancer and need to be investigated (19). However, our results support that in classic FAP with severe phenotype the main genetic contribution is mutations in the APC gene.

CNV are important polymorphisms that can influence gene expression and phenotypic variation. PCR-based detection of target gene CNV using the TaqMan® copy number assay offers a reliable method to measure copy number variation in the human genome (20) and in this study allowed us to validate the results of the aCGH methodology, which identified, in two members of a family with FAP, the allelic loss of the *NOTCH2* and *BMP2* genes.

The CNV methodology confirmed the allele losses in *NOTCH2* and *BMP2* genes in two patients of the FAP2 family, mother (proband) and daughter, probably there was maternal germline segregation of the haploidy of these two genes, since this change was not found in the father family, also affected by mutation in the APC gene (Figure 1). These two genes are associated with the sporadic tumorigenesis of colorectal cancer, where both show decreased expression (21, 22).

Allelic losses of *NOTCH2* and *BMP2* were not found in the other patients from the five families in the study (FAP1, FAP3, FAP4 and FAP5). It is noteworthy that the FAP2 family was the one that presented the most aggressive form of the disease, among the five families studied, and probably the loss of an allele in *NOTCH2* and *BMP2* genes has given the FAP2 family the aggressive phenotype. For this reason, validation in many samples is imperative to confirm the germline role of these variants in colorectal cancer tumorigenesis. FAP is a syndrome with considerable genetic and phenotypic heterogeneity (23) and this phenomenon may explain the presence of genetic alterations, probably secondary, such as the allelic losses of the *NOTCH2* and *BMP2* found in the two members of the FAP2 family.

*NOTCH2* frameshift mutations were described in four families with colorectal cancer syndrome that did not have a fully defined genetic cause (24). Additionally, germline mutations in *BMP2* confers susceptibility to pulmonary arterial hypertension (25). However, a study involving patients with juvenile polyposis, an autosomal dominant syndrome that predisposes to colorectal and gastric cancer, did not find germline mutations in *BMP2* (26).

Therefore, this study provides evidence of the involvement of *NOTCH2* and *BMP2* in the germline carcinogenesis of a family with classic FAP and reveals genetic heterogeneity, as another four families did not show

changes in the copy number of these genes. However, validation in a larger number of families could confirm the presence of these secondary changes and perhaps reveal different subtypes of progression from classic FAP.

## Conflicts of Interest

The Authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or data interpretation; in the writing of the manuscript, or in the decision to publish the results.

## Authors' Contributions

Conceived and designed the experiments: DFAA, SFLJ, CFAMN, PAP, RMRB. Performed the experiments: CFAMN, DFAA. Analyzed the data: CFAMN, DFAA, RMRB. Wrote the paper: CFAMN, PPA, RMRB.

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